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When clarity is crucial: regulating ocular surface immunity

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Abstract

The ocular surface is a unique mucosal immune compartment in which anatomical, physiological and immunological features act in concert to foster a particularly tolerant microenvironment. These mechanisms are vital to the functional competence of the eye, a fact that this underscored by the devastating toll of excessive inflammation at the cornea – blindness. Recent data have elucidated the contributions of specific anatomical components, immune cells and soluble immunoregulatory factors in promoting homeostasis at the ocular surface. Here, we highlight research trends at this distinctive mucosal barrier, and identify critical gaps in our current knowledge.

What sets the ocular surface apart

As a mucosal surface, the ocular surface shares several functional properties with the mucus membranes of the gut, respiratory and urogenital tracts. However, only at the ocular surface are optical properties critically important—thus a successful immune response at the ocular surface must not jeopardize corneal clarity. In conspiring to limit blinding inflammation at the ocular surface, the immune system walks a tightrope. An insufficient immune response increases susceptibility to microbial disease, yet an overzealous response can induce corneal opacification through collateral damage to bystander cells.

The relative restraint of the ocular immune response was formally recognized in the 1940s, when Peter Medawar reported prolonged survival of allogeneic skin grafts placed heterotopically in the anterior chamber of the eye [1]. Medawar coined the term ‘immune privilege’ to describe this phenomenon, concluding that the apparent absence of lymphatic drainage resulted in the sequestration of antigenic material, thereby preventing the generation of an immune response. Our modern understanding of ocular immune privilege encompasses a diverse amalgam of anatomical attributes, physiological and

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immunoregulatory processes, that includes the relative scarcity of lymphatics described by Medawar (now known as ‘immunological ignorance’).

Similar to other immune privileged tissues in the body, such as the brain, the eye has limited regenerative capacity. For example, the corneal endothelium does not proliferate sufficiently to compensate for age-related cell loss (if at all), which is observed as a progressive decrease in corneal endothelial cell density with aging [2]. This cell layer is critical for the maintenance of corneal transparency, and diseases that accelerate cell loss (e.g. corneal dystrophies or inflammatory conditions) are potentially blinding as a result. Thus, it is vital that immune-mediated inflammation at the ocular surface does not cause excessive collateral damage. Our understanding of the anatomical, cellular and molecular factors that modulate the immune response at the ocular surface has deepened markedly over recent years; these immunoregulatory mechanisms are reviewed herein.

Components and function of the ocular surface microenvironment

Structurally, a continuous epithelium is shared by the cornea, limbus and conjunctiva. The corneal epithelium is a nonkeratinized stratified squamous epithelium, similar to the mucosa of the oral cavity and esophagus. Posterior to the corneal epithelium lays the stroma (consisting of regularly arranged collagen fibrils interspersed with sparse keratocytes) and the corneal endothelium (Figure 1). Here, we review select components that play a major role in fostering the unique immune physiology found at the ocular surface—the tear film, corneal epithelial cells, dendritic cells (DCs) and neuropeptides.

Tear film

The tear film represents a formidable barrier to potentially pathogenic inflammatory mediators, with antigens constantly being ushered away from the ocular surface by blinking and reflex tearing [3] (Figure 1). Similar to other mucosal fluids, tears contain a wide variety of antimicrobial molecules that facilitate cytolysis or prevent the growth of pathogenic organisms, such as lysozyme, lactoferrin and lipocalin [3]. The principal antibody in the tear film is secretory IgA, which functions pleiotropically to neutralize toxins, viruses and bacteria; and to regulate DC maturation [4]. Notably, the tear film also regulates the function of the epithelial cells that it bathes, with tears enhancing epithelial resistance to bacterial invasion through increased activation of NF κ B and AP-1 transcription factors [5]. Maintenance of the tear film on the ocular surface is facilitated by the epithelium-derived glycocalyx, which consists of high molecular weight heavily glycosylated membrane-associated mucins [6]. These membrane-associated mucins prevent bacterial adhesion and invasion, whilst secreted gel-forming mucins provide lubrication and trap debris for removal via the lacrimal puncta [6]. At the ocular surface, MUC5AC is the predominant secreted mucin [7]. In the respiratory and gastrointestinal tracts, MUC5AC forms large protein mucus complexes via disulfide bonds between its cysteine-rich regions [8]. However, secreted MUC5AC has a lower molecular weight at the ocular surface, presumably to preserve the optical properties of the tear film [9].

Ocular surface epithelium

Corneal epithelial cells perform multiple functions to maintain immune homeostasis (Figure 1). Constitutively, the immune response is limited both by molecules expressed at the epithelial cell surface (such as FasLigand [FasL] [10] and programmed death ligand-1 [PDL-1] [11]) and by secreted molecules (such as the endogenous vascular endothelial growth factor [VEGF] traps sVEGFR1 and VEGFR3 [12,13] and pigment epithelium-derived factor [PEDF] [14]) (Table 1). The recognition of pathogen-associated molecular patterns via Toll-like receptors (TLRs) on corneal and conjunctival epithelial cells results in the secretion of a broad range of antimicrobial peptides, such as human β -defensin (hBD)-1, hBD-3 and cathelicidin (hCAP-18/LL-37), that have broad-spectrum activity against bacteria, fungi and viruses [15]. The ocular surface epithelium is exposed to abundant TLR ligands in the external environment, and various mechanisms have been identified that curb the ensuing immune response. For example, two explanations of the unresponsiveness of corneal epithelial cells to lipopolysaccharide (LPS), a TLR4 ligand, have been proposed [16,17]. Ueta et al. have reported that the deficient expression of myeloid differentiation-2 (MD-2, a vital component in LPS-TLR4 signalling) is responsible, whilst Zhang et al. have shown that although corneal epithelial cells express copious TLR2- and TLR4-specific mRNA, the corresponding proteins are not expressed at the cell surface but intracellularly [16,17].

Ocular surface epithelial cells have been shown to confer a tolerogenic profile upon DCs, and there is evidence that goblet cells specifically contribute towards this phenomenon through the expression and activation of transforming growth factor (TGF)- β 2 in a thrombospondin-1 (TSP-1) dependent manner [18]. Corneal epithelial cells express thymic stromal lymphopoietin, another important regulator of DC function [19]. In the gut, a broad array of soluble factors secreted by the epithelium influence DC activity, including retinoic acid, prostaglandin E2 and interleukin-10 (IL-10) [20]. Further work is required to fully define the crosstalk between epithelial cells and DCs at the ocular surface.

Dendritic cells

The highest density of DCs is found in the conjunctiva, and their density decreases from the limbus to the central cornea (Figure 2) [21]. Steady state DCs found in non-lymphoid tissues process antigen sampled from the local microenvironment continuously, and play an essential role in triggering adaptive immune responses [22]. Membrane nanotubes have been demonstrated in vivo on MHCII⁺ cells in the murine cornea, and it has been proposed that these may transport antigens between the tear film and DCs, analogous to intraepithelial DCs in the respiratory and intestinal tracts [22,23]. The major DC subsets have been identified at the cornea [24,25]. The peripheral corneal epithelium has been demonstrated to contain Langerhans cells, and the peripheral anterior corneal stroma contains DC-SIGN⁺ DCs [26]. This correlates well with the distribution pattern observed in the skin, with Langerhans cells present in the epidermis and DC-SIGN⁺ DCs contained in the dermis [26]. Another important finding concerns the distribution of MHC class II⁺ Langerhans cells in the corneal epithelium – these cells are found in the limbus and the peripheral cornea, but not in the center of the uninflamed cornea [27]. Initially thought to be solely expressed by Langerhans cells (a population of DCs found in the skin and mucosa containing Birbeck

granules), it has been established that other non-Langerhans cells (frequently referred to as CD103⁺ DCs) in the corneal stroma express Langerin, which further parallels findings from the skin [28]. Interestingly, MHC class II⁻, costimulatory molecule⁻ DCs have been identified in both the central and peripheral anterior corneal stroma [29]. In the inflamed cornea, these cells mature with enhanced expression of MHC class II, CD80, CD86 and CD40, and can be found both centrally and peripherally, in contrast to the normal cornea whose center is devoid of DCs [27,30]. Siglec-H⁺ plasmacytoid DCs have also been identified in the cornea, and there is evidence that ocular surface inflammation alters Siglec-H expression by plasmacytoid DCs, resulting in expansion of Siglec-H^{low} relative to Siglec-H^{high} subpopulations [31].

A further subset of DCs that has been identified in the normal conjunctiva and posterior stroma are CD11b⁺ DCs (Figure 2) [26,32]. This phenotype has been shown to be the principal DC subset driving the accumulation of antigen-specific CD4⁺ T effector cells and Tregs following subcutaneous vaccination of the relevant antigen plus an adjuvant [33]. CD11b⁺ DCs have been identified widely in mucosal tissues, including in the respiratory and intestinal mucosae [34,35]. Indeed, reports examining allergic airway inflammation have identified CD11b⁺ DCs as the predominant antigen-presenting cell in secondary allergic reactions [36,37]. Khandelwal et al. have demonstrated the importance of CD11b⁺ DCs in ocular allergy, by showing that CD11b⁺ DCs play a dominant role in triggering allergic T cell responses in a murine model of ocular allergy with pre-sensitized T cells [32]. Although the therapeutic potential of tolerogenic DCs in the setting of corneal transplantation has been demonstrated [38], the DC phenotypes that maintain immune tolerance at the ocular surface have not been thoroughly characterized [25,39].

Neuroregulation of ocular surface immunity

Given that the cornea is the most densely innervated structure in the human body, it is perhaps unsurprising that neuropeptides (produced and released by neurons) play an important role in regulating immune homeostasis at the ocular surface. Neuropeptides have been implicated in moderating both the innate and adaptive immune responses at the ocular surface. Thus far, the rapidly developing field of ocular neuroimmunology has focused on describing the molecular and structural composition of nerve fibers in the cornea, and correlating this with their function. Select neuropeptides that have attracted particular attention at the ocular surface include substance P (SP), calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP) [40].

SP has been reported to recruit monocytes and neutrophils, promote mast cell degranulation, regulate DC maturation and promote the maturation of Th17 cells [41,42]. The severing of corneal nerves in one eye has been shown to induce SP secretion in both eyes, resulting in the inhibition of regulatory T cell function and increased rejection of subsequent corneal allografts in both eyes [43]. Mice deficient in the receptor for SP (neurokinin-1 receptor [NK1R], encoded by *Tacr1*) exhibit increased desquamation of apical epithelial cells, decreased corneal nerve density and decreased corneal DC density [44]. SP is also contained in the sensory neurons of the gastrointestinal and respiratory tracts, and *Tacr1*^{-/-} mice exhibit increased protection against acute inflammatory diseases in these tissues [45,46].

In contrast to the predominantly pro-inflammatory effects that SP exerts on immune and epithelial cells, CGRP inhibits the production of inflammatory cytokines by macrophages, the maturation of DCs and the production and proliferation of IL-2 [47]. Elevated levels of CGRP have been reported in the tears of patients following corneal surgery, and may represent a compensatory immunoregulatory mechanism [40,48]. Notably, the recruitment of neutrophils to the inflamed cornea via IL-8 has been shown to be promoted by both CGRP and SP [42,49]. In addition to modulating cytokine secretion, NPY regulates immune cell trafficking, T helper cell differentiation, NK cell activity and the generation of reactive oxygen species [50]. Furthermore, NPY has been proposed to contribute towards ocular immune privilege through the suppression of macrophage functionality [51]. VIP also has pleiotropic effects as an immune regulator; it functions to downregulate TLRs, inhibit chemokine expression, induce tolerogenic DCs and limit the release of pro-inflammatory cytokines [52]. Although the importance of these select neuropeptides has been established the ocular surface, further experimental studies are required to clarify the roles of other neuropeptides, as well as the extent of neuro-immune crosstalk in various ocular surface pathologies.

Immune response to infection at the ocular surface

Corneal opacification and neovascularization in microbial keratitis are not produced directly by pathogens, but instead result from a vigorous immune response inflicting collateral damage on bystander cells. CD4⁺ T cells play a key role in coordinating this response [53]. Here, we review recent studies investigating the immune response in viral keratitis and bacterial keratitis.

Viral infection

The corneal epithelium is a major site of primary herpes simplex virus 1 (HSV-1) infection [54]. Indeed, HSV-1 keratitis is the leading cause of infectious blindness in developed countries [55]. When the innate defences of the corneal epithelium are overwhelmed, there is a preclinical phase of HSV-1 keratitis that corresponds to the productive influx of myeloid cells that inhibit viral growth [53]. Following viral clearance, CD4⁺ T cells orchestrate an inappropriately sustained leucocytic infiltration of the cornea. It is the persistent inflammation, angiogenesis and lymphangiogenesis that is associated with this phase of the disease that jeopardizes corneal clarity [53]. In a study using DC-autophagy-deficient mice, Jiang et al. have demonstrated that mice lacking DC-autophagy exhibit suppressed activation of CD4⁺ T cells and reduced clinical disease, without affecting innate immunity or viral replication [56]. Importantly, the study illustrates how the CD4⁺ T cell response can be dampened without compromising control of the viral infection. The long-term changes to the corneal immune microenvironment that are induced by subclinical HSV-1 infection have also been investigated. Rowe et al. have demonstrated that transient corneal HSV-1 infection induces CD4⁺ T cell-dependent resistance to infection by unrelated pathogens, such as pseudorabies virus [57]. However, there is a trade-off for this heightened protection, since subclinical HSV-1 infection also rendered the cornea more susceptible to trauma-induced inflammation [57].

Bacterial infection

The Gram-negative bacterium *Pseudomonas aeruginosa* is a common cause of bacterial keratitis; indeed, it is the most common pathogen implicated in contact lens-related corneal infection [58]. In bacterial keratitis, the critical role of CD4⁺ T cells in mediating inflammation is evidenced by the fact that C57BL/6 mice depleted of CD4⁺ T cells do not undergo perforation following infection with *Paeruginosa* [59]. There is marked plasticity in the *Pseudomonas* keratitis disease course, which is determined in part by the host's genetic susceptibility to favoring a Th1/Th2 response. In C57BL/6 mice, where a Th1 response predominates, corneas perforate by 7 days post-infection [59]. In contrast, in Th2 prone hosts such as BALB/c mice, there is no perforation seen at 7 days post-infection [60]. In these mice, IFN- γ derived from NK cells has proved critical to disease resolution [61]. Moreover, the neuropeptide SP has been shown to have an important regulatory function in controlling IFN- γ production by NK cells [61].

The study of *Pseudomonas* keratitis has shed light on the immunoregulatory function of commensal organisms at the ocular surface. Kugadas et al. have demonstrated how Swiss Webster mice, typically resistant to *Pseudomonas* keratitis, become susceptible to disease in a germ free environment [4]. The authors report that monocolonizing germ free mice with *Coagulase Negative Staphylococcus* sp. cultured from conjunctival swabs adequately reestablishes resistance to infection [4]. The presence of authentic commensals at the cornea and conjunctiva has been contentious for many years, in part because of their scarcity. Indeed, whilst 10⁶-10⁸ colony-forming units (CFU) per mL are found in human saliva, tears yield only 10²-10³ CFU per mL [62]. In support of naturally existing commensals at the ocular surface, St Leger et al. have isolated the commensal *Corynebacterium mastiditis* from murine conjunctivae, and demonstrated that this organism provokes a commensal-specific IL-17 response from $\gamma\delta$ T cells in the ocular mucosa [63]. The ensuing immune response was shown to promote neutrophil recruitment and antimicrobial release into the tears, defending the eye against infection with *Pseudomonas aeruginosa* and *Candida albicans*. Providing evidence against the ocular surface supporting a substantial core microbiome, in a study of 45 healthy human subjects sampled on 3 occasions over the course of 3 months and processed using both culture-dependent and culture-independent methods, Ozkan et al. did not identify any microorganisms that were present in all subjects at all times or in all subjects at any one time [64]. Given the "paucibacterial" levels of commensals at the normal ocular surface, the extent to which these bacteria contribute to local immune homeostasis remains unclear. In contrast, the dialogue between gut microbiota and the host immune system is well-established [65]. Intriguingly, a "gut-eye" axis of immune regulation has been proposed, whereby altering gut commensals has been shown to modulate secretory IgA levels and IgA transcripts in eye-associated lymphoid tissues [66].

Autoimmunity at the ocular surface

Ocular surface autoimmune diseases include both ocular specific pathologies (e.g. dry eye disease [DED]) and systemic diseases (e.g. Sjögren's syndrome, systemic lupus erythematosus, rheumatoid arthritis). These disorders share a common immunopathogenesis associated with aberrant activation of the immune response against putative self-antigens.

Research on ocular surface autoimmune disease has focused on DED, partly due to the established murine models available, but also due to the high prevalence and societal burden of this disease [67]. Indeed, it is estimated that over 16 million adults in the US have been diagnosed with DED [68]. The T cell-mediated basis of DED was formally established by data from the Pflugfelder-Stern-Niederhorn collaboration published over a decade ago, demonstrating that DED could be induced in T cell-deficient nude mice following adoptive transfer of CD4⁺ T cells isolated from mice exposed to desiccating stress (following depletion of CD25⁺ regulatory cells) [69]. Since that time, data from our laboratory and others have substantiated the paradigm of DED as a localized mucosal autoimmune disease [70].

Immunopathogenesis of DED

DED immunopathology is triggered when the proinflammatory pathways at the ocular surface overwhelm its immunoregulatory capacity. Exposure to desiccating stress activates MAPK signalling pathways, and prompts the ocular surface epithelium to secrete a range of pro-inflammatory mediators including IL-1 β , TNF- α , and IL-8, as well as a variety of matrix metalloproteinases (MMP-1, -3, -9, -10 and -13) [70,71]. As with other mucosal autoimmune diseases, the activation of TLRs and/or NOD-like receptors (NLRs) are involved in the induction of the innate response [72]. For example, inhibition of TLR4 has been shown to reduce the mRNA expression of IL-1 β , IL-6, and TNF in the cornea stroma of mice exposed to desiccating stress, relative to untreated mice, with an attendant decrease in ocular surface epitheliopathy [73]. The critical role of APCs in linking the desiccating stress-induced innate response to chronic CD4⁺ T cell activation has been confirmed by Schaumburg et al., who demonstrated that diminishing resident ocular surface APCs with clodronate inhibited the generation of autoreactive CD4⁺ T cells [74]. The presence of MHC class II negative APCs identified at the corneal epithelium may contribute to T cell tolerance [27,75]. Recent work by Barbosa et al. using a *Spdef*^{-/-} mouse model of DED (*Spdef*^{-/-} mice lack conjunctival goblet cells) has elucidated the contribution of goblet cells to ocular surface immune tolerance [76]. The investigators demonstrated that antigens applied to the ocular surface pass into the conjunctival stroma through goblet cell associated passages in wild-type mice, but are retained in the epithelium of *Spdef*^{-/-} mice. By pulsing single cell cervical lymph node suspensions from WT or *Spdef*^{-/-} mice with OVA₃₂₃₋₃₂₉ peptide, and co-culturing the cells with OTII CD4⁺ T cells for 3-4 days, the investigators showed that OTII CD4⁺ T cells that were primed by DCs isolated from *Spdef*^{-/-} mice exhibited heightened IFN- γ production and decreased expression of Foxp3 relative to those primed by WT DCs [76]. The host of afferent immunoregulatory mechanisms that we have described previously (including anti-inflammatory cytokines, epithelial cell surface molecules, neuropeptides, and lymphangiogenic privilege) act in concert to block the induction of the DED immune response.

As with inflammatory bowel disease, DED is characterized by mucosal infiltration of T cells that secrete Th1 and Th17 cytokines. Notably, the Th17 subset has been identified as having particular importance in both of these mucosal autoimmune conditions [77,78]. A significant Th17-derived IL-17A⁺IFN- γ ⁺ population has been identified in DED, implying that Th17 cells mediate ocular surface autoimmunity through IFN- γ as well as IL-17A [79]. In a

mouse model of chronic DED, Chen et al. demonstrated that the persistent inflammation seen in the chronic phase of the disease is mediated by an effector memory Th17 population [78]. IL-7 and IL-15 have been shown to be critical in maintaining this pathogenic subset, and in vivo blockade of these signalling pathways prevents the development of chronic DED [80]. Th17 immunity in DED is also dependent on the expression of C-C chemokine receptor (CCR)-7 by APCs, which directs APC migration towards lymphoid tissue, across a C-C motif chemokine ligand 21 (CCL21) chemotactic gradient [81]. Topical blockade of CCR7 has been demonstrated to block the induction of Th17 immunity, reduce inflammatory cytokine expression at the ocular surface and decrease disease severity [81]. Th17-derived granulocyte-macrophage colony stimulating factor (GM-CSF) itself contributes to Th17 cell induction by promoting MHC-II expression by CD11b⁺ cells and CD11b⁺ migration [82]. Interestingly, CD8⁺ cells have also been shown to regulate the Th17 response in DED [83]. Zhang et al. demonstrated that CD8⁺ cell depletion promoted the generation of Th17 cells in a murine model of DED, via the activation of DCs in both the ocular surface and draining lymph nodes [83].

Immunoregulation of DED

The balance between pro-inflammatory Th17 cells and Tregs in autoimmunity has attracted considerable attention. The developmental pathways of these subsets are reciprocally interconnected, and there is notable plasticity between the two lineages [84]. At the ocular surface, Tregs have been shown to be dysfunctional in DED, with their suppression of Th17 cells being particularly impaired [85]. Increasing age is a major risk factor for DED, and it has been suggested that the altered functioning of Tregs may contribute towards this phenomenon [86]. Coursey et al. used a murine model of spontaneous lacrimal keratoconjunctivitis in NOD.B10.H2^b mice to investigate the phenotypic and functional changes in Tregs at various ages (7-14w, 45-50w, 96-100w). The investigators demonstrated that CD4⁺CD25⁺ Tregs from aged mice exhibit decreased suppressive capacity relative to Tregs from young mice [86]. Moreover, subsets of Foxp3⁺CD4⁺CD25⁺ T cells were identified that express the proinflammatory cytokines IL-17A⁺ and IFN- γ ⁺. Adoptive transfer experiments suggested that these IL-17A⁺/IFN- γ ⁺ Tregs were sufficiently pathogenic to induce disease, since young RAG1^{-/-} mice that underwent adoptive transfer of old Tregs developed inflammatory disease comparable to those that received old effector T cells [86]. There has been interest in expanding the endogenous Treg population in DED, in order to shift the immunological balance in favor of tissue protection [87]. In a murine model, Ratay et al. have increased the recruitment of endogenous Tregs to the ocular surface using a locally-situated system that releases CCL22 in a controlled manner, thereby creating a sustained chemokine gradient [87]. Tregs are selectively recruited by CCL22 due to higher expression of CCR4 (the corresponding chemokine receptor) relative to other cells [88]. Ratay et al. showed that treatment with their CCL22 controlled release system resulted in (i) abrogation of decreased tear production, goblet cell depletion and increased epitheliopathy relative to untreated mice, (ii) decreased frequency of CD4⁺ T cells in regional draining lymph nodes and (iii) reduced infiltration of CD4⁺ IFN- γ ⁺ T cells in the lacrimal gland [87]. The potential to ameliorate DED through the expansion of the host's own immunoregulatory mechanisms will likely drive further research into Treg-based therapeutics.

Modulation of alloimmunity by the ocular surface microenvironment

The cornea is the most commonly transplanted tissue in the body, with over 150,000 grafts performed annually worldwide [89]. The success rate of corneal allografts is unique in the field of solid organ transplantation, with the five-year survival rate in low-risk hosts (non-vascularized and uninflamed host beds) exceeding 90% [90]. However, in high-risk grafts with inflamed and vascularized host beds, or where there is a history of previous graft rejection, less than 50% of corneal allografts are successful [91,92]. Corneal allograft rejection is characterized by the prominence of its IFN- γ secreting CD4⁺ T cell (Th1) response—indeed, corneal allograft rejection is markedly abrogated in CD4^{-/-} mice, yet rejection is undiminished in CD8^{-/-} mice [93]. Our understanding of the phenomena that temper the immune response to allografts at the ocular surface, and how these phenomena are revoked in the high-risk setting, has deepened over recent years.

During the sensitization phase of the alloimmune response, antigen-presenting cells (APCs) are trafficked from the corneal graft site to the regional draining lymph nodes via lymphatic vessels, where they prime naïve host T cells (Figure 3). APC infiltration is limited by the constitutive expression of IL-1 receptor antagonist (IL-1Ra) by corneal epithelial cells; indeed, topical treatment with IL-1Ra has been shown to decrease APC infiltration and promote allograft survival [94]. The expression of co-inhibitory molecules such as PDL-1, FasL and glucocorticoid-induced tumor necrosis factor receptor family-related protein ligand (GITRL) by corneal epithelial and endothelium also promotes graft survival by fostering a tolerogenic milieu and inducing apoptosis of Th1 cells [95–97]. APCs have been shown to express immunomodulatory molecules at the ocular surface that promote corneal allograft survival. In a study of TSP-1^{-/-} mice, Saban et al. demonstrated that compared to a 50% rejection rate in WT corneal allografts, almost all TSP-1^{-/-} mice grafts were rejected [98]. The investigators identified greater proliferation and IFN γ ⁺ expression by T cells stimulated by TSP-1^{-/-} APCs relative to WT, with increased expression of MHCII and B7 by TSP-1^{-/-} APCs during inflammation. A diverse collection of soluble immunomodulatory factors have been identified in the aqueous humor (including α -MSH, CGRP, TGF- β ₂ and VIP) that function to promote Treg activation, induce tolerance in macrophages and DCs, as well as to inhibit effector T cells, complement and NK cell activity [99,100].

Angiogenic and lymphangiogenic privilege

The normal human cornea is devoid of both blood and lymphatic vessels. This state is actively maintained through the expression of both antiangiogenic and antilymphangiogenic factors. The VEGF family of ligands and receptors are key promoters of angiogenesis. Hemangiogenesis is driven by VEGF-A binding to VEGFR-1 and VEGFR-2, and lymphangiogenesis is promoted by VEGF-C and VEGF-D binding to VEGFR-3 [101]. An endogenous spliced variant of soluble VEGFR-2, secreted by both corneal epithelial cells and stromal cells, has been demonstrated to play a crucial role in maintaining corneal alymphaticity [102]. Albuquerque et al. showed that administration of exogenous soluble VEGFR-2 selectively inhibits lymphangiogenesis in a corneal suture injury model, and promotes high-risk corneal allograft survival. The importance of lymphatics in facilitating allosensitization is evidenced by the fact that ipsilateral cervical lymphadenectomy prior to

corneal transplantation significantly prolongs allograft survival [103]. Several endogenous inhibitors of both lymphangiogenesis and hemangiogenesis have been identified. sVEGFR-1 and VEGFR-3 are constitutively expressed by the corneal epithelium and act as decoy receptors, binding the growth factors VEGF-C and VEGF-D [12,13]. Indeed, Cursiefen et al. have demonstrated that the constitutive expression of VEGFR-3 by corneal epithelium serves as a 'sink' for VEGFR-3 ligands (VEGF-C and VEGF-D) and is critical for maintenance of corneal clarity [13]. The investigators showed that *ex vivo* blockade of syngeneic donor corneal epithelium with anti-VEGFR3 prior to layering onto the corneas of de-epithelialized BALB/c host mice significantly inhibited its antiangiogenic capacity relative to controls. Endostatin is a proteolytic fragment of collagen XVII that is constitutively expressed by the corneal epithelium, and its expression has been correlated with corneal graft survival. Furthermore, Tan et al. demonstrated that exogenous administration of endostatin inhibits angiogenesis, resulting in improved graft survival [104]. Angiostatin, a decomposition fragment of plasminogen, contributes to the maintenance of avascularity in corneal wound healing [105]. Corneal epithelial cells express PD-L1, and PD-L1^{-/-} mice exhibit a heightened angiogenic response to suture-induced corneal injury relative to wild-type [106]. Thus, corneal epithelial expression of PD-L1 has been recognized as a constitutively expressed regulator of angiogenesis. Other potent endogenous antiangiogenic factors include TSP-1, TSP-2 and PEDF [107]. Interestingly, allogeneic T cells themselves promote vascular endothelial cell proliferation, emphasizing the cross-talk between inflammation and angiogenesis [108].

Regulatory T cells

The critical contribution of regulatory T cells (Tregs) to maintaining immune privilege has been confirmed in a murine model of corneal transplantation, in which adoptive transfer of alloprimed Foxp3⁺ Tregs prior to grafting has been shown to induce tolerance [109]. Foxp3⁺ Tregs consist of multiple subsets, and our understanding of the relative contribution of these subsets towards allotolerance is increasing. In addition to thymus-derived natural Tregs (tTregs), Foxp3 expression can be induced in conventional T cells resulting in the generation of peripherally-induced Tregs (pTregs) [110]. There is evidence that these alloantigen-specific pTregs play a dominant role in mediating allograft tolerance; indeed, the frequency and function of pTregs (but not tTregs) are reduced in high-risk relative to low-risk corneal transplantation [111]. Furthermore, adoptive transfer of pTregs from mice at low-risk of graft rejection to mice at high-risk prolongs graft survival and reduces graft opacity [111]. The constitutive expression of GITRL (glucocorticoid-induced tumour-necrosis-factor-receptor-related protein ligand, also known as Tnfsf18) by the cornea has been shown to promote the generation and expansion of Tregs, thereby promoting graft survival [97]. Treg suppression of effector T cells is primarily contact-dependent, with GITR and CTLA-4 molecules playing essential roles in this process [112]. IL-17A has been shown to be necessary for the development and function of Tregs in a murine model of corneal transplantation, despite its role as a proinflammatory cytokine in the setting of autoimmunity [112]. The possibility of expanding Tregs *in vivo* has attracted attention as a tool to limit the alloimmune response, and promote long-term allograft survival [113]. However, the phenotypic plasticity of Tregs is potentially problematic for novel therapeutic strategies that seek to employ Tregs as means to promote graft tolerance. Indeed, lineage tracing studies

using Foxp3 reporter mice have identified a subpopulation of Tregs that lose their Foxp3 expression, becoming pro-inflammatory ‘exTregs’ that are phenotypically indistinguishable from effector T cells [114,115].

Concluding remarks and future directions

The preservation of optical clarity is a unique priority of the ocular mucosal immune system. Despite many similarities with other mucosal surfaces, there are specific immunological adaptations at the ocular surface that safeguard the optical properties of the cornea. This review highlighted components of the local microenvironment that modulate the immune response. Published data regarding immunoregulatory mechanisms at the ocular surface in the context of infection, alloimmunity and autoimmunity were summarized.

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Trends Box

- Immune privilege protects the eye from sight-threatening immunoinflammatory responses. The factors that orchestrate ocular immune privilege continue to be defined. Current work in the field is focused on the immunoregulatory processes that conspire to limit the effector response.
- Our understanding of epithelium-derived immunoregulatory factors, ocular surface dendritic cells, neuro-immune crosstalk, the ocular surface microbiome, memory Th17 cells in autoimmunity and CD4⁺ T cell plasticity at the ocular surface has deepened markedly over recent years.
- Recent findings suggest novel treatment strategies to suppress autoimmunity and promote allotolerance, for instance by modulating CD4⁺ T cell chemotaxis and expanding regulatory T cell populations.

Outstanding questions

What are the mechanisms by which corneal and ocular surface nerves regulate immunity?

How is the memory immune response regulated in chronic inflammatory diseases of the ocular surface?

What is the contribution of Treg and Th17 plasticity to ocular surface immunity?

How is susceptibility to ocular surface inflammation affected by age?

What is the significance of the microbiome in regulating ocular surface immunity?

Figure 1. Anatomical and functional characteristics of the ocular mucosal surface

The ocular surface comprises the cornea, limbus and conjunctiva and is protected by a non-keratinized, stratified epithelium. The epithelium constitutively expresses a range of immunomodulatory factors, including cell surface molecules (such as FasL and PDL-1) and secreted molecules (such as soluble VEGF decoy receptors [sVEGFRs] and PEDF). The epithelium is bathed in tear film, which consists of three distinct layers—an outer lipid layer (secreted by Meibomian glands in the eyelids), a middle aqueous layer (secreted by the main and accessory lacrimal glands) and an inner mucous layer (secreted by goblet cells). Secretory IgA (sIgA) is the predominant immunoglobulin in the tears, and acts in concert with a wide variety of antimicrobial molecules to facilitate cytolysis/limit the growth of pathogenic organisms. The high molecular weight heavily glycosylated membrane-associated mucins of the glycocalyx facilitate the maintenance of the tear film on the ocular surface, in addition to limiting bacterial adhesion and promoting the removal of debris via the lacrimal puncta. Corneal nerves form networks that penetrate the epithelium and stroma—these include the sub-basal and anterior stromal nerve plexuses. Neuropeptides such as CGRP and VIP moderate both innate and adaptive immunity. The stroma contains collagen-synthesizing keratocytes as well as immature CD11b⁺ antigen-presenting cells. The corneal endothelium is posterior to the stroma, and expresses a wide variety of immunoregulatory factors, including GITR and PD-L1.

Figure 2. Distribution of DCs at the ocular surface

The highest density of DCs is found in the conjunctiva, and their density decreases from the limbus to the central cornea. Langerhans cells are found in the conjunctiva and peripheral corneal epithelium. DC-SIGN⁺ DCs have been identified in the conjunctiva and peripheral anterior stroma. CD11b⁺ DCs are found in the conjunctiva and posterior stroma. Immature DCs are distributed throughout the anterior corneal stroma—these cells mature in response to inflammation with enhanced expression of MHC class II and costimulatory molecules CD80, CD86 and CD40.

Figure 3. Dominant immune pathways in ocular surface alloimmunity and autoimmunity
 Antigen presenting cells stimulate naïve CD4⁺ cells to differentiate into effector cells. The rejection of corneal allografts is principally driven by Th1 cells, which are generated in the presence of the cytokine IL-12. In contrast, a Th17 effector response predominates in ocular surface autoimmunity, with TGF-β, IL-6 and IL-23 inducing Th17 generation. Regulatory T cells modulate effector CD4⁺ T cell responses in both alloimmunity and autoimmunity.

Table 1

Select immunoregulatory factors implicated in ocular surface immunity

	Molecules	Function
Inflammatory cytokines	IL-1	IL-1, IL-6 and TNF- α are upstream inflammatory cytokines, while IL-17A, IL-17F and IFN- γ among others tend to be more specifically implicated in T cell-mediated ocular surface disease [39,99].
	IL-6	
	TNF- α	
	IL-17A, F	
	IFN- γ	
Regulatory cytokines	TGF- β	TGF- β functions pleiotropically—it inhibits the proliferation of T cells and induces Foxp3 ⁺ inducible Tregs, yet in the presence of IL-6 induces pathogenic IL-17-producing Th17 cells [116]. In addition, TGF- β plays a critical role in tissue fibrosis [117]. IL-1Ra is upregulated in ocular surface inflammation, and functions to block the actions of IL-1 and suppress leucocyte/APC activity [94].
	IL-1Ra	
Inhibitory factors	PD-L1	PD-L1, FasL and TRAIL promote apoptosis of activated immune cells, limit T cell proliferation and suppress inflammatory cytokine production [99]. TSP-1 activates the immunomodulatory cytokine TGF- β [18].
	FasL	
	TRAIL	
	TSP-1	
Neuropeptides	Substance P	Substance P functions pleiotropically—it stimulates the recruitment of monocytes and neutrophils, inhibits IL-10 production by DCs and promotes Th17 production, yet also induces Tregs [41,42]. α -MSH, CGRP and VIP dampen the recruitment of inflammatory cells, limit T cell proliferation and suppress production of inflammatory cytokines [40]. α -MSH can also promote Treg function [100]
	α -MSH	
	CGRP	
	VIP	
Chemokines		Upregulated in ocular surface inflammation, these chemokine ligands direct the homing of leucocytes across a chemotactic gradient. Examples include CCL2, CCL5, CXCL9, CXCL10, CXCL11, CCL20 and others [118–120].
Hemangiogenic and lymphangiogenic factors	sVEGFR-1	Hemangiogenesis and lymphangiogenesis are limited by the constitutive expression of VEGF decoy receptors, which are upregulated in ocular surface inflammation [12,13,101–103]. PEDF suppresses VEGF expression, and its expression is decreased in ocular surface inflammation [14,107].
	sVEGFR-2	
	VEGFR-3	
	PEDF	