Macrophage physiology in the eye.

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Word length: 6389 (excluding references and figure legends)

Number of Figures: 3

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Acknowledgments:

Funding for HRC: National Health and Medical Research Council APP1042612, PGM: National Health and Medical Research Council APP 1069979 SD: Rebecca L. Cooper Medical Research Foundation APP10470. The authors acknowledge Dr Cecilia Naranjo Golborne for her excellent confocal microscopy skills; the Florey Advanced Microscopy Facility at The Florey Institute of Neuroscience & Mental Health Facility for provision of instrumentation, training and general support and the facilities, scientific, and technical assistance of Monash Micro Imaging, Monash Histology Platform, and Monash Flow Core, Monash University, Victoria, Australia.

Key words: Macrophage, microglia, cornea, retina, uvea, eye

This article is published as part of the Special Issue on “Macrophages in tissue homeostasis”

Abstract

The eye is a complex sensory organ composed of a range of tissue types including epithelia, connective tissue, smooth muscle, vascular and neural tissue. While some components of the eye require a high level of transparency to allow light to pass through unobstructed, other tissues are characterized by their dense pigmentation, which functions to absorb light and thus control its passage through the ocular structures. Macrophages are present in all ocular tissues, from the cornea at the anterior surface through to the choroid/sclera at the posterior pole. This review will describe the current understanding of the distribution, phenotype and physiological role of ocular macrophages, and provide a summary of evidence pertaining to their proposed role during pathological conditions.

Introduction to the eye

The eye is a highly specialized and complex sense organ containing several unique, closely juxtaposed tissue types that arise from neural ectoderm, surface ectoderm and mesenchyme, most likely all of neural crest origin, each required to perform very different functions that allow clear visual images to be formed on the neural retina. Maintaining transparency and thus optimal visual function is essential for survival in most vertebrate species. The cornea is the anterior, transparent tissue of the eye, with a critical refractive function. The pigmented iris, ciliary body and choroid constitute the uveal tract or middle vascular layer of the eye. The iris regulates the size of the pupil and thus the amount of light entering the eye, and also assists in accommodation. The ciliary body functions to alter the shape of the lens in accommodation and to produce the aqueous humor, ocular fluid homologous to cerebrospinal fluid in and around the brain and spinal cord. The aqueous nourishes the cornea and lens and, along with the rigidity of the dense corneoscleral envelope, maintains intraocular pressure. Sharing many similarities with the brain, the retina is a typical neural tissue, protected behind blood-ocular barriers and consists of a complex matrix of interconnected neurons such as photoreceptors, bipolar cells and ganglion cells, supported by glial cells such as Müller cells and astrocytes. The choroid, a highly vascularized, pigmented connective tissue that forms the posterior part of the uveal tract, lies adjacent to the neural retina, supplying trophic and metabolic support to the outer retina. Within each of these microenvironments exists a diverse range of resident tissue macrophages, highly specialized to both support homeostatic functions and coordinate inflammatory responses to pathogenic or injurious stimuli. The mammalian eye offers unique opportunities to explore macrophage heterogeneity in very closely applied and functionally diverse tissues in an organ which lacks a typical lymphatic drainage system; however, due to the small dimensions of these ocular elements it presents its own distinct challenges. In this review, we discuss resident macrophages located within various ocular tissues and highlight their role in normal physiology of the eye and during pathology.

Corneal macrophages

Distribution and phenotype

The cornea is a dense, regular connective tissue consisting of precisely arranged layers of collagen, wedged between an anterior stratified, squamous, non-keratinized epithelium and a posterior endothelial monolayer. Keratocytes are the mesenchymal-derived fibroblasts of the corneal stroma, whose primary role includes secretion of extracellular matrix components, which serve to maintain the transparent nature of this refractive tissue. Despite originally considered to [contain only a few scattered lymphocytes in the stroma](#) [77], it is now accepted that the healthy cornea contains homeostatic populations of macrophages that reside throughout the anterior and posterior regions of the corneal stroma (Fig. 1A), sandwiched between the collagenous layers and closely associated with keratocytes (Fig. 1B) [104]. In the mouse cornea, most CD45⁺ bone marrow-derived cells express typical macrophage markers including F4/80 [6], Cx3cr1, Iba-1 [13], CD11b [32] and CD68 [76, 15, 12]. Approximately 30% of resident corneal macrophages co-express major histocompatibility complex (MHC) class II (Fig. 1A) [104, 12], with some laboratories reporting that a subset of these MHC class II⁺ cell populations also express CD11c, an integrin that is enriched in dendritic cells and thus may represent bona fide professional antigen presenting cells [31, 33]. Similar populations of CD45⁺ CD11b⁺ CD11c⁻ macrophages and CD45⁺ CD11b⁺ CD11c⁺ dendritic cells have been confirmed in human corneas, with the greatest difference between species being the possible higher representation of stromal dendritic cells compared to macrophages [64].

Unlike macrophage populations in other connective tissues such as the dermis of the skin, which harbors a diverse population of resident immune cells including mast cells, natural killer cells, $\gamma\delta$ T cells, as well as several well described dendritic cell and macrophage subsets [106], the normal corneal stroma appears only to host macrophages and dendritic cells, members of the mononuclear phagocyte system. Thus in the absence of a complementary repertoire of diverse leukocyte subsets that normally underlie epithelial barrier sites, understanding the role of resident macrophages as the major immunological guardians of the corneal stroma is critical.

Role in homeostasis

In healthy human [107] and mouse corneas [104] a modest proportion of CD45⁺ resident cells express the hematopoietic stem cell marker CD34. Flow cytometric analysis of 70 pooled mouse corneas by Forrester and colleagues revealed that approximately 3% of all stromal cells express CD34 [56], a number that corroborates closely with the percentage of CD45⁺ cells reported in immunomorphological studies of the normal mouse cornea [104]. Considering that the majority of studies of human and mouse corneal macrophages agree that most CD45⁺ cells in healthy corneas phenotypically resemble macrophages [6, 104, 120, 15, 43], it is possible that these macrophages have a multipotent capacity to de-differentiate into keratocytes and contribute to collagen synthesis following injury, as has been demonstrated *in vitro* using primary cultures of isolated corneal stromal cells [107].

The cornea has several anatomical and physiological specializations to ensure that corneal transparency and architecture are preserved, thus minimizing light scatter. Blood vessels, lymphatic vessels and melanocytes are absent from the central cornea and are limited to the corneoscleral junction or limbus (where cornea blends with the white sclera of the eye). Any disruption to the collagenous architecture, either through excessive infiltration of inflammatory cells, secretion of collagen-degrading proteases or transformation of normally quiescent keratocytes into fibroblastic myofibroblasts can lead to increased scattering of light which can manifest as corneal haze or scarring. Aberrant growth of limbal blood or lymphatic vessels, a feature usually only observed during severe corneal infections, injuries, or transplant rejections, can further disrupt corneal homeostasis thus compromising transparency. Macrophages located in the more peripheral regions of the cornea, close to the limbal lymphatics and blood vessels, express lymphatic endothelium hyaluronan receptor (LYVE-1) [119], a marker normally used to identify lymphatic endothelial cells [84]. *In vitro* studies have demonstrated that these LYVE-1⁺ corneal macrophages, identified via their expression of CD11b and F4/80, can induce tube-like structures that stain positively for LYVE-1 and podoplanin, suggesting that these cells were in fact capable of forming *de novo* lymphatic vessels [61]. This phenomenon was later examined *in vivo* using corneas from mice in which corneal macrophages were depleted by a local injection of the macrophage-depletion agent dichloromethylene-bisphosphonate (Cl2MBP or clodronate [111]), or in mice deficient in F4/80 or CD11b expressing cells. In all three scenarios there was a significant reduction in the number of lymphatic vessels in the limbal/peripheral cornea of normal, untreated corneas and in corneas exposed to suture injury (a common model used to induce neovascular growth and lymphangiogenesis) [62]. In another related study, Cursiefen *et al.* demonstrated that macrophages recruited to the cornea following suture injury express vascular endothelial growth factor-C and-D (VEGF-C and VEGF-D), and they too provided evidence that local depletion of macrophages abrogated corneal lymphangiogenesis, presumably due to reduced production of VEGF-C and VEGF-D [16]. These data provide strong support for a role of resident corneal macrophages in maintaining lymphatic vessel homeostasis in the normal mouse cornea and promoting lymphangiogenesis during inflammatory conditions [62].

Role in pathology

Despite an increased awareness of the presence of corneal macrophages in recent years, data demonstrating their precise role in normal corneal homeostasis are rare. By comparison, many studies have reported the critical roles macrophages play when the cornea is physiologically challenged, either through infection, injury, ocular surface stress or during transplant rejection. Consistent with their proposed role in immunity, several studies using Mafia (Macrophage Fas-Induced Apoptosis) mice, in which macrophages expressing the colony stimulating factor 1 receptor can be conditionally induced to undergo apoptosis, have provided evidence of a role for resident macrophages in generating innate inflammatory responses to pathogenic threats including the highly contagious adenovirus [87], *Pseudomonas aeruginosa* [105] and microbial products including lipopolysaccharide (LPS) [11]. Expression of the extracellular matrix proteoglycan,

lumican, on corneal macrophages is integral to the early host response to *P. aeruginosa* infection [101]. In a mouse model of *P. aeruginosa* keratitis, corneal lumican-deficient macrophages were unable to generate adequate inflammatory responses to the bacteria, resulting in impaired bacterial clearance and delayed resolution of inflammation [101].

Perhaps more compelling evidence supporting the role of corneal macrophages during pathological conditions can be found in a series of studies involving the local depletion of corneal macrophages using clodronate. Injection of clodronate liposomes into the nearby subconjunctival space effectively induced the selective apoptosis of resident corneal macrophages, whilst sparing the closely related resident corneal dendritic cells [103, 125]. In a mouse model of dry eye disease, (using autoimmune regulator [Aire] knockout mice which exhibit salivary and lacrimal gland exocrinopathy, and thus have severely reduced tear production leading to corneal drying), local depletion of corneal macrophages was associated with reduced corneal epitheliopathy and stromal fibrosis [125]. Using a similar approach in rats, Slegers *et al.*, demonstrated a role for corneal macrophages in the acute phase of corneal allograft rejection however this treatment was only effective when clodronate liposomes were administered at the time of graft transplant or within 48 hours of the surgery [102]. These studies suggest that corneal macrophages contribute to immunopathology during a range of pathological conditions affecting the cornea.

Disruption to the corneal epithelial barrier, either due directly to the wound itself or due to insufficient healing mechanisms, can make the cornea vulnerable to recurrent epithelial erosion or opportunistic infection. Owing to the dense network of sensory nerves, an improper wound healing response following corneal stromal or epithelial injury can result in pain and discomfort. Many lines of evidence have outlined the role of key players involved in corneal wound healing, including, but not limited to, neutrophil infiltration and the associated chemokines such as CXCL1 and adhesion molecules such as E- and P-selectin [54], the presence of a small populations of limbal $\gamma\delta$ T cells [53] and the production of proinflammatory cytokines that orchestrate inflammatory cell influx. In particular, macrophage-derived production of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF- α) is instrumental in mediating corneal wound healing by inducing keratocyte transformation into myofibroblasts [93]. Further providing evidence of macrophage activation following sterile corneal injury, we have reported the increased presence of long (up to 300 microns), thin (i.e. less than 200 nanometers) cellular processes emanating from the soma of macrophages in the corneal stroma (Fig. 1C, D) [14]. Whilst several studies had demonstrated these highly novel cellular features, termed membrane or tunneling nanotubes, *in vitro* [81, 91], our account of these extensive cellular processes on corneal macrophages was the first description of this cellular phenomenon in a mammalian tissue. [The remarkable length of some macrophage-derived membrane nanotubes makes it possible for distant physical connectivity between widely spaced cells, which contrasts to the well-described interdigitating cellular processes that connect adjacent keratocytes in the stroma.](#) The *de novo* formation of membrane nanotubes by corneal macrophages was captured using

time-lapse confocal microscopy of corneal explants, and was associated with cellular stresses such as serum depletion [100]. Evidence of *in vivo* membrane nanotube function has only recently been reported by Rehberg *et al.*, [90] who demonstrated transfer of nanomaterials between resident tissue macrophages in the mouse cremaster muscle. The precise role of membrane nanotubes in living mammalian tissues is an ongoing and exciting area of research.

The sensory nerves in the cornea are essential to alerting the tissue to the presence of threat or injury. The unique anatomy and architecture of the cornea has allowed our laboratory to reveal a possibly unique physical association between resident corneal macrophages and large nerve trunks in the peripheral corneal stroma [99]. In the cornea, the highest density of nerve axons and superficial terminals are located in the basal epithelium of the central cornea. A population of resident CD45⁺ cells lies immediately beneath this dense network of epithelial nerves (Fig. 1E, F). We have shown that within two hours after central epithelial injury, macrophages in the periphery disassociate from the larger nerve branches that enter the cornea at the limbus [99]. Whilst these data do not reveal a distinct function of corneal nerve-associated macrophages, their rapid mobilization within hours after distant injury suggests that they may be involved in the acute inflammatory response, which has been shown to be critical for efficient wound healing in the cornea.

MACROPHAGES IN THE UVEAL TRACT

Iris and ciliary body macrophages

Distribution and phenotype

The iris is a circular ring of contractile connective tissue, which dilates and constricts to regulate the passage of light through its central aperture, the pupil. It consists of a highly vascularized connective tissue stroma **rich in melanocytes, and on its posterior aspect it has a modified non-pigmented epithelium which forms the dilator pupillae muscle and a pigmented epithelium.** Macrophages and dendritic cells overwhelmingly predominate as the major resident leukocytes in the iris [66], with very small numbers of resident mast cells reported in human and rodent irides [63, 66]. The ciliary body, lying posterior to the iris, produces aqueous humor, which fills the anterior chamber of the eye. The tight junctions between the inner layer of ciliary epithelium forms a crucial component of the blood ocular barrier, preventing the passage of large macromolecules and circulating immune cells that could compromise transparency of the ocular media. Many of the functions of iris and ciliary body macrophages pertain to the overarching goals to limit exposure of the anterior chamber of the eye to exogenous antigens, and thus unwanted immune responses.

The immunophenotype of iris and ciliary body macrophages has been the subject of several reports [69, 66, 50] and reviews [67], particularly owing to a series of studies performed over the past twenty-five years

from our laboratories. Much of what is known about iris macrophages stems from the utility of a wholemount preparation technique [68] used to stain and examine full thickness iris tissue. Examination of wholemount tissue has demonstrated dense networks of resident macrophages in the iris stroma of the mouse [66], rat [71] and human [69]. A common feature amongst iris macrophages, that were identified based on F4/80 immunoreactivity (mouse) and ED1/ED2 (rat), was their strong expression of MHC class II. Later studies using the Cx3cr1-GFP mouse model, in which the majority of tissue-resident macrophages (and other myeloid cells) express eGFP at the locus of the chemokine receptor Cx3cr1 [36], provided confirmation of the presence of large populations of iris and ciliary body macrophages [40].

Role in homeostasis and pathology

One of the most characteristic features of macrophages is their ability to phagocytose large amounts of extracellular debris and apoptotic/necrotic cells, and participate in tissue remodeling. In the iris and ciliary body, phagocytosis of melanin granules by resident macrophages is critical to ensure excess pigment granules are retained in the tissue and not released into the anterior chamber of the eye, where they could potentially disrupt the clear visual axis or block the drainage of aqueous fluid from the anterior chamber. Indeed, some of the earliest descriptions of resident iris pigment-laden cells, known as “clump cells of Koganei”, relate to their immense ability to ingest and store large amounts of pigment released from melanocytes and atrophic iris pigment epithelial cells [117]. Excessive shedding of pigment granules from the epithelium causes a condition known as pigment dispersion syndrome, which can lead to blinding ocular hypertension and glaucoma. Migration of large, pigment-laden macrophages [obstructing outflow of aqueous humor through the trabecular meshwork and thus causing raised IOP](#) from the iris stroma into the trabecular meshwork, a specialized connective tissue that drains aqueous humor outflow from the anterior chamber of the eye, can block aqueous humor outflow and thus increase intraocular pressure [98]. Other potentially blinding conditions that involve large debris-laden macrophages include phacolytic glaucoma [108] (macrophages replete with lens debris), melanolytic glaucoma (melanin laden macrophages in uveal melanoma) [72] and hemolytic and ghost cell glaucoma (erythrocytes or ghost erythrocyte laden macrophages following hemorrhage in the eye) [108].

Choroidal macrophages

Distribution and phenotype

The choroid is the loose, vascular connective tissue rich in melanocytes that lies behind the retina, separated by a monolayer of cuboidal retinal pigment epithelium (RPE), whose barrier properties are essential to normal functioning of the adjacent neural retina (Fig 2A, B). The choroid is the sole source of nourishment for the outer retina in animals with a retinal blood supply (e.g. rodents, primates and humans) and in those without retinal vessels (e.g. guinea pigs and most herbivores). The blood flow in the choroid is amongst the highest in the human body and the capillary bed or choriocapillaris (Fig 2B) is fenestrated on the aspect immediately adjacent to the RPE. The first conclusive evidence that the choroid contained

dense networks of resident tissue macrophages came from studies in our group in the early 1990s when we developed methods for first obliquely sectioning [24] and then dissecting the choroid free from the eye cup prior to immunostaining and examination as intact sheets or wholemounts (Fig 2C) [8]. This latter novel method gave a new perspective of the density, morphology and distribution of immune cells in the rat choroid. Double immunoperoxidase staining revealed a rich ($\sim 600/\text{mm}^2$) and extensive network of ED1⁺ ED2⁺ ED3⁺ (CD68⁺ CD163⁺ and CD169⁺) macrophages in the perivascular space, which exhibited dendriform or elongated fusiform morphology (Fig 2D) [8, 45]. A small proportion of these resident tissue macrophages expressed MHC class II⁺ (OX6⁺); however, the majority of MHC class II⁺ cells were considered at the time to be putative dendritic cells, as they did not express the markers ED2 or ED3 [8]. Subsequent studies in the mouse eye revealed identical networks of F4/80⁺ (CD160) and SER4⁺ (CD169) resident macrophages in the choroid [66]. In addition, corroborative studies in human choroidal sections confirmed the presence of cells with a similar macrophage immunophenotype [10].

The availability of genetically modified fluorescent reporter mice, improvements in commercially available antibodies, and advances in confocal microscopy and multicolor flow cytometry over the last 10-15 years has seen a greater understanding of the distribution and phenotype of immune cells, including tissue macrophages, in the eye. In Cx3cr1^{gfp/+} and Cx3cr1^{gfp/gfp} reporter mice, the green fluorescent protein (*gfp*) gene replaces either one (*gfp/+*) or both (*gfp/gfp*) *Cx3cr1* alleles, which encode the fractalkine receptor that is expressed by monocyte-derived cells [36]. Studies of Balb/c Cx3cr1^{-gfp/+} and Cx3cr1^{gfp/gfp} mice in our lab 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 2E) [37]. This is in agreement with the few other studies of choroidal myeloid cells [47]. Flow cytometry of myeloid cells in the mouse uveal tract is challenging due to the accuracy needed in dissecting the distinct ocular compartments, the small volumes of the tissues and finally the resulting yield of cells. However, despite this we have recently performed detailed immunophenotypic analysis of macrophages in Balb/c Cx3cr1^{gfp/gfp} mouse choroid using large numbers of [pooled eyes \(from n = 18 mice\)](#) to obtain sufficient cell numbers for flow cytometry analysis. Our analysis shows that of the CD45⁺ cells present in the normal mouse choroid, 40% expressed F4/80 and are thus likely to represent the resident macrophage population (Fig. 3D). [In contrast, pooled iris-ciliary body preparations from the same mice revealed that 66% of all CD45⁺ cells co-expressed F4/80, suggesting that the normal iris and ciliary body are more densely populated with macrophages than the choroid.](#) Within the uveal tract, the CD45⁺ F4/80⁺ cells expressed the myeloid cell marker CD11b, but were CD11c-, indicating that were not likely to be dendritic cells. However, the vast majority of these uveal tract macrophages expressed MHC class II and the co-stimulatory molecule CD86 (Fig 3E), suggesting that they may have putative antigen presenting capabilities. This high co-expression of MHC class II supports our immunomorphological data from choroidal wholemounts (Fig 2E) and suggests our previous immunoperoxidase double staining data from rat choroid may have underestimated the number of MHC class II co-expressing macrophages which we showed co-

existed alongside MHC class II⁺ putative dendritic cells [8]. Alternatively, these discrepancies may be explained by species differences.

The precise role of tissue resident macrophages in the choroid in the normal eye has not been well studied; however, it is presumed that, in addition to immunosurveillance, these cells are likely to play an important role in maintaining homeostasis by removing debris and dead cells. Live imaging of DiI perfused [52] choroidoscleral explants from Cx3cr1-GFP mice crossed with B6 albino ((B6(Cg)-Tyr^{c-2j}/J) mice revealed that macrophages closely associated with the choriocapillaris were located on the scleral aspect of this flattened capillary bed (Fig 2F) with only occasional processes protruding through to the basal RPE [47]. A similar finding has been recently reported in the normal human choroid [65]. Kumar *et al.* [47] were the first to examine the behavior of macrophages in mouse choroid explants using time lapse confocal microscopy and revealed that their cell bodies were rather sessile however their processes exhibited repeated extension and retraction in multiple directions.

Role in pathology (AMD)

Age-related macular degeneration (AMD) is the commonest cause of vision loss in developed countries [20]. Resident tissue macrophages lie adjacent to the RPE, which sits upon a complex acellular layer of connective tissue and basal lamina known as Bruch's membrane (BrM) [10]. Changes in BrM and in the RPE underlie the pathology of AMD, which is considered to have an inflammatory [88] or 'para-inflammatory' pathogenesis [118]. A range of normal age-related changes occur in BrM including basal laminar deposits, basal linear deposits, soft drusen and hard drusen (Fig 2G). These changes can progress to RPE loss and atrophy of the neural retina, known as 'dry' AMD, whereas 'wet' AMD is characterised by choroidal neovascularisation (CNV) and formation of a disciform scar between the retina and the RPE [83, 95]. While the exact mechanisms by which sub-RPE debris accumulates with age and [how this progresses to the pathological state](#) in AMD is unknown, a number of factors are thought to contribute including: oxidative stress on neural retina and RPE [116], ischaemia due to ageing changes in the choriocapillaris, reduced phagocytic ability of RPE, and reduced clearance of Bruch's membrane debris by resident macrophages [57]. There is also a reported association of individuals with defective complement activation and susceptibility to AMD [30, 42].

Due to the putative role of macrophages in normal aging at the retinal-choroid interface and in the pathogenesis of AMD, there has been interest in the numbers and phenotype of macrophages in the sub-macular BrM and the choroid in normal human eyes and in eyes with early and advanced AMD [10, 65]. Unlike in normal ageing eyes (Fig. 1H), choroidal CD68⁺ macrophages in early AMD eyes are more closely associated with BrM and express inducible nitric oxide synthase (iNOS) [10], which suggests that these cells are classically activated macrophages (M1 phenotype). More recently McLeod *et al.* (2016) correlated macrophage shape, distribution, immunophenotype and morphology in choroidal wholemounts

from normal eyes and eyes with various grades of severity of AMD. The morphology and density ($433/\text{mm}^2$) of choroidal macrophages in normal eyes and in non-macular choroidal regions of diseased eyes was remarkably similar to previous data from mouse studies [66, 47]. These macrophages in the human choroid were Iba-1⁺ with a minor population expressing HLA-DR (MHC class II) and increased in density in submacular areas, where an increasing loss of complexity of shape (or greater roundedness) appeared to correlate with atrophy of the choriocapillaris and disease of the RPE [65]. However, immunophenotypic analysis was limited to these two markers. It seems logical that the vascular atrophy in the submacular choroid would act as a 'sterile' injury signal for activation of local choroidal macrophages which would then be involved in debris removal. Whether a defect in debris clearance plays a role in the development of AMD changes is currently the subject of intense research.

The uveal tract is one of only a few sites in the body where melanocytes exist in a rich connective tissue environment (Fig 2H), the meninges being one of the few others, where they co-exist alongside tissue macrophages [23]. Choroidal melanomas are the most aggressive tumours of the uveal melanomas and have poor prognostic outcomes (7). Oddly, the extent of inflammation is a prognostic marker for the development of metastatic disease and despite the fact that the eye is an immune-privileged site, inflammation is often described within intraocular tumours [110]. Choroidal melanomas are often characterized by increased numbers of tumor-infiltrating lymphocytes and macrophages, as well as an increased expression of Human Leukocyte Antigens (HLA) class I and II on uveal melanoma cells themselves [59]. Unlike most other malignancies, a high expression of the HLA class I and II antigens in uveal melanoma is associated with a poor prognosis [19]. The precise role of choroidal macrophages in these tumours besides phagocytosis of necrotic debris, as alluded to earlier, is still unclear but is of considerable interest as it may have a bearing in the design of novel immunotherapies.

Retinal microglia

Distribution and phenotype

The neural retina, along with the brain parenchyma and spinal cord is populated by specialized resident macrophages called microglia. Retinal microglia are normally located in three locations: the nerve fiber layer/ganglion cell layer, the inner plexiform layer and the outer plexiform layer (Fig. 3A). In the normal eye, retinal microglia exhibit a highly ramified morphology, which consists of a small cell body and multiple, branching, fine cellular processes (Fig. 3B). In response to injury or inflammation, microglia can retract their processes and display a stout morphology (Fig. 3C). [Much of our understanding of the phenotype of retinal microglia has come from studies in mice.](#) In steady-state conditions, virtually all of the CD45⁺ cells in the murine retina express low levels of the macrophage marker F4/80 (Fig. 3D), and the vast majority of these are microglia. This is in stark contrast to the mouse iris-ciliary body and choroid, which also contain rich populations of CD45⁺ F4/80⁻ resident cells, presumed to be dendritic cells (Fig. 3D). Whilst microglia are without question the most abundant immune cells present in the retina, it should

be noted that the retina also contains a small population of perivascular macrophages [73], which are located within the glia limitans of the retinal vasculature; however, these cells will not be discussed here.

Due to their location behind the blood-retina barriers, microglia exist within an 'immune privileged' microenvironment. Single-cell RNA sequencing studies in brain microglia have demonstrated that microglia are strikingly different than bone marrow-derived monocytes at the transcript level, but are closely related to perivascular macrophages [28]. Such studies have yet to be performed in the retina. In contrast, the expression of cell surface phenotypic markers on microglia overlaps significantly with that of other cells within the mononuclear phagocyte system. For example, in the normal mouse retina, microglia express CD11b, F4/80, CD115, Iba-1 and Cx3cr1 [29, 17, 80]. Microglia uniquely express low levels of CD45, which, in combination with other surface markers, enables them to be distinguished from other leukocyte populations using flow cytometry [80]. In steady-state conditions, retinal microglia do not express CD11c or MHC class II; however, they can upregulate these markers during retinal inflammation/injury [25, 126, 60]. In contrast, O'Koren *et al.* recently demonstrated that retinal microglia retain a CD45^{lo} F4/80^{lo} CD11c^{lo} I-A/I-E⁻ phenotype in a model of light-induced retinal degeneration [80]. Comparative studies of uveal tract resident macrophages and retinal microglia in our laboratory clearly demonstrate that microglia display a markedly different phenotype to iris and ciliary body macrophages and choroidal macrophages, and are thus a unique macrophage population within the eye (Fig. 3E).

Until recently, it was thought that microglia in the normal CNS were static, and as such they have been described as existing in a 'resting' state. However, it is now evident that their processes are highly motile and continuously sample the surrounding environment by continuous extension, retraction and remodeling [79, 48]. *In vivo* and *ex vivo* visualization of microglial behavior, made possible using Cx3cr1^{gfp/+} mice, has demonstrated that the processes of retinal microglia are highly dynamic structures that extend into the extracellular space in all directions. In contrast, their cell bodies remain relatively immobile within the adult mouse retina during steady-state conditions. The chemokine receptor Cx3cr1 plays a role in the velocity of microglial dendrite movement, but is not absolutely required for this dynamic behavior in both the normal and injured retina [55]. In the normal mouse retina, extracellular ATP regulates the morphology and motility of ramified microglia [22], potentially via P2 receptors [51]. Müller cells are likely candidates for the production of this regulatory ATP, as they have been shown to produce extracellular ATP via several pathways. Fontainhas *et al.* [22] demonstrated that inhibition of pannexin channels in *ex vivo* murine retinal explants decreased microglial process motility and morphology measurements including dendritic tree area, dendritic length and total number of branch points. It was hypothesized that neurotransmissions within the retina modulate pannexin-mediated ATP release by Müller cells, which in turn regulates the morphology and motility of microglia within the normal retina [114]. Thus, in the normal mouse retina, microglia are thought to closely interact with Müller cells.

Origin and bone marrow replenishment

The origin of microglia has been debated for many years [27]; indeed the ‘glia’ component of their name could be considered misleading as they now appear to be of a clearly different lineage from other ‘true’ glial cells such as astrocytes, Müller cells/radial glia and oligodendrocytes. Recent fate mapping studies have demonstrated that brain microglia (and thus likely retinal microglia) develop from yolk sac primitive macrophages, which arise prior to definitive hematopoiesis [26]. These progenitor cells colonize the murine neuroepithelium from embryonic day 8.5/9.5 and give rise to microglia within the developing CNS [3, 2, 26]. In human tissue, microglia precursors appear to migrate into the retina at the optic disc and peripheral margins, potentially via the developing retinal vasculature and blood vessels in the adjacent ciliary body respectively [35, 18]. As microglia precursors are present within the retina prior to retinal vascularization, it is hypothesized that initial colonization occurs via the ciliary body vessels, and then a secondary wave of microglia precursor cells invade as the retinal vasculature develops [18, 85]. However, a retinal vasculature is not absolutely required for migration of microglia precursors into this tissue, as numerous species (including fish, birds, some marsupials and many eutherian mammals) possess an avascular retina, yet still have rich populations of retinal microglia [78, 34 and unpublished data]. Studies in mice, rabbits and zebrafish suggest that microglia precursors may also enter the retina from the vitreous and its temporary vasculature [5, 34, 94].

Prior to evidence from fate mapping studies demonstrating that microglia had yolk sac origins it was initially believed that they were replenished by bone marrow-derived precursors during adulthood. Several studies have investigated retinal microglia ‘turnover’ in bone marrow chimera models, using fluorescent reporter mice as donors. We have previously demonstrated that bone marrow-derived cells are recruited to the retina following lethal irradiation, albeit at a much slower rate than the uveal tract [39]. Similar results have been reported in other studies [37, 75, 41, 44], suggesting that (under certain conditions) bone marrow-derived cells are capable of infiltrating the retina and contributing to the pool of microglia. Whilst these data provide interesting comparisons of the ‘turnover’ of resident myeloid cells between different sub-compartments of the eye and other tissues, they should not be interpreted to mean that microglia are replenished from the bone marrow in physiological conditions, as whole-body irradiation has been reported to cause neuroinflammation and dysregulation of the blood-brain and blood-retinal barriers [74, 4]. To circumvent the issues associated with irradiation and bone marrow transplantation, parabiotic mice have been used to examine the contribution of circulating hematopoietic cells to the pool of brain microglia. In contrast to previous bone marrow chimera models, parabiosis experiments have demonstrated that microglia are a stable population of cells that are maintained throughout adulthood by self-renewal without reconstitution from the blood stream [1, 26]. Given that the retina is comprised of neural tissue and is considered to be an extension of the brain, it is reasonable to assume that these findings can be extended to retinal microglia. Thus, there is an emerging consensus that during physiological conditions, bone marrow-derived cells are not likely to contribute to the pool of retinal microglia.

Physiological importance

Microglia perform several important functions in the retina, including phagocytosis of debris and apoptotic cells, modulating vasculogenesis, synapse maintenance and responding to inflammation. In a recent study, Wang *et al.* examined the effects of microglia depletion on the normal mouse retina, and demonstrated that microglia are not required for the maintenance of retinal architecture, nor the survival or organisation of retinal neurons. Conversely, sustained microglia depletion led to synaptic degeneration and atrophy within the outer plexiform layer and reduced visual function [115]. These findings suggest that retinal microglia are required for the maintenance of healthy neuronal synapses and are consistent with the known synaptic pruning role of microglia in the normal brain [82]. Microglia have also been implicated in vasculogenesis within the developing CNS [46]. Due to their close interactions with blood vessels, it has been hypothesized that certain macrophage populations may act as ‘bridges’ between islands of developing vessels [21]. Rymo *et al.* provided evidence that retinal microglia may support retinal vasculogenesis in the developing eye by demonstrating that: (i) retinal microglia are frequently located at sites of endothelial tip-cell anastomosis, and (ii) microglia ablation resulted in a reduced number of filopodia-bearing vascular sprouts in the retina. Furthermore, in a rat aortic ring culture model, the addition of microglia was shown to stimulate vessel sprout formation in a contact-independent manner [92]. Combined, these data demonstrate that microglia play a critical role in retinal development and homeostasis.

Role in pathology

Microglia are highly responsive to disruptions within the CNS microenvironment caused by injury, inflammation or infection. Immune responses in the CNS must be tightly regulated, therefore microglia are responsible for inducing and balancing pro- and anti-inflammatory responses to rapidly resolve neuroinflammation whilst maintaining the homeostatic conditions required for neuronal function. Microglia are thought to play key roles in a number of retinal diseases, including diabetic retinopathy, oxygen-induced retinopathy, experimental autoimmune uveoretinitis and glaucoma [89, 9, 49, 112]. Upon detection of danger signals, microglia rapidly upregulate pro-inflammatory markers, and release cytokines and effector molecules. In retinal degeneration, microglia migrate from the inner retina to the subretinal space, where they associate with the retinal pigment epithelium and phagocytose apoptotic or stressed photoreceptors [124, 122]. Accumulations of microglia in the subretinal space are also observed in the aging retina, and it is hypothesized that age-related changes in retinal microglia may contribute towards the pathogenesis of AMD [58].

Recent studies have demonstrated that in a range of disease models (lipopolysaccharide challenge, light injury, optic nerve crush and excitotoxicity), retinal microglia upregulate the mitochondrial protein, translocator protein (TSPO) [38, 113, 97]. In retinal microglia, administration of the TSPO agonist XBD173 significantly reduced the expression of the pro-inflammatory genes CCL2, IL-6 and iNOS. Furthermore, XBD173 reduced the migratory capacity of retinal microglia, increased their phagocytic

capacity and reversed their amoeboid morphology [38] suggesting that TSPO may be a promising target for immunomodulatory therapies [97]. Wang *et al.* have also recently identified that the endogenous ligand for TSPO, diazepam-binding inhibitor (DBI), is expressed by Müller cells in the retina. Interestingly, TSPO-DBI signaling in the retina was shown to negatively regulate microglial reactive oxygen species production, TNF- α production and proliferation in a range of inflammation/injury models. These findings suggest that microglia-Müller cell interactions promote dampening of the inflammatory response, possibly facilitating a return to steady-state conditions [113].

Other ocular macrophages

Whilst in the adult eye the lens is devoid of macrophages (or indeed other cell types besides lens epithelial cells), macrophages play a critical role in removal of the tunica vasculosa lentis and pupillary membrane, which are temporary vascular networks that nourish the developing lens until that role can be assumed by the aqueous humor [23, 70]. Many of these macrophages appear to be retained in the eye as vitreal macrophages or ‘hyalocytes’, which express characteristic macrophage/myeloid cell markers such as F4/80, Iba-1, CD169 and CD11b in mice, and ED2 in rats [86, 109]. Hyalocytes are primarily located between the inner limiting membrane of the retina and the vitreous membrane, which is the encapsulating layer of condensed vitreal collagen. The precise role of hyalocytes in the adult eye is unclear, but their morphology, activation status and increased number can be an early indication of pathological changes in models of diabetes and systemic exposure to infective agents [109]. The sclera, the outer protective layer of the eye, also contains resident macrophages [119, 96]. A recent study of the normal human sclera demonstrated that the episclera contained a high number of CD68⁺ CD11b⁺ MHC class II⁺ LYVE-1⁺ CCR7⁺ cells, presumed to be M1 macrophages [96]. To date, the significance and role of scleral macrophages in normal physiology of the eye and during ocular disease is not known.

Concluding remarks

The eye has several unique challenges to preserve optical clarity and maintain adequate function of the neural retina. There is a growing interest in the role of ocular macrophages during homeostasis and pathological conditions, particularly in limiting unwanted inflammation in the normally clear cornea and the retina, where excessive production of pro-inflammatory cytokines or growth factors can cause neurotoxicity and impact visual function. Though not covered in this review, attention is starting to be directed towards the spectrum of macrophage activation states, in particular the M1 vs M2 paradigm, in various ocular conditions such as dry eye disease [121], uveal melanoma [7] and the pathogenesis of AMD [123]. The ability to influence macrophage activation in the eye may offer future therapeutic avenues to treat ocular diseases with a known inflammatory basis.

References

1. Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM (2007) Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci* 10:1538-1543. nn2014 [pii]10.1038/nn2014
2. Alliot F, Godin I, Pessac B (1999) Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. *Brain research Developmental brain research* 117:145-152.
3. Alliot F, Lecaen E, Grima B, Pessac B (1991) Microglial progenitors with a high proliferative potential in the embryonic and adult mouse brain. *Proc Natl Acad Sci U S A* 88:1541-1545.
4. Alt C, Runnels JM, Mortensen LJ, Zaher W, Lin CP (2014) In vivo imaging of microglia turnover in the mouse retina after ionizing radiation and dexamethasone treatment. *Invest Ophthalmol Vis Sci* 55:5314-5319. 10.1167/iops.14-14254
5. Ashwell K (1989) Development of microglia in the albino rabbit retina. *J Comp Neurol* 287:286-301. 10.1002/cne.902870303
6. Brissette-Storkus CS, Reynolds SM, Lepisto AJ, Hendricks RL (2002) Identification of a novel macrophage population in the normal mouse corneal stroma. *Invest Ophthalmol Vis Sci* 43:2264-2271.
7. Bronkhorst IH, Jager MJ (2013) Inflammation in uveal melanoma. *Eye (Lond)* 27:217-223. 10.1038/eye.2012.253
8. Butler TL, McMenamin PG (1996) Resident and infiltrating immune cells in the uveal tract in the early and late stages of experimental autoimmune uveoretinitis. *Invest Ophthalmol Vis Sci* 37:2195-2210.
9. Checchin D, Sennlaub F, Levavasseur E, Leduc M, Chemtob S (2006) Potential role of microglia in retinal blood vessel formation. *Invest Ophthalmol Vis Sci* 47:3595-3602. 10.1167/iops.05-1522
10. Cherepanoff S, McMenamin P, Gillies MC, Kettle E, Sarks SH (2010) Bruch's membrane and choroidal macrophages in early and advanced age-related macular degeneration. *Br J Ophthalmol* 94:918-925. 10.1136/bjo.2009.165563
11. Chinnery HR, Carlson EC, Sun Y, Lin M, Burnett SH, Perez VL, McMenamin PG, Pearlman E (2009) Bone marrow chimeras and c-fms conditional ablation (Mafia) mice reveal an essential role for resident myeloid cells in lipopolysaccharide/TLR4-induced corneal inflammation. *J Immunol* 182:2738-2744. 10.4049/jimmunol.0803505
12. Chinnery HR, Humphries T, Clare A, Dixon AE, Howes K, Moran CB, Scott D, Zakrzewski M, Pearlman E, McMenamin PG (2008) Turnover of bone marrow-derived cells in the irradiated mouse cornea. *Immunology* 125:541-548. 10.1111/j.1365-2567.2008.02868.x
13. Chinnery HR, Leong CM, Chen W, Forrester JV, McMenamin PG (2015) TLR9 and TLR7/8 activation induces formation of keratic precipitates and giant macrophages in the mouse cornea. *J Leukoc Biol* 97:103-110. 10.1189/jlb.3AB0414-216R
14. Chinnery HR, Pearlman E, McMenamin PG (2008) Cutting edge: Membrane nanotubes in vivo: a feature of MHC class II+ cells in the mouse cornea. *J Immunol* 180:5779-5783. 180/9/5779 [pii]
15. Chinnery HR, Ruitenberg MJ, Plant GW, Pearlman E, Jung S, McMenamin PG (2007) The chemokine receptor CX3CR1 mediates homing of MHC class II-positive cells to the normal mouse corneal epithelium. *Invest Ophthalmol Vis Sci* 48:1568-1574. 10.1167/iops.06-0746
16. Cursiefen C, Chen L, Borges LP, Jackson D, Cao J, Radziejewski C, D'Amore PA, Dana MR, Wiegand SJ, Streilein JW (2004) VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest* 113:1040-1050. 10.1172/jci20465
17. Dando SJ, Naranjo Golborne C, Chinnery HR, Ruitenberg MJ, McMenamin PG (2016) A case of mistaken identity: CD11c-eYFP(+) cells in the normal mouse brain parenchyma and neural retina display the phenotype of microglia, not dendritic cells. *Glia* 64:1331-1349. 10.1002/glia.23005
18. Diaz-Araya CM, Provis JM, Penfold PL, Billson FA (1995) Development of microglial topography in human retina. *J Comp Neurol* 363:53-68. 10.1002/cne.903630106
19. Ericsson C, Seregard S, Bartolazzi A, Levitskaya E, Ferrone S, Kiessling R, Larsson O (2001) Association of HLA class I and class II antigen expression and mortality in uveal melanoma. *Invest Ophthalmol Vis Sci* 42:2153-2156.

20. Evans JR (2001) Risk factors for age-related macular degeneration. *Prog Retin Eye Res* 20:227-253. S1350-9462(00)00023-9 [pii]
21. Fantin A, Vieira JM, Gestri G, Denti L, Schwarz Q, Prykhozhij S, Peri F, Wilson SW, Ruhrberg C (2010) Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood* 116:829-840. 10.1182/blood-2009-12-257832
22. Fontainhas AM, Wang M, Liang KJ, Chen S, Mettu P, Damani M, Fariss RN, Li W, Wong WT (2011) Microglial morphology and dynamic behavior is regulated by ionotropic glutamatergic and GABAergic neurotransmission. *PLoS One* 6:e15973. 10.1371/journal.pone.0015973
23. Forrester JV, Dick AD, McMenamin PG, Lee WL (2002) *The Eye: Basic Sciences in Practice*. Edinburgh: Saunders.
24. Forrester JV, McMenamin PG, Holthouse I, Lumsden L, Liversidge J (1994) Localization and characterization of major histocompatibility complex class II-positive cells in the posterior segment of the eye: implications for induction of autoimmune uveoretinitis. *Invest Ophthalmol Vis Sci* 35:64-77.
25. Gallego BI, Salazar JJ, de Hoz R, Rojas B, Ramirez AI, Salinas-Navarro M, Ortin-Martinez A, Valiente-Soriano FJ, Aviles-Trigueros M, Villegas-Perez MP, Vidal-Sanz M, Trivino A, Ramirez JM (2012) IOP induces upregulation of GFAP and MHC-II and microglia reactivity in mice retina contralateral to experimental glaucoma. *Journal of neuroinflammation* 9:92. 10.1186/1742-2094-9-92
26. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M (2010) Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 330:841-845. 10.1126/science.1194637
27. Ginhoux F, Lim S, Hoeffel G, Low D, Huber T (2013) Origin and differentiation of microglia. *Frontiers in cellular neuroscience* 7:45. 10.3389/fncel.2013.00045
28. Goldmann T, Wieghofer P, Jordao MJ, Prutek F, Hagemeyer N, Frenzel K, Amann L, Staszewski O, Kierdorf K, Krueger M, Locatelli G, Hochgerner H, Zeiser R, Epelman S, Geissmann F, Priller J, Rossi FM, Bechmann I, Kerschensteiner M, Linnarsson S, Jung S, Prinz M (2016) Origin, fate and dynamics of macrophages at central nervous system interfaces. *Nat Immunol* 17:797-805. 10.1038/ni.3423
29. Gregerson DS, Yang J (2003) CD45-positive cells of the retina and their responsiveness to in vivo and in vitro treatment with IFN-gamma or anti-CD40. *Invest Ophthalmol Vis Sci* 44:3083-3093.
30. Hageman GS, Anderson DH, Johnson LV, Hancox LS, Taiber AJ, Hardisty LI, Hageman JL, Stockman HA, Borchardt JD, Gehrs KM, Smith RJ, Silvestri G, Russell SR, Klaver CC, Barbazetto I, Chang S, Yannuzzi LA, Barile GR, Merriam JC, Smith RT, Olsh AK, Bergeron J, Zernant J, Merriam JE, Gold B, Dean M, Allikmets R (2005) A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A* 102:7227-7232. 10.1073/pnas.0501536102
31. Hamrah P, Huq SO, Liu Y, Zhang Q, Dana MR (2003) Corneal immunity is mediated by heterogeneous population of antigen-presenting cells. *J Leukoc Biol* 74:172-178.
32. Hamrah P, Liu Y, Zhang Q, Dana MR (2003) Alterations in corneal stromal dendritic cell phenotype and distribution in inflammation. *Arch Ophthalmol* 121:1132-1140. 10.1001/archoph.121.8.1132
33. Hamrah P, Liu Y, Zhang Q, Dana MR (2003) The corneal stroma is endowed with a significant number of resident dendritic cells. *Invest Ophthalmol Vis Sci* 44:581-589.
34. Herbomel P, Thisse B, Thisse C (2001) Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epidermis through a M-CSF receptor-dependent invasive process. *Dev Biol* 238:274-288. 10.1006/dbio.2001.0393
35. Hume DA, Perry VH, Gordon S (1983) Immunohistochemical localization of a macrophage-specific antigen in developing mouse retina: phagocytosis of dying neurons and differentiation of microglial cells to form a regular array in the plexiform layers. *J Cell Biol* 97:253-257.
36. Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, Littman DR (2000) Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol* 20:4106-4114.
37. Kaneko H, Nishiguchi KM, Nakamura M, Kachi S, Terasaki H (2008) Characteristics of bone marrow-derived microglia in the normal and injured retina. *Invest Ophthalmol Vis Sci* 49:4162-4168. 10.1167/iovs.08-1738

38. Karlstetter M, Nothdurfter C, Aslanidis A, Moeller K, Horn F, Scholz R, Neumann H, Weber BH, Rupprecht R, Langmann T (2014) Translocator protein (18 kDa) (TSPO) is expressed in reactive retinal microglia and modulates microglial inflammation and phagocytosis. *Journal of neuroinflammation* 11:3. 10.1186/1742-2094-11-3
39. Kezic J, McMenamin PG (2008) Differential turnover rates of monocyte-derived cells in varied ocular tissue microenvironments. *J Leukoc Biol* 84:721-729. 10.1189/jlb.0308166
40. Kezic J, Xu H, Chinnery HR, Murphy CC, McMenamin PG (2008) Retinal microglia and uveal tract dendritic cells and macrophages are not CX3CR1 dependent in their recruitment and distribution in the young mouse eye. *Invest Ophthalmol Vis Sci* 49:1599-1608. 10.1167/iops.07-0953
41. Kezic JM, McMenamin PG (2013) The effects of CX3CR1 deficiency and irradiation on the homing of monocyte-derived cell populations in the mouse eye. *PLoS One* 8:e68570. 10.1371/journal.pone.0068570
42. Klein RJ, Zeiss C, Chew EY, Tsai JY, Sackler RS, Haynes C, Henning AK, SanGiovanni JP, Mane SM, Mayne ST, Bracken MB, Ferris FL, Ott J, Barnstable C, Hoh J (2005) Complement factor H polymorphism in age-related macular degeneration. *Science* 308:385-389. 1109557 [pii] 10.1126/science.1109557
43. Knickelbein JE, Watkins SC, McMenamin PG, Hendricks RL (2009) Stratification of Antigen-presenting Cells within the Normal Cornea. *Ophthalmol Eye Dis* 1:45-54.
44. Koso H, Tsuchiko A, Lai CY, Baba Y, Otsu M, Ueno K, Nagasaki M, Suzuki Y, Watanabe S (2016) Conditional rod photoreceptor ablation reveals Sall1 as a microglial marker and regulator of microglial morphology in the retina. *Glia* 64:2005-2024. 10.1002/glia.23038
45. Krause L, Coupland SE, Hoffmann F (1996) The behaviour of ED1- and ED2-positive cells in the rat iris and choroid following penetrating keratoplasty and cyclosporin A therapy. *Graefes Arch Clin Exp Ophthalmol* 234 Suppl 1:S149-158.
46. Kubota Y, Takubo K, Shimizu T, Ohno H, Kishi K, Shibuya M, Saya H, Suda T (2009) M-CSF inhibition selectively targets pathological angiogenesis and lymphangiogenesis. *J Exp Med* 206:1089-1102. 10.1084/jem.20081605
47. Kumar A, Zhao L, Fariss RN, McMenamin PG, Wong WT (2014) Vascular associations and dynamic process motility in perivascular myeloid cells of the mouse choroid: implications for function and senescent change. *Invest Ophthalmol Vis Sci* 55:1787-1796. 10.1167/iops.13-13522
48. Lee JE, Liang KJ, Fariss R, Wong WT (2008) Ex vivo Dynamic Imaging of Retinal Microglia using Time-lapse Confocal Microscopy. *Invest Ophthalmol Vis Sci* 4169-4176. 10.1167/iops.08-2076
49. Li L, Eter N, Heiduschka P (2015) The microglia in healthy and diseased retina. *Exp Eye Res* 136:116-130. 10.1016/j.exer.2015.04.020
50. Li X, Shen S, Urso D, Kalique S, Park SH, Sharafieh R, O'Rourke J, Cone RE (2006) Phenotypic and immunoregulatory characteristics of monocytic iris cells. *Immunology* 117:566-575. 10.1111/j.1365-2567.2006.02333.x
51. Li Y, Du XF, Liu CS, Wen ZL, Du JL (2012) Reciprocal regulation between resting microglial dynamics and neuronal activity in vivo. *Dev Cell* 23:1189-1202. 10.1016/j.devcel.2012.10.027
52. Li Y, Song Y, Zhao L, Gaidosh G, Laties AM, Wen R (2008) Direct labeling and visualization of blood vessels with lipophilic carbocyanine dye DiI. *Nature protocols* 3:1703-1708. 10.1038/nprot.2008.172
53. Li Z, Burns AR, Rumbaut RE, Smith CW (2007) gamma delta T Cells Are Necessary for Platelet and Neutrophil Accumulation in Limbal Vessels and Efficient Epithelial Repair after Corneal Abrasion. *Am J Pathol* 171:838-845.
54. Li Z, Burns AR, Smith CW (2006) Two waves of neutrophil emigration in response to corneal epithelial abrasion: distinct adhesion molecule requirements. *Invest Ophthalmol Vis Sci* 47:1947-1955.
55. Liang KJ, Lee JE, Wang YD, Ma W, Fontainhas AM, Fariss RN, Wong WT (2009) Regulation of dynamic behavior of retinal microglia by CX3CR1 signaling. *Invest Ophthalmol Vis Sci* 50:4444-4451. 10.1167/iops.08-3357
56. Lu JM, Zhou ZY, Zhang XR, Li XL, Wang HF, Song XJ (2010) A preliminary study of mesenchymal stem cell-like cells derived from murine corneal stroma. *Graefes Arch Clin Exp Ophthalmol* 248:1279-1285. 10.1007/s00417-010-1367-0
57. Luhmann UF, Robbie S, Munro PM, Barker SE, Duran Y, Luong V, Fitzke FW, Bainbridge JW, Ali RR, MacLaren RE (2009) The drusenlike phenotype in aging Ccl2-knockout mice is caused by an

- accelerated accumulation of swollen autofluorescent subretinal macrophages. *Invest Ophthalmol Vis Sci* 50:5934-5943. 10.1167/iovs.09-3462
58. Ma W, Wong WT (2016) Aging Changes in Retinal Microglia and their Relevance to Age-related Retinal Disease. *Adv Exp Med Biol* 854:73-78. 10.1007/978-3-319-17121-0_11
 59. Maat W, Ly LV, Jordanova ES, de Wolff-Rouendaal D, Schalijs-Delfos NE, Jager MJ (2008) Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma. *Invest Ophthalmol Vis Sci* 49:505-510. 10.1167/iovs.07-0786
 60. Maneu V, Noailles A, Megias J, Gomez-Vicente V, Carpena N, Gil ML, Gozalbo D, Cuenca N (2014) Retinal microglia are activated by systemic fungal infection. *Invest Ophthalmol Vis Sci* 55:3578-3585. 10.1167/iovs.14-14051
 61. Maruyama K, Ii M, Cursiefen C, Jackson DG, Keino H, Tomita M, Van Rooijen N, Takenaka H, D'Amore PA, Stein-Streilein J, Losordo DW, Streilein JW (2005) Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *J Clin Invest* 115:2363-2372. 10.1172/jci23874
 62. Maruyama K, Nakazawa T, Cursiefen C, Maruyama Y, Van Rooijen N, D'Amore PA, Kinoshita S (2012) The maintenance of lymphatic vessels in the cornea is dependent on the presence of macrophages. *Invest Ophthalmol Vis Sci* 53:3145-3153. 10.1167/iovs.11-8010
 63. May CA (1999) Mast cell heterogeneity in the human uvea. *Histochem Cell Biol* 112:381-386.
 64. Mayer WJ, Irschick UM, Moser P, Wurm M, Huemer HP, Romani N, Irschick EU (2007) Characterization of antigen-presenting cells in fresh and cultured human corneas using novel dendritic cell markers. *Invest Ophthalmol Vis Sci* 48:4459-4467.
 65. McLeod DS, Bhutto I, Edwards MM, Silver RE, Seddon JM, Luty GA (2016) Distribution and Quantification of Choroidal Macrophages in Human Eyes With Age-Related Macular Degeneration. *Invest Ophthalmol Vis Sci* 57:5843-5855. 10.1167/iovs.16-20049
 66. McMenamin PG (1999) Dendritic cells and macrophages in the uveal tract of the normal mouse eye. *Br J Ophthalmol* 83:598-604.
 67. McMenamin PG (1997) The distribution of immune cells in the uveal tract of the normal eye. *Eye* 11 (Pt 2):183-193.
 68. McMenamin PG (2000) Optimal methods for preparation and immunostaining of iris, ciliary body, and choroidal wholemounts. *Invest Ophthalmol Vis Sci* 41:3043-3048.
 69. McMenamin PG, Crewe J, Morrison S, Holt PG (1994) Immunomorphologic studies of macrophages and MHC class II-positive dendritic cells in the iris and ciliary body of the rat, mouse, and human eye. *Invest Ophthalmol Vis Sci* 35:3234-3250.
 70. McMenamin PG, Djano J, Wealhall R, Griffin BJ (2002) Characterization of the macrophages associated with the tunica vasculosa lentis of the rat eye. *Invest Ophthalmol Vis Sci* 43:2076-2082.
 71. McMenamin PG, Holthouse I, Holt PG (1992) Class II major histocompatibility complex (Ia) antigen-bearing dendritic cells within the iris and ciliary body of the rat eye: distribution, phenotype and relation to retinal microglia. *Immunology* 77:385-393.
 72. McMenamin PG, Lee WR (1986) Ultrastructural pathology of melanolytic glaucoma. *Br J Ophthalmol* 70:895-906.
 73. Mendes-Jorge L, Ramos D, Luppo M, Llombart C, Alexandre-Pires G, Nacher V, Melgarejo V, Correia M, Navarro M, Carretero A, Tafuro S, Rodriguez-Baeza A, Esperanca-Pina JA, Bosch F, Ruberte J (2009) Scavenger function of resident autofluorescent perivascular macrophages and their contribution to the maintenance of the blood-retinal barrier. *Invest Ophthalmol Vis Sci* 50:5997-6005. 10.1167/iovs.09-3515
 74. Mildner A, Schmidt H, Nitsche M, Merkler D, Hanisch UK, Mack M, Heikenwalder M, Bruck W, Priller J, Prinz M (2007) Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. *Nat Neurosci* 10:1544-1553. 10.1038/nn2015
 75. Muther PS, Semkova I, Schmidt K, Abari E, Kuebbeler M, Beyer M, Abken H, Meyer KL, Kociok N, Joussen AM (2010) Conditions of retinal glial and inflammatory cell activation after irradiation in a GFP-chimeric mouse model. *Invest Ophthalmol Vis Sci* 51:4831-4839. 10.1167/iovs.09-4923
 76. Nakamura T, Ishikawa F, Sonoda KH, Hisatomi T, Qiao H, Yamada J, Fukata M, Ishibashi T, Harada M, Kinoshita S (2005) Characterization and distribution of bone marrow-derived cells in mouse cornea. *Invest Ophthalmol Vis Sci* 46:497-503.
 77. Naumann GOH, Apple DJ (1986) *Pathology of the Eye*. New York: Springer Verlag.

78. Navascues J, Moujahid A, Quesada A, Cuadros MA (1994) Microglia in the avian retina: immunocytochemical demonstration in the adult quail. *J Comp Neurol* 350:171-186. 10.1002/cne.903500203
79. Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 308:1314-1318. 10.1126/science.1110647
80. O'Koren EG, Mathew R, Saban DR (2016) Fate mapping reveals that microglia and recruited monocyte-derived macrophages are definitively distinguishable by phenotype in the retina. *Scientific reports* 6:20636. 10.1038/srep20636
81. Onfelt B, Nedvetzki S, Yanagi K, Davis DM (2004) Cutting edge: Membrane nanotubes connect immune cells. *J Immunol* 173:1511-1513.
82. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, Giustetto M, Ferreira TA, Guiducci E, Dumas L, Ragozzino D, Gross CT (2011) Synaptic pruning by microglia is necessary for normal brain development. *Science* 333:1456-1458. 10.1126/science.1202529
83. Penfold P, Killingsworth M, Sarks S (1984) An ultrastructural study of the role of leucocytes and fibroblasts in the breakdown of Bruch's membrane. *Australian journal of ophthalmology* 12:23-31.
84. Prevo R, Banerji S, Ferguson DJ, Clasper S, Jackson DG (2001) Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. *J Biol Chem* 276:19420-19430. 10.1074/jbc.M011004200
85. Provis JM, Leech J, Diaz CM, Penfold PL, Stone J, Keshet E (1997) Development of the human retinal vasculature: cellular relations and VEGF expression. *Exp Eye Res* 65:555-568. 10.1006/exer.1997.0365
86. Qiao H, Hisatomi T, Sonoda KH, Kura S, Sassa Y, Kinoshita S, Nakamura T, Sakamoto T, Ishibashi T (2005) The characterisation of hyalocytes: the origin, phenotype, and turnover. *Br J Ophthalmol* 89:513-517. 10.1136/bjo.2004.050658
87. Ramke M, Zhou X, Materne EC, Rajaiya J, Chodosh J (2016) Resident corneal c-fms(+) macrophages and dendritic cells mediate early cellular infiltration in adenovirus keratitis. *Exp Eye Res* 147:144-147. 10.1016/j.exer.2016.05.016
88. Ramkumar HL, Zhang J, Chan CC (2010) Retinal ultrastructure of murine models of dry age-related macular degeneration (AMD). *Prog Retin Eye Res* 29:169-190. 10.1016/j.preteyeres.2010.02.002
89. Rao NA, Kimoto T, Zamir E, Giri R, Wang R, Ito S, Pararajasegaram G, Read RW, Wu GS (2003) Pathogenic role of retinal microglia in experimental uveoretinitis. *Invest Ophthalmol Vis Sci* 44:22-31.
90. Rehberg M, Nekolla K, Sellner S, Praetner M, Mildner K, Zeuschner D, Krombach F (2016) Intercellular Transport of Nanomaterials is Mediated by Membrane Nanotubes In Vivo. *Small* (Weinheim an der Bergstrasse, Germany) 12:1882-1890. 10.1002/smll.201503606
91. Rustom A, Saffrich R, Markovic I, Walther P, Gerdes HH (2004) Nanotubular highways for intercellular organelle transport. *Science* 303:1007-1010.
92. Rymo SF, Gerhardt H, Wolfhagen Sand F, Lang R, Uv A, Betsholtz C (2011) A two-way communication between microglial cells and angiogenic sprouts regulates angiogenesis in aortic ring cultures. *PLoS One* 6:e15846. 10.1371/journal.pone.0015846
93. Saika S, Ikeda K, Yamanaka O, Flanders KC, Okada Y, Miyamoto T, Kitano A, Ooshima A, Nakajima Y, Ohnishi Y, Kao WW (2006) Loss of tumor necrosis factor alpha potentiates transforming growth factor beta-mediated pathogenic tissue response during wound healing. *Am J Pathol* 168:1848-1860. 168/6/1848 [pii]
94. Santos AM, Calvente R, Tassi M, Carrasco MC, Martin-Oliva D, Marin-Teva JL, Navascues J, Cuadros MA (2008) Embryonic and postnatal development of microglial cells in the mouse retina. *J Comp Neurol* 506:224-239. 10.1002/cne.21538
95. Sarks S, Cherepanoff S, Killingsworth M, Sarks J (2007) Relationship of Basal laminar deposit and membranous debris to the clinical presentation of early age-related macular degeneration. *Invest Ophthalmol Vis Sci* 48:968-977. 10.1167/iovs.06-0443
96. Schlereth SL, Kremers S, Schrod F, Cursiefen C, Heindl LM (2016) Characterization of Antigen-Presenting Macrophages and Dendritic Cells in the Healthy Human Sclera. *Invest Ophthalmol Vis Sci* 57:4878-4885. 10.1167/iovs.15-18552
97. Scholz R, Caramoy A, Bhuckory MB, Rashid K, Chen M, Xu H, Grimm C, Langmann T (2015) Targeting translocator protein (18 kDa) (TSPO) dampens pro-inflammatory microglia reactivity in

- the retina and protects from degeneration. *Journal of neuroinflammation* 12:201. 10.1186/s12974-015-0422-5
98. Schraermeyer M, Schnichels S, Julien S, Heiduschka P, Bartz-Schmidt KU, Schraermeyer U (2009) Ultrastructural analysis of the pigment dispersion syndrome in DBA/2J mice. *Graefes Arch Clin Exp Ophthalmol* 247:1493-1504. 10.1007/s00417-009-1146-y
 99. Seyed-Razavi Y, Chinnery HR, McMenamin PG (2014) A novel association between resident tissue macrophages and nerves in the peripheral stroma of the murine cornea. *Invest Ophthalmol Vis Sci* 55:1313-1320. 10.1167/iovs.13-12995
 100. Seyed-Razavi Y, Hickey MJ, Kuffova L, McMenamin PG, Chinnery HR (2013) Membrane nanotubes in myeloid cells in the adult mouse cornea represent a novel mode of immune cell interaction. *Immunol Cell Biol* 91:89-95. 10.1038/icb.2012.52
 101. Shao H, Scott SG, Nakata C, Hamad AR, Chakravarti S (2013) Extracellular matrix protein lumican promotes clearance and resolution of *Pseudomonas aeruginosa* keratitis in a mouse model. *PLoS One* 8:e54765. 10.1371/journal.pone.0054765
 102. Slegers TP, Broersma L, van Rooijen N, Hooymans JM, van Rij G, van der Gaag R (2004) Macrophages play a role in the early phase of corneal allograft rejection in rats. *Transplantation* 77:1641-1646.
 103. Slegers TP, van der Gaag R, van Rooijen N, van Rij G, Streilein JW (2003) Effect of local macrophage depletion on cellular immunity and tolerance evoked by corneal allografts. *Curr Eye Res* 26:73-79.
 104. Sosnova M, Bradl M, Forrester JV (2005) CD34+ corneal stromal cells are bone marrow-derived and express hemopoietic stem cell markers. *Stem Cells* 23:507-515.
 105. Sun Y, Karmakar M, Roy S, Ramadan RT, Williams SR, Howell S, Shive CL, Han Y, Stopford CM, Rietsch A, Pearlman E (2010) TLR4 and TLR5 on corneal macrophages regulate *Pseudomonas aeruginosa* keratitis by signaling through MyD88-dependent and -independent pathways. *J Immunol* 185:4272-4283. 10.4049/jimmunol.1000874
 106. Tay SS, Roediger B, Tong PL, Tikoo S, Weninger W (2014) The Skin-Resident Immune Network. *Current dermatology reports* 3:13-22. 10.1007/s13671-013-0063-9
 107. Thill M, Schlagner K, Altenahr S, Ergun S, Faragher RG, Kilic N, Bednarz J, Vohwinkel G, Rogiers X, Hossfeld DK, Richard G, Gehling UM (2007) A novel population of repair cells identified in the stroma of the human cornea. *Stem Cells Dev* 16:733-745. 10.1089/scd.2006.0084
 108. Ueno H, Tamai A, Iyota K, Moriki T (1989) Electron microscopic observation of the cells floating in the anterior chamber in a case of phacolytic glaucoma. *Japanese journal of ophthalmology* 33:103-113.
 109. Vagaja NN, Chinnery HR, Binz N, Kezic JM, Rakoczy EP, McMenamin PG (2012) Changes in murine hyalocytes are valuable early indicators of ocular disease. *Invest Ophthalmol Vis Sci* 53:1445-1451. 10.1167/iovs.11-8601
 110. van Essen TH, van Pelt SI, Bronkhorst IH, Versluis M, Nemati F, Laurent C, Luyten GP, van Hall T, van den Elsen PJ, van der Velden PA, Decaudin D, Jager MJ (2016) Upregulation of HLA Expression in Primary Uveal Melanoma by Infiltrating Leukocytes. *PLoS One* 11:e0164292. 10.1371/journal.pone.0164292
 111. Van Rooijen N, Sanders A (1994) Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 174:83-93.
 112. Wang JW, Chen SD, Zhang XL, Jonas JB (2016) Retinal Microglia in Glaucoma. *Journal of glaucoma* 25:459-465. 10.1097/ijg.0000000000000200
 113. Wang M, Wang X, Zhao L, Ma W, Rodriguez IR, Fariss RN, Wong WT (2014) Macrogliia-microglia interactions via TSPO signaling regulates microglial activation in the mouse retina. *J Neurosci* 34:3793-3806. 10.1523/jneurosci.3153-13.2014
 114. Wang M, Wong WT (2014) Microglia-Muller cell interactions in the retina. *Adv Exp Med Biol* 801:333-338. 10.1007/978-1-4614-3209-8_42
 115. Wang X, Zhao L, Zhang J, Fariss RN, Ma W, Kretschmer F, Wang M, Qian HH, Badea TC, Diamond JS, Gan WB, Roger JE, Wong WT (2016) Requirement for Microglia for the Maintenance of Synaptic Function and Integrity in the Mature Retina. *J Neurosci* 36:2827-2842. 10.1523/jneurosci.3575-15.2016
 116. Winkler BS, Boulton ME, Gottsch JD, Sternberg P (1999) Oxidative damage and age-related macular degeneration. *Mol Vis* 5:32.

117. Wobmann PR, Fine BS (1972) The clump cells of Koganei. A light and electron microscopic study. *Am J Ophthalmol* 73:90-101.
118. Xu H, Chen M, Forrester JV (2009) Para-inflammation in the aging retina. *Prog Retin Eye Res* 28:348-368. 10.1016/j.preteyeres.2009.06.001
119. Xu H, Chen M, Reid DM, Forrester JV (2007) LYVE-1-Positive Macrophages Are Present in Normal Murine Eyes. *Invest Ophthalmol Vis Sci* 48:2162-2171. 10.1167/iovs.06-0783
120. Yamagami S, Usui T, Amano S, Ebihara N (2005) Bone Marrow-Derived Cells in Mouse and Human Cornea. *Cornea* 24:S71-S74.
121. You IC, Coursey TG, Bian F, Barbosa FL, de Paiva CS, Pflugfelder SC (2015) Macrophage Phenotype in the Ocular Surface of Experimental Murine Dry Eye Disease. *Archivum immunologiae et therapiae experimentalis* 63:299-304. 10.1007/s00005-015-0335-0
122. Zabel MK, Zhao L, Zhang Y, Gonzalez SR, Ma W, Wang X, Fariss RN, Wong WT (2016) Microglial phagocytosis and activation underlying photoreceptor degeneration is regulated by CX3CL1-CX3CR1 signaling in a mouse model of retinitis pigmentosa. *Glia* 64:1479-1491. 10.1002/glia.23016
123. Zandi S, Nakao S, Chun KH, Fiorina P, Sun D, Arita R, Zhao M, Kim E, Schueller O, Campbell S, Taher M, Melhorn MI, Schering A, Gatti F, Tezza S, Xie F, Vergani A, Yoshida S, Ishikawa K, Yamaguchi M, Sasaki F, Schmidt-Ullrich R, Hata Y, Enaida H, Yuzawa M, Yokomizo T, Kim YB, Sweetnam P, Ishibashi T, Hafezi-Moghadam A (2015) ROCK-isoform-specific polarization of macrophages associated with age-related macular degeneration. *Cell reports* 10:1173-1186. 10.1016/j.celrep.2015.01.050
124. Zhao L, Zabel MK, Wang X, Ma W, Shah P, Fariss RN, Qian H, Parkhurst CN, Gan WB, Wong WT (2015) Microglial phagocytosis of living photoreceptors contributes to inherited retinal degeneration. *EMBO molecular medicine* 7:1179-1197. 10.15252/emmm.201505298
125. Zhou D, Chen YT, Chen F, Gallup M, Vijmasi T, Bahrami AF, Noble LB, van Rooijen N, McNamara NA (2012) Critical involvement of macrophage infiltration in the development of Sjogren's syndrome-associated dry eye. *Am J Pathol* 181:753-760. 10.1016/j.ajpath.2012.05.014
126. Zinkernagel MS, Chinnery HR, Ong ML, Petitjean C, Voigt V, McLenachan S, McMenamin PG, Hill GR, Forrester JV, Wikstrom ME, Degli-Esposti MA (2013) Interferon gamma-dependent migration of microglial cells in the retina after systemic cytomegalovirus infection. *Am J Pathol* 182:875-885. 10.1016/j.ajpath.2012.11.031

Figure Legends

Figure 1: Confocal microscopy images demonstrating the distribution and phenotype of corneal macrophages and their close association with keratocytes and epithelial nerves.

(A) *En face* view of resident Cx3cr1-GFP⁺ macrophages (green) in the mouse corneal stroma, some of which also express MHC class II (red). Z-profile view shows the localization of the majority of cells in the stroma, with a few double positive dendritic cells visible in the epithelium. (B) MHC class II⁺ macrophages (red) embedded between corneal stromal keratocytes in the normal mouse cornea (F-actin; green). (C) Long, wandering MHC class II⁺ membrane nanotubes and bridging nanotubes between two cells (D) in the corneal stroma 24 hours after lipopolysaccharide induced inflammation. (E) *En face*, anterior view of a rendered confocal z-series showing the central corneal epithelial nerve plexus (β III-tubulin; grey) with CD45⁺ macrophages (red) visible beneath the nerves. (F) Posterior view of the same CD45⁺ macrophages in the anterior corneal stroma. Z profile beneath panel E and F shows the close association of CD45⁺ macrophages with the epithelial nerve plexus. Scale bars: A, 150um; B-F, 40um.

Figure 2: Distribution and phenotype of macrophages in the choroid.

(A) Semithin resin section of the normal human retina and choroid. (B) Higher power view of the RPE and choriocapillaris, separated by Bruch's membrane (arrows). (C) Summary of the method used to isolate the choroid for wholemount staining. (D) Light micrograph of immunoperoxidase stained rat choroidal wholemount. Note all macrophages co-stain purple illustrating that they co-express both ED2 and ED3. (E) Confocal microscopy image of a large region of a choroidal wholemount from Balb/c Cx3cr1-^{gfp/gfp} mouse stained with MHC class II (red). GFP/green fluorescence indicates that all myeloid cells are Cx3cr1⁺ with a significant majority co-expressing MHC class II (yellow/orange). Note the similarity of the pattern of distribution between rat and mouse. (F) The scleral aspect of the choriocapillaris (red) in Balb/c Cx3cr1-^{gfp/gfp} mice as visualised by Imaris software using confocal microscopy data. (G) Light microscopy of post-mortem 'normal' aged (83 years old) human eye showing extensive drusen (green arrowheads) under the RPE but above BrM (black arrow). (H) The choroid from a normal 53 year old human subject which has been deliberately obliquely sectioned (hence the RPE appearing multilayered and the choroid being apparently 'thicker' in profile) and stained with Iba-1 (red). Note the large numbers of pleomorphic Iba-1⁺ macrophages in the choroid beneath the RPE and interspersed between the melanocytes (brown). CC = choriocapillaris; GCL = ganglion cell layer; INL = inner nuclear layer; IPL = inner plexiform layer; NFL = nerve fibre layer; ONL = outer nuclear layer; OPL = outer plexiform layer; PRL = photoreceptor layer; RPE = retinal pigment epithelium. Scale bars: A, 100 um; B, 20 um; D-E, 100 um; F-G, 20 um; H, 10 um.

Figure 3: Distribution and phenotype of retinal microglia, and comparison with uveal tract macrophages.

(A) Orthogonal view of a mouse retinal wholemount demonstrates that microglia (green) are distributed within the ganglion cell layer/nerve fiber layer (GCL/NFL), inner plexiform layer (IPL) and outer plexiform layer (OPL). MHC class II⁺ cells (red) are observed in the choroid immediately underneath the retinal pigmented epithelium (RPE). Nuclei (blue) are stained with Hoechst. (B) Retinal microglia in the OPL of the normal Balb/c Cx3cr1-*gfp/gfp* mouse retina exhibit a highly ramified morphology, whereas 24 hours following systemic lipopolysaccharide challenge (C), retinal microglia begin to retract their processes and adopt a stout morphology. (D) Flow cytometric analysis of CD45⁺ cells in the Balb/c Cx3cr1-*gfp/gfp* mouse retina reveals that the iris-ciliary body and choroid contain F4/80⁺ resident macrophages, and F4/80⁻ leukocytes, most likely dendritic cells. In contrast, virtually all of the CD45⁺ cells in the retina display a unique CD45^{lo} F4/80^{lo} phenotype, which is a distinctive feature of microglia. Numbers shown in flow cytometry plots indicate the percentage of CD45⁺ cells within the gated regions. (E) Comparison of gated uveal tract macrophages and retinal microglia from (D) demonstrates the distinctive immunophenotype of microglia (CD45^{lo} F4/80^{lo} CD11b⁺ MHC class II⁻ CD86⁻) compared to iris-ciliary body and choroidal macrophages (CD45⁺ F4/80^{hi}, CD11b⁺, MHC class II⁺, CD86⁺). Numbers shown in flow cytometry plots indicate the percentage of positive cells based on fluorescence minus one controls. INL = inner nuclear layer; ONL = outer plexiform layer; PRL = photoreceptor layer. Scale bars in (C) and (D) are 100 μ m.