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Dendritic cell physiology and function in the eye

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Summary: The eye and the brain are immunologically privileged sites, a property previously attributed to the lack of a lymphatic circulation. However, recent tracking studies confirm that these organs have good communication through classical site-specific lymph nodes, as well as direct connection through the blood circulation with the spleen. In addition, like all tissues, they contain resident myeloid cell populations that play important roles in tissue homeostasis and the response to foreign antigens. Most of the macrophage and dendritic cell (DC) populations in the eye are restricted to the supporting connective tissues, including the cornea, while the neural tissue (the retina) contains almost no DCs, occasional macrophages (perivascularly distributed), and a specialized myeloid cell type, the microglial cell. Resident microglial cells are normally programmed for immunological tolerance. The privileged status of the eye, however, is relative, as it is susceptible to immune-mediated inflammatory disease, both infectious and autoimmune. Intraocular inflammation (uveitis and uveoretinitis) and corneal graft rejection constitute two of the more common inflammatory conditions affecting the eye leading to considerable morbidity (blindness). As corneal graft rejection occurs almost exclusively by indirect allorecognition, host DCs play a major role in this process and are likely to be modified in their behavior by the ocular microenvironment. Ocular surface disease, including allergy and atopy, also comprise a significant group of immune-mediated eye disorders in which DCs participate, while infectious disease such as herpes simplex keratitis is thought to be initiated via corneal DCs. Intriguingly, some more common conditions previously thought to be degenerative (e.g. age-related macular degeneration) may have an autoimmune component in which ocular DCs and macrophages are critically involved. Recently, the possibility of harnessing the tolerizing potential of DCs has been applied to experimental models of autoimmune uveoretinitis with good effect. This approach has considerable potential for use in translational clinical therapy to prevent sight-threatening disease caused by ocular inflammation.

Keywords: *dendritic cells, tolerance, autoimmunity, cell trafficking, in vivo imaging, transplantation*

Introduction

The eye reveals a novel perspective on homeostatic mechanisms, as it has evolved in such a way that it combines higher order light-sensing processes with vegetative functions, aimed at both maintaining normal cellular physiology as well as warding off attack by invasive foreign microorganisms with the minimum of fuss (immune privilege). The evolutionary

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ancestral link between the eye as an outpouching of the brain has meant that the protective meningeal coverings of the brain have homologs in the three basic layers of the eye, namely the outer protective corneo-scleral envelope and the uveal tract (middle vascular layer) providing critical vegetative support to the innermost layer, the delicate neural retina (*Fig. 1*). One example of the adaptation of physiology within the eye is represented by the blood flow in the choroid (*Fig. 1*) which has the highest flow rate per square millimeter of any tissue in the body. Indeed, blood travels at such a speed through this vascular bed that it is only partly deoxygenated when coursing from the arterial to the venous system. Thus, despite their very high metabolic activity, the retinal photoreceptors do not have enough time to extract all the available oxygen (1).

Accordingly, myeloid sentinel and scavenger cells [dendritic cells (DCs) and macrophages] in the eye are exposed to a unique environment in the choroid (one component of the uveal tract) (*Fig. 1*) layer, which contrasts starkly with the entirely avascular corneal microenvironment, where such cells are instead exposed to lower temperatures and the direct effects of short-wavelength ultraviolet (UV) light. It might be expected that for these reasons alone, cells trespassing through the disparate ocular tissues will behave differently depending on the circumstances.

The eye and the brain are often described together in the context of immune privilege, a term coined to describe the modified immune responses to allograft or foreign antigen when placed into the anterior chamber of the eye (2, 3). However, the eye comprises much more than central nervous system (CNS)-derived neuro-epithelial tissue (the retina), and it is a misconception to ascribe 'privilege' equally to all its components. Indeed the term privilege is a relative one and can be graded by the strength of immune responses in many different situations. Thus, 'immune privilege' is a property also attributed to certain solid tumors (4) and to surviving vascularized allografts (4, 5). Paradoxically, there is even a price to be paid for immune privilege in the eye, which may render the eye more at risk of severe damage once the immunological barriers are breached (2, 6).

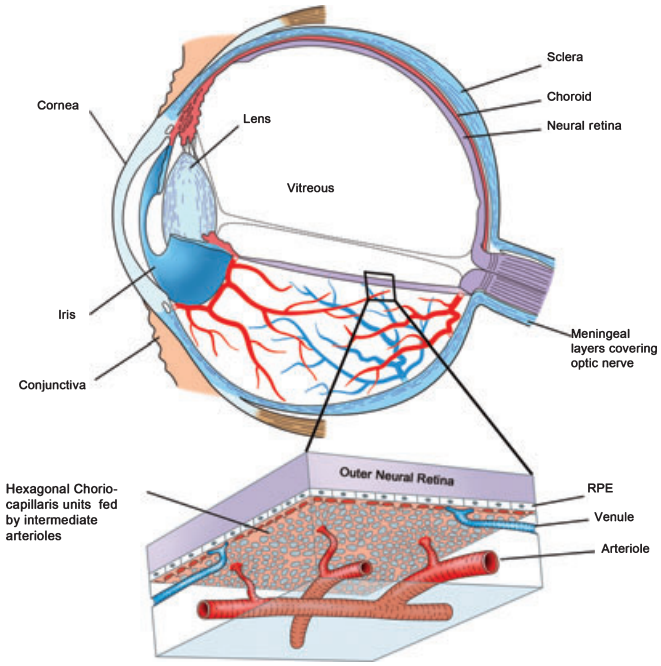

***Fig. 1.* Outline diagram of anatomy of the human eye.** The three main layers of the eye are shown: the outer coat of cornea/sclera, the middle layer of uveal tract comprising the iris, ciliary body, and the choroid, and the inner layer of the neural retina. The optically clear vitreous gel occupies the central compartment and contains the central hyaloid canal. Blood flow in the choroid is the highest of any tissue in the body. The choriocapillaris is a modified capillary network, in effect forming a blood-filled lake which serves the highly metabolic outer retinal photoreceptors. The majority of ocular dendritic cells reside in the uveal tract.

Myeloid cells in ocular tissues

The spectrum of myeloid cells

Innate immune cells responding to pathogen-associated molecule patterns (PAMPs) represent the first line of defense in the immunological battlefront in any tissues. Bone marrow-derived cells constitute one major subset of innate immune cells and have a common progenitor cell generating two main lineages, myeloid and lymphoid subsets (reviewed in 7–11). Myeloid cells develop into two major categories, macrophages and DCs, which leave the bone marrow as precursor cells and populate the tissues. In secondary lymphoid tissues such as the spleen, Flt3 receptor^{+} progenitor cells may continue to divide *in situ* providing a constant low self-sustaining population of DCs (7). The evidence for such *in situ* proliferation of myeloid precursor cells in non-lymphoid tissues is less convincing, and replenishment of tissue-resident myeloid cells such as in those that reside in the skin is believed to be directly from the bone marrow (12). In addition, there is a potential considerable recruitment of 'inflammatory' DCs from monocytes in times of need (13) both to the tissues, which then ultimately migrate to the draining lymph node, and also directly from the blood to the secondary lymphoid tissues (14). This process is not fail-safe, as under certain circumstances bacteria and bacterial products can prevent the conversion and maturation of monocytes to DCs (15). This has important implications for induction of 'tolerogenic' DCs by lipopolysaccharide (LPS) (see later).

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'Lymphoid' lineage DCs derive from bone marrow-derived lymphoid precursors and colonize the primary (thymus) and secondary lymphoid tissues (thymus, spleen, liver, and lymph nodes) and reside there (reviewed elsewhere in this volume). By contrast, myeloid lineage cells, which are present in non-lymphoid peripheral tissues, constantly migrate to the secondary lymphoid organs, carrying immunological information to maintain homeostasis (16, 17). Interestingly, each tissue or site in the body, such as the cornea, has its 'regional' site-specific lymph node that is restricted to receiving migratory myeloid cells from that anatomically defined region (18). In addition, myeloid cells migrating to their site-specific lymph node normally end their travels at this site and do not recirculate, unlike naive T cells, both in the inflamed tissue and in the steady state, although different signaling processes may be activated for each condition (19–21). Specific molecules such as the Ca^{+}-activated channel protein TRMP4, also regulate migration separately from activation of DCs (22). DCs have a more defined migratory role in transporting antigen to the secondary lymphoid tissues than macrophages which, after recruitment to the tissues, may live and die *in situ*. Both sets of cells respond to specific cues (chemokines and other mediators) (23–25). In humans, it has been suggested that this difference in behavior of macrophages from DCs is related to the expression of cell surface adhesion receptors such as CD312 (26).

The ocular surface

The ocular surface has many similarities to the skin and comprises two major components: the conjunctiva and the cornea (reviewed in 1). The conjunctiva is a loose tissue covering the globe and the inner surface of the eyelids, and consists of a stratified non-keratinized epithelium, with specialized structures such as goblet cells, overlying a fine vascular connective tissue stroma, which has parallels in both the dermis of the skin and the submucosa of the respiratory tract. The conjunctival epithelium contains intraepithelial lymphocytes and DCs (*Fig.* 2), while the stroma contains several types of immune cells including monocytes, macrophages, mast cells, and other innate cells. There are few lymphocytes or focal lymphoid aggregates in the normal rodent conjunctiva, although in humans, there are collections of non-encapsulated conjunctiva-associated lymphoid tissues (CALT), especially in older eyes.

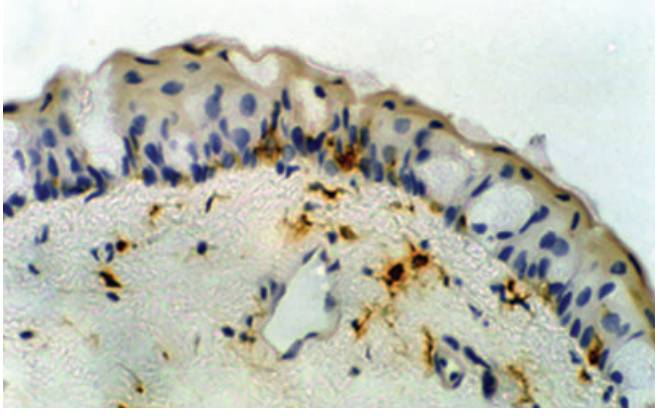

***Fig.* 2. Conjunctival DCs in the rat.** Both intraepithelial and stromal DCs are shown expressing MHC class II (Ox6) (immunoperoxidase stain). DCs, dendritic cells; MHC, major histocompatibility complex.

The conjunctiva is highly vascular and is essentially the only part of the 'eye' that contains lymphatics. These drain to the superficial cervical lymph node in the mouse, the same lymph node that drains the periocular skin. This is strictly separate from the eye-draining lymph node, which is the submandbular node (18). The conjunctiva forms one part of the mucosa-associated lymphoid system (CALT, see above) (27), and immunological tolerance can be induced by administration of soluble antigens to the ocular surface (28). In addition, the conjunctiva develops large leukocytic aggregates (nodules) during corneal graft rejection, similar to lymphoid aggregates in the gut (29).

The cornea and the conjunctiva-covered sclera are continuous through a very important junctional region termed the limbus, important because it is believed to be the source of epithelial stem cells required for repair of corneal epithelial defects (30, 31). In addition, the limbus is the site where the conjunctival surface vessels terminate, leaving the cornea an avascular structure. During development, there are important signals derived from factors such as vascular endothelial growth factors (VEGF), which inhibit corneal vascularization and lymphangiogenesis (32, 33).

The cornea comprises a stratified epithelial layer atop a basement membrane (Bowman's membrane) and overlying the tough connective tissue stroma, which comprises the bulk of the cornea (*Fig.* 3). Its type I/type III collagenous structure is highly ordered to permit the transmission of visible light waves. The posterior surface is lined by a single monolayer of specialized endothelial cells, whose function is to pump fluid out of the cornea in a posterior direction into the anterior chamber. The corneal stroma is thus relatively dehydrated compared with other extracellular matrices and is additionally supported by a specific glycosaminoglycan, keratan sulfate. This arrangement ensures the transparency of the cornea by maintaining regular spacing of the uniquely sized and uniform corneal collagen fibrils (1).

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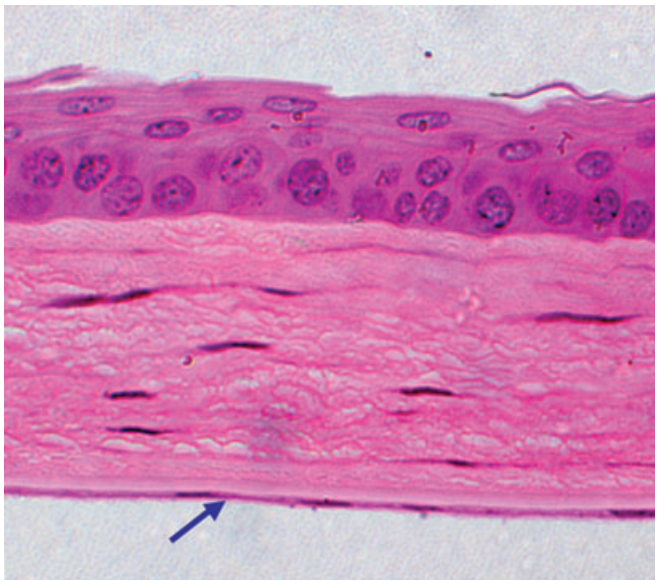

Fig. 3. Section of normal mouse cornea showing the anterior surface epithelium (top), the stroma containing keratocytes and the posterior surface endothelial monolayer (arrow) (H&E).

Throughout the stroma there are scattered fibrocytes (keratocytes) which maintain the stromal matrix components. In addition, there is a significant population of recently discovered leukocytes, the DC component of which decreases in number from the periphery towards the center. There has been considerable controversy over the nature of these cells. Initial studies suggested that the central cornea was devoid of major histocompatibility complex (MHC) class II^+ cells, and this was considered one explanation for the immune-privileged status of the cornea (34). However, recent studies have confirmed that the central corneal stroma does indeed contain MHC class II^+ cells, but in the mouse at least, these cells are $CD11b^+CD11c^-$ (35) (Fig. 4). Some of these cells also express $CD45^+CD68^+CD169^+$ and CX3CR1 (36). An earlier set of studies suggested that the mouse corneal stroma contained a population of MHC class II^-, $CD11c^+$ cells which became MHC class II^+ after migration to the draining lymph node (37, 38). However, we and others have not been able to

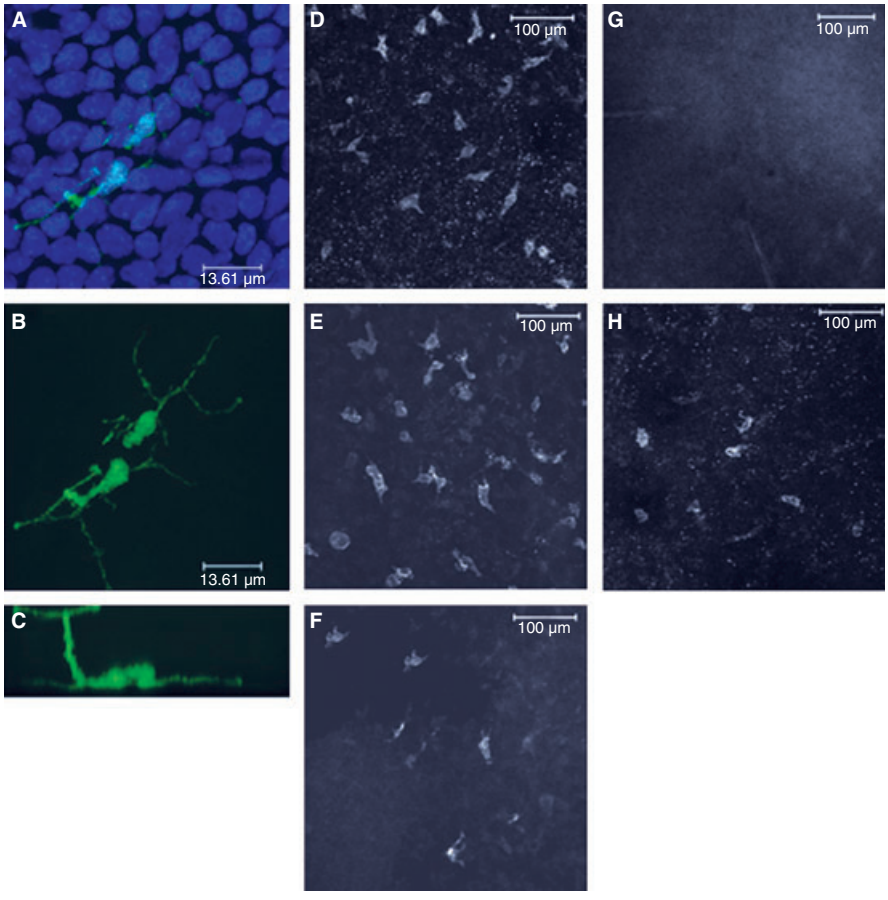

Fig. 4. Views of the cornea. (A, B) Confocal microscopy of corneal wholemount demonstrating DCs in the corneal epithelium of CX3CR1-gfp transgenic mouse; 'plan views' showing the ramified processes spreading laterally [3A; epithelia nuclei – blue (DAPI)]; (C) one of the DCs from (A, B) viewed in Z-profile sending a process towards the apical surface; (D–F) Corneal wholemount preparations showing intrastromal leukocytes (D: CD45; E: CD11b: F: MHC class II); (G, H) Corneal wholemount staining for CD 11c before (G) and 24 h after (H) epithelial abrasion. DCs, dendritic cells; MHC, major histocompatibility complex.

repeat these studies (35, 39, 40). Instead, we find that the population of leukocytes in the central corneal stroma comprise two subsets: an MHC class II^+ population (approximately 40%) that are $CD11b^+$ $CD11c^-$ (i.e. macrophages) and an MHC class II^- population that are $CD34^+$ and are probably myeloid precursor cells (40). There is also a small population of $B220^+CD11c^{lo}$ cells which appear to be plasmacytoid DCs. Interestingly, it has been suggested that the $CD11b^+$ cells have the potential to differentiate into Lyve-1^+ cells and form lymphatics when the cornea is inflamed (41). Recent observations (42) have revealed that MHC class II^+ $CX3CR1^+CD45^+$ cells in the corneal stroma have extremely fine (<0.8 μm) cellular processes (*Fig.* 5), which extend several hundreds of microns laterally and bear the hallmarks of membrane or tunneling nanotubes (43). These unique cellular processes, previously only observed *in vitro*, were seen occasionally to form cellular bridges between other MHC class II^+ cells. Their function in the cornea is yet to be determined; however, they do increase in number following injury alone and injury plus LPS exposure (42). It has been speculated that they form an immunological syncytium and act as a means for distantly separated cells to communicate in this relatively sparsely populated tissue (43).

The corneal epithelium, considered as an immunological entity discrete from the stroma, also contains a population of MHC class II^+ cells that are less numerous towards the center (*Fig.* 4). Variability in immunostaining in the cornea using anti-CD11c monoclonal antibody experienced by different researchers has led to some uncertainty concerning the presence of DCs in the corneal epithelium, but recent studies of the CD11c-enhanced yellow fluorescence protein (eYFP) transgenic mice show that intraepithelial MHC class II^+ cells co-express $CD11c^+$ (44, 45, H.R. Chinnery, unpublished data) confirming their DC lineage. There are few if any $Gr1^+$ cells in the resting corneal epithelium or stroma, most of which were also $B220^+$, indicating they were probably plasmacytoid DCs (pDCs) (40). Recent studies have shown that the MHC class II^+ cells reside in the basal epithelial layer (46) migrating into this site from the peripheral cornea but not from the stroma (47). In addition, in the epithelium some DCs project processes (periscopes) anteriorly towards the surface tear film where it has been speculated that they might sample antigens from the environment (42, 48) (*Fig.* 4). In this manner, they are analogous to similar intraepithelial DCs in the intestinal and respiratory tracts (49–51).

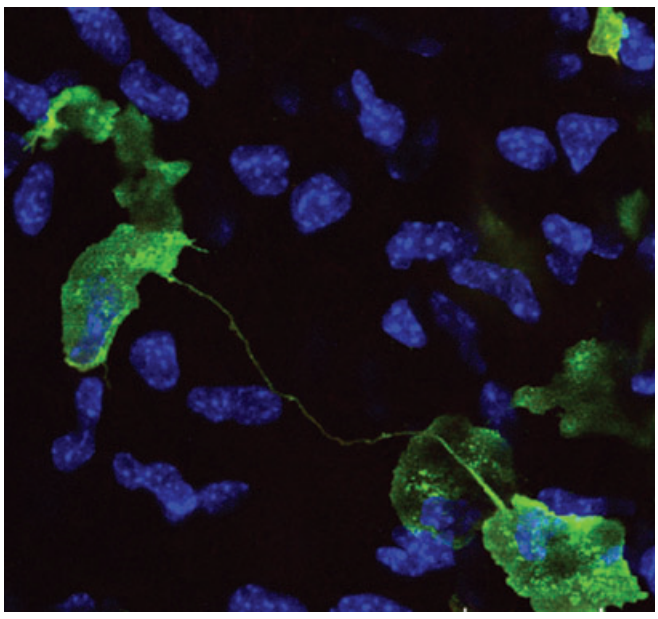

***Fig.* 5. Two MHC class II^+ DCs (green) in the corneal stroma in communication via a long fine membrane nanotube.** Keratocyte nuclei in the corneal stroma are clearly evident (blue, DAPI). MHC, major histocompatibility complex; DCs, dendritic cells.

Corneal and conjunctival intraepithelial leukocytes most likely represent the equivalent of skin Langerhans cells, and indeed all corneal MHC class II^+ cells are rather loosely described as Langerhans cells (52). In the skin, some Langerhans cells express the C-type, mannose-specific lectin Langerin/CD207 as well as CD1a, while dermal DCs express a different C-type lectin, DC-SIGN; there is also a separate subset of Langerin$^+$ dermal DCs (12). In addition, Langerhans cells in the skin constitutively express E-cadherin, which can bind homotypically to other Langerhans cells or to the epithelium and can also bind to α_E-integrin$^+$/$CD103^+$ intraepithelial lymphocytes (12).

Recent studies of the human cornea have confirmed that the central corneal epithelium lacks DCs but the peripheral cornea contains Langerin/$CD207^+$ Langerhans cells, and the peripheral anterior corneal stroma, equivalent to the dermis of the skin, contains DC-SIGN/$CD209^+$ DCs (53). Few of the peripheral Langerhans cells expressed DC-LAMP, a marker of DC maturation. Interestingly, the cornea is negative for E-cadherin, while the conjuctiva is positive and is also the site where intraepithelial $CD8a^+$ T cells reside (54). The central corneal stroma, however, contains a small population of MHC class II^+ macrophages (*Fig.* 4). In the mouse there is a fairly extensive and evenly distributed population of $CD45^+CD11b^+F4/80^+$ macrophages (36). These human and mouse studies are of considerable importance to the mechanism of corneal allograft rejection, which unlike with vascularized grafts, is almost exclusively mediated via indirect allorecognition (see below).

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In the mouse, no Langerin$^+$CD11c$^+$ cells have been detected in the conjunctiva and only occasional Langerin$^+$CD11c$^-$ cells (55). In addition, in the normal conjunctiva there are almost no pDCs present, but these cells infiltrate the conjunctiva in large numbers during allergic inflammation (55). Few studies have been performed on conjunctival DCs in humans (54). Expression of the FCεR1 is characteristic of intraepithelial DCs and the numbers of such cells are also greatly increased in allergic conjunctivitis (56) as well as DCs expressing IgE (57), as might be expected. Interestingly, chronic application of topical therapies (e.g. anti-glaucoma medications) to the ocular surface is also associated with an increase in migratory Langerhans cells and may even induce a low level of inflammation (58). There have been no studies reported on epithelial cell adhesion molecule (EpCAM) in ocular surface cells.

The differences in corneal and conjunctival Langerhans cells, particularly in the expression of E-cadherin, might be reflected in their function in the steady state: in the skin, expression of E-cadherin is downregulated on migratory Langerhans cells, which may apply also to cells in the conjunctiva. The lack of E-cadherin in the cornea probably reflects the relative paucity of these cells in this tissue.

The intraocular compartment

Early studies suggested that the eye had no lymphatic vessels and no immune cells. All immune cell traffic was considered to come via the blood stream and the uveal tract was considered to be a modified lymphovascular tissue (59). However, when the role of tissue leukocytes, and DCs in particular, in the induction of immune responses became understood, tissue distribution analyses suggested that most tissues contained such cells, but that neural tissue and in particular the CNS was devoid of antigen-presenting cells (APCs) (60, 61). However, this notion was modified when it became clear that the lining tissues of the CNS (the meninges and their perivascular extensions) contained rich populations of resident DCs and macrophages (62, 63), while the brain's complement of bone marrow-derived microglial cells are now recognized to play a role in tissue-specific immunoregulation (64–66). These studies were extended to the eye, in which the uveal tract (comprising the iris, ciliary body, and choroid; *Fig. 1*) of all species studied was shown to contain similar extensive networks of macrophages and DCs (*Fig. 6*). In the iris and ciliary body, these cells were seen to be distributed throughout the stroma and seemed to be sessile cells with little evidence of migration out of these tissues when challenged intraocularly with antigen (67). However, in related studies, it was clear that antigen delivered to the anterior chamber of the eye (*Fig. 7*) is readily transported as soluble antigen via extracellular spaces that eventually communicate with lymphatic channels; it is possible, even likely, that some of this antigen is cell-associated antigen inside DCs and macrophages, as demonstrated for antigen tracking from the cornea (see below) (68, 69).

Similar populations of CD11b$^+$CD11c$^+$ DCs and macrophages are present in the choroid (70, 71), where they appeared to be draped along the length of the medium and larger sized vessels (*Fig. 6*), as well as in close apposition to the basal surface of the retinal pigment epithelium (RPE). Here, MHC class II$^+$ CD11c$^+$ cells appeared to insert dendritic processes close to the basal aspect of the RPE cell, an area where there is good evidence that shed photoreceptor debris accumulates with age possibly representing a source of (auto)antigen. This close relationship may underlie one aspect of the sentinel role of DCs in regulating autoimmune pathology such as uveoretinitis (see below) in addition to the role played by peripheral retinal DCs (71).

Uveal tract DCs are phenotypically CD8α$^-$CD11b$^+$CD11c$^+$, i.e. conventional myeloid DCs. Few if any B220$^+$ pDCs have been found, nor have CD8α$^+$ DCs been found, which usually

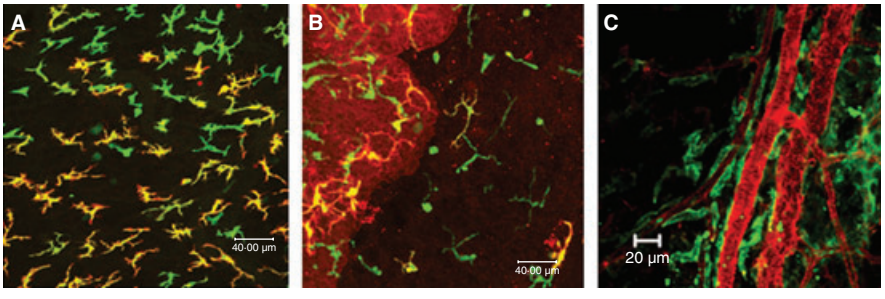

***Fig. 6.* Views of the iris.** (A) Iris wholemount from CX3CR1-gfp transgenic mouse stained with anti-MHC class II antibody (red). Note the mixture of CX3CR1$^+$ MHC class II$^+$ cells (orange, putative DCs) and MHC class II$^-$ Cx3cr1$^+$ cells (green only, putative macrophages); (B) Similar preparation showing intraepithelial MHC II$^+$ CX3CR1$^+$ DCs in the ciliary body (orange); (C) Choroidal wholemount from wildtype mouse stained for F4/80 (green) and phalloidin (red) showing distribution of perivascular macrophages. MHC, major histocompatibility complex; DCs, dendritic cells.

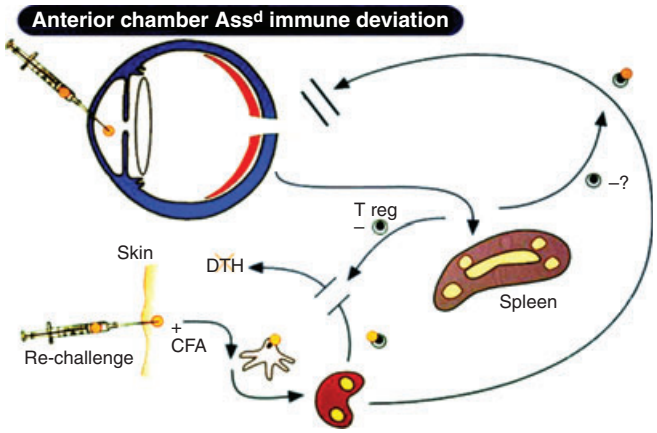

Fig. 7. Anterior chamber-associated immune deviation (ACAID). The diagram shows antigen inoculation into the anterior chamber of the eye inducing a 'deviant' immune response, in that, when the organism is re-challenged with the same antigen at a distant site, there is suppression of the delayed-type hypersensitivity response to this antigen but no suppression of cytotoxic T-cell or complement fixing antibody responses. ACAID is antigen specific, i.e. immune tolerance to a third party antigen cannot be induced by this technique. ACAID requires an intact spleen, involves the thymus, and is mediated via NKT cells, F4/80 macrophages, and $CD8^+$ regulatory T cells. Unusually, $CD4^+$ T cells are also required. Its teleological role is unclear.

are restricted to secondary lymphoid tissue. Uveal tract macrophages are predominantly $CD11b^+$, $F4/80^+$ cells with few signs of activation markers in the resting state (Fig. 6). A proportion of the choroidal macrophages are also $CD169^+$ (71–73).

It is possible to culture DCs and macrophages from explants of uveal tissue, including the human, and such cells morphologically sort into two types, a small highly motile and strongly MHC class II^+ population and a smaller population of DCs with extensive veil-like projections. Expression of CD80/86 on these cells is normally low, but their antigen-presenting function can be significantly enhanced if co-cultured with macrophages (74). Previous studies had shown that iris-resident macrophages, unlike their resident counterparts in the lung, were more pro-inflammatory than immunosuppressive (74, 75).

Initial searches for DCs in the retina using classical immunohistochemical staining of tissue sections were negative (71). The predominant leukocytic cell in the retina was shown to be the microglial cell, a $CD11c^+CD45^{lo}F4/80^+$ cell (76) (Fig. 8), which was considered initially to have antigen-presenting capacity, both in the retina and in the brain (see above). However, subsequent extensive investigations of the microglial cell have concluded that its main role is an immunosuppressive one, at least in the non-inflamed retina (77–79). Instead, a second very small population of perivascular MHC class II^+ DCs and macrophages that line the retinal vessels in meningeal extensions were thought to be cells with the potential to initiate retinal inflammation (80) (Fig. 8). These cells are analogous to the perivascular $CD11c^+$ cells in the brain parenchyma, which, in elegant conditional knockout studies, have been shown to be required for induction of experimental autoimmune encephalitis (EAE) in mice (81).

However, most recently, using flat mounts of the retina, a second small subpopulation of MHC class II^+ $33D1^+$ cells has been found in the retinal periphery and surrounding the optic nerve (82). These cells are located at sites where the initial signs of inflammation appear in autoimmune uveoretinitis and are also those sites where activated retinal antigen-specific T cells initially accumulate prior to disease onset (82). In addition, they are prominent participants in autoimmune retinal inflammation (Fig. 9). It has recently been shown that the monoclonal antibody 33D1 is a marker for the DC receptor DCR1 expressed selectively on splenic MHC class II^{hi} DCs in the marginal zone and associated with stimulation of immune responses (83). Their location in peripheral sites in the retina is therefore intriguing, particularly as the expression of 33D1 was strain dependent, strongly positive in the EAU-resistant Balb/c (H2d) mouse but negative in the resting retina of the B10RIII (H2r) mouse (82). No CD8α, DEC205, B220, CD80/86 positive DC was found in the normal retina (82).

Steady-state turnover of myeloid cells in the eye

'Resident' bone marrow-derived cells in the tissues are replenished in the adult at tissue-specific variable rates from precursor/progenitor cells which seed the periphery (84, 85). In addition, monocytic cells from the blood stream can rapidly enter inflamed sites and differentiate into both macrophages and DCs (86). The eye participates in this process and each ocular tissue is repopulated at slightly different tempos. In addition, the molecular signals required to repopulate the tissues may be different in the resting and in the inflamed state.

The chemokine receptor CX3CR1 appears to mediate homing of MHC class II^+ DCs to the cornea but does not appear critical for recruitment of $CD45^+CD169^+CD11b^+$ macrophages (36). In addition, studies of mouse chimeras using the GFP-CX3CR-1 mouse cell lines have shown that the major part of the corneal myeloid cell population is replenished over a period of 8 weeks (36). Myeloid cells, particularly macrophages, have long been implicated in corneal blood vessel angiogenesis, and more recently $CD11b^+$ macrophages have been identified as precursors of lymph vessels in the cornea

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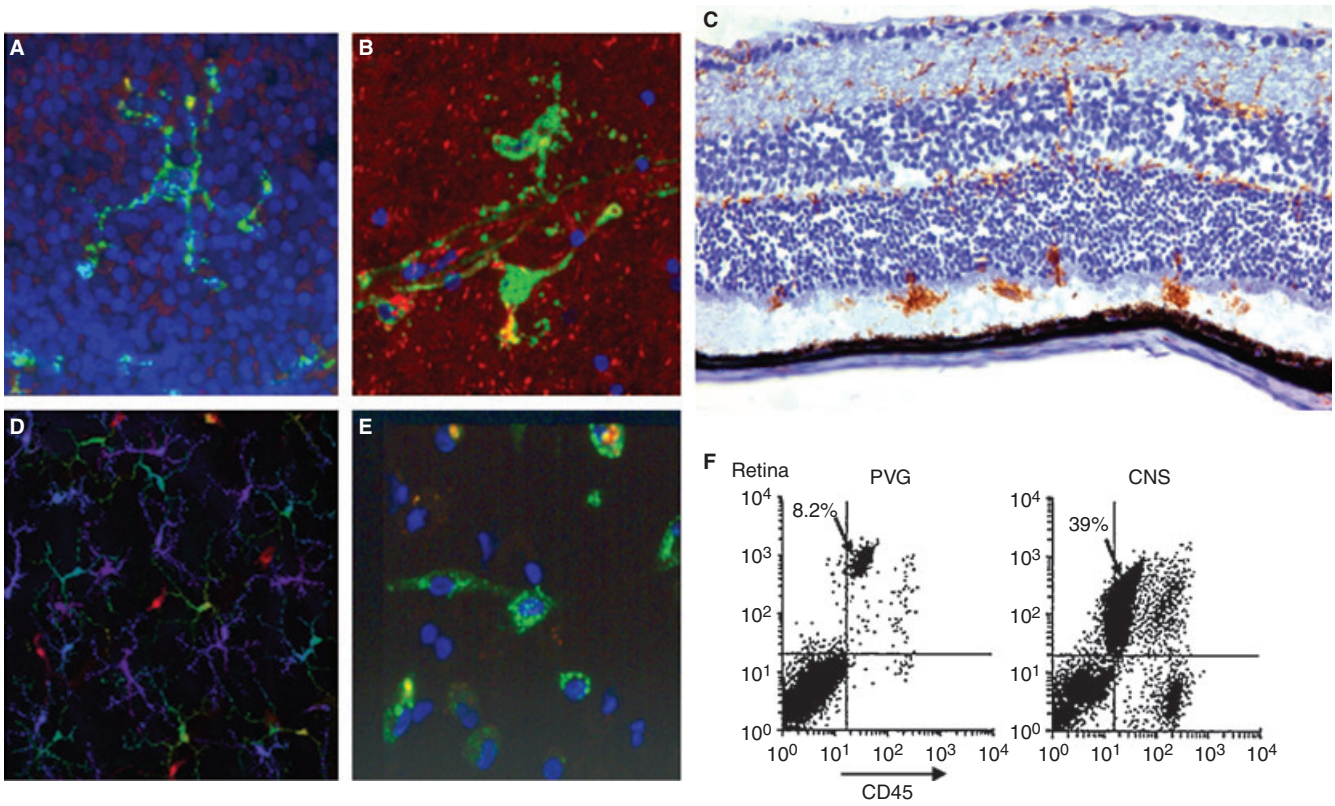

***Fig. 8.* Retinal microglial cells.** (A) Wholemount human retina showing $CD45^{lo+}$ microglial cells (green), DAPI (Blue); (B) wholemount human retina, showing $CD45^{+}$ perivascular leukocytes with processes contacting the venule; (C) section of retina from retinal degeneration mouse (rds mouse) showing large numbers of activated microglia throughout the retinal layers (F4/80 stain); (D) retinal microglia in wholemount from CX3CR1-gfp transgenic mouse; the layers of microglia throughout the depths of the retina in this confocal stack of the entire retina are revealed through color-coded depth projection; (E) microglia cultured from rat retinal explant; (F) flow cytometry of microglia from retina and brain (CNS) showing characteristic CD45/CD11b/c staining (x-axis shows $CD11c^{+}$ cells gated on CD11b).

(41). Furthermore, evidence has been provided that $CX3CR1^{+}$ macrophages/DCs might prevent new vessel formation, while CCL2-responding macrophages may promote new vessel growth (87). Interestingly, angiogenesis-promoting macrophages appear to require the surface molecule CEACAM-1 (88). The cornea is normally devoid of both blood and lymph vessels, a process which appears to be regulated by VEGF/VEGF receptor interaction. Recently, a splice variant isoform of the VEGFR2 has been described to inhibit lymphangiogenesis in the cornea (33). The control of the resident myeloid cell population in the cornea has significant impact on its normal avascular physiology.

None of the above studies examined the conjunctiva; however, in the skin, chimeric model experiments have shown that a proportion of Langerhans cells are self-renewing (12), and while this may apply to the conjunctiva, there is little evidence of this effect in corneal APCs, as irradiation led to a marked depletion and repopulation almost entirely with bone marrow-derived cells within 2 weeks postirradiation (Xu H, Forrester JV, unpublished data). It is more likely, however, that conjunctival Langerhans cells are predominantly if not exclusively bone marrow derived, as they are in other mucosal epithelia (89).

Turnover of myeloid cells in the retina has also been studied for many years, initially using radiolabeling and autoradiographic histological techniques (90, 91). More recently, bone marrow chimera studies using the same CX3CR1 model have shown that turnover of microglial cells in the neural retina is considerably slower than corneal myeloid cells, taking up to 6 months to completely restore retinal subpopulations, and is not dependent on the expression of CX3CR1 (73, 92). In addition, normal aging as well as certain photoreceptor retinal degenerations are associated with the accumulation of microglial cells in the subretinal space, a site in which they are normally absent, particularly if there is no light damage (93) (McMenamin PG, unpublished data) (*Fig.* 10). Interestingly, studies of other systems have revealed that $CX3CR1^{+}CSF\text{-}1R^{+}$ myeloid precursor cells respond differently in their turnover rates depending on whether it is basal

homeostatic turnover or whether it is turnover and recruitment associated with an inflammatory response. Thus, the use of CX3CR1 as a marker for DC/macrophages may not reveal the complete picture (86, 94).

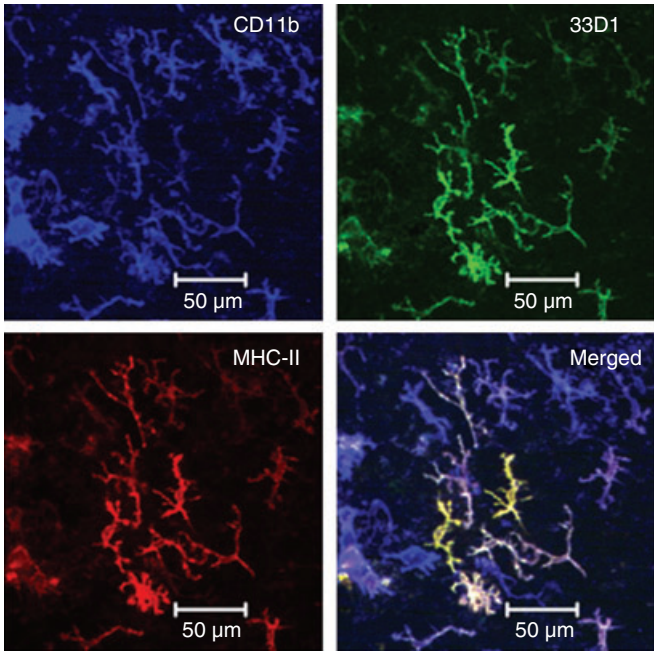

***Fig. 9.* Triple staining of retinal flat mounts for CD11b (blue), DCR1$^+$ (33D1 green), and MHC Class II (red) in EAU**. Note strong co-positivity between DCR1 staining and MHC class II (merged). MHC, major histocompatibility complex; EAU, experimental autoimmune uveoretinitis.

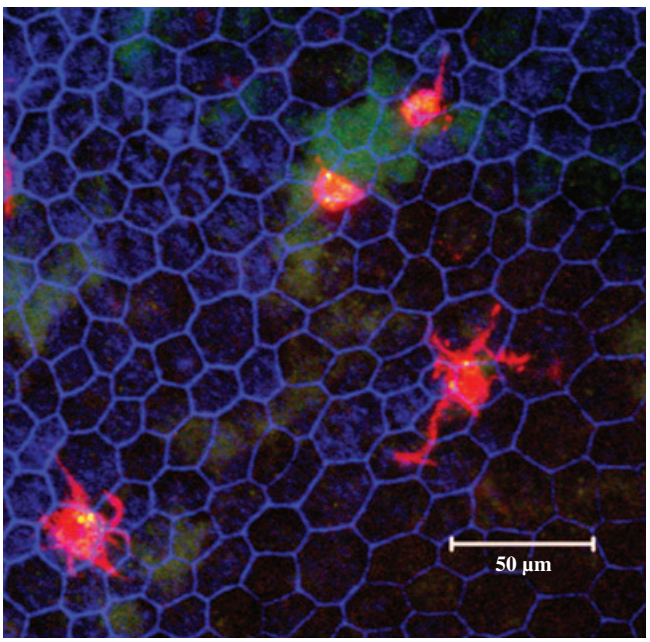

***Fig. 10.* Subretinal microglia stained with antibody Iba1 in aged mouse retina.** Hexagonal outlines of the retinal pigment epithelium are shown in blue (phalloidin).

In contrast to the retina, the turnover of DCs in the uveal tract is rapid (95). This rate is more in line with DC turnover in lymph nodes and spleen and might be expected therefore of populations of APCs that will respond rapidly to new antigen.

Resident DCs and macrophages in the eye have overlapping but complementary functions

The mononuclear phagocyte system and the eye

The mononuclear phagocyte system (MPS) in most tissues is geared towards maintaining homeostasis (96). Resident DCs patrol the tissues, sampling self-antigen while on the watch for foreign antigen. To the former they maintain tolerance and to the latter they induce immunity, after transporting the respective antigens to the secondary lymphoid tissues (16, 97, 98). Resident macrophages meanwhile mostly function locally by silently removing dead and dying cells during the normal homeostatic process of tissue renewal (a housekeeping role). In individual tissues, resident DCs and macrophages are customized to vary these functions: in the skin, Langerhans cells, originally thought to mediate immune responses, are more likely to promote tolerance, while dermal DCs are programmed for immunity (12). Macrophages in the skin perform the classical role of tissue repair and debris clearance and protection against microorganisms that breach the epidermal barrier. In the lung alveolar macrophages act as a large net to trap inhaled microorganisms and are thus the first site of attack of organisms such as *Mycobacterium tuberculosis* (99, 100). The eye presents an interesting variation on the above: the corneal and conjunctival epithelial myeloid cells behave somewhat like their skin and lung counterparts, but corneal stromal myeloid cells, apart from being mostly macrophages, also have a novel anti-angiogenic role (see above). By contrast, the role of the infrequent resident retinal DCs, situated at potential entry sites of inflammatory cells into the retina, i.e. the marginal zones of the retina, is not known, while the F4/80$^+$ microglia probably have an immunosuppressive role in the steady state (101).

Immune privilege

Immune privilege as a concept developed from the seminal work of Medawar who showed that skin and tumor allografts when placed in the anterior chamber of the eye were not rejected (102, 103). Initially, this phenomenon was attributed to tight blood–tissue barriers, then to a lack of blood or lymphatic vessels (thus preventing both the afferent and efferent responses), followed by a lack of ocular APCs, all of which

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proved ill-founded (2). Currently, it is believed that immune privilege is a relative property, can be selective of different aspects of the immune response, is present in tumors and other tissues (4), and is inducible for instance in accepted vascularized grafts (104). Much of this is now attributed to properties of the tissue and indeed tissue-centered regulation of immune responses is now an accepted concept and is dependent on the micro-environment or context in which an immune response occurs.

Anterior chamber-associated immune deviation

Despite this more recent rationalization of experimental phenomena, some concepts remain unexplained. One of these is anterior chamber-associated immune deviation (ACAID), which describes the induction of antigen-specific tolerance by inoculation of soluble antigen into the anterior chamber of the eye (*Fig. 7*). This process requires an intact spleen, and it has been suggested that F4/80$^+$ macrophages transport the inoculated antigen to the spleen, where they further interact with NKT cells, CD4$^+$ T cells, and B cells to induce a subset of suppressor CD8$^+$ T cells (reviewed in 105). Definitive proof that spleen-seeking eye-derived F4/80$^+$ cells exist, has not been shown and, while there is clearly a reproducible phenomenon demonstrable [originally described by Medawar in the skin graft experiments (102)], its mechanism remains obscure. Interestingly, ACAID-inducing properties of aqueous humor can be abolished in both eyes by application of retinal laser thermal burns to one eye, adding further complexity to the phenomenon (106). However, its relationship to immune privilege proper, which describes the downregulation of the immune response in the privileged tissue (2), is not obvious. F4/80$^+$ macrophages themselves are immunosuppressive (101), and the dense networks of these cells in the eye (cornea, retina, and uveal tract) probably provide some degree of local privilege. In addition, in conditions of induced privilege, local infiltrating myeloid cells appear to adopt this role.

Microenvironment and functional plasticity

It is likely therefore that the ocular microenvironment promotes immune tolerance by acting directly on ocular myeloid cells. There are several mediators and molecules of the 'immuno-suppressive microenvironment' (*Table 1*), and it is likely that many more will be discovered. Factors such as vaso-active-intestinal peptide (VIP), hepatocyte growth factor (HGF), pituitary adenylate cyclase activating polypeptide (PACAP), and many others, either directly promote tolerogenic DCs and/or immunosuppressive macrophages (107, 108). In particular, TGF-β present not only in abundance as latent cytokine but also as the active moiety in the aqueous fluid appears to have a major immunosuppressive role. Expression of programmed cell death domain-1 and its ligand (PD-1 and PDL-1) in ocular tissues also has an important immunosuppressive effect (109, 110). Some of these factors induce tolerogenicity in DCs via indoleamine oxidase (IDO) (111–113). It is thus not impossible that if such tolerogenic DCs trafficked through the ocular compartments to the secondary lymphoid system, they may retain the potential to promote systemic tolerance.

Trafficking of antigen from the eye

The above considerations raise the question of how antigen is transported from the eye. Current notions of self-tolerance propose that self-antigen is 'sampled' by sentinel DCs in the steady state and transported to the secondary lymphoid tissues where it transmits a non-immune, tolerizing signal to potential autoreactive T cells (114, 115). Early experiments of this model in the eye used intracameral injection of ovalbumin into mice adoptively transferred with transgenic OT-1 cells

***Table 1.* Mediators involved in immune privilege in the eye**

Type of factor	Example	T-cell function	Macrophage activity	Antigen presentation	Cell killing/apoptosis
Neuropeptide	VIP, PACAP, CGRP melanocortin, Substance P	Inhibits	Inhibits promotes		
Neurotransmitter	GABA, glycine	Inhibits			
Cytokine	TGF-β, IL-10		Promotes*		
Chemokine	MCP-1, SDF-1				
Membrane ligand	FasL, CD200, TRAIL, APRIL				Promotes
Membrane receptor	TLRs	?Inhibits	Promotes		
Cellular enzymes	IDO, L-arginase	Inhibits		Inhibits	Promotes
MHC protein	Qa-1			Inhibits	
Prostaglandins	PGE2, LTAb 4	Inhibits		Inhibits	
Cannabinolds		Inhibits		?Inhibits	?Promotes
Free radical	Peroxynitrate		Promotes	Inhibits	Promotes

*Scavenger activity?

(116). There was specific induction of OT-1 cells only in the submandibular eye-draining lymph node (SM DLN). In later experiments, labeled soluble antigen was tracked through the lymphatic system and found to traffic to the spleen and even as distantly as the mesenteric lymph node (68). In addition, the speed of transport to the secondary lymphoid tissues was very rapid (hours). Similar experiments have recently been performed using soluble antigen applied to the denuded corneal stroma and, once more, the speed of antigen transport was very rapid, being detected within minutes in the SM DLN (*Fig. 11*). However, from the cornea, soluble antigen only trafficked to the eye-draining lymph node in its first pass and then via the circulation to the spleen, and it did not traffic generally throughout the secondary lymphatics until it had drained from the venous circulation to commence a second pass through the lymphatics (Zexu D, Kuffova L, Forrester JV, unpublished data). By contrast, intracameral injection of soluble antigen led to early general distribution to most secondary lymphoid organs (68). Most recently, cell-associated antigen was tracked using a system that allowed plasmid antigen uptake into APCs but did not permit secretion of the antigen due to lack of a signal sequence on the transcribed protein (39). These studies showed that antigen from the cornea is taken up by corneal DCs, which infiltrate the cornea after minor abrasion, take up and process the antigen within a few hours, and transport it selectively to the eye-draining lymph node and the spleen, from which it is cleared over a period of 72 h. The potential therefore for immunity or tolerance to be induced by eye-derived APCs is clearly present, is probably initiated in the secondary lymphoid tissues, and regulation of the developing effector response is likely to take place in the eye, when the eye-seeking T cells return to the site of antigen origin.

Cytokines and chemokines are important in the regulation of this migratory DC behavior, and CCR7 is recognized to be an important receptor for DC migration both in the steady state and during inflammation (117). CCR7 is upregulated in the iris in endotoxin-induced inflammation and is expressed on corneal APCs in the corneal periphery in response to injury in syngeneic grafts (118).

Role of tissue-specific DCs in the induction of tolerance

The possibility therefore that the microenvironment regulates immune responses has taken on a new level of interest. As indicated above, ocular immune privilege is a long-recognized concept, and there is an extensive and growing list of the many mediators which might modify DC behavior, thus promoting tolerance (*Table. 1*). Local production of immune mediators now has specific relevance to the induction of regulatory T (Treg) cells *in situ* in many sites, for instance in response to infection by organisms such as *Leishmania*, and may vary depending on the tissue (119). Such ideas are not very far removed from the original notions surrounding immune privilege and emphasize once more the importance of tissue regulation, upwards or downwards, of the immune response. These concepts have a special relevance to ocular infections such as onchocerca, herpes simplex keratitis and retinitis, and ocular toxoplasmosis.

Recruitment of myeloid DCs during ocular inflammation

Types of ocular inflammation

Ocular inflammation can be considered in anatomical and pathological terms. The ocular surface is frequently subject to a range of infectious and allergic diseases, while the uveal tract, the sclera (*Fig. 1*), and the retina are more susceptible to autoimmune and infectious (viral and parasitic) diseases. Initiation of infectious disease in the intraocular compartments is usually through a systemic inoculation of microorganisms, inducing a local lymph node involvement and finally an ocular

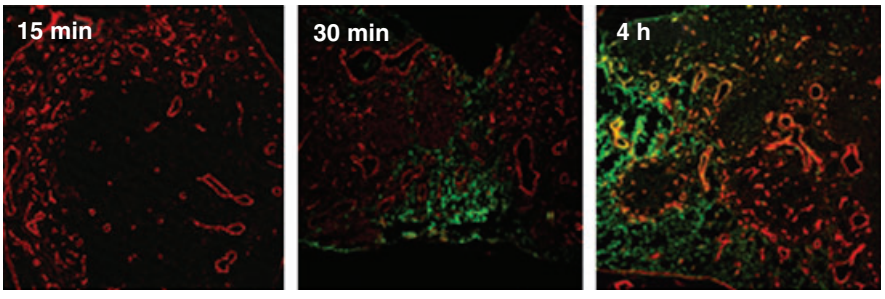

***Fig. 11.* Tracking of EαGFP protein applied to the abraded corneal surface at different times up to 4 h post application.** Sections of submandibular draining lymph node stained with anti-perlecan antibody (red); EαGFP (green). Note the appearance of EαGFP staining 30 min after the application of labelled protein to the abraded cornea and the percolation of the protein through the perlecan^{+} draining lymph node conduits which communicate with the high endothelial venules and ultimately the venous circulation.

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infection. Entry directly to the eye can also occur at the ocular surface (e.g. by trauma or contact lens wear), and in these cases the inflammation is often cleared by local innate immune responses with a later adaptive immune response producing a non-infectious immune attack as seen at a later stage in corneal herpes simplex disease. Occasionally, infection may spread from another site, e.g. the lungs in tuberculosis, inside trafficking infected macrophages, and a new secondary tuberculosis focus ensues. This 'Trojan horse hypothesis' has been proposed as one means by which *Toxoplasma gondii* enters the eye (120). Intravenous metastatic infection, e.g. of *Candida* retinitis, may occur in immunocompromised individuals.

The ocular surface

Corneal blindness is common worldwide, through conditions such as trachoma (*Chlamydia trachomatis* infection), river blindness (*Onchocerca* filarial infection), and viral infections such as herpes simplex and the measles virus. Inflammatory disease of the cornea and conjunctiva leads to the upregulation of many pathogen recognition receptors (PRRs) including Toll-like receptors (TLRs) (121), NOD-like receptors (NLRs), and other components of the inflammasome (122). In experimental models of bacterial and fungal keratitis, TLRs in particular, expressed on both surface corneal epithelium and on infiltrating DCs and macrophages, have a specific role in the induction of the immune response of the cornea to *Fusarium* (123). In some corneal diseases, the mechanisms of DC priming of T cells have been extensively detailed. For instance, endosymbiotic *Wolbachia* bacteria have been identified as the pathogenetic element in *Onchocerca* filarial keratitis (124). An extract of this organism, *Brugia malayi* female worm extract (BMFE), signals via TLR2/6 (125). This effect has been attributed to a 20mer peptide of *B. Malayi Wb* peptidoglycan-associated lipoprotein (wBmPAL). The diacyl peptide induced systemic TNF-α production as well as a neutrophil-mediated keratitis. Interestingly, this peptide moiety primed DCs to induce a non-polarized Th1/Th2 cell response (126). Similarly, an important role for resident myeloid DCs and macrophages in the regulation of the response to LPS/TLR4-mediated keratitis has been demonstrated using chimeric and conditional ablation models (127).

The mechanism of herpes simplex viral (HSV) keratitis (HSK) presents an interesting unresolved conundrum. HSV infects the cornea, travels to the trigeminal ganglion, and establishes latency. Initial infection of the corneal epithelial cells is followed by an early HSV-specific Th1 response, which induces a second stage of stromal keratitis about 10 days after the initial infection. However, it is not known whether HSK due to the immune response is induced locally or in the secondary lymph nodes with trafficking of antigen-specific T cells to the site of infection (*Fig.* 12). Both $CD4^+$ and $CD8^+$ T-cell responses are involved and vary in pathogenetic importance dependent on the strain of virus used to infect the cornea. For instance, the RE strain of HSK mediates HSK via $CD4^+$ T cells and DCs (possibly migratory Langerhans cells) (*Fig.* 12) are early players in this response. However, direct infection of DCs in the cornea during the initial epithelial infection has not been demonstrated. In similar experiments on skin infection, DCs migrate to the DLN and after cross-presentation of HSV antigen to resident lymphoid DCs, induce antigen-specific T cells which then home to the site of infection (128). This is the likely route of HSK disease induction, but it is possible that *in situ* antigen presentation can also occur by way of cornea-resident memory T cells as has been shown in the skin (129). An interesting associated site of $CD8^+$ T-cell activation in this model has been identified to occur in the latently infected neurons of the trigeminal ganglion. These $CD8^+$ T cells appear to have a significant role in the maintenance of latency, paradoxically via direct release of granzyme B into the neuronal cell body (130). Presumably this process requires $CD8^+$ T-cell activation via an APC, but the nature of this cell and its source are currently unknown. It has been suggested that 'satellite' glial cells may perform this function (131).

DC trafficking in corneal graft

There is considerable information on the role of donor and host leukocytes in corneal allograft rejection. Corneal allografts enjoy a reputation for high levels of acceptance, even when HLA matching is not performed, but this is somewhat misplaced (132). Low-risk grafts (i.e. grafts performed in patients who have a corneal opacifying type of dystrophic disease that does not involve prior inflammation) have an excellent 1-year success rate. However, high-risk grafts (i.e. grafts in which there has been previous infection such as herpes simplex or in which there is extensive vascularization associated with the corneal opacity) fare much less well, and indeed, the 5-year survival of such grafts is lower than that for matched vascularized grafts such as the kidney or the heart (133, 134). Models of corneal graft have been developed in experimental animals which explore the mechanism of graft rejection (reviewed in 135).

Corneal donor grafts normally utilize the central portion of the tissue (6–8 mm diameter in the human, 3 mm in the rat,

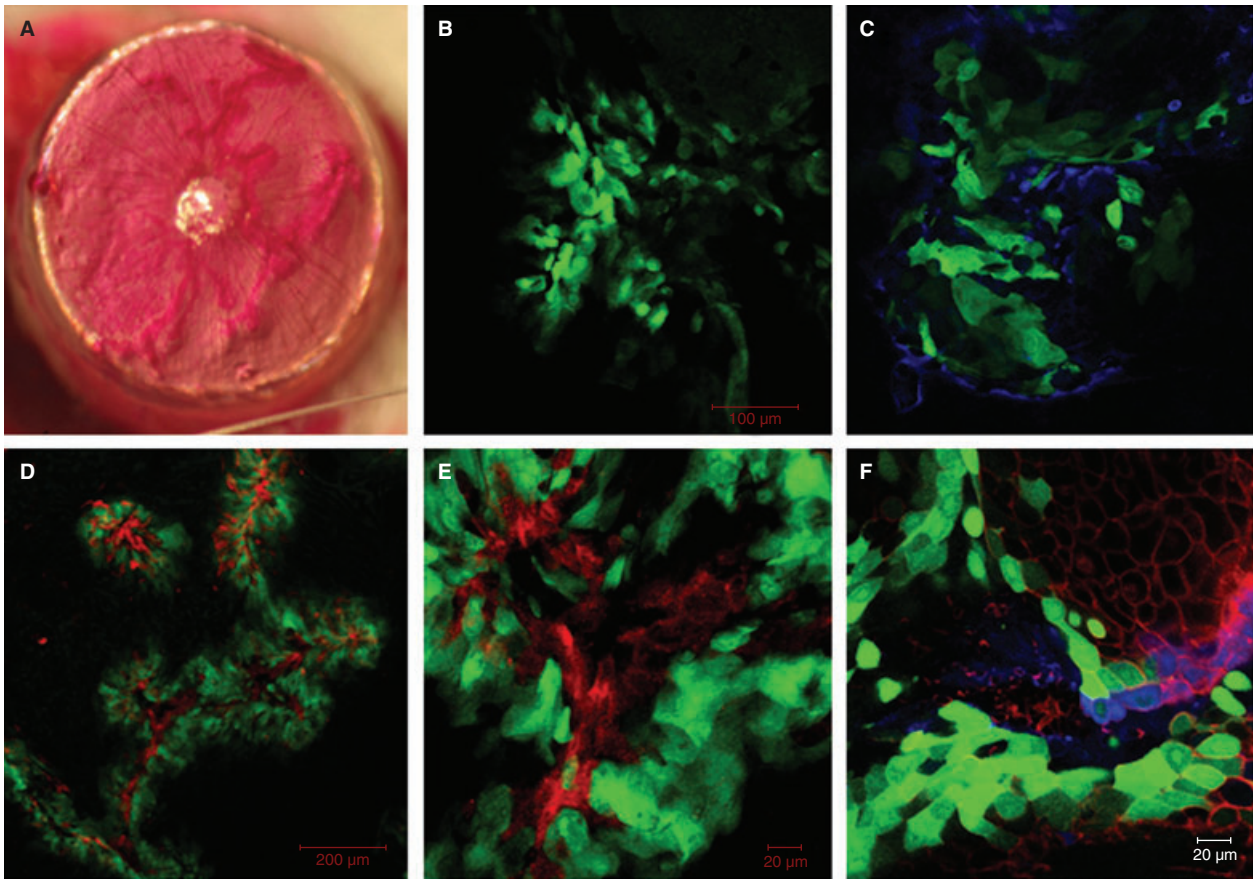

Fig. 12. Corneal epithelial herpes simplex infection (dendritic ulcer) in the mouse using an eGFP recombinant human herpes virus-1: day 2 postinfection. (A) Clinical *in vivo* appearance showing dendritiform corneal epithelial ulcer; Rose Bengal dye stains the infected/necrotic cells (red); (B) confocal microscopy image of fixed cornea showing eGFP HSV-infected cells; (C) confocal image combining eGFP HSV-infected cells with $CD45^+$ leukocytes (blue); (D) confocal image showing eGFP HSV-infected epithelial cells and non-infected $F4/80^+$ macrophages/DCs; low-power view of an entire clinical 'dendrite'; (E) detail of a groove of the dendrite showing $F4/80^+$ non-infected (red) and eGFP-infected cells (green); (F) similar image to (E) showing $CD11b^+$ macrophages/DCs (blue), $actin^+$ uninfected epithelial cells (red), and eGFP HSV-infected epithelial cells (green), located inside the herpetic dendrite. GFP, green fluorescent protein; HSV, herpes simplex virus; DCs, dendritic cells.

2 mm in the mouse), and the density of leukocytes in the central cornea is low, although there are some MHC class II^+ macrophages (see above: Myeloid cells in ocular tissues – the ocular surface section). Accordingly, direct allorecognition via passenger leukocytes is minimal, while indirect allorecognition represents the major if not the exclusive route whereby allograft rejection occurs (136–140). Experiments using donor grafts, previously infected either with a non-secretory green fluorescent protein (GFP)-labeled plasmid or with a non-secretory C3′-expressing plasmid, to Rag knockout mice which have been reconstituted with C3′-specific T cells have shown that antigen from donor cornea is taken up and presented by host DCs which traffic to the site-specific draining lymph node (SM DLN) and cross-present antigen on MHC class II to naive T cells (39). The process begins within 6 h of corneal grafting. As indicated above, soluble antigen from the donor cornea is transferred even more rapidly to the secondary lymphoid tissues (30 min after application to the cornea), but specific priming of T cells takes place in the eye-draining SM DLN only when the cell-associated antigen arrives. Recent studies suggest that initial activation of T cells occurs when soluble antigen is taken up by resident lymphoid DCs, but that proliferative T-cell responses are induced when the second wave of cell-associated antigen arrives some hours later via migratory DCs from the tissues (141, 142). During the next several days, waves of T-cell expansion and contraction occur in the SM DLN, and finally graft rejection takes place about 12–15 days after grafting (143). Interestingly, although there is considerable myeloid cell and T-cell infiltration of the host, infiltration of the donor with large numbers of cells does not take place until around 9–10 days after grafting (143) (Fig. 13). It appears therefore that accumulation and retention of T cells within the graft has to reach a certain level before rejection can occur. Graft rejection is accompanied by much

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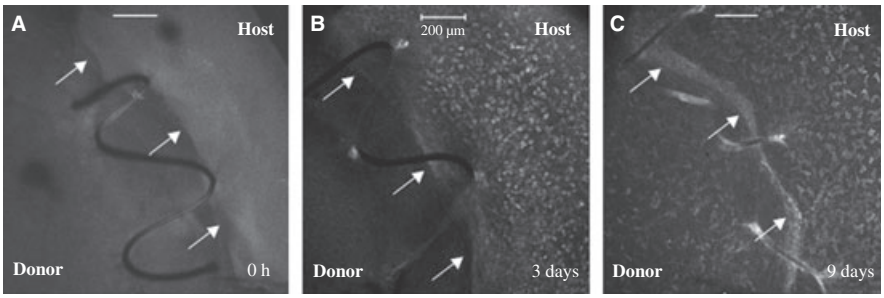

Fig. 13. Influx of CD11c$^+$ DCs into the transplanted mouse corneal graft. (A) 0 h, (B) 3 days and (C) 9 days postgraft. Note the marked infiltration of CD11c$^+$ cells in the host corneal bed by 3 days but absence of such cells in the donor graft; by 9 days there was considerable CD11c$^+$ infiltration in the donor tissue as well as in the host. Arrows - recipient/graft interface, DCs, dendritic cells.

of the expected upregulation on adhesion molecules and chemokines some of which are necessary for rejection (144–146).

Understanding why some grafts are rejected and others are not is difficult. Uniquely, the cornea allows the mechanism of indirect allorecognition of a single antigen disparity to be evaluated (147, 148), and experiments indicate that between 20% and 50% of donor grafts using both X–Y disparity and the transgenic expression of a single foreign antigen such as hen egg lysozyme (Hel antigen) are rejected, albeit slowly (Vitova A, Kuffova L, Holan V, Cornall RC, Forrester JV, *et al.*, unpublished observations). Limiting factors include antigen-specific T-cell precursor frequency and APC priming through innate immune mechanisms.

The intraocular compartment

Despite the evidence for the privileged status of the eye, intraocular inflammation (uveitis, uveoretinitis, and uveoscleritis) is common and ranks fourth as a cause of blindness in the Western world (149). About 50% of cases are due to infectious causes, and the full gamut of microorganisms can be implicated. Prominent are mycobacterial infections, parasitic infections particularly toxoplasmosis, and viral diseases such as cytomegalovirus (CMV) and herpes (simplex and zoster). Recent and resurgent additions to this list are spirochetal infections (Lyme disease and syphilis) and unusual viral infections such as dengue fever. Many of these diseases are recognized as 'opportunistic' infections that develop in immunocompromised individuals. Thus, they have become more frequent as a result of the acquired immune deficiency syndrome epidemic. Diseases such as CMV or herpes-induced acute retinal necrosis (ARN) were almost unknown prior to human immunodeficiency virus infection (reviewed in 150). In one sense, such diseases reflect the fact that immune privilege of the eye comes at with some cost (6), and this organ is thus more liable to attack and damage by microorganisms that would be cleared with full restoration of function by other tissues.

The remaining non-infectious cases of intraocular inflammation are considered to be autoimmune or at least immunologically mediated. This is based on clinical observations of conditions such as sympathetic ophthalmia, probably one of the first autoimmune diseases described, in which inflammation develops in 2–3 months in the healthy fellow eye following penetrating injury to the first eye (70). In classical thinking, release from the injured eye of autoantigen 'sequestered' from the immune system during development and thus not 'seen' as self leads to the activation of autoreactive T cells systemically, which then home to the sites of autoantigen in the healthy eye and cause inflammation. Initial studies of uveal tissue for the elusive antigen led eventually to the discovery of the retina as the repository of several potent autoantigens (151, 152), mainly as photoreceptor antigens, and several have now been sequenced and pathogenic epitopes identified (153). A model of experimental autoimmune uveoretinitis (EAU) has been described with considerable mechanistic analogs in similar models of encephalomyelitis and collagen arthritis, and the genetics of species and strain susceptibility have been documented (153).

Accordingly, questions relating to the pathogenesis of EAU are the same as those relating to other models of autoimmunity, such as how and where do autoreactive T cells become activated? Do activated T cells home to the retina? Can activated T cells cross the blood–retinal barrier? Which APCs induce the disease and where do they do this?

Current concepts in pathogenesis of inflammatory disease suggest that initial priming and activation of effector T cells occur during the first 6–72 h of interaction with specific antigenic peptide–MHC class II (pMHCII) complexes in the

secondary lymphoid tissue, where the T cells are retained by increased expression of CD62L and downregulation of sphingosine-1R (154). During this time they undergo clonal expansion. They then depart the LN after re-expression of sphingosine-1R and home to the tissues. Once more they are retained in the tissues by cells expressing specific pMHCII complexes, usually but not necessarily DCs (reviewed in 155). In the target tissues, however, there is little or no proliferation of T cells; rather, there is secretion of pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-17, which recruit and activate pathogenic cells, particularly pro-inflammatory macrophages. In addition, there are several sets of regulatory cells recruited to the retina during autoimmune inflammation including suppressive macrophages, which are likely to inhibit proliferation but not accumulation of pathogenic T cells (156). Other regulatory cells (157) either in the tissues or in the secondary lymphoid tissue probably act to downregulate the production/function of pathogenic T cells (see later).

There are several candidate resident pMHCII-expressing APCs in the uvea/retina that might undertake this secondary cytokine-producing activation of T cells. In particular, in the retina we have shown that T cells accumulate in the initial stages of EAU around the $33D1^+$ cells in the retina at the peripheral margins (*pars plana*) and around the optic nerve (82). In the later stages, there is migration of T cells into the retina via the retinal vessels and active presentation of pMHC (*Fig.* 14). Similar experiments have been performed in rat EAU, using a GFP-labeled retinal S antigen-specific T-cell line (Williams K, Linington C, Forrester JV, unpublished data). Adoptive transfer of these cells intraperitoneally was followed by an initial 'disappearance' of the cells which then re-appeared in the eye and in the spleen. However, only in the eye were they activated (high expression of OX40), and such activated T cells were initially localized to the peripheral retina/uveal tract where these two tissues meet.

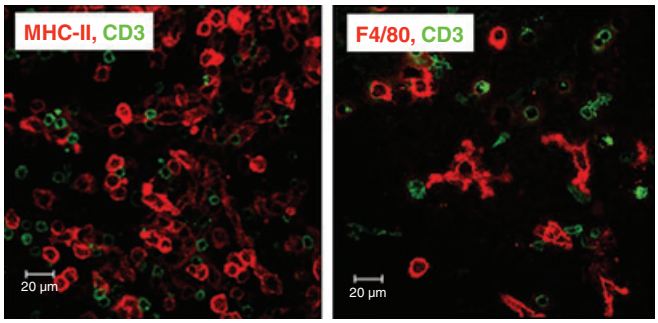

***Fig.* 14. Close encounters between MHC class II^+ (left panel) and $F4/80^+$ (right panel) DCs with $CD3^+$ T cells in flat mount preparations of retina in EAU at the peak of the disease.** MHC, major histocompatibility complex; DCs, dendritic cells; EAU, experimental autoimmune uveoretinitis.

Although such 'resident' APCs are likely to receive and activate trafficking primed autoreactive T cells egressing the lymph node, a sustained inflammatory response on the one hand requires further recruitment of potential APCs, while downregulation of the response requires induction of regulatory mechanisms including Tregs. In adoptive transfer experiments, it has been shown that myeloid cells are continually recruited to the retina during the active stages of EAU (158). However, only fresh bone marrow-derived monocytes are recruited, not cells which have been activated *in vitro* prior to transfer or cells which have previously trafficked through a tissue, e.g. peritoneal macrophages/DCs. In addition, adoptively transferred fresh bone marrow cells do not immediately enter the inflamed retina but require several hours of recirculation before they can enter the tissues. During this time, they upregulate a range of adhesion molecules, such as CD44 and chemokine receptors such as CCR7, which prepare them for duty (158).

Most of the models of EAU and indeed other autoimmune models utilize antigen emulsified in an adjuvant, inoculated into the dermis. This approach allows activation of DCs through innate immune receptors and activation of antigen-specific T cells in the skin-draining LN. In a spontaneous model of EAU and also in models in which central tolerance is impaired, 'spontaneous' activation of T cells in the periphery leads to EAU (159, 160). An unanswered question therefore remains. Does antigen from the retina traffic to the secondary lymphoid tissues, and if so, how and when does this occur? Spontaneous EAU can be induced in a double transgenic mouse model in which foreign antigen, hen egg lysozyme (Hel) is expressed in the retina under the control of the promoter for IRBP and crossed to the T-cell receptor mouse for Hel (double transgenic Hel-IRBP:TCR or IRBP-Dbl Tg mice) (161). These mice develop EAU 21 days after birth, which progresses into adulthood with complete retinal destruction despite considerable central deletion of antigen-specific T cells. As Hel antigen is bound to the membrane of the photoreceptor outer segment in this model, no soluble Hel antigen escapes to the periphery. However, sufficient cell-associated Hel antigen must have escaped to activate T cells in the periphery and permit them to traffic to the retina to cause damage. Experiments are in progress to detect the cell-associated antigen, and this investigation should reveal considerable information on how T cells are educated in the periphery both for tolerance and its breakdown (autoimmunity).

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DCs in clinical ocular disease

Ocular surface disease

As might be expected, DCs form part of the inflammatory cell exudate in many forms of ocular surface disease (162–164), but no systematic clinical studies have been carried out to determine their role. DCs are also found in the stroma of rejected human corneal grafts (165) and can even be imaged by *in vivo* confocal microscopy in patients with keratitis (166). They are also seen in keratoconus, a condition in which there may be minimal, if any, inflammation (*Fig.* 15).

Intraocular inflammation

DCs have been identified in pathological specimens from patients with severe uveitis and very recently have been detected using flow cytometry in aqueous fluid samples taken from patients with active chronic panuveits (Dennison A, Murray P, Curnow J, personal communication). DCs have also been studied in the blood of patients with various forms of uveitis, including that associated with Behçet's disease. Such patients have been found to have reduced levels of circulating pDCs (167, 168), as has been found in many patients with autoimmune disease and particularly SLE (169). In patients with non-Behçet's uveitis, reduced levels of pDCs have also been detected, and interestingly, pDCs in these patients appeared to be unable to elaborate IFN-α in response to CpG when they were cultured *in vitro*, if they were concomitantly being treated with IFN-α for control of their uveitis (168). Despite this endogenous failure of their pDCs to secrete IFN-α, systemic treatment with IFN-α led to an increase in circulating $CD4^+CD25$ $Treg^+$ cells as well as increased levels of IL-2 (Yeoh J, Kuffova L, Forrester JV, unpublished data).

The role of DCs in infectious uveitis has not been directly studied. However, recent studies of extrapulmonary tuberculosis suggest that infected DCs and macrophages from infective granulomas in the lung can migrate away from the lung lesion as part of a highly dynamic cellular process. Infected DCs then have the potential to lodge in small blood vessels and lymphatics in tissues such as the choroid of the eye and produce miliary tubercular lesions. Further study of this potential mechanism is in progress (100, 170).

Retinal degeneration

Age-related macular degeneration (AMD) has recently been highlighted as a disease that may develop through possible innate immune dysregulation. In part, this view has evolved from the evidence indicating that mutations in some complement components as well as other proteins greatly enhance the risk of contracting AMD. Excessive deposition of complement components at the retinal pigment epithelial/outer retinal interface (*Fig.* 16), particularly concentrated in deposits of waste material (termed drusen), may act as triggers for inflammatory macrophage activation and induction of focal regions of aberrant angiogenesis, which have devastating effects on visual acuity if they are located close to the fovea (reviewed in 171). The role of resident myeloid cells in this

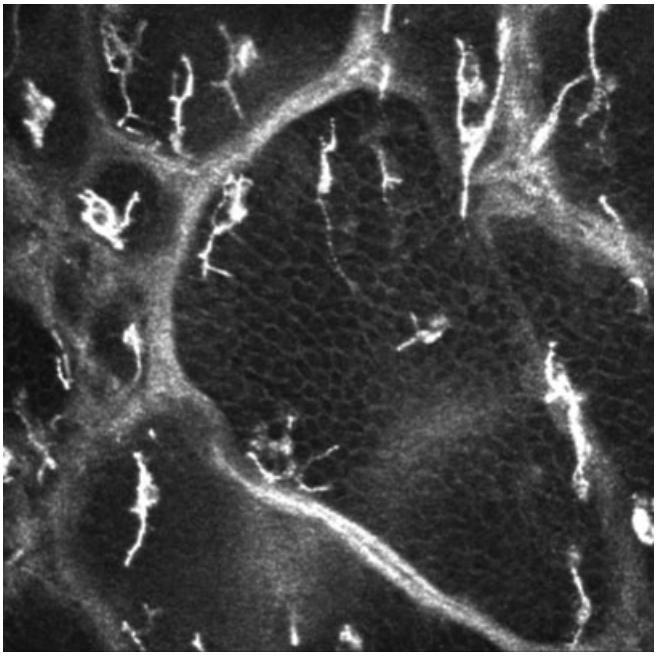

***Fig.* 15. Human corneal dendritiform cells, presumed leukocytes, imaged *in vivo* by reflectance confocal microscopy in a case of keratoconus.** The acute accumulation of fluid in this previously extremely thin cornea, clinically presenting as 'acute corneal hydrops', permitted good resolution of the infiltrating cells (photograph courtesy of DV Patel and CN McGhee, Auckland University).

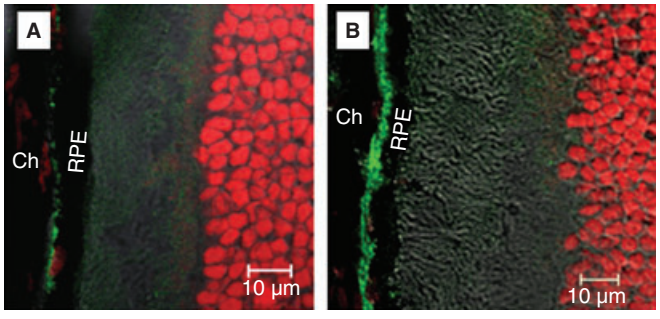

***Fig.* 16. Complement deposition at the retina/choroidal interface in aged mice.** (A) Six-week-old mouse retina; (B) 18-month-old mouse retina. Complement (green), red (PI). Ch, choroids; RPE, retinal pigment epithelium.

process has been investigated. As indicated above, the retina proper does not have DCs except in the peripheral regions. However, endogenous retinal microglia become activated with age and disease and migrate into the subretinal space (*Figs 8 and 10*) where they accumulate lipofuscin (92, 93). In addition, it is likely that there is increased recruitment of infiltrating cells (? microglial precursors). In other forms of retinal degeneration, sialoadhesin^{+} microglia infiltrate the retina (172) as part of a low-grade pro-inflammatory response (173, 174) (*Fig. 8*). This process has been reported to be even more prominent in the CCL2/CCR2$^{-/-}$ mice (175), in Cx3cr1$^{-/-}$ mice (176), and in double knockouts (177). These mice have been reported to develop a retinal degeneration and also to develop 'drusen-like deposits' in their outer retina (*Fig. 16*), although these are actually intraretinal, unlike drusen in the human eye which are sub-RPE (178). Recent studies suggest that there may be similarities in the changes in these mouse models with those found in aging and light-induced damage. Drusen act as sinks for complement deposition and also contain many other retinal proteins and lipids in a partially degraded state. Recent reports have suggested that carboxyethylpyrrolle derivatives of photoreceptor membrane lipids may be taken up by choroidal DCs, which lie immediately below the RPE, and are presented as atypical antigenic material, possibly on CD1d, to T cells, thus initiating a low-grade autoimmune inflammatory response (179). This may then promote the later infiltration of inflammatory macrophages and the subsequent angiogenic response.

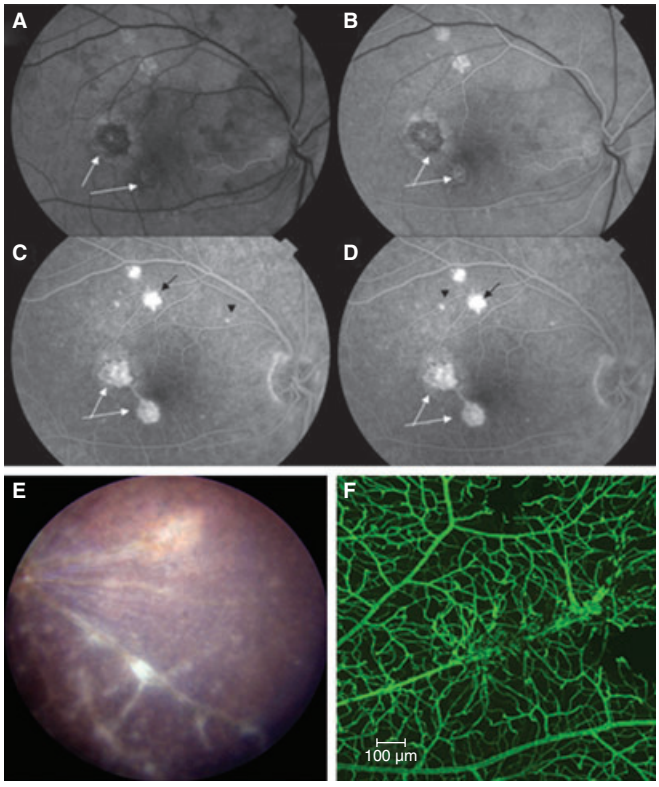

***Fig. 17.* Neovascularization at the retinal/choroidal interface.** (A–D) Sequences taken from a fluorescein angiogram in a patient with a subretinal neovascular membrane typical of age-related macular degeneration (wet type) or in the chronic stages of choroido-retinal inflammatory eye disease (uveitis); the images show the progressive tracking of intravenously injected fluorescein dye through the retinal vessels with accumulation and leakage of dye in the neovascular complex (arrow). (E, F) Similar neovascular complex in the subretinal space in the mouse in the very late stages of experimental autoimmune uveoretinitis. (A) red-free fundus photograph; (B–D) time sequences of fluorescein angiograms as the dye passes through the retinal vasculature. (E) Clinical fundus image of the mouse eye; (F) flat mount preparation of mouse retina stained with collagen type IV showing two neovascular complexes.

Similarities between AMD and atherogenesis have been drawn on many occasions, and recently the possibility that aortic valve DCs may play a part in such processes has been raised (180). This has much in common with the development of AMD as outlined here. In addition, this process in the eye has a direct immunological analog: in humans with low-grade posterior uveitis and in mice in the resolving phase of EAU, subretinal neovascular responses causing visual loss in precisely the same manner as wet AMD develop, which in the case of EAU are induced by autoimmune responses to photoreceptor degradation products (*Fig. 17*).

DCs as therapy for ocular disease

As DCs have a central role in maintaining and regulating both tolerance and immunity, one approach has been to target endogenous DCs using antibody fusion proteins that bind to DC-specific cell surface antigens. This has been used to promote both tolerance in models of autoimmunity and immunity against tumors (16, 181). Different DC molecular targets have been used to tip the balance in favor of immunity versus tolerance, for instance CD205 to promote tolerance and DCR1 to promote immunity. Both approaches have had some success in experimental models but have not yet reached use in clinical studies (84, 182–184).

An alternative approach has been to use DCs as a cellular vaccine: once more this has been promoted in developing vaccines against human immunodeficiency virus but also to restore tolerance, as in autoimmune diseases. In several experimental models particularly autoimmune diabetes, the administration of antigen-primed DCs to mice has been shown to prevent the onset of disease (16, 182, 185). Similarly, antigen-primed DCs have been shown to induce tolerance in EAE (186) and in collagen arthritis (187, 188). Antigen-primed DCs have most recently been used to prevent EAU

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and, as for other models, appear to do so by expanding antigen-specific Tregs (189). Interestingly, while pMHCII complexes appear to be required to induce tolerance, their main function appears to be in ensuring egress of $CD62L^+$ Tregs from the DC-draining lymph node rather than inducing clonal expansion of Tregs, which appears to be possible in a non-specific manner. The expanded Tregs are then available to home to secondary lymphoid tissue where T effectors are expanding after immunization with peptide (189). By contrast, CCR7 appears to be necessary for Tregs to home to the inflamed tissue, but their ability to inhibit disease at the site of inflammation appears to be less than in the secondary lymphoid tissue, i.e. during priming (190, 191). Questions relating to the site of action of Tregs and their clonal expansion and functional capacity are important in relation to regulation of autoimmune inflammation, and direct tracking studies suggest that Tregs at the site of inflammation have suppressive effects of some magnitude (142).

From a clinical perspective, preventing the onset of disease has less immediate therapeutic relevance compared with treating active disease. In this regard, models of spontaneous autoimmune disease have considerable value, and models of diabetes in which disease is initiated through a prodromal insulitis have shown that it is possible to regulate disease when T-effector cell priming has clearly occurred (185). Recently, in the model of spontaneous EAU developed using the double transgenic Hel-IRBP:TCR mouse described above (161), mice develop EAU as a focal inflammation affecting the retina–choroid which progressively develops over 4–6 weeks to complete retinal destruction. In preliminary studies, we have observed that in mice in which Hel-pulsed DCs have been administered subcutaneously at d21 postnatally, the disease can be halted. These are encouraging early results and further experiments are in progress.

Immune cell vaccine therapy is not without potential side effects: in trying to induce immunity against tumors, autoimmunity against cross-reactive self-antigens can be induced, for instance against melanin-associated antigens leading to vitiligo after DC therapy. A recent report in a patient with metastatic skin melanoma described the development of full-blown signs of Vogt–Koyanagi–Harada disease (vitiligo, alopecia, hearing loss, poliosis, and bilateral panuveitis) after administration of autologous Treg tumor-infiltrating lymphocytes (192). In addition, the theoretical risk of inducing tumors in patients being treated for autoimmune disease is possible, but this therapy has not yet reached the clinic.

Conclusion

DCs pervade and reside in all tissues including the eye. In the eye, an 'immunosuppressive' microenvironment probably confers on DCs those special properties that contribute towards what is widely recognized as immune privilege. However, these effects on DCs in the eye are fundamentally not different from equivalent effects on DCs in other tissues: it is merely a matter of context. Accordingly, there is much to be learned from studying DC behavior in tissues such as the eye and the brain, as they allow greater understanding of immunopathological processes. They also allow the construction of novel therapeutic approaches to control the disease, be it to promote immunity and inhibit tumors and infectious disease or to restore regulatory control over autoimmune disease.

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