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The Impact of Oxidative Stress and Inflammation on RPE Degeneration in Non-neovascular AMD

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Abstract

The retinal pigment epithelium (RPE) is a highly specialized, unique epithelial cell that interacts with photoreceptors on its apical side and with Bruch's membrane and the choriocapillaris on its basal side. Due to vital functions that keep photoreceptors healthy, the RPE is essential for maintaining vision. With aging and the accumulated effects of environmental stresses, the RPE can become dysfunctional and die. This degeneration plays a central role in age-related macular degeneration (AMD) pathobiology, the leading cause of blindness among the elderly in western societies. Oxidative stress and inflammation have both physiological and potentially pathological roles in RPE degeneration. Given the central role of the RPE, this review will focus on the impact of oxidative stress and inflammation on the RPE with AMD pathobiology. Physiological sources of oxidative stress as well as unique sources from photo-oxidative stress, the phagocytosis of photoreceptor outer segments, and modifiable factors such as cigarette smoking and high fat diet ingestion that can convert oxidative stress into a pathological role, and the negative impact of impairing the cytoprotective roles of mitochondrial dynamics and the Nrf2 signaling system on RPE health in AMD will be discussed. Likewise, the response by the innate immune system to an inciting trigger, and the potential role of local RPE production of inflammation, as well as a potential role for damage by inflammation with chronicity if the inciting trigger is not neutralized, will be debated.

Keywords

Age-related macular degeneration; complement; inflammation; mitochondrial dynamics; Nrf2; oxidative stress; retinal pigment epithelium

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1. Introduction

While aging is a decline in the ability to respond and adapt to the cumulative impact of different exposures (Jones, 2015), age-related disease develops when cellular dysfunction from impaired cytoprotective pathways is severe enough to cause tissue injury. Age-related macular degeneration (AMD) is one such disease. It is the leading cause of vision loss among the elderly in the United States and western societies (Congdon et al., 2004) and is a major public health problem that costs $30 billion annually in the United States (Brown et al., 2005a). Over 1.75 million people in the United States have advanced AMD, and as the "baby boomers" age, it is estimated that 3 million people will be affected by 2020 (Friedman et al., 2004), and 1 in 3 people in their 8^{th} decade of life with early AMD will develop advanced disease over the next decade (Gehrs et al., 2006; Mukesh et al., 2004). Currently, 8.7% of the world-wide population has AMD, and the projected number of people afflicted with AMD by 2020 is 196 million, increasing to 288 million by 2040 (Wong et al., 2014). As a result, the cost of AMD is predicted to increase to $59 billion over the next 20 years (Centre for Eye Research Australia, 2006). The impact of AMD on an individual's quality of life is high. For example, Brown et al found that the decrease in quality of life from early AMD is similar to a person with symptomatic HIV, and with advanced AMD, to one with metastatic prostate cancer having poorly controlled pain (Brown et al., 2005b). With vision loss, a person with AMD is less active (Wysong et al., 2009), and is at higher risk of depression (Augustin et al., 2007; Brody et al., 2001) or anxiety (Berman and Brodaty, 2006) than unaffected elderly people.

The retinal pigment epithelium (RPE) is a highly specialized, polarized epithelial cell whose apical side is in intimate contact with photoreceptor outer segments, and its basal side is adherent to Bruch's membrane, a specialized, pentalaminar extracellular matrix that separates the RPE from the choriocapillaris (Hogan, 1972). Some of the RPE's essential functions that maintain the health and function of both the photoreceptors and choriocapillaris include the daily phagocytosis of photoreceptor outer segments, light absorption to optimize vision, heat exchange, vitamin A metabolism, and secreting vascular endothelial growth factor (VEGF), which works as a trophic factor to sustain the health of the choriocapillaris endothelium (Saint-Geniez et al., 2009). With Bruch's membrane, it maintains the outer blood retinal barrier to provide selective entry and removal of metabolites and molecules to and from the retina and systemic circulation (Strauss, 2005). Along with its remarkable and diverse functions, its sandwiched location makes it pivotal for maintaining normal vision, and in particular, central visual acuity that is provided by the macula. As a result, RPE impairment contributes significantly to AMD.

Oxidative stress has long been considered a major influence on the RPE in AMD pathophysiology. Both environmental and genetic factors support this theory. The macula lives in a high oxidative stress environment, which can be magnified by those who choose to smoke, making it the strongest non-genetic risk factor for AMD aside from chronological aging (Rahman and MacNee, 1996; Rangasamy et al., 2004; Smith and Hansch, 2000). A high fat diet (HFD) intake, which also induces oxidative stress as reviewed in (Uranga et al., 2010), is another preventable AMD risk factor (Chiu et al., 2014; Mares-Perlman et al., 1995; Seddon et al., 2003; Seddon et al., 2001). Over the past decade, nearly 40 genetic

variants have been identified that are associated with AMD risk, and include genes that encode proteins involved in oxidative stress, which strengthens the premise that oxidative stress has an etiologic role. For example, since mitochondria are an abundant source of reactive oxygen species (ROS) formation, the identification of polymorphisms in mitochondrial MTND2*LHON4917G, NADH dehydrogenase subunits, and mitochondrial superoxide dismutase 2 (SOD2), that are associated with AMD risk, suggests a pathogenic role for oxidative stress (Canter et al., 2008; SanGiovanni et al., 2009). Likewise, the LOC387715 locus polymorphism that is associated with AMD risk is higher in smokers than either smoking or the polymorphism alone, is additional evidence of a pathogenic role of oxidative stress (Schmidt et al., 2006).

A decade ago, the discovery of polymorphisms in complement factor H (CFH) with up to a sevenfold increased risk of AMD introduced innate immunity as a pathophysiologic factor (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). Shortly after, genetic abnormalities in other complement pathway genes that influence the risk for AMD were identified such as C3 (rs2230199), factor I (rs10033900), and the C2/factor B locus (rs9332739, rs547154, rs4151667, and rs641153) (Fagerness et al., 2009; Gold et al., 2006; Spencer et al., 2007; Yates et al., 2007). Since these discoveries, scientists around the world have been working to delineate greater understanding of how innate immunity influences AMD pathogenesis. Despite this intensive research in both oxidative stress and innate immunity, several questions remain unanswered. To what degree does oxidative damage contribute to AMD? How does the macula protect itself from oxidative damage? How does innate immunity protect the macula and to what degree does tissue injury from either exaggerated or chronic innate immune response contribute to AMD? Does oxidative damage trigger innate immunity? Given the central anatomic and functional role of the RPE in the macula, in this review, we will take a critical look at the role of oxidative stress and innate immunity on RPE degeneration in non-neovascular AMD pathobiology.

2. Clinical Definition of AMD

According to the American Academy of Ophthalmology, AMD is defined by the presence of at least intermediate-size drusen (63 μm or larger in diameter), or yellowish accumulations of debris within Bruch’s membrane; RPE abnormalities such as hypopigmentation or hyperpigmentation; reticular pseudodrusen or subretinal deposits; and/or the presence of any of the following features: advanced non-neovascular disease or geographic atrophy (GA), which is a circumscribed area or areas of RPE cell loss, and neovascular disease (exudative, wet) including choroidal neovascularization, polypoidal choroidal vasculopathy, or retinal angiomatous proliferation (https://www.aao.org/preferred-practice-pattern/age-related-macular-degeneration-ppp-2015).

3. Histopathologic Changes in Non-neovascular AMD

The clinician defines the macula as the central 6 mm diameter area located between the major retinal vascular arcades while the anatomist defines it as the retina with more than one layer of ganglion cell nuclei (Orth et al., 1977). The macula contains two distinct sub-regions composed of different photoreceptors. The central region or fovea, is 0.8 mm in

diameter (2.75° in visual angle), and is dominated by cones. The parafovea surrounds the fovea and is rod-dominated (Curcio et al., 1990). In the macula, rods actually outnumber cones by 9:1. Thus, while the central macula is cone dominated, the rod-dominated parafovea makes the macula cone enriched and not cone-dominated.

In early AMD, patients complain of poor vision despite 20/20 visual acuity, with impaired macular sensitivity, contrast sensitivity, light exposure recovery, and dark adaptation (Chen et al., 1992; Eisner et al., 1992; Eisner et al., 1991; Midena et al., 1997; Owsley et al., 2015), which are due to diminished parafoveal rod responses (Hood et al., 2002; Owsley et al., 2016; Parisi et al., 2007). In the aging parafovea, the region of high rod density, the RPE are hexagonal, enlarged, and have the highest frequency of multinucleated cells (Starnes et al., 2016). Of the possible causes of multinucleation, such as endoreplication, cell fusion, or incomplete cell division, Chen et al found that oxidative stress generated during the phagocytosis of oxidized outer segments, induces RPE proliferation, but cytokinesis failure, indicating that incomplete cell division is likely the primary etiology for multinucleation (Ach et al., 2014; Chen et al., 2016). In early AMD, the RPE degenerates and assumes a mesenchymal morphology while photoreceptors can appear intact (Ach et al., 2015; Hartmann et al., 2011; Iwama et al., 2010; Sulzbacher et al., 2012; Vogt et al., 2011; Wu et al., 2014). With progressive RPE changes, parafoveal rods die before either the RPE or cones (Starnes et al., 2016), and surviving rods often lack outer segments (Curcio et al., 1996; Curcio et al., 1993). The RPE undergoes progressive degeneration as AMD advances with the most severe changes in GA (Ach et al., 2015; Vogt et al., 2011). In the transitional zone surrounding GA, RPE cells have severely irregular shape and pigmentary changes, can dissociate from Bruch's membrane to become multilayered or they can migrate into the retina or "subduct" into Bruch's membrane (Guidry et al., 2002; Sarks et al., 1988; Vogt et al., 2011; Zanzottera et al., 2015). These changes are typical of epithelial mesenchymal transition (EMT) type II, where epithelial cells undergo multiple molecular changes to assume a mesenchymal phenotype in an attempt to regenerate (Kalluri and Neilson, 2003), resulting in loss of polarity, loss of epithelial function, increased mobility, and resistance to cell death (Kalluri and Weinberg, 2009; Zeisberg and Neilson, 2009). Collectively, these RPE changes suggest RPE dysfunction has a central role in rod dysfunction and death in both early and late stage AMD.

Coincident with RPE changes, Bruch's membrane undergoes a series of histopathologic changes known as basal deposits, or accumulations of heterogeneous debris, as classically categorized by Sarks (Sarks, 1976). The composition and location define whether basal deposits are associated with aging or AMD. Beginning early in life, Bruch's membrane develops outer collagenous layer deposits, which account for much of Bruch's membrane's age-related thickening (van der Schaft et al., 1991). Basal laminar deposits (BlamD), which accumulate between the RPE cell and its basement membrane, are associated with aging when they are thin and similar in composition to the RPE basement membrane, including collagen IV, laminin, and heparan sulfate proteoglycans (van der Schaft et al., 1994). When BlamD become thickened and contain heterogeneous debris such as long spacing collagen, inflammatory molecules, and lipids, they are associated with AMD. Basal linear deposits (BlinD), which form within the inner collagenous layer of Bruch's membrane, are associated with AMD. Histopathologically, BlinD and large drusen (>125microns in diameter) are

different morphologic forms of the same lesion (Abdelsalam et al., 1999; Bressler et al., 1994; Curcio and Millican, 1999; Green and Enger, 1993; Spraul et al., 1996).

The RPE appears to be centrally involved in basal deposit and drusen formation. The accumulation of apolipoprotein B100 (apoB100) containing lipoproteins, which are reminiscent of very low density lipoproteins (VLDL) and low density lipoproteins (LDL), is an important antecedent event to basal deposit and drusen formation (Curcio et al., 2005). As the Curcio and Handa labs have demonstrated, these lipoproteins likely originate primarily from the RPE rather than the systemic circulation, and may be secreted in response to lipid overload by the RPE (Curcio et al., 2010; Fujihara et al., 2014; Wu et al., 2010). These lipoproteins transit from the RPE through Bruch's membrane in linear tracks (Pikuleva and Curcio, 2014), and due to aging related structural changes to Bruch's membrane, first accumulate in the inner collagenous layer adjacent to the elastic layer, and then toward the RPE, eventually forming a "lipid wall". Because the lipid wall prevents the passage of material, inflammatory and cellular debris accumulates, forming basal deposits and drusen. These observations correlate with the hypothesis made by Hageman et al, in 2001, that drusen are biomarkers of immune-mediated processes at the RPE-Bruch's membrane triggered by cellular debris and lipids that accumulate in Bruch's membrane (Hageman et al., 2001).

Besides drusen, deposits also form in the subretinal space between the photoreceptors and RPE. Originally identified by Mimoun et al. in 1990 (Mimoun et al., 1990), reticular pseudodrusen were first clinically observed in AMD. Zweifel et al., using optical coherence tomography (OCT), showed that these deposits were located in the subretinal space (Zweifel et al., 2010) which have been confirmed by histopathological analysis (Rudolf et al., 2008; Sarks et al., 2011). While long ignored in part, because they can be challenging to visualize on clinical exam, reticular pseudodrusen are now a fundamental criteria for diagnosing and monitoring AMD progression due to improvements in imaging modalities such as OCT and fundus autofluorescence, and recognition that these lesions are a marker for disease advancement (Arnold et al., 1995; Cohen et al., 2007; Hamel et al., 2009; Lee et al., 2012; Zweifel et al., 2010). While the pathogenesis of these lesions is unknown, unesterified cholesterol, apolipoprotein E, CFH, and vitronectin have been found in reticular pseudodrusen (Rudolf et al., 2008). As will be discussed below, our lab found that the C5b-9 complex regulator CD59, is also found in reticular pseudodrusen, and CD59 is secreted in a combination of exosomes and apoptotic blebs by the RPE (Ebrahimi et al., 2012). These results raise the possibility that complement activation by the RPE could contribute to reticular pseudodrusen formation.

The choroid and RPE have a symbiotic interaction whereby the RPE is dependent upon the choriocapillaris for survival. In early AMD, Seddon et al recently found that choriocapillaris loss, using EC-binding lectin from Ulex europaeus (UEA-I) to distinguish live from ghost vessels, preceded RPE cell atrophy (Seddon et al., 2016). The Mullins laboratory, also using UEA-I, found that loss of choriocapillaris area and an increase in ghost vessels, correlated with increased drusen size and density, implicating a role for choriocapillaris dysfunction in drusenogenesis (Mullins et al., 2011). These changes correspond to functional changes such as prolonged filling of the choroid in early AMD with indocyanine green or fluorescein

angiography (Pauleikhoff et al., 1999) and decreased choroidal blood volume and flow by laser Doppler flow studies (Berenberg et al., 2012).

4. Oxidative stress: when does a friend become a foe?

The generation of ROS has long been considered to have harmful consequences on the RPE. However, ROS have a physiological role that is essential for normal cellular function. Redox signaling can act as an important regulator in both physiological and pathological processes, and can be generated from several sources, as reviewed in (Finkel, 2011), including NADPH-dependent oxidases, mitochondrial electron transport chain (ETC) complexes, and other cellular enzymes including xanthine oxidase, cyclooxygenases, lipoxygenases, and cytochrome p450 enzymes. ROS signal a wide range of cellular responses that protect the RPE, such as feedback regulation after excess metabolism (Brunelle et al., 2005; Guzy et al., 2005), response to hypoxia via HIF-1α (Guzy et al., 2005), regulation of autophagy through ATF4 (Scherz-Shouval et al., 2007), and regulation of inflammatory responses (Bulua et al., 2011; Nakahira et al., 2011; Zhou et al., 2011).

The macula lives in a high oxidative stress environment in part, because it has unique sources of oxidative stress. The RPE has a high metabolic demand that produces high levels of physiologic ROS for signal transduction, but also substantial ROS produced from cellular metabolism to meet its multiple functions. To meet this metabolic demand, the macula receives some of the highest blood flows in the body. As a result, the RPE is exposed to high ambient oxygen partial pressures of 70–90 mm Hg (Winkler et al., 1999).

Due to its high metabolic activity, RPE are enriched with mitochondria, and as a result, is a major source of ROS in the RPE (Jager et al., 2008). Compared with young cells, aging mitochondria generate more ROS, and furthermore, aging cells are progressively susceptible to mitochondria derived ROS. While it has been known since the 1960s that mitochondria produce ROS (Jensen, 1966), the production of ROS by mitochondrial ETC complexes had not been fully elucidated until relatively recently (Yaniv et al., 2013; Zorov et al., 2005). Under physiological conditions during ATP synthesis, the ETC complexes generate ROS that leak out of the mitochondrial membrane (Chen et al., 2012; Liu et al., 2002; Murphy, 2009). Under pathological conditions that compromise the ETC components, the generation of ROS is significantly increased, principally at complexes I (NADH complex) and III (cytochrome complex). Not surprisingly, mutations in the genes that code for these complexes account for more than 50% of all mitochondrial ROS related pathological conditions (Zorov et al., 2005). Additionally, the decrease in mitochondrial membrane potential facilitates the leakage of ROS into the cytoplasm (Korshunov et al., 1997).

The RPE has unique sources of oxidative stress. For example, the RPE has the unusual responsibility of the routine phagocytosis of nearly 30,000 photoreceptor outer segments each day (Ershov and Bazan, 2000). During outer segment phagocytosis, intracellular H_2O_2 from NADPH oxidase in the phagosome or β-oxidation of outer segment lipids in peroxisomes is generated to the same magnitude that occurs during phagocytosis by macrophages (Miceli et al., 1994; Tate et al., 1995). Photo-oxidative stress from processing light for vision is perhaps the most unique and additional source of oxidative stress. Since

the work of Ham et al in 1978 (Ham et al., 1978), photo-oxidative stress has been linked with oxidative damage to the retina, RPE, and choroid, and multiple other works have substantiated this observation, as reviewed in (Beatty et al., 2000). These data correlate with the Chesapeake Bay Watermen studies showing the epidemiologic link of sunlight exposure and AMD risk (Taylor et al., 1990; Taylor et al., 1992).

The oxidative burden contributing to the oxidative stress load on the RPE is enhanced by the western life style. Cigarette smoking clearly adds to the oxidative stress burden because it contains over 4700 chemical components, many of which are strong oxidants (Rangasamy et al., 2004; Smith and Hansch, 2000), and each puff contains 10^{15} free radicals (Rahman and MacNee, 1996). While the exact role of oxidative damage caused by cigarette smoking in AMD is unknown, chemical oxidants in cigarette smoke deplete tissues of ascorbic acid and protein sulfhydryl groups, causing the oxidation of DNA, lipids and proteins (Cross et al., 1993; Lykkesfeldt et al., 2000; O'Neill et al., 1994). Many of these molecular changes such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and advanced glycation endproducts (AGE), have been identified in AMD, and indicate that oxidative damage is an important factor in the mechanism of disease development. These observations correlate well with the multiple studies concluding that smoking is the strongest environmental risk factor for AMD (Rahman and MacNee, 1996; Rangasamy et al., 2004; Smith and Hansch, 2000). To a lesser extent, a high fat diet (HFD) intake also induces oxidative stress, and represents another source of oxidative stress and risk factor for AMD (Chiu et al., 2014; Mares-Perlman et al., 1995; Seddon et al., 2003; Seddon et al., 2001; Uranga et al., 2010). At present, it is unclear when the burden of oxidative stress extends beyond physiological signaling and becomes pathologic, or to what extent the accumulated exogenous and preventable sources of oxidative stress overwhelm the RPE cell's antioxidant system.

5. How does the RPE protect itself from oxidative stress?

In general, cells have developed several mechanisms to protect themselves from the potentially damaging effects of oxidative stress, yet at the same time, enable vital signaling through physiological ROS. For example, oxidant-producing enzymes are located adjacent to the intended target, or oxidants have a regulated entry, such as H_2O_2 through aquaporin channels (Bienert et al., 2007). Along with the short half-life of ROS, their transient production, such as during a NOX-dependent oxidant burst, these mechanisms topographically limit ROS to a specific locale where they can be neutralized if excessive. Oxidative stress induces a cellular antioxidant response, including in RPE cells, which has been linked to activation of autophagy through the p62/Keap1/Nrf2 pathway (Azad et al., 2009; Filomeni et al., 2010; Johansson et al., 2015; Wang et al., 2014a). Autophagy is a protective, homeostatic mechanism designed to remove faulty cellular components including those generated by oxidative damage. Importantly, compromised autophagy can result in dysfunctional RPE has been shown to induce an AMD like phenotype in mice (Yao et al., 2015), and is a contributing factor in AMD (Mitter et al., 2014; Viiri et al., 2013; Wang et al., 2009).

5.1 Mitochondrial dynamics maintain a homeostatic level of mitochondrial ROS

As discussed above, a large proportion of ROS is derived from mitochondria. Since RPE cells are predominantly post-mitotic, damaged mitochondria are not removed during cell division (Cai et al., 2000). Instead, mitochondrial health and function rely on a dynamic process of mitochondrial fission and fusion followed by removal of damaged mitochondria (Westermann, 2010). Through fission, damaged mitochondria are sequestered and then degraded through dynamin -1- like protein (Drp1) (Twig et al., 2008). The damaged mitochondrial fragments are selectively removed by mitophagy, a specialized type of autophagy. When the mitochondrial membrane potential ($\Delta\Psi_m$) is decreased, the Pink1-Parkin mediated signaling system is initiated (Lin and Kang, 2008), ultimately leading to ubiquitination and lysosomal degradation of the affected mitochondria. Pink1 is a nuclear encoded mitochondrial serine-threonine kinase. In healthy mitochondria, Pink1 transits through the mitochondrial membranes into the matrix where it is degraded by protease presenilin-associated rhomboid-like protein (PARL), its antagonist (Meissner et al., 2011). With a decrease in $\Delta\Psi_m$, Pink1 remains on the mitochondrial surface, where it phosphorylates a wide range of proteins, most notably Parkin and Ubiquitin. Phosphorylated Parkin in turn ubiquitinates proteins of the outer mitochondrial membrane, eventually resulting in LC3b mediated lysosomal phagophore formation and degradation (Kitagishi et al., 2017; Lazarou et al., 2015; Rub et al., 2017).

Mitophagy protects the mitochondria from environmental insults like chronic oxidative stress, and is highly sensitive to fluctuations in oxidative stress. Under mild oxidative stress that is unable to induce generalized autophagy, mitophagy is induced through mitochondrial fission in a Drp1 dependent manner (Frank et al., 2012). Because any deficiency in mitophagy can result in elevated mitochondrial ROS (Kitagishi et al., 2017; Narendra et al., 2008), impaired mitophagy and mitochondrial dynamics in the RPE have been implicated in early AMD (Feher et al., 2006; Karunadharma et al., 2010). The excessive mitochondrially derived ROS has significant implications that can further injure the mitochondria and threaten the overall health of the RPE (Kinugasa et al., 2015; Zhou et al., 2009) because mitochondrial DNA (mtDNA) are especially susceptible to injury due to the lack of an efficient proof-reading and repair mechanism, and the lack of introns in mitochondrial genes. Thus, any mutation in mtDNA will directly affect the gene product (Blasiak et al., 2013). Since all mitochondria encoded genes are involved in oxidative phosphorylation (OXPHOS), mitochondrial damage leads to defects in OXPHOS, resulting in impaired metabolism and further increases in ROS production, which further damages mitochondria and other cellular elements (Liang and Godley, 2003). In fact, Golestaneh et al recently found that iPS RPE cells derived from AMD patients had mitochondria that were significantly damaged, which reduced ATP production and enhanced glycolysis (Golestaneh et al., 2016). Furthermore, the AMD derived RPE cells were highly susceptible to oxidative stress and produced high levels of ROS under such stress. These changes correlated with decreased Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a regulator of mitochondrial biogenesis and function, and NAD-dependent deacetylase sirtuin1 (SIRT1).

Direct evidence for mitochondrial damage in AMD has been documented in several studies. Feher et al found decreased mitochondrial mass with ultrastructural alterations (Feher et al., 2006) in AMD specimens. The RPE of AMD affected patients have increased mtDNA damage compare to RPE from age matched controls (Blasiak et al., 2013), macular RPE have more mtDNA damage than peripheral RPE (Nordgaard et al., 2008), and mtDNA damage occurs at stages prior to overt visual loss (Karunadharma et al., 2010; Lin et al., 2011). mtDNA damage is correlated with a decrease in 8-oxoguanine glycosylase, a marker for mtDNA repair capacity (Lin et al., 2013) and an increase in disease severity (Blasiak et al., 2013). Importantly, Terluk et al have recently shown that mtDNA damage preferentially develops in the RPE, but not the retina to illustrate the importance of mtDNA damage in the RPE as an early event in AMD progression (Terluk et al., 2015). The mtDNA damage affects regions required for transcription and replication of mitochondrial proteins, and electron transport chain (ETC) complex I and II subunits (Terluk et al., 2015). With damaged mitochondria, the cell has compromised energy production, as well as an imbalance in pro and anti apoptotic signals, leading to RPE dysfunction and death, the two key features of AMD pathogenesis (Kanda et al., 2007; Kinnunen et al., 2012). However, with mitochondrial injury necessitating a severe mitophagic response, EMT or even apoptosis can result (Higgins and Coughlan, 2014; Redza-Dutordoir and Averill-Bates, 2016). Given that the RPE undergoes degeneration reminiscent of EMT type II, and apoptosis is one proposed mechanism of RPE cell death in AMD, future studies should consider the role of mitophagy and mitochondrial dynamics in the pathogenesis of RPE degeneration.

5.2 The Nrf2 cytoprotective signaling system and its decrease in the RPE

In general, the RPE has a complex series of antioxidant systems that protect cells against oxidative damage. These systems are regulated through various transcription factors including Nf-κB, AP1, the FoxO family, Wnt, or PGC-1α. Perhaps, the most comprehensive transcription system is mediated through Nuclear factor erythroid-2 related factor 2 (Nrf2), a basic leucine zipper transcription factor (Biswas and Rahman, 2008). Nrf2 regulates a coordinated transcriptional program that allows cellular redox homeostasis, yet protects the cell from oxidative injury (Nguyen et al., 2003; Rangasamy et al., 2004; Thimmulappa et al., 2002) (Fig 1). Nrf2 is normally sequestered in the cytosol by interacting with Kelch-like ECH-associated protein 1 (Keap1). Keap1 also functions as a substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase complex, which also helps to maintain steady-state levels of Nrf2 via proteolysis of Nrf2 by the ubiquitin-proteasome pathway (Adams et al., 2000; Kobayashi et al., 2004; Yu and Kensler, 2005; Zhang et al., 2004). Without oxidative stress, Keap1 constitutively suppresses Nrf2 signaling by degrading Nrf2 to prevent Nrf2 from translocating to the nucleus, which results in a low baseline expression of antioxidant genes that are regulated by other transcription factors (Thimmulappa et al., 2002). With acute oxidative stress, Keap1 undergoes a conformational change after multiple cysteine residues interact with ROS, releasing Nrf2, which inhibits Keap1-mediated proteasomal Nrf2 degradation. The released Nrf2 then translocates to the nucleus, where it dimerizes with Maf proteins, and binds to the antioxidant response element (ARE) in the promoters of its target genes to initiate transcription (Dinkova-Kostova et al., 2005; Kobayashi and Yamamoto, 2005; Wakabayashi et al., 2003). The Nrf2 signaling response regulates both an

early acute phase through actions of the “direct” enzymes, such as catalase or SOD, which neutralize H_2O_2 and superoxide, respectively, and a chronic phase by regulating glutathione and thioredoxin levels, as well as xenobiotic metabolism enzymes that produce reducing equivalents, such as NADPH quinine oxidoreductase (NQO-1) (Osburn et al., 2008). When cellular glutathione is depleted by oxidative stress, cells can die by oxidatively mediated apoptosis (Rahman et al., 2005; Walsh et al., 1995; Will et al., 1999). Several studies have identified Nrf2 as an essential signaling system in the RPE (Gao and Talalay, 2004; Nelson et al., 2002; Nelson et al., 1999; Tanito et al., 2005).

In addition to its antioxidant protection, Nrf2 signaling has recently been found to play an unexpected role in maintaining mitochondria and metabolism. Nrf2 controls the expression of several tricarboxylic acid cycle (TCA) enzymes, which increases glucose flux through the TCA cycle to generate NADH, an essential substrate for ETC complex I to promote ATP production (Dinkova-Kostova et al., 2015; Singh et al., 2013). Likewise, Nrf2 also increases the expression of fatty acid oxidation enzymes to provide FADH2 as a substrate for ETC complex II (Dinkova-Kostova et al., 2015). Nrf2 signaling can also increase glycolysis by inducing the expression of several key components (Singh et al., 2013). Finally, Nrf2 promotes the expression of alcohol dehydrogenase, aldehyde dehydrogenase, or NADPH alenol oxidoreductase, which break down 4-HNE modified proteins in ETC complex I and II to rejuvenate mitochondrial function (Miller et al., 2013). Future studies will need to clarify the relative contribution of impaired antioxidant protection to metabolism with Nrf2 failure.

The accumulation of oxidatively damaged molecules in the macula with AMD suggests that the antioxidant response is insufficient. Aging can reduce Nrf2 mRNA or protein, which impairs Nrf2 signaling. Suzuki et al showed an age-dependent decline in Nrf2 from cigarette smoking (Suzuki et al., 2008). These results have also been observed in mice, where aging suppressed the induction of Nrf2 and its target genes in alveolar macrophages in response to cigarette smoking (Suzuki et al., 2008). Our lab has shown that aging impaired the Nrf2 response in the RPE after acute oxidative stress in mice (Sachdeva et al., 2014). While the RPE of young mice elicit a robust induction of Nrf2 mediated antioxidant gene expression after exposure to the chemical oxidant sodium iodate, the RPE of old mice had a blunted antioxidant response that resulted in the accumulation of MDA. MDA damage was reversed by genetic rescue of Nrf2 signaling by conditionally knocking down Keap1.

Nrf2 also declines in several diseases. For example, in emphysema, Nrf2 mRNA and protein, and Nrf2-dependent antioxidants and glutathione levels are reduced, and oxidative stress is increased in diseased compared to normal lungs (Malhotra et al., 2008; Suzuki et al., 2008). These same findings are seen in Nrf2 deficient mice after they develop an emphysema phenotype following chronic cigarette smoke exposure (Rangasamy et al., 2004). We have also provided evidence that Nrf2 is decreased in AMD (Wang et al., 2014b). In maculas of early AMD, Nrf2 immunolabeling was increased with nuclear labeling in morphologically normal RPE compared to the RPE of non-AMD maculas, which is compatible with induced Nrf2 signaling from a high oxidative stress microenvironment of the AMD macula. In dysmorphic RPE cells from AMD maculas, Nrf2 immunolabeling was decreased relative to morphologically normal RPE cells (Fig 2). This finding suggests that Nrf2 signaling has failed in diseased areas, and with this failure, the narrow balance between physiological and

pathological oxidative stress is upset, resulting in tissue damage. However, inadequate Nrf2 signaling may not sufficient to induce advanced stage disease. For example, our lab found that C57Bl6J mice treated with chronic cigarette smoking have impaired Nrf2 signaling in the RPE, which was associated with marked RPE degeneration, but without progression to more advanced stages such as neovascular AMD or geographic atrophy (Cano et al., 2010; Fujihara et al., 2008). Similarly, Zhou et al. found that $Nrf2^{-/-}$ mice developed elements of AMD including drusen and RPE abnormalities, but again, no progression to advanced disease (Zhao et al., 2011). It is possible that impairment of more than one cytoprotective pathway in the RPE is necessary to induce further dysfunction and cell death during advanced stages of AMD.

6. The role of inflammation on the RPE

Inflammation is the body's response to damage of its cells and tissues in a series of processes designed for the eventual clearance of pathogens and repair of damaged tissue. Acute inflammation is a short-term process that involves leukocyte infiltration, removal of the trigger, and tissue repair. Chronic inflammation is a prolonged response that can result in tissue injury or destruction if the inciting trigger is not neutralized. Persistent inflammation is thought to underlie many diseases including AMD. The inflammatory response includes activation of both innate and adaptive immunity (Patel et al., 2005). At this time, the relative protective or pathologic responses during disease development have not been clearly delineated. In response to oxidatively damaged lipids, macrophages polarized toward an M1 phenotype accumulated in the subretinal space of mice immunized with carboxyethylpyrrole (CEP) prior to lesion development while macrophages and an AMD phenotype were absent in Ccr2-deficient mice, implicating macrophages in tissue injury (Cruz-Guilloty et al., 2013). Furthermore, subretinal mononuclear phagocytes or IL-1β might contribute to cone photoreceptor loss late stage AMD (Eandi et al., 2016) in part, because activated mononuclear phagocytes might resist elimination by the RPE with production of TNF-α, which downregulates OTX2, an important transcription factor that regulates genes that are essential to RPE function (Mathis et al., 2017). In the choroid, macrophages are recruited to basal deposits and drusen (Cherepanoff et al., 2010). Recently, mast cell degranulation has been identified in the choroid of all phases of AMD, which can release proteolytic enzymes that could result in Bruch's membrane degradation, RPE death, choriocapillaris cell death, and choroidal thinning during AMD development (Bhutto et al., 2016). In the RPE, inflammation involves the innate immunity arms of complement and the inflammasome. The rest of this review will focus on these two arms of immunity.

6.1 Complement: when does a protective response become dysfunctional?

Complement components and its regulatory elements are typically synthesized in large abundance by the liver, and then released into the bloodstream for delivery to tissues in need of a complement defense response. In fact, CFH is one of the most abundant plasma proteins (Zipfel and Skerka, 2009). Indeed, systemically derived complement appears to play a role in AMD. Scholl et al showed that complement activation products C3a, C3d, Ba, C5a, and SC5b-9 were significantly elevated in plasma from AMD patients compared to controls (Scholl et al., 2008). This was especially true for chronic activation markers Ba and C3d

($p<0.001$) and factor D, which collectively, provided better discriminative accuracy for AMD risk than genetic markers of the complement system. Reynolds et al provided additional evidence of systemic alternative complement pathway component involvement in AMD where increased plasma Bb and C5a were associated with AMD (Reynolds et al., 2009). These studies indicate that systemic complement exerts a role in AMD.

However, a low level of local complement production is necessary for immune surveillance. The RPE is capable of producing complement components and several membrane-bound and soluble regulators to prevent excessive complement activation (Perez and Caspi, 2015). The RPE robustly expresses the classical and alternative pathway components and regulators, but limited production of lectin or terminal pathway components (Anderson et al., 2010). The choroid has a higher expression of classical and alternative complement components and regulators than the RPE (Anderson et al., 2010), and can provide complement components to the RPE in response to an inflammatory trigger, and regulate this response by supplying complement regulators if the RPE is unable to synthesize these components except for C5 and C7, which are not expressed by the RPE or choroid. For C5b-9 formation, the RPE and choroid must rely on systemic components. This interaction between the local microenvironment and the systemic circulation clearly takes place since C5b-9 is found in the choroid as early as 2 years of age (Mullins et al., 2014). C5b-9 localizes to the choriocapillaris rather than the retina or RPE, and increases in the choriocapillaris with aging (Hageman et al., 2005; Seth et al., 2008). In addition, patients homozygous for the 402H CFH AMD risk allele have elevated C5b-9 in the choriocapillaris along with choroidal atrophy compared to low risk eyes. These observations suggest that C5b-9 mediates choroidal atrophy in AMD (Mullins et al., 2014), which can result in hypoxia or reduce the delivery of glucose, a pivotal substrate for the RPE and photoreceptors, to jeopardize the health of both the RPE and photoreceptors due to their extremely high metabolism. Proof of this possibility is that hypoxic RPE cells have increased glucose consumption, which contributes to photoreceptor atrophy (Kurihara et al., 2016).

The alternative complement pathway is distributed differently in AMD than in aging. In normal eyes, C3 immunolocalizes to the choriocapillaris like factors B and D, while in non-neovascular AMD, C3 is additionally found in the RPE and sub-RPE space overlying drusen, which suggests C3 complement activation in the RPE (Anderson et al., 2010). Whether this altered pattern is protective or pathologic is unclear at this time, but studies on CFH immunolabeling indicate that CFH, which is normally confined to the choriocapillaris, intercapillary pillars, and Bruch’s membrane, is decreased in both early AMD and geographic atrophy (Bhutto et al., 2011). CFH is mainly secreted apically by the RPE (Chen et al., 2007; Kim et al., 2009), suggesting a regulatory role in the interphotoreceptor matrix adjacent to photoreceptors, and either systemically or choroidally produced CFH may localize to the basal RPE. Its decrease does not necessarily imply that complement is dysregulated because the C3 regulators CD55 and CD46 may compensate. However, CD55, a 70 kDa a glycophosphatidylinositol (GPI) anchored membrane protein that interferes with the conversion of factor B to Bb by factor D to prevent C3 convertase formation, is minimally expressed by the RPE (Vogt et al., 2006; Yang et al., 2009). CD46, a transmembrane protein, is a cofactor for the serine protease factor I, which cleaves and

inactivates surface-bound C3b, to regulate C3. In contrast to CD55, several labs including ours, have found that in normal eyes, CD46 is abundant and localizes to lateral and basal RPE surfaces (Ebrahimi et al., 2012; McLaughlin et al., 2003; Vogt et al., 2006). The basal distribution suggests that CD46 plays a regulatory role of C3 convertase in the basal RPE. Vogt et al found that CD46 is decreased before identifiable AMD changes to the RPE and Bruch's membrane (Vogt et al., 2011). We found that in intermediate disease, basal CD46 labeling was lost in areas overlying large drusen along with the immunolabeling of extracellular vesicular elements within drusen (Ebrahimi et al., 2012) (Fig 3), observations that were made also by Johnson et al (Johnson et al., 2001). In geographic atrophy, CD46 immunolabeling in the RPE was decreased (Vogt et al., 2011). This work coincides with our discovery that oxidized lipids reduce CD46 by RPE cells due in part, to apoptotic shedding and exosomal release of CD46 into the supernatant (Ebrahimi et al., 2012). Other studies indicate that oxidative stress can reduce complement regulators (Chen et al., 2007; Ebrahimi et al., 2012; Thurman et al., 2009; Wu et al., 2007). Thurman et al found that H_2O_2 reduced the surfaced accumulation of CFH, CD46, and CD55, which disrupted the barrier function of RPE cells (Thurman et al., 2009) while Wu et al found that CFH expression is decreased by FOXO3 acetylation (Wu et al., 2007). Chen and Forrester found that oxidized lipids reduced CFH production by RPE cells (Chen et al., 2007). Collectively, these studies suggest that the decrease in multiple C3 regulators by oxidative stress could result in dysfunctional C3 activity in AMD. The accumulation of the anaphylatoxins C3a and C5a in drusen of early AMD patients suggests that C3 is abnormally regulated in the basal RPE/Bruch's membrane (Nozaki et al., 2006).

CD59 is a glycoprotein regulator that attaches to cells by a GPI anchor that halts C5b-9 formation by preventing C9 forming with the C5b-8 complex. Our lab found that CD59 immunolabeling in the RPE of normal and early AMD eyes is minimal, but strong in the choroidal vascular endothelium, which suggests an important role in controlling the age-related C5b-9 choroidal deposition (Ebrahimi et al., 2012). In early AMD, CD59 labeling is observed at the apex of morphologically normal RPE and within reticular pseudodrusen, and is decreased in dysmorphic RPE overlying drusen. In the transition zone of geographic atrophy, CD59 immunolabeling is weak in morphologically preserved and dysmorphic RPE cells. Like CD46, we found that oxidized lipids induced the release of CD59 in both apoptotic blebs and exosomes, suggesting a mechanism for its decrease in the RPE and accumulation in reticular pseudodrusen (Ebrahimi et al., 2012). Using polarized primary RPE cells and the ABCA4$^{-/-}$ Stargardt mouse model, Tan et al found two early protective responses of CD59 that preserve RPE cell health (Tan et al., 2016). Accelerated recycling of CD59 to the RPE cell surface inhibited C5b-9 formation and fusion of lysosomes with the RPE plasma membrane limited calcium release and preserved mitochondrial function. This process could involve cholesterol accumulation induced by oxidized lipids or vitamin A dimers to activate acid sphingomyelinase, which increased tubulin acetylation to impede organelle traffic. Thus, defective CD59 recycling and lysosomal exocytosis after sublytic C5b-9 could induce mitochondrial fragmentation and RPE dysfunction. Collectively, with reduced CD46, CFH, and CD59, it is possible that the impairment of multiple regulators and not any single factor is needed for complement dysregulation to contribute to AMD lesion formation.

Compelling mechanistic evidence of a role for complement dysregulation was lacking until the recent work by Toomey et al (Toomey et al., 2015). Using aged $CFH^{+/-}$ mice given a high fat diet, this group found that decreased CFH led to increased basal deposit formation and decreased vision, as quantified by ERG changes. Mechanistically, CFH competes with lipoproteins for binding sites in Bruch's membrane. With decreased CFH, lipoproteins accumulate in Bruch's membrane, to trigger basal deposit formation. Importantly, basal deposits did not form in $CFH^{-/-}$ mice where complement activity is minimal due to secondary consumption, indicating that dysregulated complement in heterozygous mice, contributed to lesion formation. Thus, decreased CFH contributed to complement activation, leading to RPE damage and visual impairment, and basal deposit formation. Whether other complement regulators were also impaired is unknown in this model, but is certainly possible given the high oxidative stress environment generated by the combination of aging and HFD.

This work illustrates a non-complement regulatory function of CFH, of competing with lipoproteins for binding to Bruch's membrane (Clark et al., 2010; Clark et al., 2013; Sarrazin et al., 2011). CFH binds to heparan sulfates in Bruch's membrane at its short consensus repeats (SCR) 6-8 (Clark et al., 2010; Clark et al., 2013). Because the 402H polymorphism occurs within the SCR6-8 region, heparan binding is reduced with 402H CFH. In addition, Keenan et al have shown that the quantity of heparan sulfate decreases in Bruch's membrane with aging, resulting in fewer binding sites for CFH, which especially affects the ability of the 402H CFH variant to bind Bruch's membrane (Keenan et al., 2014). Thus, either an age-related reduction in heparan sulfate or the reduced binding by 402H CFH would encourage lipoprotein accumulation in Bruch's membrane, a key event prior to basal deposit and drusen formation (Curcio et al., 2011). The ocular "response to retention" hypothesis theorizes that retained lipoproteins become oxidized due to the macula's high oxidative stress environment, and elicit a pro-inflammatory response. Indeed, we previously showed that oxidized lipoproteins accumulate in Bruch's membrane including drusen in AMD, and that oxidized lipoproteins induce a pro-inflammatory transcriptional response by RPE cells (Yamada et al., 2008).

Pentraxin-3 (PTX-3) is an additional factor that regulates CFH. The pentraxins are an essential component of humoral innate immunity that function as pattern recognition receptors (PRR) which includes the short pentraxin, C-reactive protein (Garlanda et al., 2005). PTX3, which is typically produced by local tissue following a pro-inflammatory signal (Breviario et al., 1992), serves as a PRR that binds to microbes, P-selectin, and complement components. Our lab recently showed that PTX3 is induced in the RPE by oxidative stress, and that PTX3 preferentially binds to CFH over complement C1q (Fig. 4) (Wang et al., 2016). We found that PTX3 deficiency in vitro and in vivo magnified complement activation induced by oxidative stress with increased C3a, FB, and C3d, but not C5b-9 formation. The increased C3a, by interacting with C3aR, induced IL-1β mRNA expression and secretion of its activated form. In addition, PTX3 deficiency augmented NLRP3 inflammasome activation that enhanced IL-1β, but not IL-18 production by the RPE. With PTX3 deficiency, the complement and inflammasome pathways worked in concert to produce IL-1β in sufficient abundance to recruit macrophages to the choroid. These results demonstrated that PTX3 acts as an essential brake for complement and

inflammasome activation by regulating the abundance of CFH in the RPE, and provide critical insight into the complex interplay between oxidative stress and innate immunity in the early stages of AMD. Since PTX3 binds equally to the 402H and 402Y CFH alleles (Deban et al., 2008) and competes with heparan for binding to CFH at SCR19-20 (Blackmore et al., 1998; Blackmore et al., 1996), PTX3 could compensate for impaired 402H CFH binding with heparan, and provide a tissue protecting supply of CFH. Under normal conditions, heparan sulfate proteoglycans likely play the main regulatory role on CFH by insuring an adequate supply. Immunohistochemical studies showed that PTX3 localizes to inner Bruch's membrane, and thus, acts as a reservoir for functional CFH to modulate complement at the RPE. Since PTX3 production is low without stress, PTX3 is unlikely to have a substantive role during unstressed conditions, but increases with oxidative stress. While an age-related decline or maldistribution of heparan or heparan sulfation could alter CFH distribution and hence, complement regulation, PTX3 could compensate for these changes since CFH and PTX3 co-localized during oxidative stress. However, our lab in immunohistochemical studies, showed that PTX3 is decreased in inner Bruch's membrane and dysmorphic RPE overlying basal deposits and drusen in AMD (Yamada et al., 2008), which suggests that PTX3 could decline with aging or in AMD. If true, the protective reservoir of CFH would decrease with decreased PTX3 and could result in either lipoprotein accumulation or dysregulated complement activation at C3 during AMD development.

CFH has another non-complement function. The Binder lab, with our collaboration, showed that CFH, mediated through SCR7 and SCR20, binds to MDA, which is contained in oxidized LDL (Weismann et al., 2011). In AMD specimens, CFH and MDA co-localized in Bruch's membrane including drusen, along with C3d, a cleavage product of iC3b, indicating cofactor activity at the same sites. Functionally, CFH binding to MDA prevented a pro-inflammatory response by the RPE that was elicited by the oxidized epitopes. The 402H CFH polymorphism significantly reduced the ability of CFH to bind MDA, thereby suggesting a causal link to AMD etiology. Thus, besides its complement regulatory function, CFH can influence both lipoprotein accumulation and neutralize the deleterious inflammatory effects of MDA by the RPE.

6.2 The inflammasome and its interaction with complement

The inflammasome has emerged as another immunity related factor in non-neovascular AMD pathobiology since the seminal work by Tarallo et al (Tarallo et al., 2012). Inflammasomes are multi-protein complexes that detect cellular danger and implement innate immune defense through activation of IL-1β and IL-18 (Martinon et al., 2002), and recently inactivation of IL-33 (Cayrol and Girard, 2009). The inflammasome can be activated by a number of triggers, notably oxidative stress (Dostert et al., 2008), C5b-9 (Laudisi et al., 2013; Triantafilou et al., 2013), and aluRNAs. Inflammasomes require a priming signal to transcriptionally increase its components (NLRP3, ASC, procaspase-1), and a second activating signal that oligomerizes these components, resulting in caspase-1 activation that cleaves IL-1β and IL-18 for secretion (Schroder and Tschopp, 2010). Tarallo et al demonstrated that alu RNAs, through reduced DICER1, activate the inflammasome in the RPE, with increased IL-18, leading to geographic atrophy (Tarallo et al., 2012). This group followed up with a subsequent study showing that DICER1 deficiency with Alu RNA

accumulation resulted in increased IL-18 that led to RPE cell death via caspase-8 activation through a Fas ligand-dependent mechanism (Kim et al., 2014). Doyle et al, on the other hand, reported that inflammasome activation with increased IL-18 protected against choroidal neovascularization in an acute model of AMD (Doyle et al., 2012).

Undoubtedly, inflammasome activation, like most inflammatory responses, is initially protective. What converts its response to tissue injuring? Three factors may be relevant in generating a pathologic response. First, the IL-1β and IL-18 balance is important. IL-1β is a powerful and dominant cytokine that is tightly regulated both at the transcriptional level and by the inflammasome (Dinarello, 1996; Rider et al., 2011). IL-18, which is constitutively expressed and is regulated by the inflammasome (Jordan et al., 2001; Okamura et al., 1998), restrains the pro-inflammatory effects of IL-1β. For example, IL-18 controls the inflammation generated by IL-1β during infection to prevent gastric epithelial cell injury (Hitzler et al., 2012). Secondly, the source (s) of the inflammasome can be relevant. While Inflammasome activation in colon epithelium repairs damaged cells, inflammasome activity by macrophages causes tissue damage (Zaki et al., 2010). This scenario is possible in early AMD where the RPE activates the inflammasome as an initial protective response, and recruits macrophages that accumulate in Bruch's membrane, potentially adding additional inflammasome activity (Cherepanoff et al., 2010; Kauppinen et al., 2012; Killingsworth et al., 1990; Penfold et al., 1985; Sarks, 1980; Tarallo et al., 2012). Several high quality studies indicate that the NLRP3-inflammasome is activated by the RPE after oxidative stress, such as after exposure to A2E (Anderson et al., 2013) or oxLDLs (Gnanaguru et al., 2016). Tseng et al showed that lysosomal destabilization activated the inflammasome with IL-1β release, which was linked to RPE dysfunction and pyroptotic cell death (Tseng et al., 2013). This finding is significance because autophagy impairment is linked with AMD pathobiology (Kaarniranta et al., 2013; Mitter et al., 2014). Activation of the P2X7 receptor is a widely supported mechanism for inflammasome activation (Rathinam et al., 2012; Schroder and Tschopp, 2010). As in other cell types, Kerur et al showed that P2X7 receptor activation stimulates the inflammasome in the RPE after exposure to Alu RNAs (Kerur et al., 2013). These three triggers, oxidative stress, lysosomal destabilization, and P2X7 receptor activation, like in other cell types, may work in concert to activate the inflammasome in the RPE (Bartlett et al., 2013; Cruz et al., 2007; Rathinam et al., 2012; Schroder and Tschopp, 2010; Wang and Sluyter, 2013). Liu et al recently showed that impaired autophagy, or possibly mitophagy in RPE cells led to recruitment of macrophages with inflammasome activation, which promoted RPE and photoreceptor degeneration (Liu et al., 2016). Thirdly, the duration of inflammasome activity is an important determinant. With inadequate resolution of the inciting insult, chronic inflammasome activity converts the acute protective response into disease. Part of this conversion is mediated through IL-18, which has opposite effects through IL-11 (i.e. pro-proliferative) and IFN-gamma (i.e. anti-proliferative) during acute and chronic inflammation, respectively (Dupaul-Chicoine et al., 2010; Nava et al., 2010; Reuter and Pizarro, 2004; Zaki et al., 2010). The inflammasome, through IL-18, prevented neovascularization using the acute laser model (Doyle et al., 2012), but contributed to RPE cell loss during a chronic model of geographic atrophy (Tarallo et al., 2012). The different outcomes by IL-18 might be explained by the acute and chronic duration of these experiments. Both of these models had low IL-1β since they lacked an

obvious IL-1β priming signal. This is in contrast to our work showing increased IL-1β after C3a generation with PTX3 deficiency. Since Cao et al have shown strong IL-1β and IL-18 in the RPE of AMD (Cao et al., 2016), the additional induction of IL-1β could influence the AMD phenotype by upsetting the IL-1β/IL-18 balance to generate a deleterious pro-inflammatory microenvironment.

While many studies focus on the impact either of complement or inflammasome activation, several studies indicate that these arms interact with each other. For example, C3a induces ATP release (Asgari et al., 2013) which prompts the P2X7 receptor to associate with NLRP3 and activate the inflammasome (Franceschini et al., 2015). Given the impaired C3 regulators, C3a abundance is a plausible link to inflammasome activation. Likewise, Brandstetter et al have recently shown that C5a acts as a priming signal for RPE cell inflammasome activation with IL-1β secretion after lipofuscin mediated photo-oxidative damage (Brandstetter et al., 2015). This same group also showed that C5a can activate the inflammasome in RPE cells, which increases its susceptibility to photo-oxidative mediated cell death through pyroptosis (Brandstetter et al., 2016). Finally, sublytic C5b-9 is known to activate the inflammasome (Laudisi et al., 2013; Triantafilou et al., 2013), providing an additional possible link between complement and inflammasome activation. Given the accumulation of C5b-9 in the choriocapillaris with aging (Mullins et al., 2014), the potential for sublytic C5b-9 to induce mitochondrial fragmentation and lysosomal exocytosis with RPE dysfunction from defective CD59 recycling (Tan et al., 2016), the documented reduction in CD59 in the RPE in AMD (Ebrahimi et al., 2012), C5b-9 mediated inflammasome activation warrants further investigation. Figure 5 summarizes the elements of the alternative complement pathway, its potential impact on AMD lesion formation, and its potential interaction with the inflammasome.

7. Does Oxidative stress cause inflammation or does inflammation induce oxidative stress?

While inflammation can produce oxidative stress, compelling evidence indicates that oxidative stress induces inflammation during the development of AMD. Inadequately neutralized oxidative stress can lead to oxidatively damaged proteins, lipids and DNA, which results in structural changes to these molecules. These oxidative modifications can generate novel “oxidation-specific epitopes” (OSE), which can induce pro-inflammatory responses in vitro and in vivo (Berliner et al., 2001). OSEs have been identified in the macula with AMD. In the retina, docosohexanoic acid, the most abundant fatty acid in photoreceptor tips, is specifically oxidized to carboxyethylpyrrole (CEP), which “tag” oxidatively damaged photoreceptors in AMD (Gu et al., 2003; Sun et al., 2005). The RPE contains multiple OSEs including MDA and 4-HNE adducts, as well as AGEs (Schutt et al., 2003; Schutt et al., 2002), while Bruch’s membrane, including drusen, contains AGEs, MDA, CEP, and oxidized phospholipids (OxPL) (Crabb et al., 2002; Farboud et al., 1999; Sun et al., 2005; Suzuki et al., 2007).

The identification of these OSEs is significant because OSEs are recognized as “danger signals” by PRRs (Chou et al., 2008). Indeed, OSEs can activate both innate and adaptive

immunity (Binder et al., 2004; Chou et al., 2008). This initial response is intended to neutralize the OSE in order to prevent tissue injury, and is thus, protective. However, inadequate neutralization of the OSE can convert the protective immune reaction into a pathologic response. For example, as mentioned above, by binding MDA, CFH neutralizes the pro-inflammatory response generated by MDA. However, the 402H CFH polymorphism, which confers increased risk for AMD, has reduced binding to MDA, potentially inducing a chronic pro-inflammatory response that can be tissue injuring (Weismann et al., 2011).

Our lab has been interested in the impact of oxidized lipoproteins that accumulate in Bruch's membrane in early AMD (Yamada et al., 2008). We showed that AGEs induced RPE cell lipoprotein lipase production, which after being secreted into Bruch's membrane, retains lipoproteins (Cano et al., 2011) which encourages their oxidation. Oxidized phospholipids (OxPL), a component of oxidized lipoproteins, are a prominent consequence of lipid peroxidation of the highly polyunsaturated fatty acids found in the photoreceptors, and are formed in cells undergoing apoptosis, a known mechanism of cell death in AMD (Chang et al., 2004). One OxPL species is the oxidation of the phosphocholine (PC) moiety of phospholipids, which are detected and bound by the IgM natural antibody E06, but not to the PC moiety of unoxidized phospholipids (Friedman et al., 2002; Shaw et al., 2000). We found that this particular OxPL, which is highly abundant in oxidized lipoproteins, accumulates in Bruch's membrane in early AMD (Handa et al., 2015). One possible PRR that can neutralize this OxPL in Bruch's membrane is lipoprotein (a), or Lp(a), which is composed of apolipoprotein (a) [apo(a)] covalently bound to apolipoprotein B-100 (apoB) (Dube et al., 2012; Hoover-Plow and Huang, 2013; Kronenberg and Utermann, 2013). Using "mutant Lp(a)" mice with a defective apo(a) lysine binding site for oxidized phospholipids, we found that these mice, when fed a high fat diet for 12 months, developed RPE cell degeneration, basal deposits, and drusen. These changes were associated with increased OxPL, decreased antioxidant genes, increased complement, and decreased complement regulators in the RPE compared to wild-type Lp(a) mice, which developed age-related changes when given a high fat diet. Importantly, Lp(a) appears to localize in Bruch's membrane including drusen, with OxPL in AMD specimens.

8. Therapeutic Considerations: Current Treatment Recommendations and Future Directions

In the United States, the current recommended treatment for dry AMD is based on the two Age-related Eye Disease Study (AREDS) trials. For patients with early AMD, no treatment is recommended because the AREDS found no benefit with high dose antioxidant micronutrients (Age-Related Eye Disease Study Research, 2001). For patients with intermediate AMD, the AREDS found that daily doses of vitamin C (500 mg), vitamin E (400 IU), β-carotene (15 mg), zinc (80 mg as zinc oxide), and copper (2 mg as cupric oxide) reduced the progression rate to advanced AMD by 25% at 5 years, and the risk of losing vision of three or more lines (doubling of the visual angle) by 19% (Age-Related Eye Disease Study Research, 2001). The AREDS was followed up with AREDS2, where patients were randomized to receive the AREDS formulation with supplemental lutein and zeaxanthin, supplemental omega-3, or the original formulation alone (2013). A secondary

randomization to four variations included elimination of β-carotene, lower zinc levels (25 mg), or both. The AREDS2 found no treatment benefit between the original formulation and the AREDS2 modifications. Because the simultaneous use of β-carotene with lutein and zeaxanthin decreases the absorption of the nutrients, plus the higher incidence of lung cancer seen in the β-carotene group that was not observed with lutein and zeaxanthin supplementation, the study concluded that lutein and zeaxanthin represent an appropriate substitute for β-carotene in the supplement, and that there was no detrimental effect of lowering the zinc levels (25 mg). The current recommendation for patients with intermediate AMD is to use the AREDS2 formulation.

The use of antioxidant micronutrients is not uniformly endorsed. A meta-analysis of clinical trials from three large, high quality studies that involved over 23,000 patients with AMD found that antioxidants did not prevent AMD (Evans, 2008). Several components of the AREDS formulation can have unanticipated health risks. For example, vitamin E was associated with heart failure in patients with vascular disease or diabetes mellitus in the Heart Outcomes Prevention Evaluation Study (Lonn et al., 2005) and increased prostate cancer after 7 years in the Selenium and Vitamin E Cancer Prevention Trial (Klein et al., 2011). Elevated zinc has been associated with neurosensory retinal cell death after light exposure (Sheline et al., 2010), and in proteins recovered from drusen (Lengyel et al., 2007) and amyloid plaques of Alzheimer's patients (Bucciarelli et al., 2002; Lee et al., 1999). In Alzheimer's disease, zinc chelation has been proposed as a therapy (Lee et al., 2004) in contrast to zinc supplement in the AREDS formulation, which was chosen for its potential antioxidant properties. Given the presence of zinc in drusen and its role in retinal neurotoxicity, some researchers have questioned the use of zinc supplementation for AMD treatment. While considered the best formulation among experts at the time, the AREDS composition was empiric and was not derived from pre-clinical investigation.

Preclinical investigation of inhibiting complement activation has translated into clinical trials. Currently, several trials at different phases are testing the benefit of complement inhibition for non-neovascular AMD. Since the FDA now allows geographic atrophy growth as a surrogate to visual acuity as a primary endpoint, most studies are evaluating geographic atrophy rather than early AMD. At present, the most promising drug is lampalizumab (Roche, Inc.), a humanized anti-factor D monoclonal antibody. In the phase II trial, which was presented at the 31st Annual Meeting of the American Society of Retina Specialists, there was a 20.4% reduction (unconventional $p=0.2$ significance) in the rate of geographic atrophy growth after 18 months of monthly intravitreal injections of lampalizumab without unexpected or unmanageable serious adverse events. In patients with the factor I AMD risk allele, the GA progression rate was decreased by 44 percent ($p<0.005$) at 18 months. These data prompted progression to the phase III trial, which is ongoing. APL-2 (Apellis Pharmaceuticals) inhibits C3 activation and thus, blocks all three arms of the complement pathway. APL-2 is a reformulated version of POT-4 (Potentia), which was found to be safe and well tolerated after intravitreal injection in phase I testing. A randomized phase II trial testing APL-2 by monthly or every other month injections for 1 year is now being investigated for treatment of geographic atrophy. LFG316 (Alcon), an anti-C5 monoclonal antibody, is being currently being investigated as either monotherapy, or in combination with CLG561, a properdin inhibitor for the treatment of geographic atrophy. Of note, CLG561

alone, did not slow geographic atrophy enlargement in a phase II study, which was presented at the 2016 Angiogenesis, Exudation, and Degeneration meeting.

What does the future hold? Will targeting elements of oxidative stress replace the antioxidant micronutrients of the AREDS? Oxidative stress targets that rejuvenate endogenous intracellular antioxidant systems have conceptual merit because the oxidative stress can be neutralized within cells where ROS are generated. Nr2 as a target has enjoyed some success with dimethyl fumarate, an Nrf2 activator, in the DEFINE study, which reduced the proportion of patients who had a relapse, the annualized relapse rate, the rate of disability progression, and the number of lesions on MRI for the treatment of relapsing multiple sclerosis (Gold et al., 2012). On the other hand, Bardoloxone, a triterpenoid that also activates Nrf2, was halted in a phase III trial that tested whether it reduced the risk of end-stage renal disease (ESRD) or death from cardiovascular causes among patients with type 2 diabetes mellitus and stage 4 chronic kidney disease because of unacceptable adverse effects (de Zeeuw et al., 2013). These systemic complications could potentially be minimized with intravitreal injections for treating AMD. With the development of nanoparticles, the possibility of more targeted treatment with sustained release is possible. For example, dendrimers can be formulated to specifically target either RPE cells or macrophages/microglia (Kambhampati et al., 2015). When Triamcinolone acetonide (TA) is incorporated into dendrimers, the anti-inflammatory activity is improved 100-fold more than free TA in macrophages. Will other anti-inflammatory targets of complement, PTX3, or the inflammasome emerge? Will future treatment target both oxidative stress and inflammation at the same time? These questions will emerge as we develop a deeper understanding of the role of oxidative stress and inflammation in the RPE.

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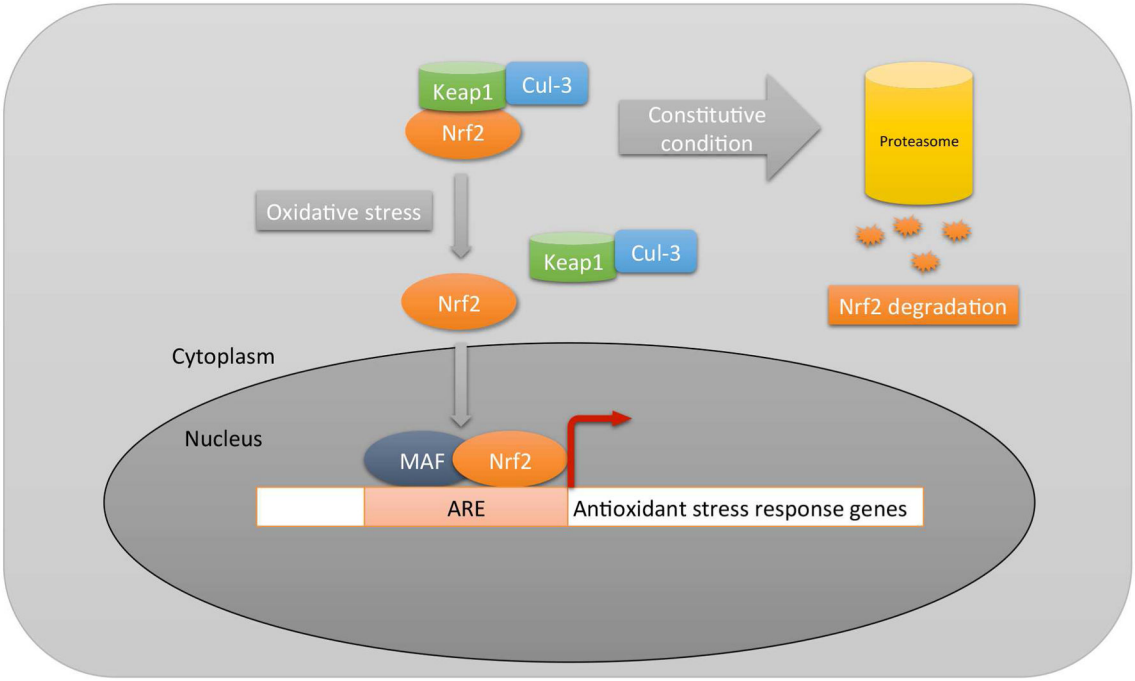

Figure 1.

Diagram of Nrf2 signaling. In the absence of oxidative stress (ROS), Nrf2 is degraded through the proteasome by a Keap1/Cul3 mechanism. With oxidative stress, the ROS induce a conformational change in cytoplasmic Keap1, which releases Nrf2 for transit to the nucleus where it interacts with Maf proteins and binds to antioxidant response elements (ARE), inducing a comprehensive antioxidant response.

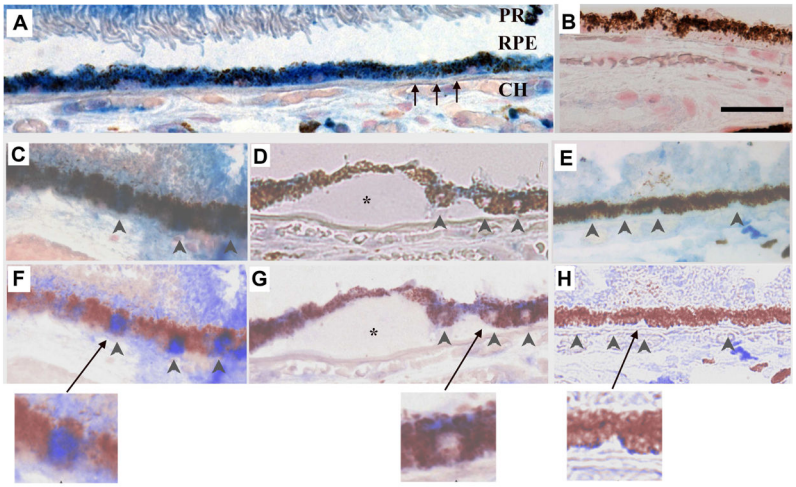

Figure 2.

Nrf2 immunolabeling in a 60-year-old Caucasian female with early AMD. A) Macular RPE with normal, cuboidal morphology have prominent cytoplasmic Nrf2 labeling (blue). B) IgG control. C) Normal macular RPE with both cytoplasmic and nuclear Nrf2 labeling (arrowheads). D) Dysmorphic macular RPE overlying drusen have lighter cytoplasmic labeling than cuboidal RPE from the same section. Nuclei (arrowheads) do not stain for Nrf2. E) Peripheral RPE with minimal Nrf2 labeling in the cytoplasm and no nuclear labeling (arrowheads). F–H) Same images as C–E, respectively, after Nuance software has converted melanin to maroon to improve visualization of Nrf2 labeling in the RPE. The arrow points to the inset of an enlarged image showing nuclear Nrf2 staining in a normal macular RPE cell (F), lack of nuclear Nrf2 staining in a dysmorphic macular RPE cell overlying a druse (*) (G), and lack of Nrf2 staining in a peripheral RPE cell (H). CH, choroid, RPE, retinal pigment epithelium, Black arrows point to Bruch's membrane. Bar=25μm. Reprinted with permission from Free Radical Biology Medicine.

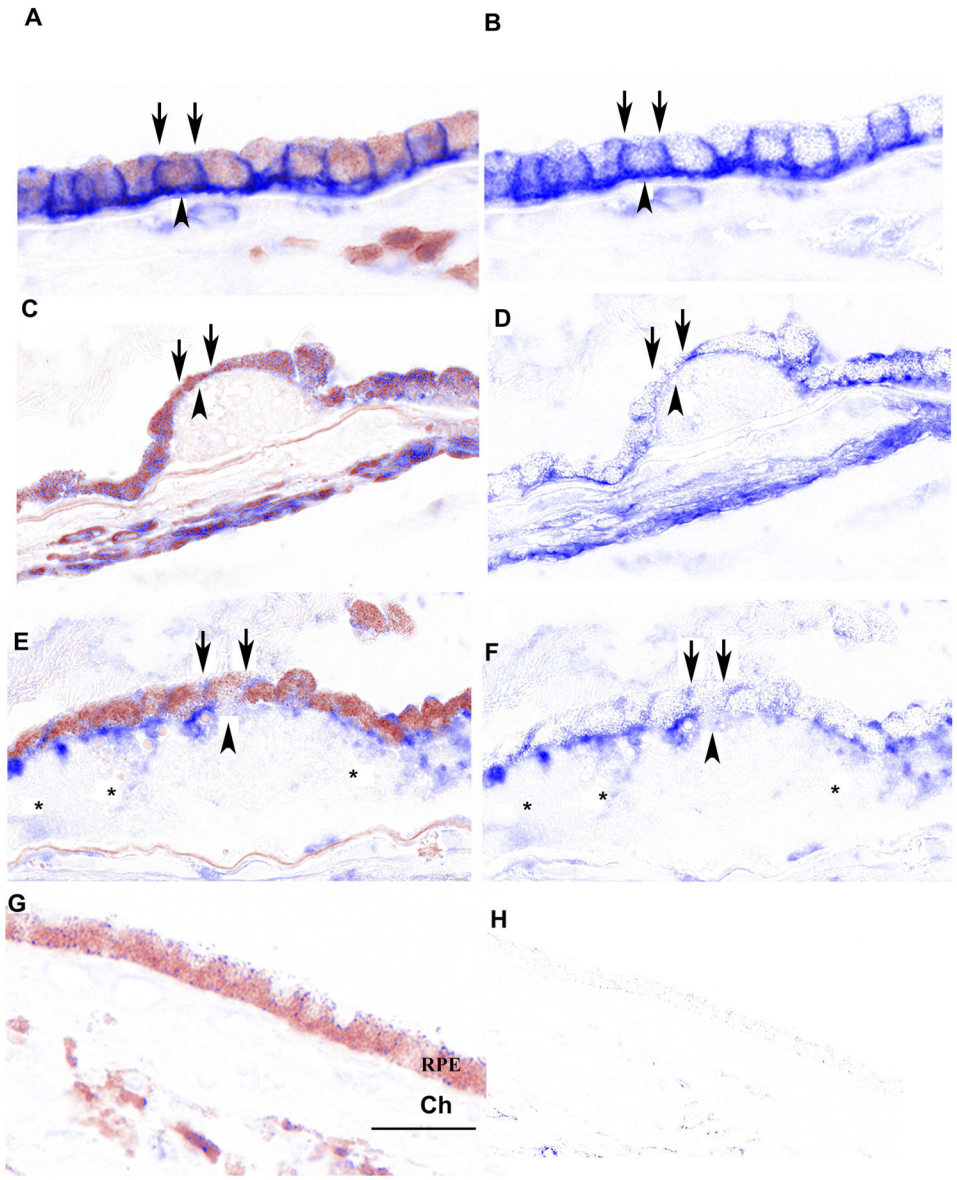

Figure 3.

Distribution of CD46 in the RPE of early AMD eyes. A. Peripheral RPE/choroid from a 94 yr. old Caucasian female with early AMD. CD46 labeling appears as a prominent line at the basal (arrowhead) and lateral (arrows) surfaces of morphologically normal RPE cells. C. Dysmorphic macular RPE cells, from the same patient, adjacent to small druse with reduced (thinner line) or undetectable CD46 labeling at their lateral surface (arrows). The basal line of CD46 labeling (arrowhead) is preserved, but thinned over the small druse. E. 96 year old Caucasian male with early AMD and a large druse (>500 μm in length). RPE cells overlying the large druse are dysmorphic and the uniform line of lateral (arrows) and basal (arrowhead) CD46 labeling is lost. CD46 labeling is diffusely distributed throughout the druse (*). G. IgG control. B, D, F, H. Same images after RPE melanin pigment is subtracted by Nuance software. Ch, choroid; Dr, druse; RPE, retinal pigmented epithelium. Bar=50μm. Reprinted with permission from Journal of Pathology.

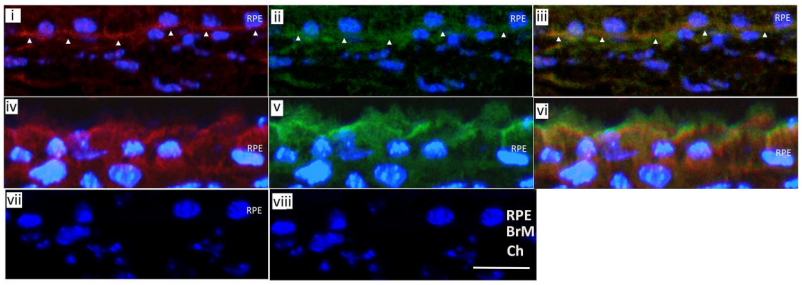

Figure 4.

Immunofluorescence confocal microscopy of a WT mouse given IVit vehicle (n=4) shows linear FH (red) staining (i) and PTX3 (green) staining (ii) in inner BrM (white arrowheads). (iii) Merged image of (i) and (ii) shows FH and PTX3 (yellow) staining overlap. WT mouse given 5 μg 4-HNE IVit (n=4) has diffuse staining in the RPE for FH (red) (iv) and PTX3 (green) (v). (vi) Merged image of (iv) and (v) shows FH and PTX3 (yellow) staining overlap. IgG isotype control for FH (vii) and PTX3 (viii). Reprinted with permission from Journal of Pathology.

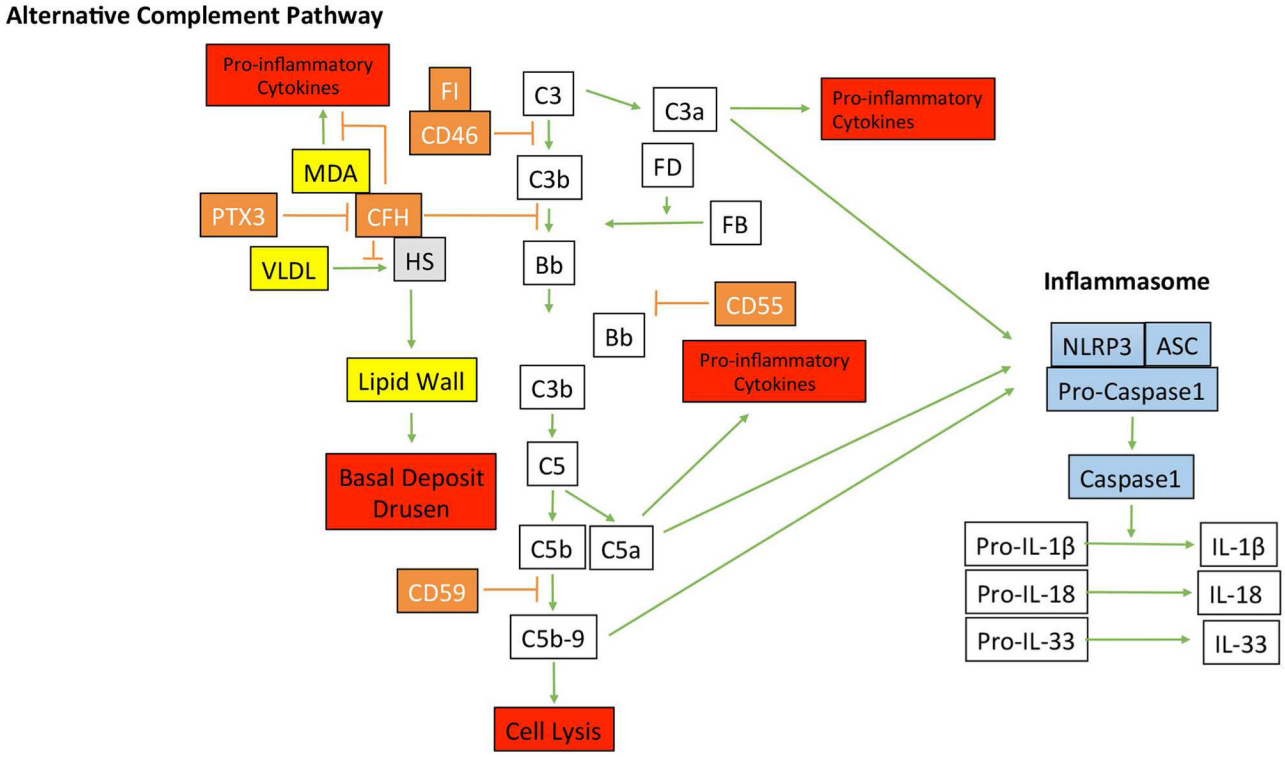

Figure 5.

Diagram of the alternative complement pathway and the inflammasome. Activation of the alternative complement pathway is regulated by factor H (CFH), CD46, CD55. PTX3, by binding CFH, controls the abundance of CFH. Besides its regulation of complement, CFH binds to malondialdehyde (MDA) to control MDA-mediated pro-inflammatory cytokine production, and heparan sulfates (HS) to compete with very low density lipoproteins (VLDL) that can become deposited in Bruch's membrane prior to basal deposit or drusen formation. Complement activation can lead to anaphylatoxins generation of C3a and C5a, which can induce pro-inflammatory cytokine production, and C5b-9 complex formation, which is regulated by CD59. C3a, C5a, and C5b-9 complexes are implicated in activating the inflammasome, composed of NLRP3, ASP, and Pro-caspase1, which upon activation, is cleaved to convert pro-IL-1β, pro-IL-18, and pro-IL-33 into their active forms.