HHS Public Access

Author manuscript

Nat Rev Immunol. Author manuscript; available in PMC 2018 May 01.

Published in final edited form as:

Nat Rev Immunol. 2017 May ; 17(5): 322–332. doi:10.1038/nri.2017.13.

New insights into mononuclear phagocyte biology from the visual system

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Abstract

Major advances in mononuclear phagocyte biology have been realized, yet key questions pertinent to health and disease remain, including in the visual system. One problem concerns how dendritic cells can trigger immune responses from certain tightly regulated immune privileged sites of the eye. Another, albeit separate, problem involves whether functional specializations exist for microglia versus monocytes in neurodegenerating retinas. We examine novel insights in eye immune privilege and, separately, review recent inroads concerning retinal degeneration. Both themes have been extensively studied in the visual system, and exhibit parallels highlighted here with recent findings in CNS mononuclear phagocytes and in the periphery.

The last decade has seen big advancements in our understanding of cells of the mononuclear phagocyte system (MPS), which include monocytes, macrophages, and dendritic cells (DCs)[1]. While their central role in triggering and/or shaping immune responses has been appreciated, key advances regarding their respective developmental origins have only recently been established [2]. From these studies, it is now understood that the ontogeny of tissue-resident macrophages is largely distinct from that of 'classical' (or conventional) DCs, signifying that these respective MPS populations are indeed unique[3] (notwithstanding monocytes, which can take on functionalities of either [4]). Other landmark studies have recently discovered the embryonic basis of adult microglia (CNS-resident macrophages[5, 6, 7, 8, 9]), addressing the long-standing debate regarding the origins of these cells[10].

Key questions regarding MPS functionality still remain. For one, the tissue-specific cues that enable MPS cells to effectively trigger adaptive immune responses in tightly regulated, immune-privileged sites are poorly understood, which we focus on here in the context of certain compartments of the visual system. Wayne Streilein described immune privilege as "nature's way" [11] of providing protection from deleterious immune responses to highly delicate and vital organ systems. These include the visual, central nervous, and reproductive

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Competing interest statement

The authors declare no competing interest.

(testis and pregnant uterus) [12, 13] systems. Certain mechanisms of immune privilege vary between these systems, and, as seen in the eye (Fig 1), even vary across different compartments within a given system[14, 15]. Some facets of immune privilege have also been recognized in the pathogenesis of certain diseases, including the immune evasion in tumor settings or by pathogens [16, 17]. Thus, this area of research has broad implications in health and disease, as previously reviewed by Niederkorn [18].

A separate, but also important, problem involves potential specialized functions for distinct MPS populations in certain neurodegenerative diseases of the CNS, including in the retina (Fig 1). Although immune privilege (or loss thereof) has been related to the pathogenesis of neurodegeneration, and has been addressed elsewhere [19, 20, 21, 22, 23, 24], we do not focus on this area here. Instead, we examine recent findings establishing that monocyte-derived cells are recruited to the CNS parenchyma in disease and are derived from the bone marrow [25, 26], whereas adult microglia are largely derived from erythro-myeloid progenitors formed during primitive hematopoiesis [5, 6, 7, 8, 10, 27]. More specifically, we will evaluate the question of whether these cells are intrinsically different at a functional level given their distinct ontogenies [9].

In the visual system, which we concentrate on here, both the themes of immune privilege and neurodegeneration have been extensively studied. In this Review, we first explore newly appreciated pathways that may explain how MPS cells activate adaptive immune networks with respect to the immune privileged cornea and retina. We then shift topics, to examine recent findings regarding MPS cell function in neurodegenerative diseases of the retina, such as glaucoma, retinitis pigmentosa, and age-related macular degeneration (AMD). Both parts of the Review also draw parallels to new findings concerning MPS biology in the CNS and periphery.

Eye immune privilege breach and the MPS

The concept of immune privilege has been revised in recent years, as highlighted in Box 1. New questions have arisen regarding how MPS cells function in immune privileged sites. On the one hand, subverting immune privilege is permissive to autoimmunity, as recently reviewed in Ref [28]; on the other hand, subversion promotes immune defenses that are needed in the context of infectious diseases. Recent findings in the eye suggest several ways in which DCs may accomplish this feat in certain compartments of the eye. The types of DCs and macrophages that are harbored in the cornea have been reviewed elsewhere [29, 30]. Additionally, note, that not all insults to the eye result in a permanent loss to immune privilege[14, 31], but we do not directly address this area here. Instead, we concentrate on novel concepts that include lymphatic vessel outgrowth (that is, lymphangiogenesis) into the immune privileged cornea (which is not part of the CNS), and peripheral nerve signals that are capable of causing a breach in this immune privilege. Finally, we also review recent data implicating gut commensal microorganisms in T cell activation and consequent autoimmune attack of the retina (which is part of the CNS). We discuss the role of DCs in these contexts and the homeostatic and pathological implications in humans.

Lymphangiogenesis overcomes immune privilege in the cornea

While there is a paucity of lymphatics within immune privileged sites (except for the testis [32]) under normal physiological conditions, access to blood vessels or drainage pathways in lymph vessels of adjacent structures has been recognized, as recently reviewed [14]. Examples include drainage from: cerebrospinal fluid through meningeal lymphatics [33, 34]; eye anterior chamber into extraorbital lymphatic vessels [35]; and the maternal–fetal interface into uterine lymphatics [36, 37]. Hence, we use the term 'pauci-lymphatic' as a designation for such sites. The cornea is one such example of a pauci-lymphatic [38], immune privileged site [39]. Specifically, the cornea proper is completely avascular, whereas the limbus (the very periphery of the cornea) is amply vascularized (Fig 2).

Numerous mechanisms that maintain this 'angiogenic privilege' [40] have been defined, such as the repression of certain pro-lymphangiogenic factors. One example is via suppression of vascular endothelial growth factor C (VEGF-C) expression, mediated in part by membrane type 1-matrix metalloproteinase signaling that suppresses macrophage production of VEGF-C [41]. Likewise, the matricellular protein thrombospondin 1 (TSP1) also suppresses macrophage expression of VEGF-C, as described by Masli and colleagues [42]. Another mechanism involves inhibiting the expression of the VEGF-C receptor, VEGFR2, which is mediated in part through forkhead box protein C1 (FOXC1) – a member of the large Forkhead box transcription factor family involved in vascular development [43]. Finally, VEGF-C decoy receptors also inhibit lymphangiogenesis by sequestering VEGF-C away from lymphatic endothelial cells [44, 45].

Subversion of these inhibitory processes occurs most notably under certain inflammatory settings, as we have shown in the allergic eye disease (AED) model [46, 47] and others have shown in models of corneal transplantation [48], HSV-1 keratitis [49], and dry eye disease [50]. In such settings, an outgrowth of lymphatics from the limbus invades the cornea (Fig 2). In some settings, there is also a concurrent invasion of blood vessels (Fig 2). For these reasons the cornea has been a widely utilized model site to study the mechanisms underpinning pathologic neovascularization – a noteworthy example is the work in tumor angiogenesis by Judah Folkman [51]. To overcome the aforementioned inhibitory activities in the cornea, certain factors are switched on [52]. Such factors, which are expressed during inflammation, include the carbohydrate-binding protein galectin-8 [53] and interleukin-17 (IL-17) [50], and contribute to corneal lymphangiogenesis through lymphatic endothelial cell sprouting and proliferation, respectively. Additional factors involved in overcoming immune privilege in the cornea have been reviewed elsewhere [54].

Evidence of limited drainage in the cornea was shown by Collin.[38] With respect to restricted migration of antigen-laden DCs to the lymph node, these ideas are largely derived from observations in the inflammatory lymphangiogenic setting [55], where their ability to migrate and consequently activate T cells is augmented. The latter was demonstrated in the context of abrogated immune privilege in the corneal transplant model [56]. However, the isolated role of lymphangiogenesis might not be identified in this system because inflammation also results in increased frequencies of DC maturation and egress into the lymphatics [57, 58], as well as recruitment of monocyte-derived cells [59]. One study demonstrated that lymphangiogenesis can be experimentally inhibited (using an integrin inhibitor) in the

presence of corneal inflammation [60]. In so doing, the authors observed that consequent T cell activation is reduced despite the abrogation of corneal immune privilege [60]. However, the authors did not quantify DC migration to the lymph node. Other evidence that corneal lymphangiogenesis, and possible migration of DCs, may lead to a breach in immune privilege can be garnered from reports showing the presence of corneal autoimmune T cell responses associated with lymphangiogenesis [61]. Examples were demonstrated in a Sjogren's syndrome-like model of corneal disease, and in models of dry eye disease [42, 46, 62, 63, 64].

In short, significant advances have been made in understanding how the immune privileged cornea maintains its pauci-lymphatic state, and how its subversion is associated with augmented DC migration and T cell responses. However, current methods used to stimulate lymphangiogenesis require an inflammatory insult, which in and of itself may trigger augmented DC migration. Work from our group and others helped to reveal the role of CC-chemokine receptor 7 (CCR7) in migration of corneal DCs to the lymph nodes [29, 46, 65, 66, 67, 68], which could be another way that DC migration may be regulated independently of lymphangiogenesis [66]. Corneal abrasion was also demonstrated to increase antigen drainage to the lymph nodes, signifying a key role for the epithelium in maintaining immune privilege [69]. Finally, the relative contribution of other factors, such as neuropeptides [70], and corneal nerves in immune privilege is also important, as we discuss in the next section.

Peripheral nerve regulation in breaching corneal immune privilege

Understanding how immune responses are altered through the hardwiring of the nervous system's reflex arcs is an emerging field,[71, 72, 73] and recent evidence suggests a role for this process in breaching immune privilege. This hard-wired activity can facilitate rapid adaptation to environmental perturbations for maintenance of tissue homeostasis. It was recently shown how this neuro-immune reflex in the gut lumen regulates macrophage function distally in the muscularis externa, as pathogens activate extrinsic sympathetic neurons that innervate this site [72]. With respect to the eye, another fascinating study revealed the possible existence of a putative neuro-immune reflex capable of breaching corneal immune privilege [74]. The authors showed that a certain nerve injury in one cornea causes the loss of immune privilege in the fellow cornea, and showed further data possibly suggesting that breach of immune privilege in both eyes is coupled through such a reflex response (Fig 3).

Authors of this cornea study took advantage of the unique acceptance rates in the mouse corneal transplant model [75] to examine the loss of immune privilege. Certain fully MHC mismatched donor cornea to host mouse combinations enjoy a spontaneous 50% acceptance rate, which is attributed to immune privilege. By contrast, the same donor-to-host combination placed in conventional sites, like the skin, yields 0% acceptance. Corneal immune privilege also exists in humans, as corneal transplantation is the only type of solid organ/tissue graft where HLA tissue typing is not standardly performed [76]. When the authors severed the nerves in a certain manner in one cornea, the expected spontaneous graft acceptance in the fellow eye was abolished in mice, which was associated with increased

allogeneic T cell activity. Hence, severing corneal nerves in one eye breaches immune privilege of the unmanipulated contralateral cornea. A similar finding was corroborated by a study involving laser burn injury to the retina in mice [77]. Intriguingly, there is an analogous human condition called sympathetic ophthalmia, in that a penetrating injury to one eye results in a uveitic inflammation in both eyes [78], although a neurogenic etiology for sympathetic ophthalmia has not been established.

Multiple lines of evidence suggest that this bilateral immune privilege breach of the cornea is a neuro-immune reflex. First, the factor required to cause this response was shown to be the neuropeptide, substance P[74]. Second, elevated levels of substance P were detected in the contralateral eye more than twelve days following the severing procedure, whereas increased neuropeptide in the serum was only detectable for several days. Thus, rather than a possible role for systemic or circulating substance P, the data suggest that the nerves in the unmanipulated contralateral cornea are the source of this neuropeptide. If true, it may implicate a putative reflex that may travel via afferent axons, perhaps by way of the trigeminal ganglia (Fig 3). Intriguingly, this response does not follow normal circuitry, a previously recognized feature reviewed by Tracey [79]. However, this specific pathway in the cornea has yet to be definitively proven.

The exact mechanism by which substance P-producing nerves in the contralateral cornea cause augmented T cell responses during immune privilege breach remains an open question. One possibility is that substance P causes qualitative pathogenic changes in corneal mononuclear phagocytes, as we have previously hypothesized [80]. For example, it was demonstrated that skin sensory nerves drive dermal DCs to trigger a psoriasiform disease [81]. Similarly, another group showed that expression of calcitonin gene-related peptide by nociceptive fibers activated dermal DCs to promote cutaneous candidiasis resistance [82]. In the gut, one study identified a microbiota-driven crosstalk between muscularis macrophages and enteric neurons that regulate peristalsis [83]. In the eye, a close proximity between nerves and myeloid cells in the cornea has also been documented [80, 84, 85], and recent findings suggest that DC production of ciliary neurotrophic factor contributes to corneal nerve regeneration [84]. Hence, it is conceivable that substance P-expressing corneal nerves that cause a breach in immune privilege may do so by altering mononuclear phagocytes in a manner that promotes activation of T cells upon certain provocation.

The possible evolutionary advantage for this putative neuro-immune reflex may be in the augmentation of host immune defense against blinding bilateral corneal infection. Acanthamoeba keratitis causes corneal nerve damage [86], and thus a unilateral infection may trigger an immune privilege breach in the contralateral eye. Such a result would potentially prevent a host from succumbing to bilateral corneal blindness, and there is some evidence in human Acanthamoeba keratitis to support this concept [87]. Future work is needed to address such a possibility. Also needing to be resolved is if commensal microorganisms may differentially modulate this response, which we will review in the next section.

The gut microbiota enables evasion of immune privilege in the retina

The underpinning mechanisms for why the gut microbiome has such a profound effect on host physiology is another fascinating and timely area of investigation, and may be relevant to how DCs activate T cell responses that affect the immune privileged retina [88]. However, for reasons not understood, the surface of the normal eye has an extraordinarily low density and diversity of commensal bacteria, which is dissimilar to any other barrier site (reviewed in Ref [89]). For example, whereas human saliva yields 10^6–10^8 CFUs/mL [90], human tear fluid yields as low as 10^2–10^3 CFUs/mL [91]. Furthermore, Gadjeva pointed out that other work using 16S rRNA sequencing does not indicate whether these data represent live bacterial colonization or instead transiently existing live or dead bacteria [89].

By contrast, the normal gut microflora has been shown to influence retinal health and the CNS at large. Recent evidence suggested that gut microbiota-dependent signals activate Th17 cells specific for the retinal antigen interphotoreceptor retinoid-binding protein (IRBP) that cause experimental autoimmune uveitis (EAU) [92]. This particular model results in spontaneous development of uveitis by two months. A similar phenomenon was shown with encephalomyelitic T cells in a model of experimental autoimmune encephalomyelitis (EAE) [93], which is induced by active immunization with the myelin-related antigen myelin oligodendrocyte glycoprotein (MOG). Likewise, it was reported that normal gut flora is associated with increased Th17 cell responses and clinical disease in EAE [94]. Interestingly, the latter report showed that the same augmented disease response occurred when the gut flora was monocolonized with the specific commensal, segmented filamentous bacteria (SFB), and thus implicated this bacterium in EAE immunopathogenesis. Similarly, the mouse colony in the EAU study mentioned above harbored SFB [92]. Furthermore, R161 T cell receptor transgenic T cells, which are specific for IRBP, were activated independently of their cognate antigen, which could suggest a similar role for SFB. However, the authors found that SFB depletion with the antibiotic vancomycin did not abrogate EAU. Hence, the gut commensal species that produces the signals that lead to the activation of uveitogenic T cells in this model remains unknown.

To summarize, the MPS has various ways in which to activate adaptive immune responses in eye immune privilege. In the cornea, evidence suggests that the presence of lymphangiogenesis amplifies the migration of antigen-laden DCs to the lymph node for consequent activation of T cell responses. Alternatively, (somatosensory) nerves in the cornea can release neuropeptides that breach corneal immune privilege and permit activation of T cells. Potentially, this pathway enables corneal DCs to activate T cells in the lymph node, but the latter needs to be confirmed. Finally, studies mentioned above suggest that commensal-derived antigens from non-privileged distal sites lead to the activation of T cells that can, in turn, attack host cells in immune privileged tissues. The DCs in the gut lamina propria are possibly stimulating these T cells in EAU, but this point also needs to be tested. Additionally, pathogens can stimulate immune responses via innate lymphoid cells or via γδ T cells[95]. Finally, gut commensals can affect the CNS, via altering microglial activity, which we also do not address here [9697]. However, the focus of the next section is on the role of microglia (as well as monocyte-derived cells) in the context of retinal degenerative diseases.

The MPS and neurodegeneration in the retina

The recent paradigm shift that adult microglia are derived from fetal origins as opposed to adult definitive hematopoiesis is reshaping our knowledge about the isolated role of these cells in neurodegenerative diseases. Within the CNS parenchyma, including the neural retina, microglia comprise the majority of the MPS (Fig 4). Efforts have shifted to better understand the relationship between positive and negative impacts of microglia and monocyte-derived cells in neurodegeneration, including in the retina. Such work may lead to novel therapies to preserve neuronal function through individually targeting these populations [98].

This section examines the role of microglia and monocyte-derived cells in retinal neurodegenerative disease, although other immune and non-immune cells also play a role. We specifically address blinding diseases not widely considered to have an immune etiology. One such disease is glaucoma, which involves axonal degeneration and dropout of retinal ganglion cells (RGCs) (Fig 4), leading to blindness. Also emphasized here is animal work on retinitis pigmentosa, which is an umbrella term for numerous inherited conditions that are characterized by degeneration of photoreceptor cells (Fig 4). We also examine AMD – a condition where clinical disease begins at the level of the choroid, which is just below the neural retina (Fig 4). For these diseases, we review new reports indicating a central role for mononuclear phagocytes in disease pathobiology, and examine the possible distinct roles of microglia and monocyte-derived cells in such settings.

Microglia and the classical complement cascade in RGC degeneration

Our understanding of microglia has evolved tremendously, and in addition to appreciating their roles as immune sentinels of the CNS, we are now aware of their vital role in synaptic maturation and maintenance [99, 100, 101, 102]. In health, the involvement of microglia and the classical complement cascade has emerged as a central factor in synaptic plasticity, as investigated in the visual system[103]. An important study by Barres and colleagues helped initiate this line of research through their demonstration that complement component 1q (C1q), which is the initiating protein in the classical complement cascade, is needed for eliminating inappropriate synaptic connections during postnatal development [104]. Stevens and colleagues then went on to show that microglia carry out this elimination process, as demonstrated by examining the anatomical refinement of connections between the retina and geniculate ganglion [105]. Specifically, they demonstrated that neuronal derived C1q tags excess synapses and consequently selects them for elimination by microglia. These studies proved that microglia are crucial to maintaining healthy synapse function and identified the mechanism by which this activity occurs at the level of RGCs and retinogeniculate connections (Fig 4).

The classical complement cascade also plays a pathologic role, as recently discovered in certain neurodegenerative diseases. In mice, the healthy adult CNS has a low presence of C1q, but in the adult retina, C1q becomes upregulated and synaptically relocalized in RGCs in DBA/2J mice [104]. This strain spontaneously develops a glaucoma-like disease, which includes degeneration of RGCs[106], and genetic deletion of C1q helps protect them from this fate [107]. A remarkably similar finding in Alzheimer's disease models was recently

discovered[108], possibly suggesting parallel mechanisms in glaucoma. Authors showed that C1q associated with synapses before overt plaque deposition and that abrogating the classical complement cascade reduces the extent by which microglia eliminate early synapse loss in the hippocampus [108]. A similar mechanism was demonstrated in a neuroinvasive disease mouse model of West Nile virus infection[109].

Of note, dysregulation of complement, albeit the alternative cascade, is implicated in AMD - a different neurodegenerative condition[110, 111]. However pathogenesis in AMD is thought to involve upstream accumulation of pathologic lipid deposits under the retina, as opposed to synapse elimination. This topic has been reviewed elsewhere [112].

These studies underscore a dichotomous nature of microglia. On the one hand, C1q enables microglia to shape healthy neuronal connections in the visual system, while on the other hand, the inappropriate reactivation of C1q in adulthood is suspected to make microglia pathogenic in glaucoma-like neurodegeneration. It is undefined as to whether this mechanism is involved in photoreceptor degeneration, which we will focus on in the next section. In addition, infiltrated monocytes are also involved in retinal degeneration, including in glaucoma models[113]. Hence, discriminating such recruits versus microglia, and parsing out their respective unique involvements in retinal degeneration, is another important area of research.

Phenotypical differences of microglia vs. monocyte-derived cells in the retina

While it is now understood that microglia have distinct origins from monocyte-derived macrophages that are recruited into the inflamed CNS[4], distinguishing between the two populations is technically challenging. Their different origins were demonstrated in a seminal study that used parabiosis to demonstrate that microglia are not replaced by circulating monocytes, but are instead a long-lived, self-renewing population [25, 26]. Subsequent findings by another group showed that adult microglia are seeded *in utero* by yolk sac-derived progenitors[5]. Of note, this ontogeny is in contrast to the origins of macrophages that reside in the periphery, as macrophage populations from peripheral tissues show mixed ontogenies (that is, they can be comprised of fetal liver-derived monocytes and/or adult bone marrow-derived monocytes)[27, 114, 115, 116, 117].

The retina in degenerative conditions is permissive to the recruitment of monocytes, which differentiate into macrophages[113, 118, 119, 120, 121]. These recruits share phenotypical markers with microglia. Numerous approaches have been devised to study these two populations independently, but many have notable drawbacks (Table 1). These approaches include mice bearing a green fluorescent protein (GFP) reporter gene knockin under the promoter control of the fractalkine receptor CX_3CR1 ($Cx3cr1^{GFP/WT}$), and a red fluorescent protein (RFP) under the chemokine receptor CCR2 promoter ($Ccr2^{RFP/WT}$). However, microglia and monocyte-derived macrophages are both CX3CR1(+) and CCR2(-)[119], and thus this approach cannot faithfully discriminate between the two populations. Another approach utilizes bone marrow chimeras to distinguish between microglia (host-derived) and recruited monocytes (donor-derived). However, the combination of irradiation and bone marrow transplantation (BMT) results in low-grade pathology and the recruitment of monocyte-derived cells (or their precursors) into the retina[26, 119, 121, 122]. Parabiosis is another

approach that can be used to circumvent this problem [26], but drawbacks include partial blood chimerism and technical challenges.

Only a Cre-Lox approach has been shown to faithfully identify the two compartments simultaneously, as devised independently by two groups [100, 116], and we subsequently established in the retina[119]. The strategy utilizes *Cx3cr1*^{CreER} transgenic mice for conditional Cre-LoxP-mediated modifications of CX3CR1-expressing cells, including microglia, monocytes and macrophages[100, 116, 119]. The long-lived microglia retain those modifications while short-lived monocytes are replaced by non-modified monocytes within a few weeks (Fig 5). Our group has mated the *Cx3cr1*$^{YFP\text{-}CreER}$ mouse to a R26^{RFP}Cre reporter[119]. After tamoxifen activation of Cre and a short 'wash out' period, we were able to faithfully discriminate RFP-labeled microglia from non-labeled monocyte-derived cells in the light-induced model of retinal degeneration. Furthermore, we found that retinal microglia have a unique surface marker phenotype (that is, CD45^{lo}, F4/80^{lo}, CD11c^{lo}, MHC class II^{lo}), which is preserved in the degeneration setting. In contrast, recruited monocyte-derived macrophages are CD45^{hi}, F4/80^{hi}, CD11c^{hi}, MHC class II^{hi} [119], which is consistent with respect to CD45 expression shown in earlier work[123]. We also identified a small but significant population of MHC class II^{hi} macrophages in the retina, similar to what was previously shown[124]. Intriguingly, we showed that these MHC class II^{hi} cells are long-lived, albeit radiosensitive, macrophages[119]. The respective roles of microglia versus monocyte-derived macrophages in retinal degenerative are further reviewed in the next section.

Microglia and monocyte-derived cells in photoreceptor degeneration

Exploration of the non-redundant contributions of microglia and monocyte-derived cells in neurodegenerative diseases has only recently begun, but early studies suggest important differences[125] . In a model of light-induced retinal degeneration, our lab used the *Cx3cr1*$^{CreER\text{-}YFP}$:R26^{RFP} mouse described above to definitively discriminate RFP-labeled microglia from non-labeled monocyte-derived macrophages[119] (Fig 5, Table 1). Interestingly, these two cell types localized to different regions of the photoreceptor cell. Whereas the majority of microglia moved to below the photoreceptor outer segment, monocyte-derived macrophages appeared throughout the depth of the degenerating retina, including next to the photoreceptor soma. Hence, it may be possible that microglia are removing distal debris of the outer segment, whereas monocyte-derived cells may be phagocytosing around the photoreceptor soma; but this remains to be proven. Evidence of differential localization of MPS cells in the spinal cord was also recently demonstrated in EAE. Authors showed that monocyte-derived macrophages made axoglial contact, whereas microglia processed debris at a more distal location[125].

The functions of specific MPS populations in retinochoroidal disease was also recently reported[126]. The authors used a mouse model that mimics certain features of AMD pathogenesis. In this model, a laser burn is administered to rupture Bruch's membrane, which separates the retinal pigment epithelium (RPE) and the choroid. This perturbation leads to the outgrowth of new blood vessels and vascular leakage into the retina, similar to what is seen in neovascular AMD. The authors used *Cx3cr1*^{CreER} mice to delete the floxed gene for the interferon-α/β receptor (*Ifnar1*) prior to laser injury in retinochoroidal

macrophages, which include retinal microglia and putative CX3CR1+ macrophages in the choroid. Strikingly, the severity of choroidal neovascular lesions and vascular leakage was worsened by conditional deletion of IFNAR1 in retinochoroidal macrophages, suggesting a central protective role for IFNAR1 signaling. While these findings are important, it was not determined whether the effect was due to conditional loss of IFNAR1 in microglia, in choroidal macrophages or from both populations (Table 1). This question is significant because the injury originates at the choroid and consequent vascular leakage is due to choroidal neovessels. Regarding human disease, while IFNα2 therapy has been tried in patients with AMD with little success [127], not much is known about IFNβ therapy in this regard.

With respect to retinitis pigmentosa, it was shown that MPS cells actively phagocytose injured photoreceptors[128]. The authors generated a *Cx3cr1*^{CreER};R26^{DTA} (diphtheria toxin A) mouse for tamoxifen-induced selective depletion of all CX3CR1+ cells, which includes microglia and monocyte-derived cells (Table 1). This transgene combination was bred onto mice homozygous for the retinal degeneration 10 (rd10) mutation of phosphodiesterase 6B (*Pde6b)*, which develop photoreceptor degeneration within the first three weeks of life. With continuous depletion of CX_3CR1^+ phagocytes, mice showed delayed and reduced degeneration out to post-natal day 50. However, methods used could not discriminate whether microglia and/or monocyte-derived cells were the effector phagocytes because both populations would be depleted in this system (Table 1). Furthermore, depletion was not started until post-natal day 21, a time when monocyte recruitment presumably had already begun since retinal levels of CCL2 (which signals via CCR2 to promote monocyte recruitment) are significant at this time-point[129]. Nonetheless, these findings suggest that the mononuclear phagocytes have important pathogenic roles in certain forms of inherited degeneration.

Other reports suggest an isolated contribution of monocyte-derived cells to the pathobiology of photoreceptor degeneration in mouse models, although the effector mechanisms are not completely understood. It was demonstrated that monocyte-derived cells, recruited through CCR2, contribute to the spontaneous age-related photoreceptor degeneration that occurs in CX3CR1-deficient mice [120], and a similar pathway was also found to be relevant in human AMD and photoreceptor degeneration [130]. Likewise, another study showed that CCR2-deficient mice bred onto *Pde6b*^{rd10} mice reduced photoreceptor degeneration [129], although the effect was less dramatic than what was seen in the aforementioned report using *Cx3cr1*^{CreER};R26^{DTA} mice [128].

Finally, the type 2 cytokine, IL-33, has recently emerged in the literature as a player in retinal degenerative disease. New findings suggest that pathogenic monocyte recruitment in photoreceptor degeneration is mediated by IL-33, which is produced by Müller glia [131]. For reasons not fully understood, another group found that IL-33 was not relevant in recruitment activity, albeit using a different model system in laser induced choroidal neovascularization model. In this system, IL-33 played a central role in regulating pathologic neovascularization at the level of the choroid[132]. In contrast, IL-33 does have a role in recruitment of monocytes following optic nerve crush in mice [133] (which models aspects of glaucoma), as well as in experimental spinal cord injury. Intriguingly, this monocyte-

recruitment activity played a reparative role in the disease setting, as previously shown in a model of glutamate intoxication of RGCs[113]. Hence, monocyte-derived cells are not universally pathogenic in all forms of retinal degeneration.

To summarize, in the steady state, microglia are important for maintaining healthy synapses. In degenerative diseases, microglia are not always protective and monocyte-derived cells are not always pathogenic. Furthermore, recent findings show that both the local environment and epigenetics shape macrophage identity and function in the steady state[134, 135, 136, 137], which may suggest that monocyte-derived cells can take on microglial functionality in neurodegeneration, or vice versa. Hence, future work is required to continue to parse out these different compartments, and to help determine if and how therapies can be designed to specifically target either cell types [98].

Concluding remarks

The advent of new techniques for assessing the ontogeny of MPS cells has increased our understanding of the roles of these cells in breaching immune privilege and in the pathobiology of neurodegenerative disease. Ontogeny research has helped show that DCs are indeed a unique branch of the MPS, and in turn will facilitate the discovery of DC-specific mechanisms in the formation of adaptive immunity relevant to privileged sites, including the cornea and retina. Ontogeny studies have also helped distinguish microglia from monocyte-derived cells and the non-redundant functions of these cell types in neurodegeneration (including in that of the retina) are now being addressed. Though complexities and open questions still remain, the reciprocal relationship between research advances in ontogeny and function, as outlined here, is augmenting our understanding of the MPS. In so doing, the vast implications of these cells in health and disease promises to become fully realized, for the visual system and throughout the human body.

Acknowledgments

Grant Funding: R01 (Saban), RPB (Saban), F32 (Reyes)

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Key points

1. Pathological lymphangiogenesis of the adult cornea is associated with breach of corneal immune privilege, and is permissive for MPS cells in triggering T cell responses.
2. Severing corneal nerves in one eye causes a breach in corneal immune privilege in both eyes, suggesting the presence of a neuro-immune reflex that may involve MPS cells.
3. Commensals in the gut, and perhaps gut MPS cells, are capable of triggering T cells that cause autoimmune of the immune privileged retina.
4. While microglia play a key role in shaping healthy synapses in the visual system, these cells may also contribute to of retinal ganglion cell dysfunction in glaucoma.
5. Microglia and monocyte derived cells are both present in the retina in a model of photoreceptor degeneration, and these distinct MPS lineages possess phenotypical differences, as confirmed in Cx3cr1^{Cre} systems.
6. Early studies using such Cx3cr1^{Cre} systems implicate the possibility for functional specializations of microglia versus monocyte-derived cells in photoreceptor degenerative diseases.

Box 1. Evolution of immune privilege

Sir Peter Medawar coined the term ‘immune privilege’ in the 1940s, using it in the context of transplant immunology. In contrast to allogeneic grafts placed within conventional sites like the skin, those placed in the brain parenchyma or the eye anterior chamber were protected from the fate of immune rejection for extraordinarily long intervals or even indefinitely, in the absence of neovascularization [138]. He also made the observation that privileged sites lacked the presence of patent lymphatic vessels, which at the time explained his theory for how these sites were afforded “privilege” from certain adaptive immune responses. New findings, however, suggest that non-conventional pathways of drainage permit antigen from these sites to access lymphatic networks or the spleen [34, 36, 78, 139, 140]. Yet still, this level of access is limited [38] and thought to be preferential to activation of regulatory immune networks [141], such as anterior chamber associated immune deviation (ACAID) described by Wayne Streilein and colleagues [11]. Furthermore, innate immune responses are suppressed in immune privileged sites [142], and breach of the blood brain barrier by T cells is relatively limited in the steady state [15].

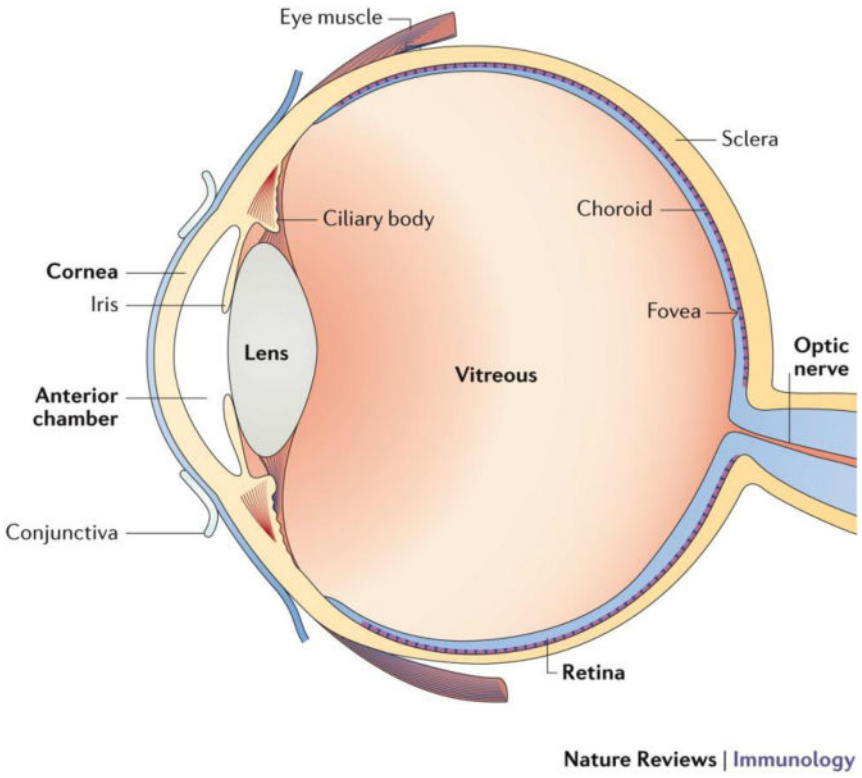

Figure 1. Immune privilege sites of the eye

The eye is divided into an anterior and a posterior segment. The anterior segment includes the cornea, anterior chamber, iris, and lens. The posterior segment includes the vitreous, retina, and choroid. Immune privileged sites and tissues of the eye include the cornea, anterior chamber, lens, vitreous, retina, and subretinal space. MPS cell types in the eye include: Cornea (DCs and macrophages [29]); iris (DCs and macrophages [30]); cilliary body (macrophages [30]); choroid (macrophages [30]); retina (microglia [119]).

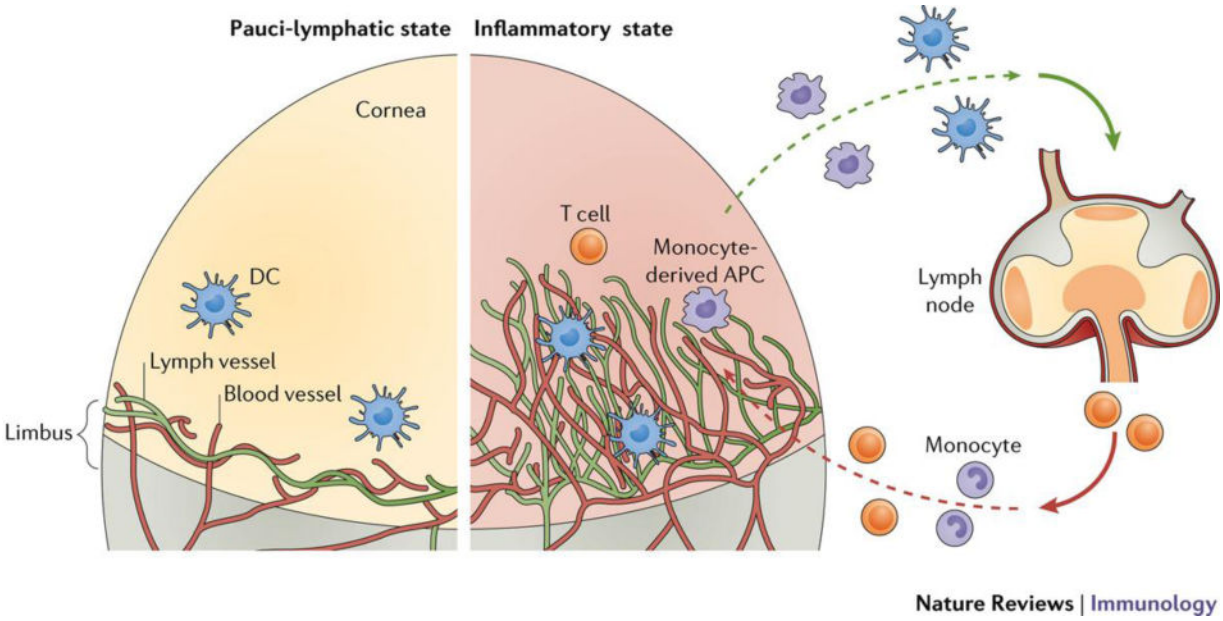

Figure 2. Pauci-lymphatic state and corneal immune privilege

Under normal physiological conditions, drainage is limited, as lymphatic (and blood) vessels are restricted to the limbus. Several key factors have been identified that retain the cornea in this pauci-lymphatic state (see text). These mechanisms are subverted in certain inflammatory settings, and lymphangiogenesis results in lymphatic vessel invasion of the cornea. The latter is associated with amplification of DC egress and consequent T cell activation in the draining LN.

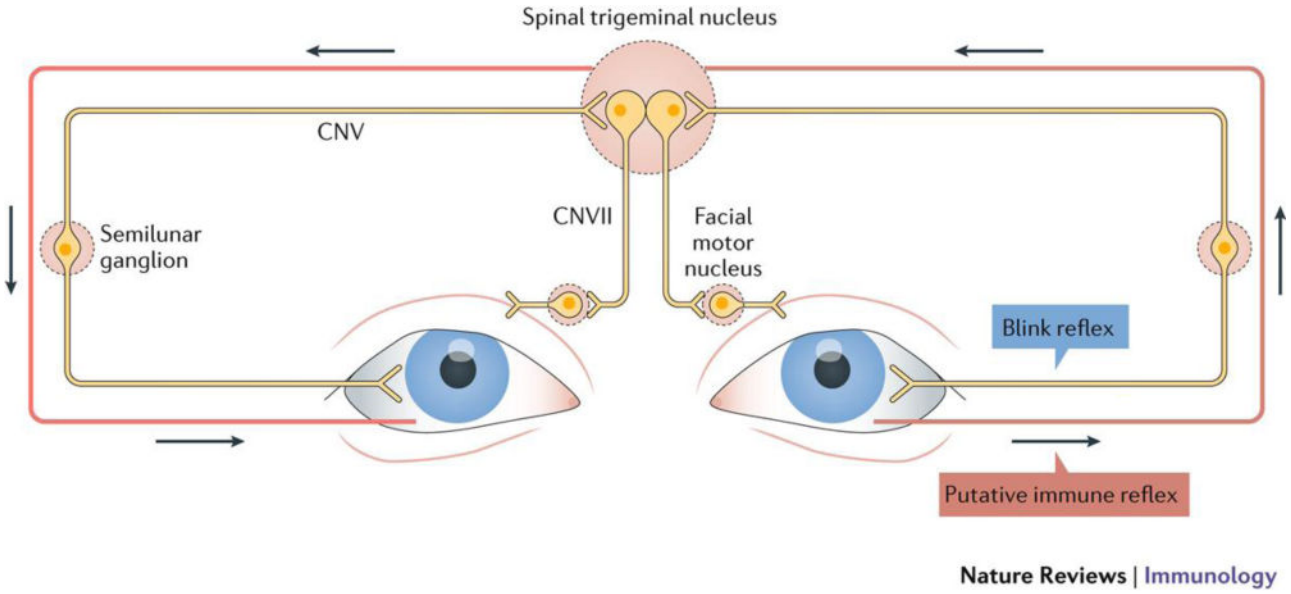

Figure 3. Putative neural response in bilateral breach of corneal immune privilege

The somatosensory nerves of the blink reflex arc (outlined in grey) may serve as the conduit for bilateral breach in corneal immune privilege (outlined in red). The blink reflex arc is initiated from corneal stimulation such as touch, which signals through cranial nerves (CN), V to VII via the trigeminal nucleus, resulting in blinking in both eyes. Severing corneal nerves in a certain manner in one eye results in a breach of corneal immune privilege in the fellow eye, which is mediated by substance P. Breach in immune privilege through this pathway may be beneficial in the setting of infection, where infection in one eye may prepare the bilateral eye by allowing it to mount an adaptive immune response.

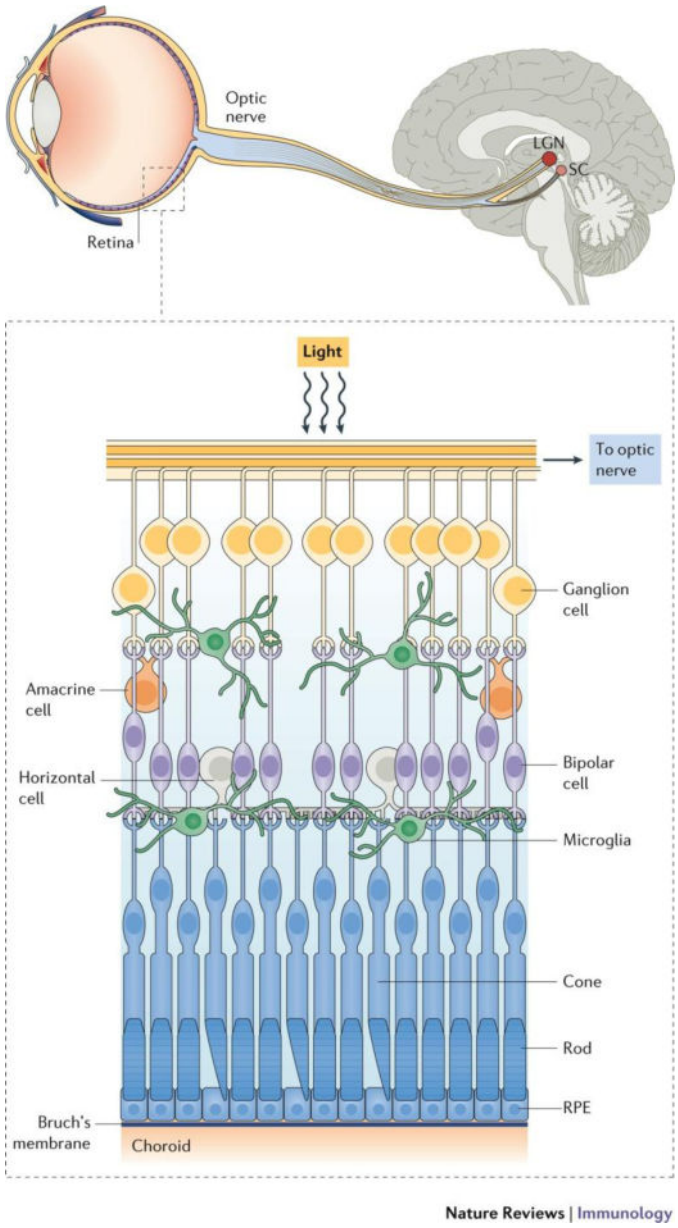

Figure 4. Visual circuit and cell types in the retina

Light travels through the cornea and lens to the retina, where photoreceptor cells (PR) translate light into signals that are relayed from bipolar cells to the retinal ganglion cells (RGC). Axons extend outside of the retina through the optic nerve to the lateral geniculate nucleus (LGN), where the signal is passed to neurons that carry it to the visual cortex. Microglia reside in and extend their processes throughout the synaptic regions in the neural retina. Retinal pigmented epithelium (RPE) is very important for the visual cycle function, and has tight junctions and other factors that aid in the maintenance of retinal immune privilege. The area between the RPE and PRs is designated the subretinal space. Beneath the RPE are the choroid and sclera which comprise the larger outer structure of the eye. In AMD, pathogenesis begins at the level of the choroid, whereas photoreceptors is the primary

site of degeneration in retinitis pigmentosa. In contrast, the RGCs and their axons degenerate in glaucoma.

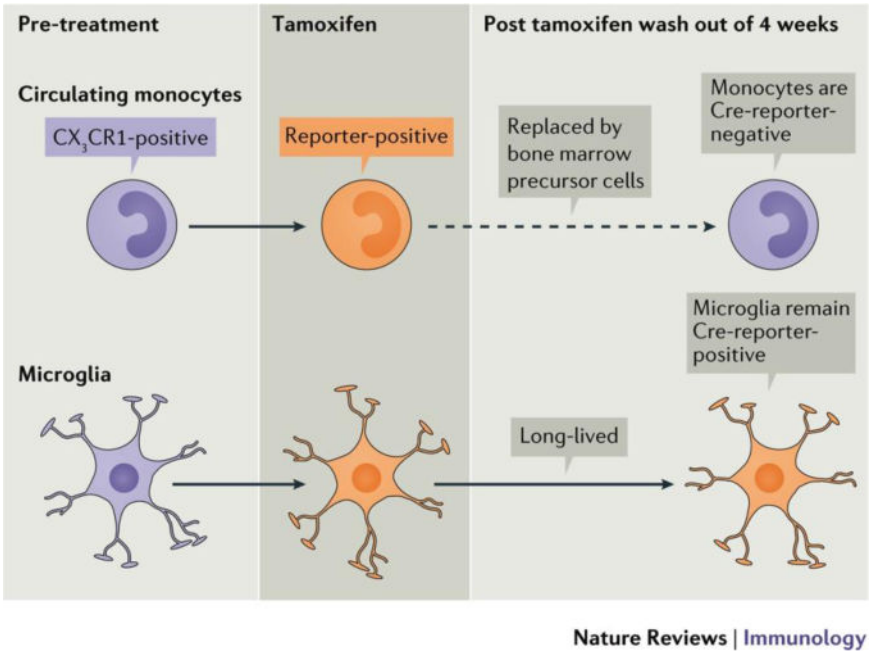

Figure 5. Definitive discrimination of microglia vs blood monocytes

Use of Cx3cr1^{CreER};Reporter^{flox} (e.g. RFP, DTR, etc.) mice allows faithful visualization of microglia, which requires the following time considerations depicted in this figure. After tamoxifen (Tam) administration, microglia retain reporter expression indefinitely, whereas circulating monocytes retain expression for 4 weeks. We refer to this time-frame as the "wash out" period, which correlates with monocyte turnover rates from the bone marrow. Hence, faithful reporter visualization in microglia alone requires inclusion of the wash out period.

Table 1

Advantages and disadvantages of numerous mouse models used to study microglia

Common techniques for studying microglia	*Utilization and Advantages*	*Disadvantages*	*Ref.*
Chimeras			
Bone marrow (BM) chimera	• Full blood chimerism • Broad repertoire for donor genotype (e.g. reporters, KO, etc.) • Faithful identification of donor-derived cells (e.g. GFP)	• Altered steady state due to irradiation • Temporary disruption of blood retina barrier • Out-population of microglia by recruited monocytes	26, 119, 121, 122
BM chimera with lead shielding of head	• Faithful identification of donor-derived cells (e.g. GFP) • Broad donor repertoire (see above) • Preserves retina in the steady state	• Cannot achieve full blood chimerism	119, 122
Parabiosis *	• Faithful identification of donor-derived cells (GFP) • Broad donor repertoire (see above) • Preserves CNS in the steady state	• Cannot achieve full blood chimerism • Technically challenging	25, 26
Null Reporter mice			
$CX3CR1^{eGFP/eGFP}$ or $CX3CR1^{eYFP/eYFP}$	• Efficient deletion of Cx3cr1 gene • Heterozygotes are good for MPS visualization • Homozygotes model age-related photoreceptor degeneration	• Not specific for microglia, as monocytes and macrophages express Cx3cr1	19, 119, 120
$CCR2^{RFP/RFP}$	• Efficient genetic deletion of Ccr2 • Heterozygotes good for imaging classical monocytes • Homozygotes have inhibited entrance of classical monocytes into the circulation from BM	• Not good for imaging macrophages, which lose CCR2 expression • Spares non-classical monocytes	119, 120, 122
Cre-Lox combinations			
$CX3CR1^{CreER}$ or $CX3CR1^{eYFP\text{-}CreER}$	• “Wash out” (see Fig 5) following tamoxifen pulsing in Cre reporter mice (e.g. RFP^{flox}) allows faithful and simultaneous	• Long-lived tissue resident macrophages (CX3CR1+) in the periphery are also labeled, such as	100, 116, 119, 126

Common techniques for studying microglia	*Utilization and Advantages*	*Disadvantages*	*Ref.*
	visualization of microglia vs. macrophages • Wash out (see above) allows specific conditional gene deletion specific to microglia • Non-invasive	putative choroidal macrophages	
CX3CR1^{CreER}; DTA ^{flox}	• Efficient depletion of MPS • Does not require DTx	• Depletes both monocytes and microglia • Tissue resident macrophages (see above) are also depleted	128
CX3CR1^{CreER}; DTR ^{flox}	• Efficient depletion of microglia alone following washout	• Requires DTx administration • Depletes long-lived macrophages in the periphery (see above)	100

* Never been done in the retina; only brain and spinal cord

Abbreviations: CCR2, CC-chemokine receptor 2; CNS, central nervous system; CreER, Cre recombinase – estrogen receptor; CX3CR1, CX3C-chemokine receptor 1; DTx, diphtheria toxin; eYP, enhanced yellow fluorescent protein GFP, green fluorescent protein; KO, knockout; MPS, mononuclear phagocyte system; RFP, red fluorescent protein;