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Immune Mechanisms in Inflammatory and Degenerative Eye Disease

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Abstract

It has recently been recognized that pathology of age-associated degenerative eye diseases such as adult macular degeneration (AMD), glaucoma and diabetic retinopathy, have strong immunological underpinnings. Attempts have been made to extrapolate to age-related degenerative disease insights from inflammatory processes associated with non-infectious uveitis, but these have not yet been sufficiently informative. Here we review recent findings on the immune processes underlying uveitis and those that have been shown to contribute to AMD, discussing in this context parallels and differences between overt inflammation and para-inflammation in the eye. We propose that mechanisms associated with ocular immune privilege, in combination with paucity of age-related antigen(s) within the target tissue, dampen what could otherwise be overt inflammation and result in the para-inflammation that characterizes age-associated neurodegenerative disease.

Introduction

The eye is a prototypic immune privileged tissue that resists immunogenic inflammation through multiple mechanisms [1][2]. Inflammatory and immune-mediated diseases in the eye must therefore be viewed against the backdrop of ocular immune privilege. Nevertheless, the eye is subject to inflammatory and para-inflammatory processes. Non-infectious uveitis describes a group of potentially blinding inflammatory ocular conditions of obscure etiology; disease progression in uveitis is thought to be driven at least in part by autoimmune mechanisms. Current concepts in ocular inflammation and the mechanisms that drive it stem largely from studying uveitis in animal models. More recently, it has been recognized that processes that had once been believed to be purely degenerative, such as adult macular degeneration (AMD), diabetic retinopathy and glaucoma, also involve inflammatory and immune elements [3]. Moreover, studies in patients and in animal models

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have implicated autoimmune processes in degenerative diseases of the eye [4, 5], suggesting some anti-inflammatory therapies that are effective for uveitis may be useful for the treatment of AMD.

However, the inflammation observed in uveitis is very different from that associated with degenerative conditions in the eye. While uveitis has a major adaptive immune component, AMD and similar degenerative conditions primarily involve innate immune elements [6, 7, 8]. While uveitis is associated with overt inflammation, AMD is slow and insidious (para-inflammation), and acute inflammation is characteristically absent. Here we critically examine the processes of inflammation and para-inflammation in the eye, comparing and contrasting the associated cellular and molecular mechanisms [9, 10]. Synthesis of the available evidence suggests that *(i)* autoimmune processes are involved as drivers (if not etiologic triggers) of both the overt inflammatory disease known as uveitis, and the para-inflammatory disease typified by AMD; *(ii)* unlike retinal antigens, the target AMD antigen(s) in the retina are scarce, which limits the adaptive immune response but not innate immune processes; *(iii)* the inhibitory ocular microenvironment as part of ocular immune privilege is able to dampen innate immune responses, but is less effective in limiting the function of effector T cells. This in turn enables effector T cells that encounter abundant target antigen in the eye to break down ocular immune privilege and precipitate the development of overt inflammation typical of uveitis.

Ocular immune privilege as a throttle of inflammation in the eye

Immune responses affecting the eye and vision must be viewed against the backdrop of ocular immune privilege. The term has been coined in the 1940s by Sir Peter Medawar [11]. It has since been studied intensively, with major conceptual contributions by the late J Wayne Streilein and his colleagues [1, 2, 12, 13]. The concept that has emerged, and that continues to guide the field today, is that the ocular environment has evolved to limit local immune and inflammatory responses in order to preserve vision. Although specifics are still being debated, it appears clear that immune privilege involves a complex combination of local and systemic mechanisms. These can be thought of as constituting successive layers of defense that are deployed as they are needed. The first line of defense is separation between the immune system and the eye by an efficient blood-retinal barrier and little, or no, direct lymphatic drainage of the inside of the globe, which is maintained as long as the eye is intact. If that is breached (as in the case of physical trauma to the eye) and immune cells from the blood enter the eye, the immuno-inhibitory ocular microenvironment, composed of diverse soluble and cell-bound molecules, steps in to control them. If that is not sufficient and an inflammation develops, the eye elicits systemic regulatory mechanisms, experimentally modeled by anterior chamber associated immune deviation (ACAID) and post-recovery eye dependent tolerance, that can limit the damage. These aspects of immune privilege have been reviewed thoroughly [1, 2, 12, 13, 14, 15] and are summarized in Table I.

The concept that we wish to bring forward is that, as part of immune privilege, the inhibitory ocular microenvironment serves as a throttle of inflammation in the eye, exerting significant measure of control over both innate and adaptive immune elements. Past and recent studies

have uncovered inhibitory effects of the ocular microenvironment on both innate and adaptive immunity. However, a difficulty has been that many of the studies from which the central concepts of immune privilege arose had been performed *in vitro* with ocular fluids and cells, extrapolating to an *in vivo* situation. This was an inevitable consequence of the lack of appropriate tools for an *in vivo* readout. Nevertheless, where available, we will try to point out conclusions based on *in vivo* data. In keeping with the issues examined in this review, which emphasize the local expression of immunity in the eye, we will concentrate on those aspects of immune privilege that act within the ocular microenvironment, rather than systemic aspects.

Immune privilege vs. innate immunity

Activation and function of innate immune elements such as NK cells, monocyte-macrophages, neutrophils and complement are all dampened by the ocular microenvironment [reviewed by 16, 17]. Ocular fluids contain at least two factors that suppress NK cell function: macrophage migration inhibitory factor (MIF) and TGF-β2. These can be shown to turn off NK cells *in vitro*. It is hypothesized that, together with nonclassical MHC antigens that are expressed by ocular cells, they may also turn off NK cells *in vivo*, although direct evidence to support this is lacking. Indirect support is provided by the well documented fact that MHC-unmatched corneal grafts, which by all expectations should have activated NK cells to kill the corneal cells, nevertheless persist and are accepted with high frequency both experimentally and in the clinic [13]. A particularly interesting molecule that has been connected to ocular immune privilege *in vivo* is FasL, which interacts with both innate and adaptive immune cells. Membrane-bound FasL is expressed in the cornea and retina (RPE). It causes apoptosis of Fas-expressing effector leukocytes and promotes tolerance to antigens within the eye [18]. Ocular fluids also contain α-MSH and may contain soluble FasL, a cleavage product of membrane FasL with antagonistic properties [19, 20]. Both these substances inhibit activation of neutrophils (although membrane FasL would activate them [21]).

A major population of innate inflammatory cells that has been described in most cases of intraocular inflammation are the macrophages [22, 23]. Their activation and function as well can be dampened by the neuropeptides alpha-melanocyte stimulating hormone (α-MSH) and calcitonin gene-related peptide (CGRP) that are present in ocular fluids. IL-10 coming from γδ T cells appears to be important for systemic manifestation of immune privilege known as ACAID [24, 25], but it is currently unknown whether a role can be ascribed to IL-10 produced by γδ T cells within the ocular milieu. The fact that IL-10 is associated with the regulation of infiltrating ocular macrophages and polarization to the regulatory M2 phenotype, reaffirms the concept of ocular immune regulation of innate responses [22, 23]. The appearance of macrophages with inhibition of neovascularization in the retina in laser induced damage models, suggest that this is the case [26]. How the role of macrophages is directed in tissue destruction vs. regeneration is still unknown, but certainly, the ocular microenvironment has the ability to signal these cells to regulate their function through messengers such as TLR ligands and macrophage specific cytokines as well.

Finally, activation of the complement cascade, either by the classical or by the alternative pathway, generates mediators which directly damage target cells by forming a membrane attack complex, and also attract and activate innate immune cells. Data indicate that a low level of complement activation is constantly present in the eye, and may be needed to protect from pathogens. To counterbalance this and control excessive complement activation that would be damaging to the tissue, the ocular fluids and cells express a number of complement regulatory proteins, including the complement factor H (CFH) and the cell bound molecules decay accelerating factor (DAF) and Crry [reviewed by 27, 28].

Immune privilege vs. adaptive immunity

The adaptive immune elements that can express effector function in the eye are CD4+ and CD8+ T cells and antibodies. It is currently unknown whether B cells, which play a systemic role in induction of ACAID, exert any function within the eye besides antibody secretion. CD8+ T cells share many effector characteristics with NK cells and may be controlled in part by similar mechanisms. CD8+ T cells also can act as regulatory cells, whose generation is at least in part driven by interactions dependent on NKT cells in the spleen [29]. The role of CD4+ T cells as effectors and as regulators in the various aspects of immune privilege has similarly been studied extensively in the past. These studies showed that processes that inhibit innate immunity in the eye can often also control adaptive immune cells, and identified new pathways, including membrane-bound FasL, PD-L1, CTLA-4 and others that act on adaptive responses. While they unquestionably yielded important insights into the ability of the eye to control adaptive immune cells, they were hobbled by lack of appropriate tools to study the relevant eye-specific responses *in vivo*. We have recently re-examined the effects of the ocular microenvironment on naïve and antigen-experienced retina-specific CD4+ T cells using T cells from T cell receptor transgenic (TCR Tg) mice expressing a retina-specific TCR, introduced into the living eye. Data indicate that the living eye efficiently converts naïve retina specific T cells, into functional Foxp3+ Tregs, or to IL-10 producing T cells (Tr1-like cells?) whose effector function is deficient [30, 31]. This requires recognition of the cognate Ag in the eye (polyclonal T cells are not converted) and is supported by the combined action of TGF-β and retinoic acid, both of which are abundant in the eye. Notably, however, T cells that have already acquired effector function outside of the eye, are insensitive to the immunoinhibitory ocular environment. They continue to express effector function and cause fulminant uveitis [31]. Moreover, the already inflamed eye is less able to convert naïve retina-specific T cells to Tregs, indicating that at least this aspect of immune privilege has been (temporarily?) lost as a consequence of inflammation. This may be due in part to presence of inflammatory mediators in the eye, which can impede Treg induction and function, as well as to depletion from the eye of anti-inflammatory and Treg-inducing factors such as TGF-β and retinoic acid from through the compromised blood retinal barrier [32, 33]. These findings could help to explain how autoimmune uveitis can occur in the face of immune privilege.

Uveitis: Inflammation characterized by the involvement of adaptive immunity

Clinical manifestations

Uveitis manifests as inflammation with a mixed ocular inflammatory infiltrate, containing elements of both the adaptive and the innate immune systems. In posterior uveitis there is typically damage to photoreceptor cells brought about by inflammation, which causes the visual deficit. Uveitis is highly variable in terms of clinical manifestations and disease course. Some types of uveitis are standalone and target only the eye (e.g., idiopathic uveitis, sympathetic ophthalmia, birdshot chorioretinopathy) whereas others are part of a more generalized systemic syndrome (e.g., uveitis accompanying Behçet's disease, Vogt-Koyanagi-Harada's disease, Juvenile idiopathic arthritis etc.) [34]. Associations with Th1 and Th17 responses have been reported and could explain some of the observed variability [35]. The autoimmune nature of these diseases is supported by lack of underlying intraocular infection, strong genetic associations with HLA Ags, central involvement of T cells in pathogenesis (evidenced by clinical efficacy of T cell targeting therapies) and immunological responses to unique retinal proteins not found elsewhere in the body. The most frequent responses seen to retinal arrestin (S-Ag) but responses to other antigens are also observed [35]. Evidence that retina-specific T cells detectable in uveitis patients have a role in pathogenesis of their disease comes from clinical data that T cell-targeting therapies, including cyclosporin, rapamycin and IL-2R blockade by Daclizumab, have a clinically positive effect on disease. Perhaps the most direct evidence comes from a double-blind placebo controlled trial in which uveitis patients who had lymphocyte responses to S-Ag were given S-Ag orally to induce oral tolerance. The patients fed S-Ag appeared to be able to be weaned off conventional therapy without experiencing a relapse better than the placebo controls [36].

Recently it has been proposed that some types of uveitis that were considered autoimmune, are in fact auto-inflammatory in nature [37]. This stems from the presence of genetic associations with allelic variants of innate immunity receptors [e.g., Behçet's disease and NLRP3/cryopyrin mutations, ref. 38] and central involvement of IL-1 in pathogenesis. However, this could be an artificial separation that reflects etiology more than pathology, because even in diseases now thought of as auto-inflammatory, including Behcet's uveitis, there are demonstrable responses to ocular antigens [39] whereas other studies report evidence for enhanced Th1 and Th17 responses (although it is not known whether the two are connected) [40, 41]. Therefore, irrespective of the initial trigger, autoimmune responses to retinal Ags released as a result of tissue breakdown may become drivers of disease.

Animal models and mechanisms

To model the complexity of human disease a number of animal models have been developed, which differ in mode of induction, clinical appearance and immunological mechanisms. Animal models of uveitis have been recently reviewed [35, 42] and a conceptual overview is presented in Table 2.

The models are based on an adaptive response to a component within the eye, usually a unique retinal protein that functions in vision and is not present in the periphery. The autoimmune models can be either induced, or spontaneous [35, 42, 43]. It should be kept in mind that animal models are invariably "amplified" to result in a relatively high incidence of disease, otherwise they would not be practical for study. However, this also makes them less than completely physiological. In the case of autoimmune uveitis, the induced models rely on stimulation of the immune system by strong bacterial adjuvants. The spontaneous models rely on a higher than normal autoimmune lymphocyte frequency. In both types of models, innate immune elements participate as ultimate and necessary mediators of tissue damage, but the retina-specific T cells are indispensable to orchestrate and direct the inflammatory response.

Among the induced models, the most studied is the "classical" model of experimental autoimmune uveitis (EAU), elicited by active immunization with a retinal antigen in emulsion with complete Freund's adjuvant (CFA) or carried on the surface of dendritic cells (DC) [44, 45]. In place of a "real" retinal antigen (IRBP or S-Ag, the same antigens shown to elicit memory responses in uveitis patients) there are models that make use of a neo-self antigen such as hen egg lysozyme (HEL) or β galactosidase (β-Gal) introduced into the retina by transgenic technology [46, 47]. T cells play a critical role in disease induction. Antibodies appear to have a disease-modifying role and exacerbate inflammation that has been elicited by T cells [48], likely through activation of the complement cascade. This may be because antibodies are excluded from the healthy eye but effector T cells can actively penetrate and break down the blood-retinal barrier, whereupon antibodies also can gain access. Among the induced models it is also worthwhile to mention "immunogenic uveitis" which is elicited by immunizing the animal to a foreign protein (e.g., BSA or OVA) in CFA, or infusing T cells sensitized to that antigen, and then the same protein is injected into the eye to serve as target [49, 50]. The model results in a strong intraocular inflammation resembling EAU, and similarly to EAU, it is driven by a vigorous adaptive response. This model will become relevant to our discussion on autoimmune mechanisms in AMD, ahead. In addition to the induced models, several spontaneous uveitis models have been developed. All are based on an excessively high frequency of retina-specific T cells, either due to a retina-specific TCR transgene [42] or to defective thymic elimination of retina-specific T cells [51, 52]. Transgenically introduced neo-self Ags into the retina can also serve as "autologous" targets when coupled with the relevant transgenic TCR [46, 47].

Pathogenic immune responses in uveitis

From data in animal models it is clear that T cells specific to retinal antigen(s) play a crucial role in uveitis. It is now well recognized that different T cell effector lineages exist, which normally deal with different types of infections, but each can also become involved in tissue pathology. Th1 and Th17 responses associated with disease have been reported in uveitis patients. In animals immunized for uveitis, both Th1 and Th17 cells specific to retina are induced. In different models of uveitis, the dominant T cell effector response may be either Th17 or Th1 [44]. Although they recruits different innate effector mechanisms (e.g., Th17 recruit neutrophils whereas Th1 recruit monocytes) both responses are tissue-destructive and independently cause pathology. This is supported by adoptive transfer experiments with *in*

vitro polarized cells, which confirm demonstrate that both T cell effector lineages are fully competent to induce uveitis, even in the absence of the reciprocal effector response in the host [44].

From numerous studies in human patients and animal models, there emerges the following sequence of events in pathogenesis of uveitis. Autoreactive T cells specific to retina, which had been activated in the periphery by unknown stimuli, circulate through the body. By chance, they happen to pass through the retinal blood vessels and adhere to the vascular endothelium. Due to their activated state they produce matrix-degrading enzymes and metalloproteinases and are able to break through the vascular tight junctions comprising the blood-retinal barrier and extravasate into the tissue. They are also producing cytokines and chemokines, which activate the (normally quiescent) local APC which then become capable of antigen presentation. The infiltrating T cells must recognize their cognate Ag *in situ* in order to induce uveitis [35, 50, 53]. It is likely that antigen recognition is necessary to maintain them in a state of activation, whereupon the proinflammatory factors they secrete start a self-amplifying inflammatory cascade: activation of the local microvasculature, recruitment of inflammatory leukocytes and antigen-presenting cells from the circulation, breakdown of the blood retinal barrier and leakage of retinal Ags into the draining LN, priming and expansion of additional autoreactive T cells and recruitment of additional inflammatory cells, enhanced tissue breakdown and antigen release, etc., etc. Thus, a robust antigen-specific effector T cell response, whether Th1 or Th17, recruits and directs the requisite innate effector mechanisms that include innate cellular elements (monocytes, neutrophils, NK cells, γδ cells) as well as adaptive and innate humoral elements (antibodies and complement), that act in concert to cause the physical tissue damage.

As discussed above, immune privilege is unable protect against a strong adaptive response. Unlike naïve T cells, primed effector T cells are (a) able to actively penetrate the BRB, (b) are not inactivated or converted to Tregs within the eye and continue to express effector function, and (c) bring about a loss of immune privilege, at least as evidenced by compromised Treg conversion in the inflamed eye, likely due to loss of molecules that maintain the inhibitory ocular microenvironment through the damaged BRB and elaboration of inflammatory mediators [31, 33].

Inflammation in age related degenerative disease: major involvement of innate immune elements

Clinical manifestations

Life-expectancy continues to increase and with it also grows a number of diseases and ailments associated with aging. AMD is a perfect example of this and represents the leading cause of legal blindness in the elderly population of the United States, where almost two-thirds of people over 80 years old are afflicted by AMD to some degree. [54] Moreover, 30–50 million individuals suffer from AMD worldwide with frequencies similar to cancer in industrialized countries.[55] The formation of drusen, an accumulation of debris (below the retinal pigment epithelium (RPE) in the macula of aging eyes, is the most common clinical sign identified by ophthalmic physicians. AMD occurs in dry and wet forms: dry AMD

relates to damage of the macula caused by atrophy whereas wet AMD involves blood vessel formation and bleeding. Although the causes of AMD are considered to be multifactorial, oxidative damage (such as the oxidative stress caused by smoking) and neo-vascularization have been identified as factors associated with progression of the disease [56, 57, 58]. That said, it is important to clarify that cause-and-effect relationships in AMD have not been clearly established, and while many of the observed hallmarks of the disease, such as drusen, are felt by many to play a role in disease pathogenesis, they could also represent its result.

A major recent advance in the research field of AMD is the association of this degenerative disease with the immune system. Activated complement factor proteins (indicative of innate immunity) have been found in drusen from AMD patients correlating with genetic polymorphisms within complement factor genes have been associated with development of AMD which has led to the notion that inflammation is an important component of this disease [59, 60, 61, 62, 63] and have implicated the immune system in the AMD disease process. In addition to the findings that innate/complement inflammatory responses may play a role in the pathogenesis of AMD, reports from our group and others implicate that adaptive immune responses also play a role [3, 4, 8]. The study of animal models of AMD, as discussed below, could provide important clues as to the intrinsic (e.g. the immune system, genetics) and extrinsic (e.g. oxidative stress) factors that modulate their development and progression, and can potentially lead to prevention or treatment regimens that by manipulating immune responses could have significant clinical benefit for this currently intractable and poorly understood disease. In a phase I/II clinical trial in which advanced wet AMD patients were treated with infliximab, rapamycin and daclizumab in conjunction with the standard of care, anti-VEGF. Both rapamycin and daclizumab (but not infliximab) decreased the number of anti-VEGF injections by approximately half [64]. These encouraging results support the notion that additional anti-inflammatories could be examined for efficacy in wet as well as dry AMD.

Animal models and mechanisms

Unfortunately the availability of animal models that are representative of AMD or other degenerative diseases of the eye is limited. Only primates and birds have a macula, and although there is an AMD model in monkeys [65, 66, 67], the disease takes years to develop and studies are difficult and expensive. Research has therefore been hampered by the relative lack of animal models of AMD. Mice, which thanks to the many genetically manipulated strains are the species of choice for basic studies, lack this structure entirely. *Therefore, while we can model processes that are believed to be involved in AMD, we cannot model the disease itself.* This is in contrast to uveitis, where reasonably good models exist that represent different aspects of human disease. Although there are diverse models of AMD, where the primary lesion is genetic or metabolic, in this review we will primarily focus on animal models of AMD with inflammatory involvement, which can be categorized into “Genetic” and “Induced Models” (Table 3). This is not to say that there is not a secondary involvement of inflammatory mechanisms in models where the primary lesion is not immunologic, but they usually have not been studied. Another inherent limitation of AMD models is that, just as models of uveitis are “amplified”, animal models of AMD are “accelerated” in order to make them amenable to study in the laboratory. It is arguable to

what extent compressing a chronic, lengthy and smoldering disease process that takes decades in humans, into a short time course able to fit into the lifespan of the mouse, is truly representative of AMD.

The “Inflammatory Gene Models” of AMD have been developed based on the association between complement factor proteins and genetic polymorphisms found in human AMD [59, 61, 62, 68]. One of them is the, $Cfh^{-/-}$ mouse, in which homozygous mice show uncontrolled C3 activation and accumulation in the retina, and photoreceptor outer segment disorganization. [69] However, contrary to AMD pathological findings, $Cfh^{-/-}$ mice show thinning of Bruch’s membrane and reduced drusen deposition instead of membrane thickening and increased drusen-like formations.

Another series of models attempt to address the role of inflammatory (or parainflammatory?) macrophages in the development of AMD. Macrophages can be found in human AMD lesions adjacent to drusen and may represent a healing and cleanup response gone awry. By altering genes for chemokines and their receptors, mouse models were created that have a deficient macrophage migration and function. Particularly noteworthy are mice deficient in CCL2/CCR2 responses, which were reported to display an AMD-like phenotype and have been the subject of intense study which led to the interpretation that defective macrophage recruitment and/or function due to lack of signaling through this axis leads to degenerative changes in the RPE and retina [22]. Retinal microglial cells share many properties with macrophages and are also thought to play a role in AMD. These cells express the CX3C (fraktalkine) chemokine receptor 1 (CX3CR1) and homozygosity of the CX3CR1 M280 and V249I alleles is associated with AMD development. [70, 71]. Aging *Cx3cr* $^{-/-}$ mice were reported to display subretinal infiltration of microglia that contain outer segment lipids and these cells show signs of intracellular drusen-like material with age. Combined *Ccl2−/−/Cx3cr1−/−* mice show accelerated AMD-like pathology that progresses to spontaneous CNV in ~15% of double mutant. Together, these data could point to a role for both microglia and macrophages in AMD pathogenesis.. Unfortunately, data obtained with these models turn out to be difficult to interpret, due to the previously unsuspected contamination of the commonly used C57BL/6N strain with the *rd8* mutation of the *Crb1* gene, which is expressed in photoreceptor cells and affects their polarity and function. Its presence causes focal degeneration that appears as white spots in the fundus, which upon clinical fundus examination look misleadingly like drusen [72]. Although full expression of the *rd8* phenotype was reported to require homozygosity, it is difficult to exclude the possibility that a heterozygous rd8 might interact with or complement expression of other ocular genes, producing a phenotype that would not otherwise be present. Therefore, interpretation of data from mice that display an “AMD-like” ocular phenotype must be qualified until confirmed to be independent of *rd8*.

In addition to the spontaneous genetic models discussed above, there are also induced models of processes believed to be involved in AMD. A common approach to model “wet” or neovascular AMD is the laser-induced model [73]. A green krypton laser is used to create breaks in Bruch’s membrane and trigger the development of corneal neovascularization (CNV). Although this model is a response to acute injury rather than a degenerative process, the resulting inflammatory responses are at least in part relevant to similar responses

accompanying AMD. For example, a role of macrophage infiltration has been established and findings can be correlated to wet AMD [23, 26]. Other models of induced disease include light-induced and smoking-Induced AMD Model [74, 75]. Interestingly, of these, in the smoking model affected mice show pathological traits reminiscent of dry AMD, including RPE loss, thickening of Bruch's membrane and accumulation of subretinal debris after 4–5 months. This has been associated with oxidative stress and triggering of immune processes in the eye [76].

To directly explore the relationship between immunity and AMD, we developed an induced immune-mediated model of AMD. This approach was based on studies of patients with AMD where carboxyethylpyrrole (CEP)-adducts were found in greater numbers in patient drusen and higher titers of anti-CEP autoantibodies in patient plasma were detected when compared to age-matched control subjects [4, 5]. CEP is an oxidative by-product from the fatty acid docosahexaenoic acid (DHA). DHA is mostly found in the retina, a tissue that is highly susceptible to oxidative damage and light exposure, and the model examines the effect of immunization of wild type (WT) mice with CEP-modified self-antigens compared to immunization controls. Over time, immunized CEP-immunized mice develop an AMD-like pathology manifesting as RPE cell hypertrophy and vacuolization, inflammatory cell recruitment in the subretinal space and outer segments, as well as RPE cell lysis. This model supports the interpretation that oxidative processes and tissue damage, irrespective of the way in which they are triggered, result in formation of new molecules that are antigenic to the immune system. These then serve as an antigenic target for adaptive immune processes that step in to participate in retinal tissue damage. In addition to supporting a role for adaptive immune mechanisms in the pathology of AMD, this model may serve as an effective way to decrease the threshold of inflammatory processes that put retinal tissue at risk of developing RPE cell dysfunction and retinal degeneration at early time points post-immunization, providing an accelerated model to study an age-related disease.

Pathogenic immune responses in AMD

In inflammatory processes such uveitis, presentation of auto-antigen in the context of infection providing a bacteria adjuvant effect, results in generation of an immune response that triggers the recruitment of inflammatory cells and mediators to the affected tissue. Interestingly, in AMD tissue stress and malfunction also induces an adaptive immune response, but the tempo and magnitude of inflammation are significantly smaller and the participating immune elements are distinct. This response, known as "para-inflammation" has been coined by Medzhitov and described to mainly rely "on tissue-resident macrophages and is intermediate between the basal homeostatic state and a classic inflammatory response" [77]. In other words, para-inflammation is implicated in chronic inflammatory conditions associated with modern human diseases and represents a tissue response to oxidative stress stuck "in limbo" between tissue repair and inflammation in order to restore functionality and stability. In contrast to uveitis, immunity to retinal antigens associated with aging produces a response that is more reminiscent of para-inflammation than inflammation.

The immunology of AMD could apply to para-inflammation in general including the role of macrophages and T cells. Macrophages have been the subject of close inspection in the

context of AMD, but our present knowledge regarding the role of T cells is surprisingly limited, mainly provided by studies of Nussenblatt and colleagues [78]. They have shown that complement components can induce IL-22 and IL-17 expression by human CD4+ T cells and that elevated levels of these cytokines are present in AMD patients. However, the identity of antigen-specific T cells that might mediate AMD pathology and how they interact with other immune cells (e.g. macrophages and B cells) remains to be elucidated.

As alluded to above, a potential link between innate and adaptive immune para-inflammatory responses in disease is oxidative stress, a known contributing factor in the development of pathological inflammatory conditions, including atherosclerosis and AMD [7]. Much of the evidence for adaptive immunity in AMD comes from the presence of anti-retinal autoantibodies in AMD patients [79, 80], which is recapitulated in our CEP mouse model mentioned above [81]. It is not clear whether the antibodies found in in human AMD patients have a primary role in AMD etiology, or if they arise in response to tissue breakdown products. Nevertheless, we would suggest that once present even as a secondary phenomenon, they can become drivers of disease,. A role for antibodies in AMD progression is supported by observations in autoimmune retinopathy (AIR), a collection of disorders that includes cancer-associated retinopathy, melanoma-associated retinopathy and non-paraneoplastic autoimmune retinopathy. Anti-α-enolase and anti-recoverin autoantibodies from CAR patients induced apoptotic cell death in retinal cells, and presence of particular antibody specificities is so strongly associated with disease that it serves as a diagnostic criterion for AIR [82, 83]. Although extensive studies have yet to be conducted for AMD, particular antibody specificities associated with AMD are beginning to emerge [84, 85]. Interestingly, despite its recognized autoimmune pathogenesis, AIR takes on the form of degeneration rather than overt inflammation.

Relevant to T cell responses, lipid peroxidation has been shown to produce oxidation-specific epitopes that can function as new antigens for immune recognition. In the context of AMD, CEP-modified proteins are known to be immunogenic and associated with development of disease. In addition, CEP acts directly and indirectly to influence macrophage polarization. On the one hand, it can directly activate macrophages, leading to M1 gene expression [8]. On the other hand, pro-inflammatory cytokine (IFN-γ and IL-17A) production by CEP-specific T cells contributes to the polarization of macrophages toward the M1 phenotype, providing a functional link between adaptive and innate immunity in the onset of disease. Malondialdehyde (MDA) is another lipid peroxidation product that serves as a marker of oxidative stress. MDA is formed upon peroxidation of polyunsaturated fatty acids present in phospholipids of low density lipoprotein (LDL), and has been associated with a number of oxidative stress-related diseases, including atherosclerosis and AMD [86]. Interestingly, it has been shown to bind to and be inhibited by complement Factor H, which has been associated with regulating susceptibility to AMD [59, 61, 62, 68, 69]. These lipid modifications of self-proteins induced by oxidative stress in the retina, such as albumin in the case of AMD, function as haptens that can induce a T cell autoimmune response. Unlike retinal antigens involved in uveitis, these seem to elicit a para-inflammatory rather than a full-blown inflammatory response.

We hypothesize that the amount of adducted retinal antigen or other AMD-related antigen available as target in the eye could at least in part account for the difference between the development of para-inflammation vs. inflammation. In the case of CEP, a likely determining factors in macrophage polarization and AMD could be differences in drusen CEP patterns, driving local foci of increased inflammation, and high vs. low retinal CEP content. It is interesting to that increased levels of CEP have been found in cone photoreceptor cells of post mortem eyes from healthy donors [8]. Since the macula (the region affected in AMD) contains the highest concentration of cone cells in the eye, it is tempting to speculate that this could be a harbinger of the propensity to develop AMD.

While age-related modifications in proteins within the retina are well documented, there is a paucity of studies on effects of aging on the local parameters of immune privilege, i.e., intactness of blood retinal barrier and the inhibitory ocular microenvironment. One study reports that FasL in the mouse retina actually becomes *upregulated* with age [87]. Nevertheless, the role of FasL in immune privilege remains somewhat controversial due to findings that FasL can be both pro- and anti-inflammatory. Interestingly, the same study reports that aging increases circulating levels of soluble FasL (a finding also reported in humans), which enhanced laser-induced CNV by attracting M2 proangiogenic macrophages into the laser lesions. However, this does not bear on immune privilege, because (a) the level of circulating sFasL is likely the result of systemic inflammatory process, (b) the lasering process itself caused downregulation of retinal FasL, which inhibits neovascularization in the eye by causing death of (Fas-expressing) new vessels [88], and (c) although CNV is used to model angiogenesis that occurs in wet AMD, it is a healing response to acute injury, not a degenerative process per se.

Concluding Remarks

Whereas in uveitis we see an overt inflammation, AMD is characterized by covert inflammation [9]. Why are these different, especially if they both have autoimmune underpinnings? Uveitis represents an aberrant response to self, brought about by a combination of two failures in self-tolerance: Ineffective central tolerance, possibly due to low expression of retinal antigens in the thymus in some individuals [89], permits retina specific T cells to escape elimination in the thymus. This is combined with inadequate peripheral tolerance, ironically as a result of immune privilege, which involves sequestration of retinal antigens in the eye and impedes peripheral tolerance [12]. On the other hand, in AMD the response to “neo-antigens” such as CEP, generated in adulthood through interaction with the environment, could be viewed as a legitimate immune response to non-self [7]. However, this by itself cannot explain why the effector mechanisms engaged in both situations should be different.

A necessary step in development of autoimmune inflammation driven by the adaptive response is recognition of the auto-antigen in the retina. A possible reason for the covert inflammation in AMD could be paucity of the target antigen in the retinal tissue. The amount of oxidative stress-induced neo-antigens in the retina as a function of age and environmental conditions has not been measured, but since it is a gradually accumulating product of metabolic processes, its amount (at least in younger animals) is probably quite

small. It is conceivable that following immunization, the activated CEP-specific T cells simply do not find sufficient target antigen to become efficiently re-activated when they enter the eye. IRBP in the cat retina is was reported to be present at >600 mg/gram protein [90] and in bovine retina ~2 mg/gram retinal tissue [91] (note that the method of quantitation differs). BG2 mice, expressing transgenic β-Gal in the retina under control of the arrestin promoter as a neo-self antigen, have 10-fold less β-Gal in the retina than IRBP (Dr. Dale Gregerson, personal communication). When crossed with β-Gal TCR Tg mice, introducing a high frequency of TCRs for the target Ag, they do not develop spontaneous uveitis, in contrast to IRBP-TCR Tg R161H mice. This could be a consequence of reduced antigen in the retina [42, 46]. However, uveitis can still be induced in these mice by immunization, so this level of expression is able to support an adaptive inflammatory response. The amount of CEP in the retina of a young mouse immunized with CEP-adducted protein is unknown, but is likely to be much less than that. We hypothesize that the slow accumulation of CEP in the retina as the mice age and sustain oxidative changes may provide a gradually increasing, but still low-abundance target, explaining the late onset and low grade of inflammatory activity in the CEP immunization model.

We further propose that intraocular levels of the molecular guardians of immune privilege (CFH, inhibitory neuropeptides, TGF-β, retinoic acid), which are rapidly lost in uveitis [33], are largely preserved in CEP-AMD, and in AMD in general, allowing the inhibitory ocular environment to exert a significant measure of control on the (mostly innate) inflammatory activity, and dampening what otherwise might become a more overt inflammation, into para-inflammation. If correct, injection of CEP-adducted protein into the eye of a CEP-immunized mouse should precipitate a more overt inflammation, similarly to the model of “immunogenic” uveitis, where injection of BSA into eyes of BSA-primed mice provides an abundant target for the adaptive response and precipitates inflammation.

The hypothesis proposed herein, aimed at outlining the immune mechanism that underlie the distinct forms of inflammation observed in inflammatory diseases of the eye, has testable predictions. We hope that interested investigators will put these premises to the test, and in this way shed light on the complex processes through which the immune system is involved in ocular homeostasis and disease.

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Highlights

- Inflammatory responses in the eye are modulated by its immune-regulatory environment.
- Distinct immunopathological processes drive uveitis and age-related degenerative diseases
- Uveitis and age-related degenerative eye disease may share autoimmune underpinnings
- Uveitis mechanisms may shed light on inflammatory processes in degenerative eye disease.
- Age-related macular degeneration may illuminate general mechanisms of para-inflammation.

Table I

The three successive layers of immune privilege [adapted from 12].

Separation	**Blood-Retinal Barrier**: prevents free traffic of cells and molecules into and out of the eye. **No lymphatic drainage** (as long as BRB is intact)
Inhibition	**Immunoinhibitory and Treg-inducing ocular microenvironment:** soluble mediators (e.g., cytokines and neuropeptides: TGF-β, α-MSH, VIP, CGRP, soluble FasL) cell-bound molecules(e.g., FasL, TSP1, PD-L1) **Soluble and cell bound complement regulatory proteins** (e.g., CFH, DAF, Crry)
Regulation	**Eye-driven systemic regulatory processes** (ACAID, post-recovery eye dependent tolerance)

Table 2

Models of uveitis

Mouse Model [ref]	Model Classification	Onset	Most common lesions	Strengths, weaknesses and Insights
Classical EAU (B10.RIII or C57BL/6 mice) [92]	Induced (immunization with IRBP in CFA)	10–12 days after immunization	Diffuse retinitis with severe photoreceptor damage, vasculitis, retinal swelling and folds, subretinal hemorrhage, vitritis, choroiditis. Anterior chamber infiltration. Predominantly mononuclear infiltrate. Heavily Th17 dependent. Disease is less severe in C57BL/6 mice.	Easily induced with high incidence, especially in B10.RIII mice, but requires use of bacterial adjuvants that may not be physiological. Has contributed important insights on basic mechanisms of the role of innate and adaptive immunity on pathogenesis. Serves as a template for experimental therapies but acute course impedes intervention after clinical onset of disease.
DC-EAU in B10.RIII mice [45]	Induced (injection of IRBP161-180 pulsed DC)	12–14 days after DC injection	Focal retinitis with punctate appearance, photoreceptor damage, retinal folds, mild choroiditis, vitritis. Th1 dependent. Prominent granulocytic infiltrate.	Less dependent on massive adjuvant stimulation than “classical” EAU, but can be inconsistent due to dependence on multiple variables. Has revealed that diverse T effector responses can result in autoimmune uveitis
R161 TCR Tg mice in B10.RIII mice [42, 52]	Spontaneous Mice transgenic for a TCR specific to IRBP	~4 weeks old	Retinitis with chronic progressive photoreceptor damage, vitritis, vasculitis, choroiditis. About half the mice develop lymphoid aggregates with characteristics of tertiary lymphoid tissue in the retina.	Spontaneous model with chronic course that permits to study natural triggers of the disease. In different founder lines, disease penetrance is dependent on the level of expression of the TCR.
AIRE$^{-/-}$ mice in B10.RIII mice [52]	Spontaneous Knockout for AIRE transcriptional regulator	4–5 wks old	Retinitis with chronic granulomatous lesions, photoreceptor destruction, choroiditis, late retinal thinning	Spontaneous model with chronic course. Provided insights on the importance of central tolerance in susceptibility to autoimmune uveitis.

Table 3

Animal Models of Inflammatory Mediated AMD

Mouse Model [ref]	Model Classification	Onset	Most common lesions	Strengths, weaknesses and Insights
CFH$^{-/-}$ [93]	Inflammatory gene knockout	12–24 mo old	Thinning of Bruch's membrane, drusen-like lesions	Spontaneous formation of retinal lesions, however, abnormal formation of many circulating auto-antibodies.
CCL2$^{-/-}$ or CCR2$^{-/-}$ [22]	Inflammatory gene knockout	9 mo old	Subretinal drusen-like lesions, Bruch's membrane thickening, ECM disruption, photoreceptor pyknosis, RPE vacuolization, CNV	Spontaneous formation of retinal lesions, however, *rd8* mutation present in these strains limits conclusions regarding degenerative vs. inherited mutation-induced changes. Long time for lesions to develop.
CX3CR1$^{-/-}$ [70, 94]	Inflammatory gene knockout	12 mo old	Subretinal microglia infiltration, retinal thinning	Retinal lesions not classical of AMD.
CCL2$^{-/-}$/CX3CR1$^{-/-}$ [94, 95]	Inflammatory gene knockout	4–6 wks old	Drusenoid lesions, retinal atrophy, RPE vacuolization, lipofuscin deposits, C3d deposition, macrophage infiltration	Spontaneous formation of retinal lesions with faster onset of disease. As with the single mutants, *rd8* mutation present in these strains limits conclusions regarding degenerative vs. inherited mutation-induced changes.
CEP [4]	Induced by immunization	40–60 d after immunization	RPE cell hypertrophy and vacuolization, inflammatory cell infiltration, RPE cell lysis, C3d deposition, Bruch's membrane thickening	Inducible immune model of Dry AMD based on human observations, i.e., presence of CEP in Drusen associated with anti-CEP auto-antibody production in patients with AMD. Hyper-immunization with CFA may alter immune responses and is not physiologic.