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Perspective: Microglia and Monocyte Distinct Roles in Degenerative Diseases of the Retina

Chen Yu¹, Christophe Rouboux², Florian Sennlaub^{2,*†}, Daniel R Saban^{1,3,*†}

¹Department of Ophthalmology, Duke University, Durham, NC 27710, USA

²Sorbonne Université, INSERM, CNRS, Institut de la Vision, 17 rue Moreau, F-75012 Paris, France

³Department of Immunology, Duke University, Durham, NC 27710, USA

Abstract

Unlike health, parenchymal macrophages in retinal degeneration are not solely comprised of microglia but may include monocyte-derived recruits. Recent studies applied transgenics, lineage-tracing and transcriptomics to decipher their distinct roles in the diseases of inherited retinal degeneration and age-related macular degeneration. Literature discussed here focuses on the disease ectopic presence of both macrophage types in the subretinal space, an extracellular site surrounding outer photoreceptor cells, which is crucially involved in the pathobiology. From these studies we propose a working model in which perturbed photoreceptor states cause a microglial dominant migration to the subretinal space for protection, whereas abundant presence of subretinal monocyte-derived cells instead drives pathogenesis and accelerated disease. The latter, we propose, is underpinned by specific genetic and non-genetic determinants that lead to a maladaptive macrophage state.

Keywords

macrophages; inherited retinal dystrophy; retinitis pigmentosa; age-related macular degeneration; AMD

Introduction

As part of the central nervous system (CNS), the retina is endowed with mononuclear phagocytes (MNP) that continually surveil its neuronal parenchyma and border tissues. These consist of microglia, a specialized type of yolk-sac derived macrophage that reside in the parenchyma [1–3] and border-associated resident macrophages [4] (i.e. long-lived retinal perivascular macrophages and short-lived choroidal macrophages adjacent to the retina [3, 5]). In certain disease states, blood derived monocytes can transiently invade the retina and differentiate into macrophages [2, 6, 7]. These monocyte-derived cells (MdCs) and retinal microglia, have received considerable research attention in retinal degenerative disease,

[†]Correspondence should be addressed to the lead contacts: Florian Sennlaub: florian.sennlaub@inserm.fr, Daniel Saban: daniel.saban@duke.edu.

*these authors have contributed equally

including inherited retinal degenerations (IRDs) and age-related macular degeneration (AMD) (Box 1). In these conditions, MNPs infiltrate the affected photoreceptor layers and other outer retinal structures, and manipulation of MNPs in mouse models can alter the disease course. Given the new appreciation that microglia and monocytes represent distinct MNP lineages [1, 8–10], the field recognizes their non-redundant functions in disease and the importance of studying these cells as separate entities. As such, the current review focuses on the distinctive roles of MdCs and microglia in retinal degenerative diseases. Here, we discuss recent studies that have elucidated differential contributions of these cells in the context of the subretinal space (SRS), delineated by the outer segments of the photoreceptors and the retinal pigment epithelium (RPE), which is crucially involved in pathobiology. From these findings in mice, we propose a unified model that classifies MNP responses as adaptive versus maladaptive, and further extrapolate this idea to retinal degenerative diseases in humans.

Roles of MNPs in Retinal Health and Photoreceptor Degenerative Disease

Type and pattern of retinal MNPs are largely homologous to brain and spinal cord parenchyma. Microglia are the principal MNPs in the normal retina parenchyma [11]. The perivascular spaces also have resident macrophages that are long-lived [12], albeit radio-sensitive [11]. Monocytes (Box 2) and dendritic cells are not thought to gain access to the retinal parenchyma in non-diseased states. Like elsewhere in the CNS, retinal microglia are highly ramified, tiled, and express C-X3-C Motif Chemokine Receptor-1 (Cx3cr1), a homeostatic microglial immune checkpoint factor that inhibits expression of inflammatory cytokines such as Interleukin-1 β (IL-1 β) [13, 14] and C-C Motif Chemokine-2 (Ccl2) [6]. However, also like in brain and spinal cord, retinal degeneration can result in large-scale monocyte recruitment, along with microglial reactivity. Yet, due to overlapping phenotypic markers of these two MNP lineages, tools to study their isolated contributions have only recently been available, which we summarize in Table 1.

Normal Physiological Conditions

The retina is highly organized into distinct laminae. Inner layers are endowed with blood vessels that have vascular endothelial cells with tight junctions making up the inner blood retina barrier (BRB). Within the inner most layers, some microglia reside around somata of retinal ganglion cell (RGC) and their axon projections within the nerve fiber layer, which connect the eye to the brain lateral geniculate nucleus and the superior colliculus via the optic nerve. However, most microglia reside within the two distinct synaptic layers, inner and outer plexiform layers (OPL and IPL, respectively). These are where photoreceptors synapse with bipolar cells and bipolar cells synapse with RGC, respectively. Recent work has revealed key differences amongst IPL versus OPL microglial pools in the steady state [3]. The maintenance of IPL microglia is mostly dependent on RGC-produced IL-34 (alternate ligand of colony stimulating factor-1 receptor, Csf1r) [15–17], whereas OPL microglia are IL-34 independent (presumably maintained by glial-derived Csf1). Importantly, these respective pools have distinct electrophysiological contributions to visual processing (Box 3).

Outer most retinal layers of adult human and pigmented rodents are physiologically devoid of MNPs and blood vessels [6, 18, 19]. This is the case throughout the photoreceptor length, from their soma (outer nuclear layer) to their ciliated segments, all the way through to the SRS and the RPE, a specialized monolayer of neural crest-derived border cells [20] (Fig.1). Absence of microglia in the SRS is of critical importance, as this site is essential for photoreceptor homeostasis and function. It contains the interphotoreceptor matrix and surrounds apical microvilli of the RPE. There, the RPE delivers neurotrophic factors, nutrients, ions, and visual cycle chromophores to photoreceptors. The RPE also phagocytoses spent photoreceptor outer segment discs in the SRS, a process carried out via conserved phagocytic machinery, such as with certain integrins [21, 22] and Mer tyrosine kinase (Mertk) [23] in a circadian-dependent manner. Lastly, RPE cells are laterally connected via tight junctions forming the outer BRB. The RPE is highly immunosuppressive [24–26], and MNPs reaching the SRS that ligate thrombospondin-1 (Tsp-1) via their CD47 receptor are eliminated by the RPE expressing Fas ligand (FasL) [26, 27] (Figure 1).

Subretinal Space in Retinal Degenerative States

Invasion by MNPs of the photoreceptor layers and SRS is a disease phenotype of IRDs and AMD [6, 14, 18, 19, 28]. It is accompanied by the loss of ramified microglia (to ameboid shaped cells) [29] and a general increase in total MNPs due to microglia replication and monocyte infiltration in certain settings [5, 30], while a Cd11c⁺ subpopulation was also described [31, 32]. Histological studies of human postmortem retinas suggest that subretinal MNPs are invariably present in intermediate and advanced (or ‘late’) forms of AMD [6, 19, 33–35] and pronounced in mouse mutants with accelerated age-associated photoreceptor degeneration [14], RPE injury model [36] and laser-induced choroidal neovascularization (CNV), an experimental model for wet AMD [37]. Subretinal MNPs have also been described in human postmortem IRD subjects [18], and in adult rodents with IRD mutations [3, 7, 38, 39], as well as other injury models [7, 11]. In fact, the presence of subretinal MNPs in IRDs was appreciated as early as the 1970’s using Royal College of Surgeons rats that have dystrophic retinas [40] - a degenerative condition later determined to be caused by a *Mertk* mutation that renders RPE incapable of phagocytosing spent photoreceptor discs [41], and *Mertk* mutation cause IRDs in humans [42]. However, it took several more decades before the distinction between endogenous microglia and recruited MdCs was definitively made [1, 9].

An important advancement to the field came in 2007 by Sennlaub *et al.* [14]. Authors demonstrated the association of a variant of *CX3CR1* with AMD, later supported in a meta-analysis [43], and that *Cx3cr1*-deficient mice develop age-related MNP infiltration of the SRS accompanied by photoreceptor degeneration and exaggerated CNV. Given that *Cx3cr1* is a homeostatic microglial checkpoint gene of MNPs [44], this phenotype revealed a link between intrinsic MNP dysregulation and disease pathogenesis. In 2013, the same group showed that AMD patients are characterized by increased intraocular CCL2 concentrations and that MdCs are present in the SRS of intermediate and late AMD patients, as well as in *Cx3cr1*-deficient mice, where they were shown to mediate photoreceptor toxicity [6]. Importantly, authors demonstrated that two key human AMD genetic risk gene variants, the Apolipoprotein E2 isoform (*APOE*) and Complement Factor H variant Y402H (*CFH*)

directly promote pathogenic inflammation in the SRS in mouse models [26, 27]. These findings emphasized the role of AMD-risk variants in inflammation and the pathogenic role of inflammation in AMD (Fig.2a).

In 2019, another advancement in understanding the cellular make-up and contribution of subretinal MNPs in degeneration was made by O’Koren and Yu *et al* [3]. Building on their study in 2016 [11], authors applied *Cx3cr1^{CreER+}* microglial lineage tracing mice and single cell RNA-seq (scRNA-seq) to decipher the ontogeny and function of subretinal MNPs in the *Cx3cr1*-sufficient setting. In a humanized IRD model [45] and in the toxic light-induced photoreceptor degeneration model (LD) [46], authors demonstrated that microglia are the dominant MNP lineage which occupies the SRS, whereas recruited MdCs were largely confined to the parenchyma. This was similarly demonstrated by another lab in a RPE injury model [36]. Moreover, authors showed direct evidence that these subretinal microglia restrict, not accelerate, disease progression. Opening the door to future elucidation of these disease restricting activities, the authors also identified gene specific inductions of subretinal microglia in these settings (Fig.2b). Collectively, their study now offers an alternative view to the cell type and function of subretinal MNPs in the course of retinal degenerative diseases.

The next sections will discuss the results that led to the independent observations of pathogenic MdCs versus protective microglia in the SRS during retinal degenerative diseases.

Pathogenic Role of MdCs in the Subretinal Space

Infiltration of MNPs in wet AMD (and laser-induced experimental CNV) characterized by a breach of the BRB (edema and subretinal hemorrhages), has long been recognized as an important factor of the pathogenesis (reviewed in [33]). In contrast, in slowly evolving geographic atrophy (GA) and IRDs, MNPs, in particular MdCs, were not commonly assumed to infiltrate the photoreceptor cell layer or inner retina, as no clinical signs of BRB rupture are visible. Over recent years, it has become increasingly clear that MNPs, including MdCs, infiltrate the outer retina in intermediate AMD and GA [33]. However, it is still passionately debated whether and how the MNP infiltration influences CNV and degeneration.

Evidence for pathogenic subretinal MNP accumulation

One of the first pieces of evidence that MNPs could be more than bystanders in retinal degeneration came from the study of *Cx3cr1*-deficient mice. *Cx3cr1* receptor is strongly expressed by MNPs, where it mediates a tonic inhibitory signal, but not on neurons or the RPE in the adult retina. *Cx3cr1*-deficient mice develop accelerated and exaggerated subretinal MNP accumulation in conditions that do not trigger inflammation in wildtype mice (such as 12–18m old mice under normal light conditions, and a non-toxic light-challenge that does not induce degeneration in pigmented wildtype mice) [6, 14, 26, 27]. In aged *Cx3cr1*-deficient mice, the prolonged presence of MNPs in the SRS and their continued ingestion of lipid-, retinol-rich photoreceptor outer segments [14] leads to sizeable, ‘foam cell’ macrophages that are visible *in vivo* by fundus examination [47] and

share a similar appearance with pseudodrusen in AMD (Box 1). Interestingly, pseudodrusen are also associated with subretinal MNPs in patients [28]. Importantly, this age-dependent accumulation of subretinal MNPs in *Cx3cr1*-deficient mice is associated with a significant degeneration of rods and cones [6, 14, 26, 27, 34, 48], but not with RPE atrophy (Fig.2A). They therefore quite accurately model a subtype of GA called incomplete outer retinal atrophy [49].

Mechanistically, *Cx3cr1*-deficient MNPs are characterized by an over-expression of APOE. The APOE excess activates toll-like receptor (TLR) signaling on MNPs in the absence of their natural ligands (damage- and pathogen-associated molecular patterns) [35]. This is likely due to APOE-induced cholesterol extraction from the lipid rafts of the plasma membrane of MNPs [33, 50]. Physiologically, TLR2 and TLR4 are separated from their co-receptor CD14 as they locate to non-raft plasma membrane. The cholesterol extraction from the rafts levitates this separation, and trigger intracellular NF- κ B activation and cytokine expression similar to ligand induced dimerization (Fig.2A') [33]. A similar mechanism has been previously described for APOA1 [51]. The resulting increased secretion of IL-6 and CCL2 observed in subretinal *Cx3cr1*-deficient MNPs reduces their RPE-induced elimination and increases monocyte recruitment, respectively (see below) [14, 35]. Additionally, *Cx3cr1*-deficient MNPs overexpress P2rx7, which constitutively activates their inflammasome and facilitates IL-1 β maturation and secretion [48, 52]. The increased IL-1 β secretion from this dysregulation mediates rod and cone degeneration, and significantly increases CNV [34, 48, 53] (Fig.2A). Collectively, *Cx3cr1* deficiency is sufficient to trigger a pathogenic non-resolving subretinal inflammation, and deletion of *ApoE* in *Cx3cr1*-deficient mice prevented the subretinal MNP accumulation, and attenuated photoreceptor degeneration and CNV after laser-injury [35].

The *Cx3cr1*-deficient mice used in these studies did not carry the *rd8*, a spontaneous frameshift mutation in *Crb1* found on C57BL6/N mice that causes late-onset photoreceptor degeneration and CNV [54]. Indeed, a number of publications describing degeneration and/or CNV formation in *Ccr2*^{-/-}, *Ccl2*^{-/-} and *Cx3cr1*^{-/-}*Ccl2*^{-/-} mice were in fact due to inadvertent *rd8* contamination.

In summary, it can be argued that *Cx3cr1*-deficiency induces a model of 'primary' subretinal inflammation, where inflammation is not the consequence of a tissue injury but solely due to 'over reacting' MNPs, triggered by a non-toxic initiating stimulus (e.g. aging, a non-toxic light challenge [6, 14]). In 'secondary' inflammation models induced by a tissue injury, *Cx3cr1*-deletion often exaggerates MNP accumulation and associated degenerative changes: e.g. laser-induced CNV [14], paraquat-induced retinopathy [55], and *rd10* mice [56, 57], a recessive IRD model.

AMD risk factors promote subretinal pathogenic inflammation

AMD is a highly heritable disease and several haplotypes encoding for protein variants have been established as risk-determinants in AMD. An isoform of *APOE*, along with a common variant of *CFH* accounts for an important part of the genetic risk [58, 59].

With respect to *APOE*, carriers of the *APOE2*-isoform, that is associated with higher APOE concentrations, are at increased risk for AMD and carriers of the *APOE4*-isoform, associated with lower APOE concentrations and impaired cholesterol transport, are protected against AMD compared to the common *APOE3*-allele [60, 61]. The MNPs of transgenic mice expressing human *APOE2* (*TRE2*-mice) were shown to express high levels of APOE that activate TLR signaling and induce inflammatory cytokines, similar to aforementioned *Cx3cr1*-deficient MNPs. Analogous to *Cx3cr1*-knockout mice, *TRE2*-mice develop age- and stress-related pathogenic subretinal MNP accumulation [26, 35]. In *Cx3cr1*-deficient mice, in the context of APOE-dependent subretinal inflammation, the *APOE4*-allele led to diminished APOE and CCL2 levels and protected against harmful subretinal MNP accumulation observed in *Cx3cr1*-deficient *APOE3*-allele carrying mice [26] (Fig.2A').

Concerning CFH, its binding to integrin CD11b/CD18 complex, strongly expressed on MNPs, curbs TSP-1 activation of its integrin-associated CD47 receptor that is necessary for MNP elimination. The AMD-associated CFH(H402) variant markedly increased this effect, promoting chronic subretinal inflammation [27] (Fig.2A'').

Taken together, these results showed that *APOE2* and the CFH(H402) variant promote subretinal pathogenic inflammation. The fact that causative genetic AMD risk variants that provide an essential trigger for disease development, promote subretinal MNP accumulation and associated degeneration, provides strong evidence for a pathogenic role of subretinal inflammation in AMD.

Evidence for subretinal MdCs as a major driver for AMD pathology

Most studies do not allow a differentiation of MdCs and microglia, as non-specific markers were used for their identification (Table 1). The receptor CCR2, strongly expressed on inflammatory monocytes, constitutes one of the few specific markers that distinguish monocytes and early MdCs, as it is not expressed by microglia [6, 62, 63]. Studies of postmortem sections showed that CCR2⁺ MdCs invariably participated in the SRS in intermediate AMD and GA [6]. Additionally, increased intra-ocular levels of CCL2, a cytokine that recruits CCR2⁺ monocytes to the tissue [62], were found in neovascular [64–69] and atrophic-AMD [6, 68].

Experimentally, *Cx3cr1*-deficient MNPs overexpress CCL2 in aged and light-challenged mice [6]. After a light-challenge of *Cx3cr1*^{GFP/GFP} *Ccr2*^{RFP/+} mice that express RFP under the *Ccr2* promoter, around 10% of subretinal MNPs were RFP⁺ and therefore monocyte-derived [6]. However, *Ccr2* is quickly down-regulated when monocytes differentiate to macrophages [3, 6, 30, 70], which leads to an underestimation of MdCs. To overcome this, Sennlaub *et al.* permanently labeled monocytes and MdCs by repeated systemic injections of the traceable nucleotide 5-ethynyl-2'-deoxyuridine (EdU). These experiments revealed that ~50% of the subretinal MNPs are MdCs [6]. Monocyte depletion with systemic clodronate liposome completely prevented EdU⁺ MdC recruitment. Concordantly, deletion of *Ccl2* or *Ccr2*, inhibition of *Ccr2*, diminished the subretinal MNPs by ~50–60% in acute and chronic models. Interestingly, the inhibition of CCR2⁺-monocyte recruitment in *Cx3cr1*-deficient mice nearly completely prevented their inflammation-associated photoreceptor degeneration,

suggesting that MdCs are responsible for the inflammation induced degeneration. Similar results were observed in the carboxyethyl pyrrole immunization-induced AMD model [71].

In laser-induced CNV, MdCs participate significantly in subretinal MNP accumulation as determined *in vivo* imaging of mice injected with an intra-peritoneal or subcutaneous depot tracer that labels monocytes and MdCs [72], and in bone marrow transplantation experiments [37, 73]. Functionally, the depletion of circulating monocytes [73–75] and the inhibition of monocyte-recruitment by genetic deletion of *Ccr2* and *Ccl2* [76–78], very significantly inhibited subretinal MNP accumulation and CNV formation [75] in numerous studies.

In IRDs, the participation of MdCs seems to largely depend on the underlying genetic defect and possibly the intensity and speed of the degeneration. For example, *Ccr2* deletion diminished MNP infiltration and had a minor protective effect in progressively degenerating *rd10* mice [79]. The faster degeneration in *Arrestin-1 (Arr1)* knockout mice [7], could not be rescued when the monocyte recruitment was prevented by CCL2 inhibition [7]. However, in *Mertk*^{-/-} mice, which lack this important phagocytosis receptor on the RPE but also on MNPs, MdCs were recruited to the SRS [38] and *Ccl2* deletion very significantly protected *Mertk*^{-/-} mice from degeneration [80].

Taken together, MdCs participate in the subretinal MNP accumulation under certain circumstances. In fast-evolving monogenetic IRDs such as in *Arr1* knockout mice, they do not aggravate the degeneration. Following laser-induced CNV, MdCs are strongly involved in CNV formation, similar to other wound-healing models. In ‘primary’ inflammation, where the genetic deficit primarily activates MNPs, such as *Cx3cr1*^{-/-} mice and in TRE2-mice, they are the main culprits of photoreceptor degeneration. *Mertk*^{-/-} mice represent a mixed situation, where the genetic deficit affects retinal homeostasis and MNP function directly. The fact that well-established causative AMD-risk variants directly affect MNP function and promote inflammation, emphasizes the role of the AMD-risk variants in inflammation and the role of MNPs, in particular MdCs, in AMD. In IRDs, this might not necessarily be the case.

Protective Role of Microglia in the Subretinal Space

With the new appreciation in the late 2000’s that microglia and MdCs represent distinct lineages [1] and early studies pointing to their possible differential functions in experimental autoimmune encephalomyelitis (EAE) and other models of CNS conditions [81–83], the need for new tools to distinguish these two cell types had become imminent. A breakthrough came in 2013 with development of *Cx3cr1*^{CreER} transgenic mice [9, 84, 85], now enabling conditional genetic manipulation of microglia by taking advantage of their long half-lives and self-renewability [84]. Importantly, these mice could be used to decipher the ontogeny of microglia versus MdCs in disease, conditionally knockout genes, or deplete microglia in an isolated fashion when paired with inducible diphtheria toxin receptor (DTR) gene (Table 1). In this section, we review recent findings showing that isolated microglia dominantly take up the SRS in *Cx3cr1*-sufficient retinal degenerative disease settings and have cytoprotective functions.

Microglial Predominance of the Subretinal Space

O'Koren *et al.* [11] was one of the first studies that applied *Cx3cr1^{CreER/+}* mice to decipher *in situ* spatial distributions of microglia versus MdCs during disease. They used confocal microscopy of retinal tissues from mice subjected to the LD model with *Cx3cr1^{eYFP-CreER/+}R26^{flox-STOP-flox-tdTomato}* mice. This approach allowed microglia to be traced as YFP⁺ tdTomato⁺ and MdCs as YFP⁺ tdTomato⁻. The findings were striking as microglia and MdCs were found distributed in distinct areas of the retina: microglia migrated to the SRS, whereas monocytes poured into the neuroparenchyma (presumably via retinal vasculature [7]). This finding was subsequently recapitulated by Wong and colleagues using the model of sodium iodate induced RPE injury, showing microglial dominance of the SRS through 30 days post injury [36]. Collectively, both studies demonstrated the spatial distinction between microglia and MdCs and now potentially uncovered a key role for microglia within the SRS in disease.

More comprehensive studies dissecting the isolated migration patterns of microglia were later carried out in a series of *Cx3cr1^{CreER/+}R26^{fl-STOP-fl-DTR}* mouse experiments by O'Koren and Yu *et al* [3]. Authors used human rhodopsin P23H knock-in mice (rhodopsin mutant model of a major autosomal dominant IRD form [45, 86]), demonstrating again that microglia predominantly occupied the SRS and were adherent to the apical aspect of the RPE [3]. These subretinal microglia were absent when administering diphtheria toxin, indicating that they are indeed DTR-expressing microglia (Fig.2B). In stark contrast, MdCs were largely incapable of migrating to the SRS in both the LD and P23H mice, even when microglia were depleted. Hence, microglia do not outcompete MdCs for the SRS, but have restricted access to it in these disease settings. The same case is also likely in the NaIO₃-induced RPE injury model [36].

A similar observation was described in the context of Alzheimer's disease (AD), where microglia encapsulate amyloid- β plaques in the cortex and hippocampus. Two groups independently demonstrated that peripheral-derived myeloid cells do not similarly cluster around these plaques and fail to modify amyloid load when microglia were depleted in the APP23 and APP/PS1 transgenic mouse models of AD [87, 88]. Another example can be gleaned in the normal mouse brain, where a distinctive microglial population, but not the choroid plexus macrophage population, was found to reside on the apical epithelium of the choroid plexus [89]. Together, these reports reveal restricted microglial access to specific parenchymal or non-parenchymal niches throughout the CNS.

Reprogramming of Subretinal Microglia

With this novel characterization of the SRS as a microglial-dominant immune niche in retinal degeneration conditions, the door now opened to addressing whether the SRS microenvironment shape the role of these microglia in disease pathobiology. Through analysis of *Cx3cr1^{YFP+}* sorted cells using scRNA-seq from retinas, authors were able to demonstrate that microglia underwent transcriptional reprogramming in the SRS [3], indicating these cells are indeed altered in this niche. Changes included upregulated antioxidant and lipid activity genes (e.g. *Srxn1*, *Apoe*, *Lpl*, *Abca1*) and downregulation of some immune response genes (e.g. *Ifngr1*, *C1qa*, *Aif1*, *Cx3cr1*, *Jun*). This profile suggests

an ability for subretinal microglia to respond to increased extracellular lipids and oxidative stress levels consistent with a neurodegenerative environment, and to do so in a manner that involves reduced pro-inflammatory responses (Fig.2B). Interestingly, downregulation of *Abca1* with senescence impairs cholesterol efflux and promotes AMD [90]. Also, supporting a protective reprogramming is the upregulation of *Ifnar1* by subretinal microglia, the loss of which increases lesion size and vascular leakage in CNV model [91]. Collectively these findings suggested against a pathogenic role for subretinal microglia.

Further countering a pathogenic role, subretinal microglia had a similar transcriptional profile with disease-associated microglial (DAM) cells. Amit and colleagues discovered this microglial subtype in the 5xFAD model of AD surrounding brain amyloid- β plaques in a *Trem2*-dependent fashion and concluded that DAM cells play a role in restricting disease progression [92]. Subretinal microglia have similar signature gene expressions, such as increased expression of *Lgals3*, *Lpl*, *Spp1*, *ApoE*, *Trem2*, and *Cstb*, and downregulated of *Tmem119*, *P2ry12*, *Cx3cr1* and *Siglech*. Furthermore, *Cx3cr1^{CreER/+}* mice utilized by O'Koren and Yu *et al.* in retinal degeneration experiments allowed to conclude that microglia, as opposed to MdCs, indeed carry this DAM signature [3]. Interestingly, a similar microglia gene signature was observed during normal postnatal brain development [93], and further thought to be crucial in normal RGC development [94]. It is tempting to hypothesize that DAM cells represent a stereotypic response that supports physiological processes under stressful environments of large-scale cell death shared in postnatal development and neurodegenerative disease. However, because a similar microglial gene profile was also reported in the EAE model which was characterized as a pathological response rather than a disease restricting one [95], more work is still needed to fully resolve the functional significance of all aspects of the DAM signature.

RPE and Photoreceptor Protection by Microglia

The discovery of microglial reprogramming in the SRS and their adherent nature to the apical RPE now begged the question as to what is the isolated contribution of microglia in degeneration. *Cx3cr1^{eYFP-CreER/+}R26^{fl-STOP-fl-DTR}* mice were used for conditional depletion of microglia in LD and P23H models to address this question. The absence of microglia resulted in massive structural damage of the RPE, thereby directly linking their subretinal localization and transcriptional reprogramming with RPE protection (Fig.2B). Moreover, the damage of RPE in this depletion setting included both lateral and/or apical defects to F-actin, resembling key features of RPE dysmorphogenesis observed in human AMD and certain forms of IRDs [96]. A better preserved RPE function is generally believed to help maintain the function and survival of photoreceptors. Hence, these findings may mean that subretinal microglia could potentially be involved in restricting degenerative disease progression.

Still, the question remained as to exactly how do microglia confer protection to the RPE. A clue was also revealed by O'Koren and Yu *et al.* [3]. They showed that the absence of microglia led to the accumulation of dead photoreceptors and debris in the SRS in both LD and P23H models. Interestingly, as MdCs are spared in this system (as are astrocytes and Müller glia ought to be as well), these findings implicated a microglial-dominant role in this

debris clearance. The same may be gleaned from a study that depleted microglia in a model of retinal detachment [97], and another study using a NMDA model of RGC excitotoxicity [98], as well as an IRD model of progressive rod-cone degeneration [99]. Also, a recent study on complement C3 and its CR3 dependent phagocytosis supports this conclusion in an IRD model of *rd10* mice [100], although there also reports suggesting that C3 (and C1q) are pathological in LD [101, 102]. Likewise, Kipnis and colleagues described microglial-dominant clearance of neuronal debris in the optic nerve crush model [103], and a similar conclusion was made by Ransohoff and colleagues in the spinal cord of EAE mice [81]. Hence, the failure to clear this debris shown by O’Koren and Yu *et al.* could have cytotoxic effects, potentially explaining the observed RPE defect. In contrast to these findings, Zhao *et al.* used continual tamoxifen administration in *Cx3cr1^{eYFP-CreER}R26^{DTA}* to deplete microglia in the *rd10* mice [39]; however, it is more likely that this effect was mostly due to tamoxifen alone [104]. Together, these findings expand our knowledge about roles of microglia beyond neuroinflammation and illuminate the differential roles between microglia and MdCs in neurodegeneration.

In summary, these studies demonstrate that the SRS is a microglial-dominant macrophage niche in *Cx3cr1*-sufficient mice in LD, P23H mice, and sodium iodate induced RPE injury, where few recruited MdCs were observed in the SRS. The net-effect of *Cx3cr1*-sufficient microglia during LD and P23H degeneration is the restriction of disease progression. Evidence suggests that this protective response is mediated at the SRS via swift clearance of otherwise toxic debris, but whether other activities that are involved in protection remains to be explored.

Unified MNP Model in Retinal Degenerative Diseases

From these studies we propose a new conceptual framework to help understand the roles of MNPs in retinal degeneration. Though the model we put forth here is based on conclusions made in different experimental settings, our thinking is guided by the understanding of microglial checkpoint genes, such as *Cx3cr1* [44], whose physiologic expression exerts a tonic inhibitory stimulus. In *Cx3cr1*-deficient mice, a normally sub-threshold stimulus (such as normal aging or a light challenge) is sufficient to drive pathogenesis [6, 14]. By contrast, in *Cx3cr1*-sufficient mice, (due to acute or progressive photoreceptor death) microglia predominantly accumulate in the SRS [3, 11], where they restrict disease progression (Fig.3). The constitutive expression of *Cx3cr1* in microglia results in responses in degeneration that have a beneficial net-effect, such as controlled clearance of otherwise toxic neuronal debris [105]. By contrast, the absence of *Cx3cr1* primes microglia to drive responses that have a harmful net-effect, such as uncontrolled proinflammatory cytokine production and further recruitment of inflammatory MdCs [6, 13]. We can therefore categorize MNP responses as beneficial/’adaptive’ versus harmful/’maladaptive’ and that a loss of certain factors, such as *Cx3cr1*, can ‘tip the scale’ from the former to the latter.

To extrapolate this model for human disease, factors that help drive maladaptive responses could include genetic variants that confer risk in developing AMD. Indeed, *CX3CR1* polymorphisms T280M or V249I in humans have been shown to be a risk factor in AMD, which may be independent or dependent on other factors [43, 106]. Other major risk factors,

APOE2 isoform and *CFH402H* variant, may also be involved in driving harmful MNP responses [26, 27]. Likewise, *TGFBR1* polymorphisms found in GWAS studies that confer AMD risk [107] may act in a similar manner. This receptor is phosphorylated by *TGFBR2* in order to propagate TGF- β 1 signaling [108], which in turn maintains microglia identity and function [109, 110]. Indeed, induced microglia conditional *Tgfr2* deletion in mice was shown to cause a degenerative disease phenotype [111]. These respective genetic factors might not be sufficient alone to drive harmful MNP responses, but possibly can with the addition of non-genetic determinants that confer risk in AMD (advanced age, western diet, and/or smoking). While AMD pathogenesis is multifactorial, MNPs are usually not driven into maladaptive in IRDs that have a monogenic Mendelian etiology involving genes of the visual system [112]. One exception may be IRDs caused by *MERTK* mutations, a gene that regulates phagocytosis of the RPE and MNPs such as microglia. The isolated role of these mutations in MNPs and their influence on retinal degeneration is not understood, but could conceivably contribute to infiltration of subretinal MdCs that may be involved in the disease phenotype.

There are limitations to our proposed model. Certain situations exist in which a robust inflammatory response could be categorized as adaptive, rather than maladaptive. For example, the control of ocular infections and the inhibition of some malignancy types likely benefit from prominent immune responses for host survival. Another limitation is that not all activities in either *Cx3cr1*-sufficient or -deficient settings are binary, i.e. solely beneficial or harmful. Microglial migration out of plexiform layers in order to take up the SRS is likely to cause synaptic deficits at the inner retina [3, 113]. Likewise, these negative changes may be curtailed by transient MdC recruitment to these evacuated plexiform niches.

Concluding Remarks

Not only is it important to study MNP responses in retinal degeneration, but it is also crucial to probe monocyte and microglial lineages as distinct entities (Outstanding Questions). We discussed how these two cell types even display functional disparities within the same microenvironment of the SRS, further highlighting the importance of ontogeny. Lastly, we proposed a unified model that classifies MNP responses in the retina as beneficial versus harmful. With this framework we posit that there are factors acting in concert which can ‘tip the scale’ from adaptive to maladaptive responses that help cause disease. Continued research is critical to better understand microglia and monocytes in health and disease, particularly given the immense disease burden that retinal degenerative diseases have on our society.

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Box 1.**IRDs and AMD**

Inherited retinal degenerations (IRDs) and age-related macular disease (AMD) are both characterized by the degeneration of rods, cones, the RPE, and the choroid, while the overlying inner retina remains morphologically intact to a certain extent. However, from an etiological and symptomatic standpoint, IRDs and AMD are very different. IRDs are rare (1/4000) monogenetic diseases [112], most often due to mutations of rod genes (rod-cone dystrophy). The lack of the essential gene (recessive e.g. phosphodiesterase 6B, *rd10* mutation) or the accumulation of a dysfunctional variant (dominant e.g. rhodopsin P23H mutation) leads to rod cell death and night blindness (age of onset 10–20 years), followed by a centripetal degeneration of the RPE and cones that lead to tunnel vision and eventually blindness. AMD is a late onset (above 55 years), prevalent, complex and multifactorial disease caused by the interplay of age, environmental, and genetic risk factors. The common AMD risk variants are not specific for the RPE or photoreceptors but instead concern factors of the complement cascade and lipoproteins, among others [107, 114]. Early AMD is characterized by lipoproteinaceous debris in the subretinal space (termed pseudodrusen, reviewed in [115]) or below the RPE (soft drusen). Advanced or ‘late’ debilitating AMD is characterized by central choroidal neovascularization (wet or ‘exudative’ AMD, late form) and ultimately a disciform scar (wet AMD end stage), or by a central, centrifugally extending lesion of the photoreceptors, RPE, and choroid that often starts parafoveally (geographic atrophy, GA, late form [33]). Loss of rods and to a lesser extent of cones, occur prior to RPE loss above pseudodrusen [116] and in a transitional zone adjacent to GA lesions [34] and disciform scars [117, 118]. Finally, the degeneration of the RPE and choroid leads to the typical atrophic lesions in the fundus appearance, which extends centrifugally. AMD only affects the central retina that contains the macula and the cone-rich fovea, necessary for high acuity vision that does not exist in mice.

Box 2.**Monocyte Derived Cells in Health and Disease**

Monocytes are bone marrow-derived myeloid cells that circulate in the blood. They are precursors of MdCs that participate in the resident MNP pool of certain organs (such as lung and gut) and are recruited during tissue damage or infection of any tissues [62]. They comprise three major subsets (classical, intermediate and non-classical) and are likely sequentially transitioning from one into another [119]. The short-lived classical monocytes constitute 90% of all circulating monocytes in humans and 60–70% in mice. They express high levels of CCR2, CD14, and Ly6C and low levels of CX3CR1 and CD16 and circulate the blood for 24h before 99% of them disappear. One percent of classical monocytes evolve into longer-lived intermediate (CCR2/CD14^{high} CX3CR1/CD16^{high}) monocytes, which finally become CCR2/CD14^{low} CX3CR1/CD16^{high} ‘patrolling’ monocytes [119]. During tissue injury, damage- and pathogen-associated molecular pattern (DAMPs and PAMPs) activate resident mast cells and resident macrophages that produce chemokines, such as CCL2 (the main ligand of CCR2), which recruit classical monocytes that differentiate into anti-microbial and pro-inflammatory macrophages. These cells release reactive oxygen species and complement components to kill, opsonize and phagocytose pathogens and secrete inflammatory cytokines (such as IL-1 β , TNF- α , IL-6, and CCL2) that alter the tissue to facilitate the immune response [120]. Although these mediators are crucial to combat infection and ensure the survival of the organism, they can also cause considerable collateral damage, in particular in the CNS (including the retina) with poor regenerative potential [33]. Once the lesion is disinfected, the infiltrating macrophages produce cytokines such as VEGF that facilitate angiogenesis and tissue repair, scar formation, and inflammation resolution [121, 122]. Finally, pro-inflammatory macrophages disappear from the site and the tissue is left with the tissue-specific resident macrophages, as before the injury [123]. If the inflammatory response is not quickly controlled, it can become pathogenic and contribute to degeneration, pathological neovascularization and fibrosis, as seen in many diseases such as metabolic diseases (obesity, atherosclerosis), neurodegenerative diseases and cancers [120, 124–126].

Box 3.**Microglial Roles in Visual Function**

There is a growing appreciation for the role of microglia in adult retinal neurophysiology. Work by Saban and colleagues dissected microglial contributions to visual processing by their distinct anatomical location in the retina [3]. Mice that lack of *IL34*, which is normally expressed by RGC, have a specific microglial loss in the IPL but not OPL. This IPL-specific defect is associated with a diminished electrophysiologic responses to light stimuli by cone bipolar cells, but normal photoreceptor responses [3]. *IL34* knockout mice have no obvious quantitative loss of synapses, thus pointing to a possible qualitative role for IL-34-dependent microglia in synaptic function within the IPL. Hence, this study established the existence of an IL-34 dependent pool of microglia restricted to the IPL that plays a key role in cone bipolar cell responses during visual processing. Prior to this study, Wong and colleagues demonstrated that transient microglial depletion leads to reduced electrophysiological responses to light stimuli in both photoreceptors and bipolar cells [113]. Interestingly, transient microglial depletion does not result in major retinal morphological changes or neuronal loss [113]. Their subsequent study showed that microglial repopulation after depletion restored the impaired visual functions [127]. Collectively, in addition to RPE [128] and Muller glia [129], these studies identified microglia as another non-neuronal cell that is involved in neuro-retinal electrophysiology.

Outstanding Questions

1. Are there markers that can distinguish subretinal monocyte-derived cells versus microglia in patients with degeneration?
2. Does an association exist between the macrophage type in the subretinal space and progression of degeneration in AMD or IRDs?
3. What is the specific ‘tipping point’ that makes a maladaptive response pathogenic in individuals with AMD genetic and non-genetic risk determinants?

Highlights

- The subretinal space — an extracellular site that hosts essential activities for photoreceptors — is invaded by mononuclear phagocyte (MNPs) in retinal degeneration, but the role of these cells in patients is controversial
- In mice, “adaptive” MNP responses in retinal degeneration are those in which reactive microglia migrate into the subretinal space to help restrict disease progression, whereas recruited monocyte-derived cells have limited subretinal access
- “Maladaptive” MNP responses are triggered in mice with certain genetic deficiencies by a normally sub-threshold insult that results in dysregulated microglia and an abundance of pathogenic monocyte-derived cells in the subretinal space
- Applying this concept of adaptive and maladaptive responses may help unravel the complex roles of subretinal MNPs in inherited retinal degenerations and age-related macular degeneration

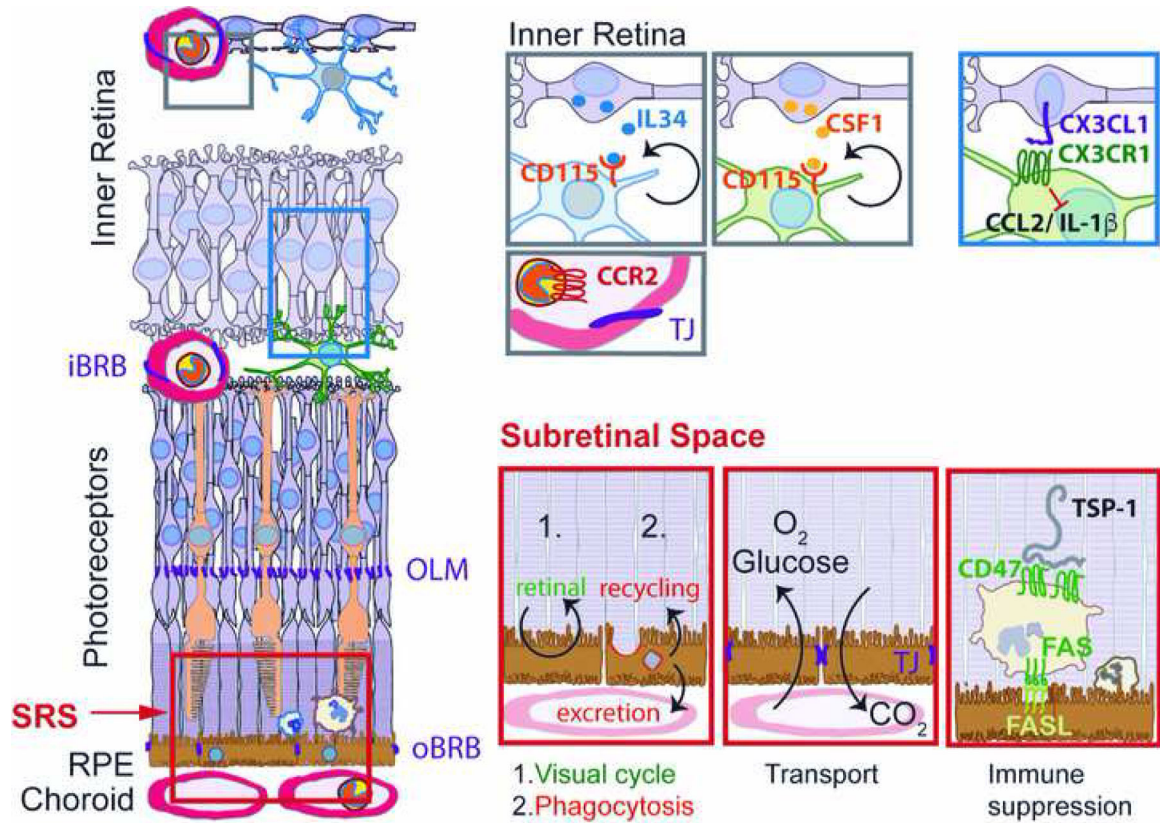


Figure 1: Physiological distribution of retinal MNPs and main functions of the subretinal space: Physiologically the retina is only populated by IL34-dependent and CSF-1-dependent microglial cells. CCR2-positive Mos are restricted to the circulation separated from the retina by the inner and outer blood retinal barrier. CX3CL1 is constitutively expressed as a transmembrane protein in inner retinal neurons and provides a tonic inhibitory signal to the microglial checkpoint factor CX3CR1, expressed by retinal MCs. The subretinal space, demarcated by the tight junctions of the outer limiting membrane and of the RPE is the site of 1.) the visual cycle; 2.) phagocytosis of used photoreceptor outer segments and excretion or recycling the material; 3.) Oxygen and glucose uptake and CO₂ excretion; 4.) immune suppression, in which FasL induces elimination of infiltrating leukocytes after activation of the integrin associated protein CD47 by thrombospondin 1 (TSP-1). iBRB: inner blood brain barrier; oBRB: outer blood brain barrier; OLM: outer limiting membrane; SRS: subretinal space; RPE: retinal pigment epithelium; IL: interleukin; CSF: colony stimulating factor; TJ; tight junction.

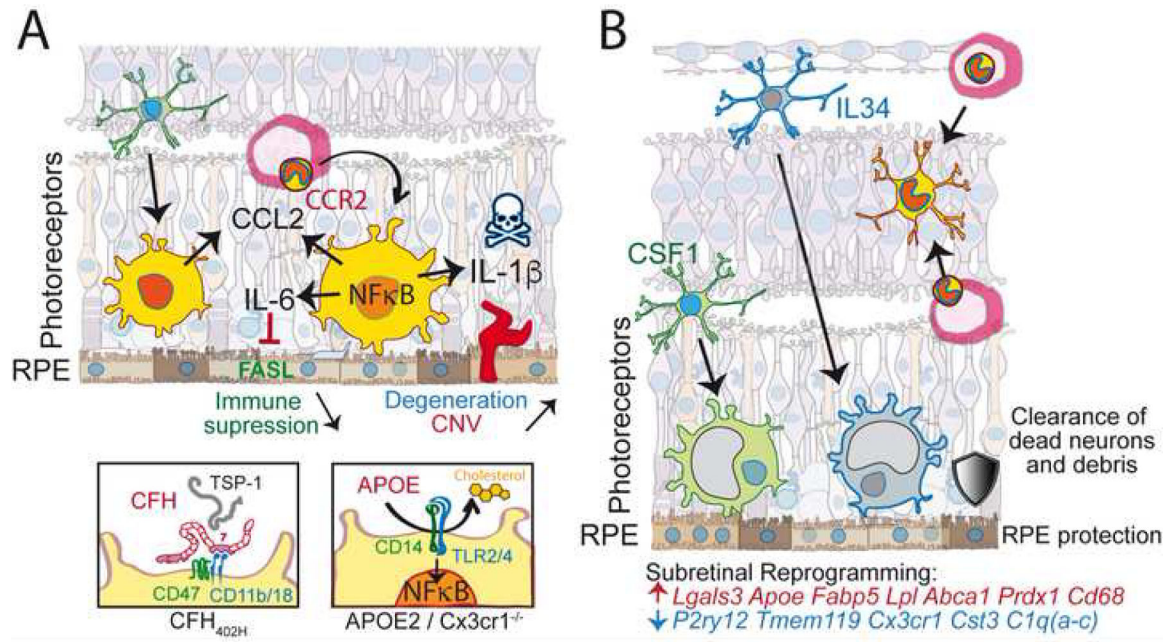


Figure 2: Molecular mechanisms of the role of MNPs in retinal degenerations:

(A) participation of mononuclear-derived cells in the infiltration of the SRS: loss of microglial checkpoint genes (such as in *Cx3cr1* deficiency) lowers the threshold of microglial activation and induces NF- κ B signaling and inflammatory cytokine secretion in conditions of normally sub-threshold stress. Increased levels of CCL2, IL-6 and IL-1 β lead to monocyte recruitment, down-regulation of FasL and immune-suppression and photoreceptor degeneration and neovascularization, respectively. (A') In AMD this NF- κ B activation can be due to the AMD-associated APOE2 allele that, similar to *Cx3cr1*-deficiency in mice, leads to increased APOE concentrations, lipid-raft destabilization of the plasma membrane and the activation of the innate immunity receptor cluster (CD14 and TLR2/4). (A'') Furthermore, the AMD-associated Complement Factor H 402H variant inhibits MNP elimination as it blocks TSP-1 mediated CD47 activation particularly efficiently. Both AMD-risk factors thereby promote pathogenic subretinal inflammation. (B) Dominance of resident microglia in the SRS in *Cx3cr1* sufficient settings: both IL34-dependent and independent microglia migrate to the SRS, which is poorly accessible to MdCs. These subretinal microglia are transcriptionally reprogrammed by downregulating homeostatic marker genes and upregulating genes involved in lipid metabolism, anti-oxidant activity and others as indicated. Functionally, these cells clear dead neurons and cellular debris and protect the RPE and photoreceptors.

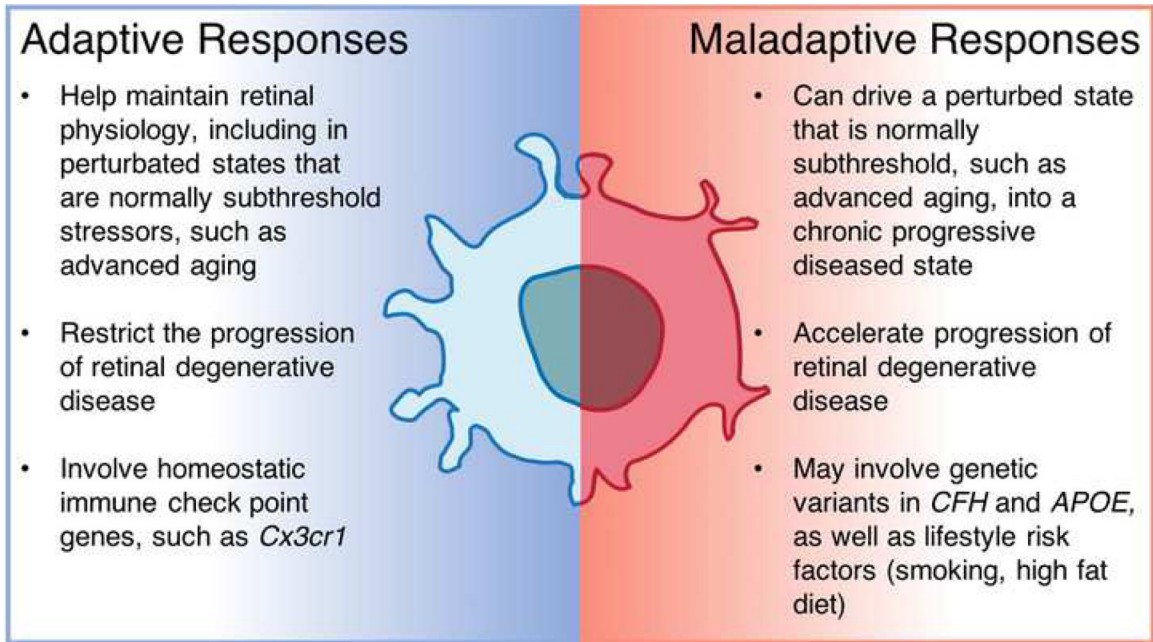


Figure 3: A unified hypothesis for MNP responses in IRD and AMD:

Adaptive responses by resident microglia express low levels of pathogenic cytokines, help eliminate toxic waste and slow down degeneration. When confounded by risk factors, such as genetic variants and environmental factors, microglia more readily express pathogenic cytokines and recruit monocytes. Such maladaptive responses by MNPs exacerbate retinal degenerative diseases.

Table 1.

Technical Challenges for Discerning MNP Lineages in Retinal Degeneration.

Tools	Pros	Cons	Refs
Marker staining			
Conventional markers, such as Iba-1, F4/80, Cd11b	<ul style="list-style-type: none"> Labels the majority of MNPs 	<ul style="list-style-type: none"> Unable to distinguish microglia and MdCs Some markers not specific for MNPs 	[2]
Microglial specific markers, such as P2ry12, Tmem119	<ul style="list-style-type: none"> Able to label specifically label microglia in steady state 	<ul style="list-style-type: none"> May not distinguish microglia versus MdCs during neuroinflammation 	[3,92,95]
Chimaeras			
Bone marrow chimera	<ul style="list-style-type: none"> Full blood chimerism 	<ul style="list-style-type: none"> Side effects of irradiation and transplantation, such as temporary disruption of BRB and myelomonocytic engraftment in the CNS 	[2]
Bone marrow chimera with lead head shielding	<ul style="list-style-type: none"> Preservation of CNS in steady state 	<ul style="list-style-type: none"> Full blood chimerism is not achievable 	[11]
Circulating monocyte-labeling			
Systemic 5-ethynyl-2'-deoxyuridine (EdU)	<ul style="list-style-type: none"> A traceable nucleotide that is integrated into the DNA of dividing cells Efficient labeling short-lived circulating monocytes and MdCs but not long-lived microglia in steady state. Nuclear stain, avoids confounding with (i) MNP auto-fluorescence and (ii) positivity due to transfer (phagocytosis) of reporter proteins 	<ul style="list-style-type: none"> Require repeated injections, which limits use for short-term study but not chronic conditions. Microglia may replicate during degeneration, local injections necessary for distinction 	[6]
Intravenously injected fluorescent tracers	<ul style="list-style-type: none"> A traceable fluorescent probe taken up by circulating monocytes and detectable in cells derived from these monocytes. 	<ul style="list-style-type: none"> Require repeated injections, which limits it for short-term study but not chronic conditions. 	[72]
Reporter mice			
<i>Cx3cr1^{GFP}</i> or <i>Cx3cr1^{YFP}</i>	<ul style="list-style-type: none"> Heterozygotes are good for labeling MNPs Homozygotes are a full deletion of <i>Cx3cr1</i> gene 	<ul style="list-style-type: none"> Unable to distinguish microglia and MdCs, as both express <i>Cx3cr1</i> 	[11,14,105]
<i>Ccr2^{RFP}</i>	<ul style="list-style-type: none"> Heterozygotes are good for labeling classical monocytes Homozygotes are a full deletion of <i>Ccr2</i> gene 	<ul style="list-style-type: none"> Not good for labeling monocyte-derived macrophages, which lose <i>Ccr2</i> expression Spares non-classical monocytes 	[6,7,34]
<i>Cx3cr1^{CreER}</i> <i>R26^{fl-STOP-fl-reporter}</i>	<ul style="list-style-type: none"> Tamoxifen treatment induces the expression of reporter and labels microglia and MdCs 	<ul style="list-style-type: none"> Other long-lived tissue-resident macrophages that maintain <i>Cx3cr1</i> expression may also be labeled 	[9,11,34,84,85]

Tools	Pros	Cons	Refs
	<ul style="list-style-type: none"> The reporter expression in monocytes is 'washed out', as they are quickly replaced by non-labeled cells once tamoxifen decayed 		
Depletion / recruitment inhibition studies			
Csf1r antagonists	<ul style="list-style-type: none"> Efficient depletion of Csf1r dependent macrophages Easy for administration 	<ul style="list-style-type: none"> Indistinguishably ablate microglia and MdCs Potential off-target effects on other tyrosine kinase receptors 	[97,98,127]
Intravenous clodronate liposome	<ul style="list-style-type: none"> Efficient depletion of monocytes and their derivatives 	<ul style="list-style-type: none"> Require repeated injections, which limits it for short-term study but not chronic conditions. Potential off-target effects 	[6,98]
<i>Cx3cr1^{CreER} R26^{fl-STOP-fl-DTA}</i>	<ul style="list-style-type: none"> Efficient depletion of MNPs Not require diphtheria toxin 	<ul style="list-style-type: none"> Indistinguishably depletes all <i>Cx3cr1</i> expressing MNPs Require multiple tamoxifen administrations 	[37,113]
<i>Cx3cr1^{CreER} R26^{fl-STOP-fl-DTR}</i>	<ul style="list-style-type: none"> Efficient depletion of microglia following 'washout' 	<ul style="list-style-type: none"> Require tamoxifen and diphtheria toxin administration Other long-lived tissue-resident macrophages may also be depleted 	[3,84]
Ccl2- and Ccr2- deletion / inhibition	<ul style="list-style-type: none"> Efficiently inhibits exit of bone marrow classical monocyte into the blood 	<ul style="list-style-type: none"> Does not inhibit non-classical monocyte-recruitment 	[6–7, 73–80]