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Immune cells in the retina and choroid: Two different tissue environments that require different defenses and surveillance

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Abstract

In the eye immune defenses must take place in a plethora of differing microenvironments ranging from the corneal and conjunctival epithelia facing the external environment to the pigmented connective tissue of the uveal tract containing smooth muscle, blood vessels and peripheral nerves to the innermost and highly protected neural retina. The extravascular environment of the neural retina, like the brain parenchyma, is stringently controlled to maintain conditions required for neural transmission. The unique physiological nature of the neural retina can be attributed to the blood retinal barriers (BRB) of the retinal vasculature and the retinal pigment epithelium, which both tightly regulate the transport of small molecules and restrict passage of cells and macromolecules from the circulation into the retina in a similar fashion to the blood brain barrier (BBB). The extracellular environment of the neural retina differs markedly from that of the highly vascular, loose connective tissue of the choroid, which lies outside the BRB. The choroid hosts a variety of immune cell types, including macrophages, dendritic cells (DCs) and mast cells. This is in marked contrast to the neural parenchyma of the retina, which is populated almost solely by microglia. This review will describe the current understanding of the distribution, phenotype and physiological role of ocular immune cells behind or inside the blood-retinal barriers and those in closely juxtaposed tissues outside the barrier. The nature and function of these immune cells can profoundly influence retinal homeostasis and lead to disordered immune function that can lead to vision loss.

Keywords

Microglia; Age-related macular degeneration; Retinitis pigmentosa; Retinal degeneration; Macrophages; Dendritic cells; Mast cells

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1. Introduction

Sharing many similarities with the brain and spinal cord, the retina is also part of the central nervous system (CNS) and likewise is protected behind specialized blood-tissue (ocular) barriers. The retina consists of a complex matrix of interconnected specialized heterogeneous types of neurons such as photoreceptors, bipolar cells (interneurons) and ganglion cells which are responsible for conversion of light from the external environment into neural impulses that are relayed to the brain where they are perceived as visual information. The neural retina is supported by glial cells such as Müller cells and astrocytes, which perform a range of critical functions essential to normal neural conduction. Microglia (MG), named at a time when it was not appreciated that they were not true glia, are yolk sac-derived specialized members of the macrophage lineage of immune cells that are interspersed within the highly layered neural retina. The neural retina is a concave hemispherical sheet of neural tissue that lies in close apposition to the other highly specialized component of the retina, the retinal pigment epithelium (RPE) (Fig. 1A–C). In addition to tight junctions between the vascular endothelial cells of retinal vessels, tight junctions between the RPE also form a critical part of the blood-retinal barrier (BRB). The RPE is neuroectoderm-derived and separates the neural retina from underlying choroid (Fig. 1C–E). The demands of maintaining an extracellular environment conducive to rapid neural conduction and the physiological and metabolic demands of a specialized thick retina (150–320 μm in humans), such as that present in primates, including man (in comparison to many animals with thinner avascular retinæ), has meant that the inner (vitread aspect) retina requires a blood supply to supplement the vascular choroid which nourishes the outer (or sclerad aspect) retina. The retinal blood supply displays highly specialized blood barrier properties identical to those seen in the brain parenchyma, namely the exclusion of large molecular weight molecules, plasma proteins, erythrocytes and leukocytes and the ability to regulate the ionic environment. The exclusion of leukocytes including granulocytes, myeloid-derived cells and lymphocytes from the normal retina contributes to what many refer to as ‘immune privilege’, which is traditionally defined as the property of a tissue to display highly attenuated responses to alloantigens. The historical context of ascribing a tissue, such as the retina, as having ‘immune privilege’ and its influence on active and passive regulation of immune responses is reviewed in detail elsewhere (Forrester et al., 2018b; Reyes et al., 2017).

The choroid contrasts sharply in structure and function to the neural retina. It is a highly vascularised, pigmented loose connective tissue that constitutes the posterior part of the uveal tract (Fig. 1A–C). It supplies trophic and metabolic support to the outer retina. It is homologous to the pia-arachnoid of the brain and its vessels do not feature the stringent barrier properties of those within the retina or brain parenchyma; indeed, the capillary bed of the choroid, the choriocapillaris, is highly fenestrated (Fig. 1D and E). The choroid lies internal to the sclera, which consists of dense irregular connective tissue, the homologue of the dura mater with which it is continuous at the optic nerve. Just as advances in the field of meningeal immunity have begun to reveal the complex nature of this compartment and its role in directing and coordination of immune cell traffic throughout the CNS (Rua and McGavern, 2018), it is likely that we are at the beginning of a similar renaissance in

awareness of the importance of the immune system in the choroid to retinal health. Like other loose connective tissues, including the leptomeninges (Chinnery et al., 2010; Coles et al., 2017; McMenamin, 1999b; Rua and McGavern, 2018), the normal mammalian choroid contains networks of tissue macrophages, dendritic cells (DCs) and mast cells (Chinnery et al., 2017; Forrester et al., 2010; McMenamin, 1997, 1999a). The recent ‘re-discovery’ of lymphatics in the dura of the mouse brain (Aspelund et al., 2015; Louveau et al., 2015, 2016) has heightened the search for uveal tract lymphatics in mammals; however, to date there is no convincing evidence of choroidal lymphatics in the mammalian eye (Schroedl et al., 2014). A more detailed discussion on the role of so-called ‘glymphatics’ in drainage of interstitial fluid in the brain parenchyma and its potential role as a route for immune cell trafficking is discussed in detail elsewhere (Forrester et al., 2018b).

Within the closely related and anatomically approximated ocular microenvironments of the neural retina and choroid exists a contrast in the nature of immune cells that are essential to immune surveillance and defense, ranging from the highly specialized MG population of the retina to the diverse array of macrophages, DCs and mast cells of the choroid. These two highly specialized defense systems must both support homeostatic functions and yet coordinate appropriate inflammatory responses to pathogenic or injurious stimuli. Whilst there may be a degree of so-called ‘immune privilege’ in the retina the occurrence of a range of diseases such as uveoretinitis, retinal vasculitis in Bechet’s disease and various pathogen driven inflammatory diseases reminds us that this protected environment can indeed be the site of vision threatening inflammatory or immune-mediated disease. Similarly, the choroid is not exempt from immune-mediated disease with autoimmune responses to melanocyte-specific antigens being the underlying cause of Vogt-Koyanagi-Harada syndrome (VKH) (Lambe et al., 2006) and choroiditis is invariably involved in inflammatory processes affecting retina (Forrester et al., 2018a). In this review, we discuss the location and function of a diverse range of immune cells within the choroid and how they have a critical role in immunoregulation of the retinal environment as a secondary consequence of their critical location so close to the neural retina. Dysregulation of choroidal immunity may contribute to a range of retinal disorders and here we speculate that it may act as surrogate ‘lymphoreticular organ’ whose function in addition to providing the obvious nutritional support to the retina may include an immunoprotective role.

2. ‘Behind the barrier’ – the retinal microglia and their biology

2.1. Distribution, phenotype and function of microglia

First introduced in 1919 by Pio del Rio-Hortega, the term ‘microglia’ was used to describe a population of non-neuronal brain cells that were morphologically and functionally distinct from astrocytes and oligodendrocytes, but still thought to be of neuro-ectodermal origin. del Rio-Hortega’s series of 1919 landmark papers was recently translated into English and published by Sierra and co-workers (Sierra et al., 2016) allowing a wide audience to appreciate the papers that launched the modern era of glial and microglial research. In his first 1919 publication, del Rio-Hortega indicated that microglia were named so to highlight the size of their soma which is significantly smaller than that of astrocytes. In subsequent publications, Rio-Hortega refined his definition of MG and argued that these cells were in

fact of mesodermal, leukocytic origin and thus did not belong to the glial cell lineage (Del Rio-Hortega, 1939; Sierra et al., 2016), which in the retina includes astrocytes and Müller cells. Other investigators provided support for the hypothesis that MG were of mesodermal origin by demonstrating that these cells displayed similar ultrastructural characteristics to macrophages (Murabe and Sano, 1982) and expressed the mouse macrophage antigen F4/80, the IgG1/2b Fc receptor and the complement receptor Mac-1 (Hume et al., 1983; Perry et al., 1985). Although their origin has been widely debated in the literature (discussed below), MG are now recognized as a specialized macrophage population within the CNS with distinct developmental origins to glial cells. Thus, the 'glia' component of their name could be considered by some to be misleading.

In the healthy retina, MG are distributed within three discrete laminae - the nerve fiber layer/ganglion cell layer, and the two neuropil layers, called the inner plexiform and outer plexiform layers (Diaz-Araya et al., 1995; Hume et al., 1983) (Fig. 2A). Normal retinal MG in these plexiform layers are highly ramified and feature a small soma projecting multiple, branching, fine cellular processes (Fig. 2B and C). Phenotypically they are CD45 intermediate (also referred to as CD45 low), CD11b⁺ and Cx3cr1⁺, identical to brain cortical MG (Fig. 2D) and elsewhere in the CNS. Also similar to brain MG, those in the retina exist in a surveillant form, in which their processes are continuously extending and retracting in all directions in a random and repeated fashion (Lee et al., 2008; Nimmerjahn et al., 2005). The activity-dependent dynamic process motility of retinal MG is thought to be driven by extracellular ATP produced by Müller cells (Wang and Wong, 2014). *Ex vivo* time-lapse confocal microscopy of retinæ from CX3CR1^{gfp/+} mice revealed that MG survey the extracellular environment of their territory several times within a period of a few minutes. Consistent with their highly organized, tiled arrangement within the retina (Fig. 2A, C), the processes of single surveillant MG cells do not cross over into neighboring MG territories (Fig. 2B); however, transient contacts between the terminal processes of neighboring MG may occasionally form (Lee et al., 2008). In the absence of fractalkine (CX3CL1-CX3CR1) signaling in CX3CR1^{gfp/gfp} mice, the process velocity of retinal MG is significantly reduced compared to mice in which fractalkine signaling is preserved, suggesting that interactions between CX3CR1⁺ MG and CX3CL1⁺ neurons partially regulate the dynamic behavior and function of retinal MG (Liang et al., 2009). In retinal MG depletion studies, whereas these cells repopulate the retina efficiently (as previously shown in brain (Elmore et al., 2014)), CX3CR1-deficient mice showed impaired MG repopulation. Thus, CX3CL1-CX3CR1 neuron-MG chemokine signaling is also involved in MG repopulation, suggesting that neuron-MG interactions are important for maintaining homeostasis (Zhang et al., 2018), perhaps by increasing proliferation and morphological maturation.

Efficient multi-tasking cells, MG have several known functions including immune surveillance, modulation of immune responses, phagocytosis, synaptic pruning, maintenance of synaptic structure and function, as well as regulating neurogenesis and axonal growth (recently reviewed by (Rathnasamy et al., 2018)). One of the most widely studied functions of MG is phagocytosis, which occurs in both the healthy and diseased CNS. The main phagocytic targets of MG in the brain and retina include pathogens, dead cells, dying cells and protein aggregates. In the brain, MG phagocytosis of apoptotic neurons is mediated by triggering receptors expressed on myeloid cells-2 (TREM2) (Kawabori et al., 2015; Kim et

al., 2017). Stimulation of TREM2 induces DAP12 phosphorylation, extracellular signal-regulated kinase phosphorylation and cytoskeleton reorganization, which facilitates phagocytosis (Takahashi et al., 2005). TREM2 has not been studied in detail in the retina; however, due to close parallels between brain and retinal MG, TREM2 may also play an important role in MG phagocytosis of apoptotic neurons in the retina. In addition to their known role phagocytosing apoptotic neurons, recent studies have demonstrated that MG are also capable of phagocytosing live (but stressed) neurons, causing death of the engulfed cell (termed ‘phagoptosis’ or primary phagocytosis) (Brown and Neher, 2014). In the rd10 mouse model, Zhao et al. showed data to suggest that retinal MG phagocytose living, non-apoptotic rods that present “eat me” signals, rather than dead and apoptotic cells, suggesting that phagoptosis may be a function of retinal MG in rd10 inherited retinal degeneration (Zhao et al., 2015). A caveat of this study is that the continual tamoxifen administration used is alone sufficient to rescue retinal structure function in rd10 mice [see (Wang et al., 2017)]. Also, the authors did not distinguish MG from infiltrating myeloid cells; however, recruited CCR2⁺ cells were predominantly located in the subretinal space, had limited contact with photoreceptors and lacked evidence of intracellular phagosomes. This may suggest that MG, rather than recruited CCR2⁺ cells, are responsible for phagocytosis of stressed rods in the rd10 model, although this remains to be confirmed as extravasated monocytes lose CCR2 expression and thus cannot be tracked using *CCR2^{RF}* mice (reviewed in (Reyes et al., 2017)).

The question of whether MG contribute to maintenance of the structure and function of the retina, and/or visual function was recently addressed by (Wang et al., 2016). These authors used *CX3CR1^{CreER}; Rosa26^{LSL-DTA}* mice to deplete CX3CR1-expressing MG in the steady state by administering repeated doses of tamoxifen, up to day 35. These studies reported no effect on the architecture, laminar organization or overall thickness of the retina, nor the survival of retinal neurons. However, MG depletion did lead to reduced functional electroretinographic responses, and these authors detected altered synaptic ribbons by transmission electron microscopy in the outer plexiform layer of depleted mice. A more precise depletion system that targets MG in the outer plexiform layer alone will be important to confirm these conclusions.

Due to their location behind the blood-retina/blood-brain barriers, MG exist within an ‘immune privileged’ microenvironment (see review, (Forrester et al., 2018b)). Although the expression of phenotypic markers (i.e. CD11b, CX3CR1, F4/80, CD115, Iba-1) on MG overlaps significantly with that of other cells within the mononuclear phagocyte system, Goldmann et al. have demonstrated that normal brain MG have a distinct RNA profile compared to bone marrow-derived monocytes (Goldmann et al., 2016). Analysis of differentially expressed genes revealed that MG expressed uniquely low levels of CD45 compared to monocytes and perivascular macrophages (PVM – see Box). These data support flow cytometry studies, which demonstrated that brain and retinal MG were CD45^{int}, whereas choroid plexus and meningeal macrophages were CD45^{hi} (Dando et al., 2016). Likewise, O’Koren et al. showed that retinal MG maintain this low/intermediate CD45 expression level in light-damage model of retinal degeneration (O’Koren et al., 2016). Single cell RNA sequencing also revealed that brain MG uniquely expressed the purinergic receptor *P2ry12* compared to monocytes and PVM (Goldmann et al., 2016). Bennet et al. confirmed

that *P2ry12* was highly expressed and enriched in brain MG; however, found that only transmembrane protein 119 (Tmem119) was expressed by brain parenchymal MG and not by choroid plexus or meningeal macrophages or PVM. These authors demonstrated that Tmem119 robustly distinguished MG from macrophages, other immune cells and neural cells in health (Bennett et al., 2016). It was recently confirmed that mouse retinal MG also express Tmem119 (Deliyanti et al., 2017); however, it is unclear whether Tmem119 is a true MG-specific marker in the disease setting.

In the early 1990s immunoperoxidase staining of post-mortem human material suggested that retinal MG constitutively express MHC class II antigens and share phenotypic characteristics with professional antigen presenting cells (APCs) (Penfold et al., 1993). However, it is now widely accepted that the majority of MG in the normal retina do not express MHC class II (Dick et al., 1995) or the co-stimulatory factors required for antigen presentation. The absence of MHC class II expression by retinal MG was also supported by recent work using *Cx3cr1^{CreER}; R26^{tdTomato}* mice (Q'Koren et al., 2016). Furthermore, functional antigen presentation studies of mouse retinal MG have clearly demonstrated that these cells have a limited capacity to act as APCs (Gregerson et al., 2004). Some studies have reported that the normal mouse retina contains very small numbers of MHC class II⁺ cells near the ciliary body-retinal junction and at the optic nerve head (Dando et al., 2016; Xu et al., 2007b) (Fig. 2E). Xu et al. suggested that these cells are putative tissue-resident DCs and are distinct from MG. The true identity, ontogeny and function of these cells remains to be investigated, although it is tempting to speculate that their location at sites where the BRB is not fully developed may influence the phenotypic characteristics of a few adjacent MG. Other studies have suggested that the normal retina contains more substantial populations of putative DCs. Using the CD11c-DTR-GFP transgenic mouse line, Lehmann et al. identified 95 ± 42 GFP⁺ cells in the normal retina and proposed that these cells represented a small population of resident DCs (Lehmann et al., 2010). Phenotypically, these cells were indistinguishable from CD45^{int} CD11b⁺ MG; however, the authors demonstrated that GFP^{hi} cells, but not MG, promoted tolerogenic responses by inducing FoxP3⁺ T cells in *in vitro* antigen presenting cell (APC) assays (Heuss et al., 2012). More recently, it was reported that GFP^{hi} cells in the CD11c^{GFP} Rpe65^{-/-} mouse retina are most likely to be of MG origin, as ablation of MG prevented the repopulation of the retina by GFP^{hi} cells. In contrast, when GFP^{hi} cells were selectively depleted and MG were intact, GFP^{hi} cells rapidly recovered from ablation (Tang et al., 2017). These findings suggest that GFP^{hi} cells in the retina may be a distinct subset of MG and not DCs.

In a separate study using a different transgenic mouse model, Dando et al. described a population of CD11c-eYFP⁺ cells in the neural retina and cerebral cortex of CD11c-eYFP *Crb1^{wt/wt}* mice (Fig. 2F). Similar to Lehmann et al., it was demonstrated that CD11c-eYFP⁺ cells within the quiescent retina could not be phenotypically distinguished from MG (Fig. 2G and H) (but were functionally distinct, Dando et al. in press, *Glia*) and it was proposed that these cells represent a subpopulation of MG rather than DCs (Dando et al., 2016), although lineage tracing studies are required to definitively confirm this. The aforementioned studies highlight that retinal MG are not likely to be a uniform population of cells, which is consistent with recent data demonstrating that the mouse brain contains subpopulations of MG with distinct RNA profiles and functional capacities. Grabert et al.

reported that the global gene expression profile of MG isolated from the healthy adult mouse brain was regionally heterogeneous, with 2527 genes found to be differentially regulated in MG isolated from the cerebellum, hippocampus, cortex and striatum (Grabert et al., 2016). Functionally, it has been demonstrated that only subsets of MG in the mouse brain may have the ability to phagocytose myelin (Scheffel et al., 2012), internalize exosomes from oligodendrocytes (Fitzner et al., 2011) and present antigens to T cells (Dando et al. in press, *Glia*). Whether Microglia and Müller heterogeneity exists within the retina has not yet been addressed.

2.2. Ontogeny of retinal microglia in the normal retina

Brain MG have their origins during the prenatal period prior to the differentiation of bone marrow-mediated ‘definitive’ hematopoiesis takes place (Ginhoux and Guilliams, 2016). It is thus likely retinal MG may share this origin. The seminal paper from Ginhoux et al. demonstrated that adult brain MG arise directly from the yolk sac (Ginhoux et al., 2010), by early erythromyeloid progenitors (EMP) (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015; Kierdorf et al., 2013). The authors used a cell lineage tracing approach whereby tamoxifen pulse-labeled embryos at E7.5 in pregnant *Runx1^{MER-CRE-MER}; R26^{LSL-YFP}* dams gave rise to YFP⁺ brain MG in the adult progeny mice (Ginhoux et al., 2010). Because *Runx1* expression during the time of tamoxifen pulsing is restricted to cells in yolk-sac blood islands (Yzaguirre et al., 2018), this experiment effectively ‘traced’ adult MG back to these primitive cells, and also indirectly demonstrated that MG are long-lived since traced MGs were found in adults. Though there is some disagreement as to the precise transcription factors that are required for MG commitment, the current understanding is that brain and spinal cord MG are embryonic-derived, long-lived and self-renewing, and maintained independent of circulating monocytic input in adults (reviewed by (Ginhoux and Guilliams, 2016)). Likewise, certain peripheral tissue resident macrophages are largely embryonic derived as well but are instead thought to arise from the fetal liver as opposed to the yolk sac (reviewed by (Hoeffel and Ginhoux, 2018)). While the types of lineage studies that established this understanding for brain MG have not been performed with respect to the retina, it has been shown that retinal MG are long-lived and self-renewing, and thus, like brain MG, are maintained independent of circulating monocytic input (O’Koren et al., 2016; Reyes et al., 2017).

The reasons for early beliefs that MG were BM-derived came from work done by Van Furth and colleagues (van Furth et al., 1972) and later studies in which investigators generated BM chimeras via whole body irradiation of wildtype hosts and subsequent transplantation of GFP⁺ donor BM (reviewed by (Mildner et al., 2013)). In this setting, GFP⁺ macrophages are found to populate CNS tissues (Mildner et al., 2013). However, it is now understood that this irradiation-transplantation regimen results in non-physiological engraftment of donor BM derived monocytes, and/or their precursor cells, that then repopulate (and eventually out-populate) host endogenous MG (Kezic and McMenamin, 2013), albeit rather slower than non-CNS tissues. Two landmark studies (Ajami et al., 2007; Mildner et al., 2007) helped shape this understanding, or perhaps ‘misunderstanding’. These authors combined irradiation and parabiosis techniques to address this question of whether MG in the spinal cord and brain had a ‘turnover’ when irradiation was or was not factored in. Their data

showed that myeloid cells provided through a GFP⁺ parabiont in the absence of irradiation, did not lead to renewal of resident MG in the GFP negative ‘host’ (Ajami et al., 2007). This suggested the ‘renewal’ of MG seen in the brain and spinal cord in irradiated-BM transplanted hosts was *not* physiological. In addition, Mildner et al. showed that engraftment involves Ccr2, the Ccl2 chemokine receptor important in egress of monocytes from the BM into the circulation suggesting that engraftment involves monocyte-derived cells (Yona et al., 2013). (Mildner et al., 2007). Interestingly, however, the follow up paper by Ajami et al. pointed to uncommitted progenitor cells (as opposed to myelomonocytic-committed hematopoietic cells) as the contributors of engrafted macrophages in irradiated-transplanted hosts (Ajami et al., 2011). Elegant work by Bruttger et al. supports this hypothesis as well (Bruttger et al., 2015). Hence, although it is established that donor BM indeed contributes to engrafted macrophages in this setting, there are still open questions pertaining to the precise precursor cells in the BM that dominantly give rise to this repopulation. Nonetheless, recent work by Cronk et al. demonstrated that during perpetual MG depletion, which is thought to create space in the CNS, pure monocytes transferred can in fact engraft in the brain to some degree (Cronk et al., 2018).

Original irradiation-transplantation studies in the eye examining the neural retina also demonstrated the appearance of donor BM-derived macrophages and showed that this process involves Ccl2-Ccr2 signaling (Chen et al., 2012; Xu et al., 2007a). At the time, however, it was still not known that this phenomenon did not represent normal turnover of retinal MG. To clarify the situation, O’Koren et al. more recently examined this question through setting up GFP⁺ BM chimeras in mice that were either protected with lead head-shielding or not during irradiation (O’Koren et al., 2016). These studies demonstrated that whilst the neural retina of head-shielded mice did *not* undergo engraftment of donor BM-derived macrophages, engraftment was readily detected in unshielded mice suggesting local irradiation damage has a role. Moreover, the authors showed that retinal MG have a CD45^{lo} F4/80^{lo} CD11c^{lo} I-A/E^{lo} phenotype, whereas engrafted monocyte-derived macrophages express CD45^{hi} F4/80^{hi} CD11c^{hi} I-A/E^{hi} under the same conditions (O’Koren et al., 2016) (Fig. 4). This study also took advantage of tamoxifen pulse-labeled *Cx3cr1*^{CreER}; *R26*^{dTomato} mice (Yona et al., 2013), a model which was used to also show that retinal MG maintain their CD45^{lo} F4/80^{lo} CD11c^{lo} I-A/E^{lo} phenotype even in the established injury setting of light damage-induced photoreceptor degeneration (Noell et al., 1966). Hence, these above studies established that retinal MG are long-lived, but also demonstrated that these cells can be phenotypically distinguishable in the light damage setting and in BM chimeras on the basis of these markers by flow cytometric analysis. A more detailed consideration of MG in retinal degenerative conditions is given below.

2.3. Microglia and recruited macrophages in retinal degeneration

Given the new knowledge that resident MG are derived from the prenatal period and maintained locally throughout life, considerable effort has now been focused on uncovering the functional differences with those monocyte-derived cells that are recruited to the CNS in certain disease states (O’Koren et al., 2016; Reyes et al., 2017; Yamasaki et al., 2014). Most retinal degenerative conditions involve increased Iba1⁺ cells in the outer layers of the neural retina and an atypically large accumulation in the subretinal space in response to a range of

initiating factors (Guillonnet al., 2017; Gupta et al., 2003; Karlstetter et al., 2015; Lad et al., 2015; Langmann, 2007; Thanos, 1992). Hence, the ability to distinguish recruited monocyte-derived macrophages from MG in the neural retina is also pertinent in certain degenerative retinal diseases, such as in types of retinitis pigmentosa and age-related macular degeneration (AMD). To differentiate these distinct macrophage compartments (i.e. MG versus monocyte-derived cells) new strategies have been brought to the fore, such as the use of *Ccr2^{RFP}* monocyte reporter mice, MG transcriptional signatures and fate mapping have also been introduced in studies of retinal degenerative disease models strategies (Bennett et al., 2016; Luckoff et al., 2017; O'Koren et al., 2016).

Application of these approaches has established that monocyte recruitment contributes to the increased number of Iba-1⁺ cells in the retina in a number of degenerative diseases (Reyes et al., 2017). Original work by Sennlaub et al. showed in *Cx3cr1* deficient mice, that *Ccr2^{RFP}* monocytes are recruited in age-related or the light-damage induced model of retinal degeneration in these knockout mice (Sennlaub et al., 2013). Similarly, Khono et al. utilized *Ccr2^{RFP}* mice to demonstrate recruitment of monocytes in the neural retina of mice with a Mer tyrosine kinase (*Mertk*) deletion (Kohnno et al., 2015), a mutation of that is associated with retinitis pigmentosa in patients thought to be due to impaired phagocytosis by retinal pigment epithelium (Gal et al., 2000). Also using *Ccr2^{RFP}* mice, Zhao et al. demonstrated recruitment of monocytes in rd10 mice (Zhao et al., 2015), which have a mutation in the *Pde6b* gene and develop retinitis pigmentosa. In some of these models, interrupting monocyte recruitment ameliorated photoreceptor degeneration to some extent, implicating a possible pathological contribution for monocytes in certain settings (Guo et al., 2012; Sennlaub et al., 2013).

Most recent work using *Cx3cr1^{CreER}; R26^{tdTomato}* lineage tracing mice suggests that recruited monocyte-derived cells and endogenous MG are differentially distributed in acute retinal disease models, possibly suggesting the existence of distinct contributions in the disease setting. O'Koren et al. demonstrated that retinal MG migrate to and accumulate in the subretinal space, as well as adhere to the RPE, whereas monocyte-derived cells were found mostly in the parenchyma, but infrequently extended to the subretinal space (O'Koren et al., 2016) (Fig. 4). This finding was made in the light damage model of retinal degeneration using *Cx3cr1^{eYFP-CreER}; R26^{tdTomato}* mice. Following this report, Ma et al. who by using the same *Cx3cr1^{eYFP-CreER}; R26^{tdTomato}* mice, likewise showed MG-dominant occupation of the subretinal space in the NaIO₃ induced model of RPE damage (Ma et al., 2017). Hence, both papers independently converge on the finding that while monocyte-derived cells are recruited to the retina in acute retinal damage, there is a MG-dominant accumulation of the subretinal space.

Interestingly, Sennlaub et al. showed in *Cx3cr1* deficient mice (*Cx3cr1^{eGFP/eGFP}*) that recruited monocytes contribute to the population of subretinal macrophages during age-related or light-damage induced models of retinal degeneration (Sennlaub et al., 2013). Investigations by McMenamin and co-workers also revealed the link between the accumulation of subretinal macrophages and age in these *Cx3cr1^{eGFP/eGFP}* mice (Chinnery et al., 2012). This study revealed that while *Cx3cr1* deficiency led to an early onset of subretinal macrophage accumulation, the data revealed that this change occurs in both aged

Cx3cr1^{gfp/+} and *Cx3cr1^{gfp/gfp}* pigmented and albino mice in the absence of marked retinal degeneration and is likely a normal response to aging. Similarly, Khono et al. also reported the presence of *Ccr2^{RFP}* monocytes in the subretinal space in retinal degeneration mice caused by *Mertk* deletion (Khono et al., 2015). However, in contrast to O’Koren et al. and Ma et al. these studies used global genetic knockout systems of *Cx3cr1* (or *Mertk*) that are expressed by and regulate MG function, and other macrophages in the periphery, which might explain the different outcomes.

Whilst many investigators have highlighted the potential migration of MG into the outer retina in a range of models of injury such as light damage or genetic defects that may influence MG migration or retinal degeneration it is worth noting that the presence of subretinal macrophages or phagocytes has been described in normal mammalian eye development in many species including humans (McMenamin, 1999c; McMenamin and Loeffler, 1990) (Fig. 5). These cells were discovered by serendipity in human fetal specimens prepared to expose the developing iridocorneal angle for scanning electron microscopy (Fig. 5A) (McMenamin and Loeffler, 1990). Large bulbous and dendriform macrophage-like cells were noted on the apical aspect of the developing RPE (Fig. 5B), which were confirmed by transmission electron microscopy to be laden with melanophagolysosomes presumably derived from dying RPE cells (McMenamin and Loeffler, 1990). Subsequent studies revealed identical cells in the subretinal space of other developing mammalian eyes (McMenamin, 1999c) (Fig. 5C and D) where they expressed markers more characteristic of choroidal macrophages (CD163, CD169) than MG, bringing into question the notion that subretinal macrophages are MG derived in development. Interestingly subretinal macrophages have been reported in the adult retina of normal fish where they have a diurnal pattern related to light exposure, an effect more evident in the albino variant of the trout species used in these studies (Allen and Hallows, 1997). Furthermore, subretinal macrophages are present in the peripheral retina of aging human eyes where cystoid degeneration is common in eyes of 50 years and above (personal observations). The presence of macrophages in the subretinal space of developing mammalian eyes, adult non-mammalian and mammalian aged eyes further underscores our incomplete understanding of subretinal phagocytes in health and disease. Hence, more research is needed to unravel whether these cells represent distinct macrophage lineages or activation of resident MG and the associated functional implications of these respective populations has become an important new area of investigation in retinal disease.

3. Protecting the neural environment: is the choroid a protective ‘lymphoreticular organ’ outside the barrier?

3.1. The pathway to the elucidation of choroidal immune cell networks in rodents

The monolayer of hexagonal RPE, a critical element of the blood retinal barrier, is separated from the choroidal vascular supply by a complex acellular layer of connective tissue and basal lamina known as Bruch’s membrane (BrM) (Fig. 1C–E). Whilst in some eutherian (placental) and non-eutherian mammals (monotremes and marsupials) both vascular (holangiotic) and partially vascularised (paurangiotic) retinae are observed (Buttery et al., 1990; Chase, 1982; McMenamin, 2007) there are many species that lack retinal blood

vessels and thus, in these species the choroid is the sole source of nourishment for the entire retina. In the context of immune defense of the retina in such species then clearly there is no retinal component to the BRB and thus the choroid (and the ciliary body) is a critical reservoir of resident immune cells and the likely pathway for infiltrating cells in infection and inflammation. Even in species with a retinal vasculature the choroid in many diseases is a critical source of inflammatory cells and thus the normal defense and surveillance at the BM-choroidal interface performed by choroidal immune cells is critical to retinal homeostasis. It was surprising therefore that for many years our knowledge of immune cells in the normal choroid was so scanty.

The first conclusive evidence that the normal choroid contained dense networks of tissue macrophages, DCs and mast cells came in the early 1990s when methods were developed for dissecting the choroid free from the eye cup as intact sheets prior to immunohistochemical staining and examination as wholemounts in conventional brightfield microscopy (Fig. 6A) (Butler and McMenamin, 1996) and later by fluorescence and confocal microscopy. This wholemount approach was to give investigators an entirely new perspective of the density, morphology, distribution and immunophenotype of immune cells in the choroid (Butler and McMenamin, 1996; Forrester et al., 1994). It revealed that the normal rat choroid contained a rich (~600/mm²) and extensive network of CD68⁺ CD163⁺ and CD169⁺ macrophages, which exhibited dendriform or elongated fusiform morphology, many with a perivascular distribution (Fig. 6A and B) (Butler and McMenamin, 1996; Forrester et al., 1994) (reviewed in detail by (Chinnery et al., 2017)). A small proportion of these macrophages expressed MHC class II⁺, however, many MHC class II⁺ cells were negative for macrophage markers leading to the proposal that the choroid contained putative DCs (Butler and McMenamin, 1996; Forrester et al., 1994). Subsequent studies of the mouse choroid revealed fewer cells with a DC phenotype but identical networks of F4/80⁺ (CD160) and CD169 tissue macrophages (McMenamin, 1999a). Follow up studies using Balb/c Cx3cr1^{gfp/+} and Cx3cr1^{gfp/gfp} mice confirmed that the choroid contained rich networks of Cx3cr1⁺ Iba-1⁺ Ib4⁺ CD45⁺ CD11b⁺ dendriform macrophages, of which a significant proportion appeared to co-express MHC class II (Fig. 6B) (Kezic et al., 2008; Kumar et al., 2014). The nature of the rich vascular bed makes it appear that many of these myeloid cells have a perivascular distribution (see below) however it was only recently revealed using 3D reconstruction of confocal microscopy data that those in the inner choroid in mice are in fact located on the scleral aspect of the choroicapillaris with only small cellular processes protruding between the dense capillary bed to the basal aspect of BrM (Fig. 6C and D). This configuration may have functional relevance and also have a bearing on the role of these cells to disease in this area (see below).

More detailed immunophenotypical analysis using flow cytometry of myeloid cells in the mouse choroid is technically challenging due to the accuracy needed in dissecting it free from the sclera, the small volumes of the tissue obtained per eye and finally the resulting yield of cells. However, despite this we have recently performed detailed flow cytometry analysis of macrophages in Balb/c Cx3cr1^{gfp/gfp} mouse choroid using large numbers of pooled eyes (~20 mice) (unpublished). The analysis shows that of the CD45⁺ cells in the normal mouse choroid, a distinct population of F4/80⁺ Cx3cr1⁺ cells are present, which likely to represent the resident macrophage population (Fig. 6E). The F4/80⁺ Cx3cr1⁺ cells

expressed the myeloid cell marker CD11b, as well as MHC class II and the co-stimulatory molecule CD86 (Fig. 6E), suggesting that they may have putative antigen presenting capabilities. This high co-expression of MHC class II supports immunomorphological data from mouse choroidal wholemounts (Fig. 6B) and suggests previous immunoperoxidase double staining data obtained from rat choroid (Butler and McMenamin, 1996; Forrester et al., 1994) may have underestimated the number of MHC class II co-expressing macrophages which co-exist alongside MHC class II⁺ putative DCs (Butler and McMenamin, 1996). Alternatively, these discrepancies may be explained by species differences, antibody specificities or other technical issues and illustrate the continuing problem in fully characterizing DC populations due of the lack of a definitive DC-specific marker. A further frustration in characterization of myeloid cells in the choroid is that the small volume of cells in the mouse choroid has until now precluded the ability to isolate and purify sufficient numbers of cells for functional assays. It seems likely new techniques such as single cell RNAseq may provide the answers to some of these unresolved issues.

3.2. Does the human choroid contain macrophages and DCs?

Studies in human choroidal sections have shown the presence of cells with a macrophage immunophenotype (Cherepanoff et al., 2010) similar to that seen in rodents. Luty and colleagues correlated macrophage shape, distribution, immunophenotype and morphology in choroidal wholemounts from normal eyes (McLeod et al., 2016). They noted the morphology and density (433/mm²) of CD68⁺ choroidal macrophages in normal eyes and in non-macular choroidal regions of diseased eyes were remarkably similar to that of the mouse (Kezic et al., 2008). These choroidal macrophages in humans were also Iba-1⁺ with a minor population expressing HLA-DR (MHC class II). The presence of cellular processes from choroidal macrophages extending to beneath the RPE, like those noted in rodents, have also been observed (Cherepanoff et al., 2010).

The presence of fully characterized DCs in the human choroid has been limited to one preliminary study by Forrester et al. (1994) and further characterization is still eagerly awaited. Difficulties in obtaining well-fixed human tissue and the availability of antibodies that definitively identify these elusive cells as described above are likely the cause of the paucity of confirmatory evidence.

3.3. Functions of macrophages and DCs in the choroid and relevance to disease

The precise role of tissue resident macrophages and DCs in maintaining homeostasis in the choroid in the normal eye has not been as well studied as those in the meninges where T cell derived IL-4 production appears to regulate meningeal macrophage function in an anti-inflammatory manner. The data revealed the fascinating observation that mice deficient in meningeal CD4 T cells had diminished learning abilities and altered social behavior (reviewed by (Rua and McGavern, 2018)). Whether similar CD4 T cells are present in the normal choroid whilst not specifically reported seems unlikely as in our hands they are extremely rare or absent in the few cases where specific attempts have been made to visualise them.

Based on our knowledge of resident macrophages in other connective tissues (Mass, 2018) choroidal macrophages are likely to be critical for immunosurveillance and detection of pathogens (via recognition of pathogen-associated molecular patterns [PAMPs])) accessing the extracellular milieu via the 'leaky' vessel. Furthermore, they phagocytose debris and dead cells following infection or sterile injury. Their role in tissue repair and angiogenesis is also likely to be similar to other connective tissue macrophages although their proximity to the neural retina may mean such functions are more tightly regulated. Live imaging of DiI perfused choroidoscleral explants from B6(Cg)-Tyr^{c-2j}/J mice (albino mice in which Cx3cr1⁺ myeloid cells express gfp) by Kumar et al. revealed that those macrophages with cellular extensions or processes protruding through to the basal RPE (see above, Fig. 6C and D) had rather sessile cell bodies but processes that exhibited repeated extension and retraction in multiple directions (Kumar et al., 2014). This bears striking similarities to the behavior of MG in the retina and brain (see discussion above) and other macrophages (Nayak et al., 2012; Rua and McGavern, 2018). The emerging role of macrophages in the meninges and their relevance for diseases affecting the brain parenchyma (autoimmune processes, stroke, migraines and head trauma) has been elegantly reviewed recently (Rua and McGavern, 2018) and in light of the homology of the leptomeninges and the choroid it provides ocular researchers with potential fruitful furrows of investigation yet to be ploughed. For example, we know that in autoimmune uveoretinitis the accumulation of macrophages in the outer retina, which is central to the photoreceptor destruction, arises in part from choroidal infiltrates (reviewed by (Forrester et al., 2018a)). Tertiary lymphoid structures are observed in the meninges of multiple sclerosis (MS) patients and in murine experimental encephalomyelitis (EAE), the most frequently used animal model of MS, where aggregates of immune cells accumulate and are associated with sub-pial cortical damage and disease progression (Pikor et al., 2015). We speculate that the focal choroidal aggregates seen in retinochoroiditis (sometimes referred to as Dalen-Fuchs nodules) may be the homologous counterpart to meningeal lymphoid aggregates and provide an environment for local processing of autoantigen in the presence of APCs (macrophages and DCs, resident and recruited) and autoreactive T cells. These events and putative interactions may be all the more critical in the uveal tract due to the absence of lymphatic drainage (Fig. 7).

What might be the role of resident DCs in the normal choroid? As eluded to above it is tempting to speculate that there may exist a subset of the Cx3cr1^{gfp+} DCs whose processes may constantly sample the tissue microenvironment that lies between the basal aspect of the RPE and the fenestrated aspect of the choriocapillaris. Similar cellular extensions that sample foreign Ag have been described in the gut epithelium (Rescigno, 2014), respiratory epithelia (Jahnsen et al., 2006) and corneal epithelium (Chinnery et al., 2017) where surveillance of antigens at external barrier sites is correlated with 'periscope' or 'snorkel'-like DC extensions. If DCs were to sample local antigens or retinal derived antigens there remains an enigma about the egress pathway that choroidal DCs may use to transport Ag-specific signals to peripheral secondary lymphoid organs as to date no proven route via lymphatics has been proven (see (Forrester et al., 2018b) for full review). Whilst it has been known for some time that lymphatics drain the dura mater around the brain, the homologous tissue in the eye, the dense connective tissue of the sclera and its continuation, the optic nerve sheath have recently been shown to lack LYVE-1 + lymphatic vessels (Troost et al.,

2018). It may be that the impaired route of access of DCs from the choroid to secondary lymphoid organs is part of the immunoprotective role of this tissue.

The role of the choroid as an entry portal for pathogens infiltrating the eye outside the BRB and the role of host immune cells, especially macrophages and DCs, in the choroid in defense against these invading and potentially vision threatening pathogens is thoroughly reviewed elsewhere (Forrester et al., 2018b).

The link between immune dysregulation and complement in the pathogenesis of age-related macular degeneration (AMD), the commonest cause of vision loss in people aged 50 and above in developed countries, has recently refocused attention on the role of macrophages not only on the retinal aspect of the RPE (the so-called subretinal macrophages - see discussion above) but also on the tissue macrophages in the choroid. Alterations in BrM and the RPE underlie the pathology of AMD, which is now considered to have an inflammatory or 'para-inflammatory' pathogenesis (Xu et al., 2009). A range of normal age-related changes occur in BrM including basal laminar deposits, basal linear deposits, soft drusen and hard drusen. These changes can progress to RPE loss and atrophy of the adjacent neuroretina, known as 'dry' AMD, whereas the more sight threatening 'wet' AMD is characterized by neovascular tissue arising from the choroid and rupturing through BrM which goes on in many cases to form a disciform fibrovascular scar between the retina and the RPE. While the exact mechanisms by which sub-RPE debris accumulates with age and how this progresses to the pathological state in AMD remains partly unclear, a number of factors, are thought to contribute including: oxidative stress on neural retina and RPE; ischaemia due to ageing changes in the choriocapillaris; reduced phagocytic ability of RPE; complement deposition; and reduced clearance of BrM debris by resident macrophages. This topic has recently been reviewed in this journal (Guillonnet al., 2017) and once again highlights the importance of our emerging understanding of the immune cells in the choroid.

A study of a small sample of AMD-affected eyes by Cherepanoff et al. revealed that unlike in normal ageing eyes choroidal CD68⁺ inducible nitric oxide synthase (iNOS)⁺ macrophages are more closely associated with BrM in early AMD which suggests that these cells are classically activated macrophages (M1 phenotype) (Cherepanoff et al., 2010). More recently McLeod et al. reported that in eyes with various grades of severity of AMD there was an increased density of macrophages in submacular areas of the choroid (compared to non-macular regions) and these cells displayed reduced complexity of shape (or greater roundedness) which appeared to correlate with atrophy of the choriocapillaris and pathological changes in the RPE suggesting an activated macrophage phenotype (McLeod et al., 2016). These authors have proposed that the changes in the vascular atrophy in the submacular choroid would act as a 'sterile' injury signal causing activation of local choroidal macrophages which would then be involved in debris removal (McLeod et al., 2016). Whether a defect in debris clearance plays a role in the development of AMD is currently the subject of intense research.

4. Mast cells in the choroid in health and disease

Mast cells which are classically divided into connective tissue mast cells (CTMCs) or mucosal mast cells (MMC), have traditionally been considered as effector cells in allergic disorders and certain immune responses to parasites. They secrete numerous mediators, including neurotransmitters, cytokines, and chemokines, that are released in response to a variety of natural and pharmacological triggers. More recently, a broader perspective of their functional heterogeneity, such as immunomodulation, angiogenesis, tissue repair and remodelling after injury has emerged (Galli et al., 2011; Gilfillan and Beaven, 2011). Classically, the release of a range of vasoactive mediators, such as histamine, upon binding of allergens to the FcεRI (high affinity IgE receptor) on mast cells was considered as the defining feature of allergic responses. The changes that accompanied this release, namely vascular leakage and erythema, tissue oedema, increased mucosal secretion and recruitment of inflammatory cells are the classical hallmarks of allergic responses. The distribution and density of mast cells near mucosal surfaces and in the dermis of the skin supports this basic tenet of mast cell function. Recent paradigm shifts in our understanding of mast cells biology has led to a better understanding of their role in initiation, development, expression, and regulation of innate and adaptive immune responses well beyond classical IgE-mediated allergic responses (Galli, 2016). Galli has suggested a less appreciated role for mast cells in protection against venomous and poisonous insects and snakes. He speculates that this may constitute a further explanation for their presence in tissues such as muscle, connective tissues not conventionally considered to be exposed to environmental allergens or part of allergic immune responses (Galli, 2016).

Unlike the retina which is devoid of mast cells, the choroid contains numerous mast cells in all species of vertebrates investigated to date (McMenamin and Polla, 2013) (Fig. 8). The earliest descriptions by Larsen (1959) identified mast cells in the choroid of albino guinea pigs, mice, rats and rabbits, and humans (Feeney and Hogan, 1961). The application of whole mount methods by Smelser and Silver in a range of species illustrated the number and perivenular arrangements of mast cells in the choroid (Smelser and Silver, 1963). However, Godfrey, also using whole mounts, in a study on the effects of age and strain on mast cell numbers, presented clear evidence that mast cells were predominantly aligned along medium sized arteries suggesting they had an effector role in regulating blood flow (Godfrey, 1987). Subsequent studies have confirmed a largely periarteriolar location in rats (Fig. 8B) but this is not as evident in other species (Fig. 8A, C, D) (McMenamin and Polla, 2013). The ubiquitous distribution of mast cells in the vertebrate choroid, but not the retina, is hardly surprising as it largely mirrors the situation in the brain and spinal cord where mast cells are present in the meninges, predominantly in a perivascular and linear pattern in the dura mater (Dimlich et al., 1991) but largely absent from the CNS parenchyma except for a few specialized regions such as the thalamus (paraventricular nuclei) and hippocampus (Brown and Weinberg, 2018).

Following the interest over many years in the potential role of mast cells in development and pathogenicity of MS and the animal model EAE (reviewed by (Brown and Weinberg, 2018) together with the suggestion by Mochizuki et al. that mast cell numbers in the choroid were also implicated in experimental autoimmune uveitis (EAU) (Mochizuki et al., 1984) we and

others explored the potential link between susceptibility of rat strains to EAU and mast cells in the choroid (Steptoe et al., 1994). Whilst the choroidal mast cells were successfully characterized immunohistochemically and histochemically as CTMCs no relationship between mast cells numbers and EAU susceptibility was found. Indeed, some rat strains (PVG/c and Brown Norway) have very few choroidal mast cells yet remain susceptible to EAU induction.

Recent research suggesting a link between mast cells and AMD has reignited interest in the role of mast cells in the healthy and diseased human eye (Bhutto et al., 2016). Bhutto and colleagues found that the number of mast cells in human choroidal wholemount samples was significantly increased in all choroidal areas in early AMD and in the paramacular area in exudative AMD and areas of geographic atrophy compared to the aged controls. They noted these mast cells were mostly located in the deeper layers (Haller's and Sattler's) which house larger choroidal vessels. Some evidence of degranulation of mast cells in the choroid in and near the macula in all forms of AMD were noted and concluded to be associated with loss of capillaries in the choriocapillaris. No clear explanation currently exists as to why choroidal mast cells would be degranulated at the time of death of these patients unless this is a chronic change in response to exposure to damage-associated molecular patterns (DAMPs) related to damaged RPE and accumulation of drusen-like material in BM. It is unlikely to be the more classical allergen or antigen-mediated event as these are rapid events more characteristic of immediate hypersensitivity or allergic responses.

5. Future directions

The eye, besides offering opportunities to perform research on immune cells relating to various ocular diseases, is also an ideal experimental platform for basic immunologists and neuroimmunologists to investigate the startling differences in immune cell function in two highly differing tissue microenvironments - the neural retina (behind the BRB) and the highly vascular connective tissue of the choroid (outside the BRB). In this review, we have compared and contrasted the phenotypic and functional differences between the MG as the primary myeloid-derived occupant of the neural retina parenchyma and the rich plethora of immune cells in the choroid. We have at several points referred to the informative homologies with the meninges surrounding the parenchyma of the brain, which should come as no surprise since the eye arises as an outpouching of the brain. Just as there has been a resurgence in interest in the immune cells in the meninges surrounding the brain which suggests they orchestrate and often precede immune responses in the CNS proper, we believe that the relatively understudied choroid with its unique location and rich populations of immune cells in such close apposition to the RPE and retina, its "leaky" vasculature facilitating ready egress of leukocytes compared to retinal vessels, is ideally equipped to sense and respond to invading pathogens and tissue damage. The data we have shown suggest that whilst the field of ocular immunology is moving forward in relation to our understanding of the role of retinal MG, choroidal immune cell function awaits further equivalent scrutiny. We propose that perhaps more attention needs to be focused on the choroid as it may serve as a protective immunological shield for the retina capable of shaping and regulating immune responses in ways that meningeal biologists and

neuroscientists are beginning to appreciate about the homologous cousins of the choroid and retina.

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Box - perivascular macrophages (PVMs)– guardians of the barrier?

Whilst microglia are without question the most abundant immune cells present in the retina, it should be noted that the retina, like the brain, also contains a small population of PVMs (Mendes-Jorge et al., 2009). These phagocytes reside in a close physical association with the neuroretinal vasculature, on the abluminal aspect of the vascular endothelial basal lamina, closely associated with pericytes but internal to the glia limitans consisting of astrocytes (in superficial retina) (Fig. 3A and B) and Muller cells in the deeper retina (Fig. 3C and D). This close association with vessel walls contrasts with MG, which are entrenched more widely in the neural parenchyma of the retina (Fig. 2A and B). In the brain, the term ‘barrier-associated macrophages’ has been used to emphasise the critical location of PVMs, as well as macrophages in the meninges, at interfaces with the BBB (Rua and McGavern, 2018). New studies suggest that PVMs in the retina are embryonic-derived, long-lived, and are ‘radiosensitive’ (O’Koren et al., 2016), similar to what was recently shown in the brain parenchyma (Goldmann et al., 2016) (reviewed elsewhere in detail (Saban, 2018)). Recently, McMenamin et al. have visualized rare PVMs in the retina of DPE-GFP transgenic mice (unpublished data, Fig. 3B). In these mice, PVMs (but not other macrophages or MG) express high levels of GFP under the control of the CD4 enhancer/promoter (Abtin et al., 2014). In the skin, PVMs express CD4 are also CD64, CD36, CD68 and CD169 + in common with other macrophages in connective tissues (Abtin et al., 2014). PVMs were described in the brain some time ago (de Groot et al., 1992; Hickey and Kimura, 1988) but have more recently become the subject of intense investigations due to their pivotal position as gatekeepers between the intravascular compartment and the brain interstitial space (Lopez-Atalaya et al., 2018; Tanabe and Yamashita, 2018). Their position relative to retinal blood vessels (Fig. 3B) may suggest similar barrier roles, including involvement in the preservation of blood-tissue barriers, immune defense against blood-borne pathogens, and bidirectional molecule transfer across the BRB. It will be intriguing to see whether the unique role of PVMs in the dermis in regulating transendothelial migration of neutrophils in venules during *Staphylococcus aureus* infection (Abtin et al., 2014) has functional parallels with CNS PVMs. Hence, future research is imperative to fully realize the importance of PVMs in retinal health and disease and their association with pericytes and their loss in diabetic retina is one area fertile for future study.

Fig. 1.

A, the anatomy of the eye with particular attention to the arrangement of the vessels in the choroid, the hexagonal units of the choriocapillaris, the anastomotic capillary bed beneath Bruch's membrane and the retinal pigment epithelium. **B**, histological view of the neural retina (NR) and heavily pigmented choroid (Ch) in toluidine blue stained semithin section of the primate (marmoset) eye. Scale bar = 50 μ m. **C**, high power view of the human outer retina in toluidine blue stained semithin section showing the approximation of the photoreceptor layer (PhR) and the choriocapillaris (cc) separated by the retinal pigment epithelium (RPE). Scale bar = 20 μ m. **D**, high power transmission electron microscopy

image of the primate (baboon) RPE, Bruch's membrane (BM) and choriocapillaris (cc). Scale bar = 2 μ m. **E**, high power view of the layers of BM and fenestrated endothelium of the choriocapillaris from '**D**'. Scale bar = 0.5 μ m *Images modified from* (Forrester et al., 2016).

Fig. 2.

A, Location of CX3CR1-GFP⁺ microglia (green) and MHC class II⁺ cells (red) in the entire thickness of the retina and choroid as seen in confocal analysis of wholemount from Cx3cr1-GFP mouse. The z-profile has been rendered with 'Imaris' software to display the layered distribution of the MG in the retina. Note the occasional subretinal macrophages in the outer retina close to the choroid which contains numerous MHC class II⁺ macrophages and DCs. GCL = ganglion cell layer, NFL = nerve fibre layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer. Scale bar = 100

µm. **B**, retinal wholemount from Cx3cr1~GFP mouse stained with Ib4 (isolectin that stains blood vessels, including capillaries, and macrophages) focused on the outer plexiform layer of microglia (green). Scale bar = 100 µm. **C**, Microglia as seen using *in vivo* scanning laser ophthalmoscopy in fluorescent mode of the Cx3cr1~GFP mouse retina (Spectralis, Heidelberg Engineering) showing the GFP⁺ microglial network (green). The image of the retinal vasculature (based on fluorescein angiography which was then false coloured red) is superimposed. **D**, Microglia in the normal mouse retina and brain parenchyma (cerebral cortex shown) are distinguished from other leukocyte populations (including perivascular macrophages and meningeal macrophages) by their intermediate expression of CD45 (top panel). Gated CD45^{int} MG express high levels of CD11b and CX3CR1 (bottom panel). **E**, The rare presence of MHC class II⁺ cells at the optic nerve head (ONH) and junction of the peripheral retina and ciliary body (CB). Scale bars = 50 µm. **F**, Retinal flatmount demonstrating the distribution of CD11c-eYFP⁺ cells in the CD11c-eYFP *Crb1*^{wt/wt} mouse retina. Controversy exists regarding the identity of these cells; however, evidence presented in panels G–I demonstrate that these cells appear to be a subpopulation of MG. **G**, Retinal CD11c-eYFP⁺ cells morphologically resemble MG as they display a ramified morphology and co-express Iba-1. Scale bar = 50 µm. **H**, Flow cytometry analysis of the CD11c-eYFP *Crb1*^{wt/wt} mouse retina demonstrates that a small subset of the total CD45^{int} MG population are CD11c-eYFP⁺, suggesting that retinal CD11c-eYFP⁺ cells are likely a subset of MG. Comparison of the expression of phenotypic markers between this CD11c-eYFP⁺ CD45^{int} MG subset and the larger population of CD11c-eYFP[−] CD45^{int} MG reveals that these two MG subsets are phenotypically indistinguishable.

Fig. 3.

A, diagrammatic summary of the blood retinal barrier components and location of the microglia (pink) and perivascular macrophages (PVM, green) in relation to the vascular endothelium (orange), the basal lamina (grey), pericytes (blue) and astrocytes (clear). Note: the PVMs can lie within the basal lamina or external to it but on the luminal aspect of the astroglial sheath. **B**, confocal stack of superficial retina only (note regular spacing of ganglion cell nuclei (DAPI, blue) in Cx3cr1~gfp mouse (green) showing GFAP⁺ astrocytes (red) in relation to Ib4⁺ blood vessels (white). Note the more rounded or ovoid myeloid cells are Cx3cr1⁺ Ib4⁺. Astrocytes extend processes around vessels although they do not appear to completely envelop them. **C**, 3D rendered confocal image (Imaris software) of the astrocytes (red) adjacent to superficial retinal vessels (Ib4⁺, white) as seen from the sclerad aspect where astrocyte interactions with vessels is less evident compared to the covering on the inner or vitread aspect which is confirmed in the Z-profile (**D**) which shows penetrating vessels passing into the deeper retina which appear devoid of astrocytes.

Fig. 4.

Microglia in the light-damage model of degeneration migrate to the subretinal space and adhere to the RPE, whereas monocyte-derived macrophages (MO-MF) are found mostly in the neural parenchyma and do not extend to the subretinal space in any significant number. Confocal microscopy of cross sections of normal (a) and light damaged retina (b) taken from *Cx3cr1^{YFP-CreER/wt};R26^{tdT/wt}* mice stained with DAPI (outer nuclear layer = ONL; outer plexiform layer = OPL; inner nuclear layer = INL; inner plexiform layer = IPL). (a) Cross-sections of normal retinas showed in the top panel contain microglia (YFP + tdT +) in the

IPL and OPL. In light damaged retinas (b) there is thinning of the ONL and this layer also contains microglia and recruited myeloid cells/MG sometimes referred to as subretinal macrophages (YFP + tdT + and YFP + tdT-, respectively), (b) *En face* flatmount confocal stacks of the apical aspect of the RPE (top) and the overlying neuroretina (bottom) from light damaged *Cx3cr1^{YFP-CreER/wt}; R26^{tdT/wt}* mice. *Adapted from* (Saban, 2018).

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Fig. 5.

A, Diagram illustrating the method used to expose the apical aspect of the retinal pigment epithelium (RPE) in the human foetal eye (*from* (McMenamin and Loeffler, 1990)) prior to scanning electron microscopy examination as shown in '**B**' which illustrates a typical subretinal macrophage replete with inclusions which are identical in size to the melanin granules in the underlying RPE. Scale bar = 5 μ m. **C**, toluidine blue stained semithin section of foetal rat retina showing a large mononuclear cell in the subretinal space. Scale bar = 10 μ m **D**, immunohistochemistry confirmed these cells expressed macrophage markers (ED2, CD169) similar to macrophages in the developing adjacent choroid (*from* McMenamin, 1999c). Scale bar = 20 μ m.

Fig. 6.

A, An early immunohistochemical stained rat choroidal wholemounts illustrating the distribution of MHC class II⁺ cells (red-brown chromagen) and CD169⁺ macrophages (blue chromagen). Double positive cells are purple (Butler and McMenamin, 1996) Scale bar = 50 μ m. **B**, confocal image of mouse choroidal wholemount from Cx3cr1-GFP mouse (myeloid lineage cells are green) stained with anti-MHC class II (red). Note the majority of cells are double positive. Scale bar = 50 μ m. **C**, choroidal wholemount from a Cx3cr1-GFP mouse perfused with DiI (red fluorescent marker) viewed from the scleral aspect illustrating the network of macrophages. Scale bar = 100 μ m. **D**, same tissue as **C** viewed from the RPE aspect showing small cellular processes protruding through the choriocapillaris. Scale bar = 50 μ m. **E**, Flow cytometric analysis of CD45⁺ cells within the CX3CR1-gfp/gfp mouse choroid demonstrates a distinct population of CX3CR1⁺ F4/80⁺ cells, which most likely

represent the resident macrophage population. Choroidal CX3CR1⁺ F4/80⁺ cells are CD11b⁺ I-A/I-E⁺ CD86⁺. In contrast, choroidal CD45⁺ CX3CR1⁻ F4/80⁻ cells are CD11b⁻ CD86⁻ and express lower levels of I-A/I-E.

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Fig. 7.

Choroidal wholemounts from DPE-GFP mice. In these mice GFP is expressed under the control of the CD4 enhancers and promoter. Although $\alpha\beta$ T cells are uniformly GFP⁺ in these mice, Weninger and co-workers recently discovered that a specific subset of cutaneous macrophages, namely PVMs, also express GFP but in higher amounts than T cells (Abtin et al., 2014). **A**, Left panel – GFP⁺ cells (green), middle panel shows larger vessels in outer retina stained with anti-Collagen Type IV (red) and right panel is a composite showing the perivascular arrangement of the CD4⁺ perivascular macrophages. **B**, the choroid in the DPE-GFP mice. The left panel is GFP + perivascular cells (green), middle panel shows Iba-1⁺ myeloid cells and right panel is a composite that demonstrates that only a small subpopulation of the choroidal myeloid cells are CD4⁺. DPE-GFP mice courtesy of Prof Wolfgang Weninger. A, x120, B, x80.

Fig. 8.

Choroidal wholemounts (**A, B**) and cross sections of the choroid (**C,D**) illustrating the number, distribution and location of choroidal mast cells close to arteries and immediately deep to the choriocapillaris. **A**, mouse choroidal whole mount stained with toluidine blue. Scale bar = 100 μm. **B**, rat choroidal whole mount stained with toluidine blue. Scale bar = 100 μm. **C**, low magnification of the human retina and choroid illustrating mast cells (white arrows), Scale bar = 50 μm shown in higher magnification in **D**. Scale bar = 10 μm. *Images from* (McMenamin and Polla, 2013).