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Immune regulation of the ocular surface

Yihe Chen, **Shudan Wang**, **Hamid Alemi**, **Thomas Dohlman**, **Reza Dana**

Schepens Eye Research Institute of Massachusetts Eye and Ear, Department of Ophthalmology, Harvard Medical School, Boston, MA 02114

Abstract

Despite constant exposure to various environmental stimuli, the ocular surface remains intact and uninflamed while maintaining the transparency of the cornea and its visual function. This 'immune privilege' of the ocular surface is not simply a result of the physical barrier function of the mucosal lining but, more importantly, is actively maintained through a variety of immunoregulatory mechanisms that prevent the disruption of immune homeostasis. In this review, we focus on essential molecular and cellular players that promote immune quiescence in steady-state conditions and suppress inflammation in disease-states. Specifically, we examine the interactions between the ocular surface and its local draining lymphoid compartment, by encompassing the corneal epithelium, corneal nerves and cornea-resident myeloid cells, conjunctival goblet cells, and regulatory T cells (Treg) in the context of ocular surface autoimmune inflammation (dry eye disease) and alloimmunity (corneal transplantation). A better understanding of the immunoregulatory mechanisms will facilitate the development of novel, targeted immunomodulatory strategies for a broad range of ocular surface inflammatory disorders.

Keywords

Immune regulation; Ocular surface; Inflammation; Autoimmunity; Alloimmunity

1. Introduction

The ocular surface consists of the corneal and conjunctival mucosal linings extending to the mucocutaneous junctions of the lid margins. The tear film - an aqueous medium enriched with lipids and mucins from meibomian glands and goblet cells, respectively, maintains the ocular interface with the external environment [1]. The ocular surface is known as an "immune-privileged" site: It holds the capability to mount effective immune defense, but it employs tight regulatory measures to prevent unnecessary local inflammatory responses to

Corresponding author: Yihe Chen, Schepens Eye Research Institute of Massachusetts Eye and Ear, 20 Staniford Street, Boston, MA 02114, USA, Phone: 617-912-0252, Fax: 617-912-0117, yihe_chen@meei.harvard.edu.

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preserve corneal integrity and function. In this review, we discuss the following regulatory mechanisms that are critical to maintaining immune homeostasis of the ocular surface: **1) Cornea avascularity:** The cornea must stay transparent and, therefore, be avascular to function normally. The lack of blood vessels limits the ingress of circulating blood leukocytes, while lack of lymphatic vessels impedes leukocytes from migrating out of the tissue. Thus, corneal avascularity essentially contributes to the immune privilege of the cornea [2]. **2) The healthy cornea is devoid of 'mature' leukocytes:** The healthy cornea is devoid of lymphoid leukocytes but is endowed with a sizable number of myeloid leukocytes [3–8], which function in both innate and adaptive immunity in response to external insults. These resident leukocytes are phenotypically "immature"; they express limited pro-inflammatory cytokines and are limited in their ability to sensitize lymphoid cells and provoke effector T cell responses. Rather, they promote induction of immune tolerance at the ocular surface [5,6,9,10]. **3) Regulatory role of the corneal epithelium:** The corneal epithelium prevents invasion of immunogenic molecules through its barrier function and actively contributes to immune quiescence regulation; this layer secretes various immunoregulatory factors that sustain the cornea's avascularity and preserve the "immature" status of resident leukocytes at the ocular surface [11–14]. **4) Neuroimmune cross-talk at the ocular surface:** More recently, immunoregulatory function of corneal nerves and their cross-talk with immune cells has drawn significant interest. Indeed, the cornea is the most densely innervated tissue in the human body, and proper corneal innervation with the physiological levels of neuropeptides at the ocular surface has been demonstrated essential to promote immune homeostasis in addition to regulating tear secretion and the blink reflex [15–17]. **5) Conjunctiva-associated lymphoid tissue (CALT) and goblet cells:** Unlike the cornea, the conjunctiva harbors a diverse group of immune cells with dominant lymphoid cells (primarily T cells) during the steady-state conditions [18]. Unique to the conjunctiva, some resident T cells aggregate in dense foci in a form of mucosa-associated lymphoid tissue (MALT) and are specifically termed conjunctiva-associated lymphoid tissue (CALT) [19]. CALT increases in size and number after ocular antigen stimulation and is implicated in the induction of mucosal tolerance and protection from infectious and non-infectious insults [20]. In addition, a special type of epithelial cell within the conjunctiva is the goblet cell, which serves as the primary source for mucins that exert antimicrobial functions and induce tolerogenic dendritic cells (DC) [21,22]. **6) Regulatory T cells (Treg) in the regional lymph nodes:** 'Intraocular' immune quiescence is primarily maintained through a phenomenon called anterior chamber-associated immune deviation (ACAID) that involves the spleen. However, the adaptive T cell immunity at the 'ocular surface' is mainly regulated within regional lymph nodes [23,24]. Indeed, suppression of effector T cells in the local lymphoid compartment is an essential mechanism maintaining the integrity of the ocular surface. In this regard, regulatory T cells (Treg) in the lymph nodes draining the ocular surface have been shown to potently suppress sensitization of naive T cells and function of activated T cells, thus preventing the loss of ocular surface immune quiescence [23,24]. Key cellular and molecular components participating in the immune regulation of the ocular surface are illustrated in Fig. 1.

2. Immunoregulatory function of corneal epithelium

The corneal epithelium is the frontline of innate ocular immunity. It forms a physical barrier against micro-organisms and environmental insults, maintained firmly by cellular structures including desmosomal junctions, hemidesmosomes, and basement membrane [25,26]. Additionally, there is increasing evidence demonstrating that the corneal epithelium has a critical immunoregulatory role in the eye. These cells constitutively express an array of immunoregulatory factors, including programmed death ligand-1 (PD-L1). Fas ligand (FasL), pigment epithelial-derived factor (PEDF), and thrombospondin-1 (TSP-1), among others [11–14].

PD-L1 binds to its receptor programmed death (PD)-1 on activated effector T cells, triggering their cell death and thereby suppressing T cell effector response [27]. PD-L1 is constitutively expressed in high levels by the corneal epithelium [11,28], but a variety of acute inflammatory insults can also transiently elevate its expression [11]. For instance, in the setting of corneal transplantation, grafts derived from allogeneic PD-L1 knockout (KO) mice are shown to be significantly more susceptible to immune rejection compared to the grafts from wild-type mice, primarily as a result of increased infiltration of effector T cells at the graft site [11]. PD-L1 KO mice show spontaneous T cell infiltration in the cornea, and these mice are prone to develop more severe dry eye disease (DED) [28]. Wild-type mice with DED show reduced expression of PD-L1 in their corneal epithelium, and blockade of PD-L1 further exacerbates ocular surface inflammation in DED [28]. These findings highlight the critical role of PD-L1 expressed by the corneal epithelium in inhibiting the ocular surface adaptive T cell immunity. Furthermore, PD-L1 expressed by the corneal epithelium binds to its other receptor, CD80, on microvascular endothelial cells and inhibits abnormal corneal blood vessel growth (hemangiogenesis), thereby maintaining the corneal avascularity [29].

FasL is a member of the tumor necrosis factor (TNF) family and functions as a negative regulator of T effectors and neovascularization. FasL mediates such roles by inducing apoptotic cell death through binding to its receptor, Fas, expressed by a variety of cells and tissues, including immune cells [30,31]. There are two forms of FasL: 1) The pro-inflammatory, membrane-bound form (mFasL); and 2) the anti-inflammatory, soluble form (sFasL) which antagonizes the function of mFasL [32]. The balance of sFasL:mFasL is a critical factor regulating their function: for instance, sFasL is the predominant form in the normal retina [33]. Although FasL is uniformly expressed by normal corneal epithelium and endothelium, it remains unclear which form(s) of FasL is predominant in the cornea [34].

PEDF belongs to the serine protease inhibitor family and is widely expressed by various ocular tissues, including the retina, choroid, ciliary body, and corneal epithelium/endothelium [14,35,36]. PEDF expressed by corneal epithelium has been shown to suppress the maturation and activation of corneal resident leucocytes effectively. Topical or systemic administration of recombinant PEDF significantly alleviates DED severity by suppressing innate inflammation, inhibiting adaptive effector T cell response, and promoting immunosuppressive functions of Treg in the local draining lymph nodes [14,37]. In mechanical and chemical corneal injury, topical application of PEDF or its derivatives

effectively promotes wound healing through enhancing limbal stem cell proliferation and inhibits corneal neovascularization through suppressing vascular endothelial growth factor (VEGF) expression [38,39].

TSP-1 is an extracellular matrix protein constitutively expressed by corneal epithelium and endothelium [13,40,41]. It regulates ocular surface homeostasis by interacting with an array of receptors, growth factors, cytokines, and proteases. TSP-1 mediates its immunoregulatory function principally by transforming the latent form of transforming growth factor-β (TGF-β) to its functional activated form, which serves as a critical anti-inflammatory cytokine [42,43]. Reactive up-regulation of TSP-1 in corneal epithelium has been observed in DED, which plays a critical role in suppressing corneal resident leukocyte activation. Furthermore, topical application of recombinant TSP-1 or its peptide derivative has been demonstrated to suppress the immune-mediated ocular surface inflammation in both DED and allergic eye disease [13,44,45]. Studies employing exogenous TSP-1 supplementation or animal models with a deletion in endogenous TSP-1 implicate the critical role of TSP-1 in the induction of Treg and their functional maintenance, which is disrupted Treg in DED [44,46]. TSP-1 mainly engages Treg indirectly through activation of TGF-β, which potently modulates Treg differentiation. Similar to PEDF, TSP-1 also serves as a potent endogenous anti-angiogenic factor. Experimental data from TSP-1 null mice show that TSP-1 suppresses both corneal hemangiogenesis and lymphangiogenesis (abnormal lymphatic vessels growth) [47,48], the pathological processes promoting DED inflammation and endangering corneal graft rejection [49,50], by binding to CD36 on monocytes regulating their expressions of VEGF [48,51].

Emerging evidence has demonstrated that corneal epithelium is also the primary source of endogenous polyunsaturated fatty acids (PUFA)-derived specialized pro-resolving mediators (SPM) at the ocular surface, including lipoxin A_4 (LXA_4), neuroprotectin D_1 (NPD_1), and resolvin D_1 (RvD_1) [52,53]. SPM is a class of bioactive lipids metabolized from the substrates of essential PUFA, including arachidonic acid (ω-6 AA), docosahexaenoic acid (ω-3 DHA), eicosapentaenoic acid (ω-3 EPA), and docosapentaenoic acid (ω-3 DPA), with specialized functions of limiting inflammation and promoting tissue homeostasis primarily through autocrine and paracrine actions on target cells that encompass ocular surface epithelial and stromal cells as well as various types of immune cells such as macrophages, DC, and T cells [54,55]. Elimination of endogenous LXA_4 has been shown to increase corneal neovascularization, and treatment with LXA_4, its analogue, or RvD_1 can effectively suppress corneal hemangiogenesis [54,56]. It has also been demonstrated that SPM promotes corneal epithelial cell proliferation and migration in vitro, and facilitates corneal wound healing and nerve regeneration in vivo [52,57–59]. As SPM are rapidly inactivated by cells in vitro, structural analogues of SPM have been designed to increase their stability and bioavailability for therapeutic studies. Topical treatment with resolvin E_1 (RvE_1) analogues or α-linoleic acid (ALA, a metabolic precursor for EPA and DHA) in pre-clinical models of DED has been demonstrated to effectively ameliorate corneal epitheliopathy, reduce goblet cell loss, and suppress inflammatory cell activation [60–62]. Pre-treatment of human donor corneas with a stable LXA_4 analogue during storage significantly increases endothelial viability [63], and treatment of hosts with a stable RvD1

analogue in a murine model of corneal transplantation effectively enhances graft survival by suppressing conventional DC activation and allosensitization [64].

The normal cornea is free of both blood vessels and lymphatic vessels, which is critical to maintaining its optical function and immune quiescence. Abnormal hemangiogenesis in host cornea, often induced by inflammation, has been determined a high-risk factor for the survival of corneal transplantation [65]. The neovascularized host bed promotes the maturation of resident myeloid cells and increases the recruitment of peripheral innate immune cells, thus enhancing the sensitization of T cells, and supporting the migration of activated T cells to the graft site inducing graft rejection [24]. Corneal transplantation also induces the formation of corneal lymphangiogenesis [50], which can provide access for activated myeloid cells at the ocular surface to regional lymphoid compartments where they prime and activate adaptive T cells, thus critically contributing to immune rejection of corneal grafts [66]. In addition, selective lymphangiogenesis has been observed in the DED cornea that starts from the peripheral cornea and advances into the central cornea with the progression of the disease, dependent on the interaction of VEGF-C and VEGF-D and their receptor VEGFR-3 [49,67,68]. These lymphatic vessels serve as an afferent pathway for activated myeloid cells to relocate to the local draining lymph nodes mediating T cell activation. Corneal epithelium produces a variety of factors, including the aforementioned, to work in concert to maintain corneal avascularity and clarity. Normal corneal epithelium constitutively expresses soluble vascular endothelial growth factor receptor-1 (sVEGFR-1, also known as sflt-1) to inhibit VEGF-A mediated new blood vessel formation by serving as an endogenous VEGF-A trap [69]. In addition, strong expression of VEGFR-3 by healthy corneal epithelium functions as a “sink” for VEGF-C and VEGF-D to prevent their interaction with VEGFR-3 on vascular endothelium, thus preventing corneal new blood and lymphatic vessels formation [70].

More recently, the immunoregulatory functions of limbal melanocytes, an essential component of the corneal epithelial stem cell niche, have been explored. In addition to their well-known protective roles in preventing limbal epithelial stem/progenitor cells from UV damages, limbal melanocytes have been shown in a series of in vitro studies to effectively suppress T cell proliferation and cytokine production primarily through cell contact-dependent mechanisms. They additionally have been shown to inhibit angiogenesis via preventing vascular endothelial cell proliferation and capillary formation [71]. These findings suggest important immunoregulatory contributions of the limbal melanocytes to limbal niche homeostasis.

Notably, the anti-inflammatory properties of corneal epithelium can be overturned in a variety of inflammatory conditions, such as DED, leading to the production and release of pro-inflammatory cytokines by the cornea, including TNF-α, IL-1β, and IL-6 [72] that lead to the disruption of immune quiescence of ocular surface.

3. Immunoregulatory function of corneal resident myeloid leukocytes

The normal cornea has no T or B lymphocytes but is endowed with bone marrow-derived principally myeloid immune cells characterized by a $CD11b^{+}CD3^{-}CD19^{-}$ phenotype,

distributed predominantly at the periphery and decreasing gradually toward the center [6,9]. These resident myeloid cells comprise a major population of $CD11b^{+}CD11c^{-}$ macrophages/monocytes in the deep stroma and $CD11b^{+}CD11c^{+}$ dendritic cells (DC) in the anterior stroma, along with a few $CD11b^{lo/-}$ $CD11c^{+}$ Langerhans cells in the epithelium [3–8]. These tissue-resident cells play essential roles in activating adaptive immune response by functioning as antigen-presenting cells (APC). Corneal APC are sentinels of the immune system at the interface of the ocular surface and outside environment, and the preponderance of them display an “immature” status characterized by low expression levels of MHC class II (MHC-II) and the absence of co-stimulatory molecules B7 (CD80 and CD86) and CD40, especially amongst those residing in the central corneal stroma [6,9], rendering them immune quiescent and even maturation-resistant thus playing regulatory functions in the setting of inflammatory insults. Those cells that possess diminished antigen presentation, production of regulatory cytokines, and generation and expansion of Treg are defined as tolerogenic APC, which are critically involved in promoting transplant tolerance and allograft survival [73,74]. Expression of TSP-1 by corneal APC has been identified as one of the mechanisms rendering these cells as tolerogenic, via preventing these cells from maturation and impairing their capacity to migrate, thus inhibiting T cell allosensitization [75]. Enrichment of tolerogenic APC in donor corneas by ex vivo treatment of corneal buttons with IL-10 and TGF-β has been successfully demonstrated to suppress allosensitization and improve the corneal allograft survival in high-risk transplantation (neovascularized host bed) [76]. However, on the other hand, immature corneal resident APC can be stimulated by a variety of up-regulated innate factors including mast cells [77] to acquire a highly mature phenotype and mobilize to regional lymphoid tissues where they prime T cells and initiate adaptive immunity, promoting the disruption of immune homeostasis at the ocular surface, in particular leading to DED and corneal graft rejection [62,78,79].

In contrast to conventional DC (cDC), plasmacytoid dendritic cells (pDC), characterized by $CD45^{+}$ $PDCA\text{-}1^{+}$ $CD45R/B220^{+}$ $CD11c^{low}$ $CD11b^{-}$ in murine and $CD45^{+}$ $BDCA\text{-}2^{+}$ $BDCA\text{-}4^{+}$ $CD11c^{low}$ in humans, are a special type of innate cell residing in the anterior stroma of cornea and limbus. pDC are primarily present in the peripheral blood and secondary lymphoid tissues, and although they are a rare population, they constitute about 15-25% of total corneal $CD45^{+}$ cells with immature phenotypes during steady state [80,81]. Originally identified as a critical source of type I IFN, pDC have been shown to play critical immunoregulatory roles. In vitro co-culture of pDC with Treg effectively sustains Treg expression of the function marker Foxp3, demonstrating that pDC are a key type of cell capable of inducing tolerance by promoting generation of Treg and preventing their dysfunction, probably through IFN-α independent mechanisms [81]. pDC also express the co-inhibitory molecule PD-L1 [81,82], which can limit effector T cell responses via interacting with PD-1 on T cells. In fact, depletion of corneal pDC in the setting of corneal transplantation has been demonstrated to increase corneal allograft opacity and effector T cell infiltration, as well as decrease Foxp3 expression by Treg in the draining lymph nodes [81]. In addition, corneal pDC contribute to neuroimmune cross-talk by maintaining the homeostasis of corneal nerves and promoting nerve regeneration, at least partially through secretion of the neurotrophic nerve growth factor (NGF) [81]. pDC residing in the

corneal limbus also play significant roles in sustaining corneal angiogenic privilege through secreting a variety of anti-angiogenic factors such as TSP-1 [81].

4. Immunoregulatory function of corneal nerves and neuropeptides

The cornea is densely innervated by the ophthalmic branch of the trigeminal nerve, which enters the corneal stroma and further branches into the epithelium [83]. The integrity of corneal nerves is essential for relaying the sensory signals and providing epithelial-trophic factors to maintain ocular surface homeostasis, including critical regulation of corneal epithelial cell and limbal stem cell survival [84–86]. Corneal neuropathy has been reported to contribute to corneal epitheliopathy in DED [87–90]. In addition, normal corneal innervation is required to suppress corneal neovascularization as corneal denervation leads to significant corneal angiogenesis and inflammation [91].

Importantly, nerve-derived neuropeptides play an important role in regulating immunity and angiogenesis. **Substance P (SP)** is one of the major neuropeptides constitutively secreted by nerve endings in the normal cornea, and a physiological level of SP is required for corneal epithelial homeostasis [15–17]. Complete abrogation of SP function by genetically knocked-out NK1R, the preferred SP receptor, has been shown to cause loss of corneal epithelial cells [92], and topical treatment with SP-derived peptide in neurotrophic keratopathy, a corneal disease characterized by impaired corneal nerve function which may lead to decreased SP production at the ocular surface, has been shown to promote the closure of the corneal epithelial defect [93]. However, the protective roles of SP can be overturned when there is an excessive amount of SP at the ocular surface that is higher than the physiological level, primarily through promoting corneal resident APC maturation and mobilization, recruiting peripheral leukocytes to infiltrate the cornea, and inducing corneal neovascularization directly on parent vessels and indirectly through activating innate immune cells including mast cells to secret VEGF-A, all of which finally leads to neurogenic inflammation and propagation of an inflammatory cascade, as reported in DED and corneal injuries [94–102]. Heightened expression of SP subsequent to the severance of corneal nerves during surgery has been reported to disrupt ocular immune privilege and promotes corneal allograft rejection [103].

Vasoactive intestinal peptide (VIP) is another important neuropeptide constitutively present in the cornea that exerts neuroimmunomodulatory function [104]. In addition, VIP has been shown effective to maintain the corneal endothelial cell integrity during donor cornea tissue storage, through preventing oxidative stress- or inflammatory cytokines-induced apoptosis, which is critical for a successful corneal transplantation. Further, intracameral administration of VIP has been demonstrated to preserve corneal endothelial cells and improve the allograft survival after corneal transplantation [105]. Calcitonin gene-related peptide (CGRP) is also abundantly expressed by corneal nerves, primarily by sensory nerves [106,107]. Reduced CGRP-expressing corneal nerves has been associated with DED progression [108].

It is well-known that **α-melanocyte-stimulating hormone (α-MSH)** present in the aqueous humor plays essential roles in modulating intraocular immunity [109,110]. Recently, β-

MSH has been detected in healthy human tears at a level about 100-fold lower than that in the aqueous humor [111,112], suggesting a role of α-MSH in regulating ocular surface immune homeostasis. In vitro treatment of peripheral blood mononuclear cells from patients with perennial allergic conjunctivitis with α-MSH significantly decreased the activated effector T cells and Th2-associated cytokines while increasing the Treg population [111]. Topical application of α-MSH in a DED model has been shown to effectively improve corneal integrity and tear secretion, preserve conjunctival goblet cells, as well as suppress the expression of inflammatory cytokines [113]. In addition, our group has shown that addition of α-MSH to standard human donor cornea storage medium effectively protects against corneal endothelial loss secondary to oxidative and pro-inflammatory cytokine-induced stresses [114]. Furthermore, subconjunctival injection of α-MSH to mice undergoing corneal transplantation significantly improves their graft survival, suppresses allosensitization of their T cells, and reduces the graft infiltration with inflammatory cells [115].

5. Immunoregulatory function of conjunctival goblet cells and CALT

Goblet cells are specialized epithelial cells in the conjunctiva serving as the primary source of mucins for tear film that covers the ocular surface [116]. Disrupted corneal barrier function is a major pathological feature in DED visualized by corneal fluorescein staining in the clinic, and decreased numbers and atrophy of conjunctival goblet cells have been consistently observed in DED [117–119]. More recently, conjunctival goblet cells have been shown to importantly contribute to local immune tolerance to foreign antigen stimulation, presumably via conditioning conjunctival APC to acquire tolerogenic properties [22]. The underlying mechanisms by which goblet cells interact with APC have yet to be determined, although studies from intestinal goblet cells suggest that goblet cell-associated antigen passages (GAPs) mediate local antigen delivery by goblet cells to APC, promoting tolerogenic tissue cDC1 [120,121]; similar GAPs have recently been identified in the conjunctiva as well [122]. In addition, MUC2, which is expressed by goblet cells in both the gut and conjunctiva [123,124] has been shown to be uptaken by intestinal DC, leading to inhibition of pro-inflammatory cytokine production and induction of tolerogenic properties of the DC [123]. Conjunctival goblet cells can produce active form of TGF-β, a critical immunoregulatory cytokine that may allow goblet cells to imprint a tolerogenic phenotype onto APC [125]. Another immunoregulatory factor secreted by conjunctival goblet cells is retinoic acid, which inhibits maturation of bone marrow-derived DC and their production of the inflammatory cytokine IL-12, through binding to the retinoid receptor X (RXR) [126,127]. Additional putative functions of conjunctival goblet cells include promotion of CALT function [128]. CALT organization consists of a lymphoid follicle with a T-zone containing $CD4^+$ cells and a B-zone with B cells and follicular dendritic cells, as well as adjacent high-endothelial venules (HEVs) and lymphatics [20]. CALT is believed to be a normal and noninflammatory component of the ocular surface [129], and is involved in the maintenance of protective mucosal immune regulation and contributes to immune tolerance at ocular surface [130]. The presence of $CD4^+CD25^+Foxp3^+$ Treg within CALT supports an immune regulatory function of CALT [20]. Experimental studies have shown an increase in number and size of CALT in ocular allergy but a decrease in DED [131]. In contrast, human

studies show increased CALT in both DED and ocular allergy patients, although these in vivo examinations need to be validated by immunohistology [132]. The specific functions of CLAT in ocular surface disease and immune regulation deserve further investigations.

6. Immunoregulatory function of Treg

Regional eye-draining lymph nodes are the primary site where adaptive immunity is activated and regulated in ocular surface inflammation. Once the immature status of ocular surface resident APC is overridden by inflammatory stimulus, these APC become activated and mobilized, migrating to the local draining lymph nodes and priming naive $CD4^+$ T cells through providing antigenic and co-stimulatory signals, as well as secreting T cell-polarizing cytokines [133–138], leading to generation of effector Th1, Th2 or Th17 cells as dominant type of central pathogenic cells mediating corneal allograft rejection, ocular allergy or DED, respectively [23,139–146]. The principal immunoregulatory mechanism that restricts and suppresses pro-inflammatory effector T cell response is mediated by the specialized $CD4^+$ regulatory T cells (Treg), characterized by high expression of CD25 (the high affinity IL-2 receptor to maintain survival and proliferation of Treg) and Foxp3 (the lineage transcriptional factor for the development and function of Treg) [147,148]. The predominant Treg population residing in the lymphoid tissues is developed during the normal process of T cell maturation in the thymus, and thus is termed thymus-derived Treg (tTreg) or “natural Treg” (nTreg). In addition, a smaller but antigen-specific population that is induced in the periphery from naive T cells in the presence of IL-2 and TGF-β or after encounters with foreign antigens, termed iTreg for *in vitro* generated or pTreg for *in vivo* generated, may play more robust regulatory functions [149–151].

Reduced number and impaired function of Treg, especially pTreg, has been reported in corneal allograft rejection, characterized by defects in both contact-dependent (decreased expression of CTLA-4) and –independent suppression (reduced secretion of IL-10 and TGF-β) [152]. In fact, hosts with accepted grafts show enhanced Treg suppressive function [153], and adoptive transfer of pTreg from these hosts has been shown to effectively prevent graft rejection in high-risk setting [152]. In addition, local subconjunctival injection of nTreg has been demonstrated to significantly suppress Th1 response and improve corneal graft survival, and the injected Treg migrate to ipsilateral cornea, conjunctiva, and draining lymph nodes, suggesting that Treg suppress both effector T cell induction in regional lymphoid tissues and function of activated Th1 cells at the ocular surface [154]. Alternatively, systemic administration of low-dose IL-2 is another promising therapeutic strategy to effectively expand Treg and enhance their function (increased CTLA-4 and IL-10 expressions), and thus improve corneal allograft survival by inhibiting effector Th1-mediated graft attack [155].

In mouse models of ocular allergy, depletion of Treg by anti-CD25 antibody treatment has been demonstrated to exacerbate the allergic disease [156,157]. On the other hand, the high dose antigen sensitization-achieved suppression of allergic conjunctivitis is dependent on the induced Treg expansion and their augmented function in suppressing Th2 response [157]. Consistently, decreased expression of Foxp3 in $CD4^+$ T cells has been reported in

patients with allergic conjunctivitis, and these Treg are defective in suppressing effector Th2 response [158,159].

Treg play an essential role in suppressing Th17-mediated inflammation and maintaining immune homeostasis. Dysfunction of Treg in suppressing effector Th17 cells critically contributes to induction of ocular surface inflammation in DED [143,160]. Increased expression of SP in draining lymph nodes has been shown as one of underlying mechanisms directly suppressing Treg function, and in DED there is increased $NK1R^+$ Treg that is functionally significantly compromised as compared to $NK1R^-$ Treg [161]. Depletion of Treg using an anti-CD25 antibody or CD25 knockout mice leads to worsened DED, while reconstitution of nTreg results in resistance to disease induction [162–165]. However, it remains to be determined whether Treg dysfunction persists over the long term (i.e. is it spontaneously reversible through dynamic microenvironmental cues) and contributes to dysregulated T cell memory that mediates the chronicity of ocular surface inflammation in DED [166]. Evidence from human study suggests a positive answer, as disruption of Th17/Treg balance in Sjogren's syndrome patients has been reported [167,168]. Further, low-dose IL-2 treatment has been demonstrated in a pilot study to successfully restore the Th17/Treg balance and Treg function, along with reduced use of glucocorticoid and disease-modifying anti-rheumatic drugs in the patients [168].

7. Conclusions and perspectives

The maintenance of ocular surface homeostasis by a variety of immunoregulatory mechanisms is critical to avoid excessive inflammation that may cause tissue damage and impairment of vision. Novel therapeutic strategies harnessing these critical mechanisms can be designed to enhance the immune regulation resulting in restoration of ocular surface homeostasis in a myriad of ocular surface diseases. In addition to the mainstay of the ocular surface-regional lymphoid compartment axis we have systemically reviewed, the potential immune regulation in lacrimal glands that contribute to tear film stability and ocular surface integrity has yet to be studied. Outside local mechanisms, systemic modulation of ocular surface health has been increasingly recognized. For example, age-related "immunosenescence" is associated with dysregulated Treg population and function that may contribute to the higher susceptibility to DED in the elderly [169]. Additionally, although the ocular surface is a relatively sterile site with low bacterial load, normal gut microbiota has been demonstrated to provide important protection in ocular surface homeostasis, and fecal microbiota transplant has been shown to dampen DED severity [170]. The understanding of systemic regulatory mechanisms is rapidly evolving, and these may provide added benefits to local immunotherapy for ocular surface disorders in translational and clinical studies.

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Highlights

- Active regulation operates within the ocular surface – regional lymph nodes axis.
- Corneal epithelium-derived factors promote avascularity and immune quiescence.
- Corneal resident ‘immature’ myeloid cells promote induction of immune tolerance.
- Corneal nerves and conjunctival goblet cells modulate ocular surface immunity.
- Regulatory T cells in regional lymph nodes critically suppress adaptive immunity.

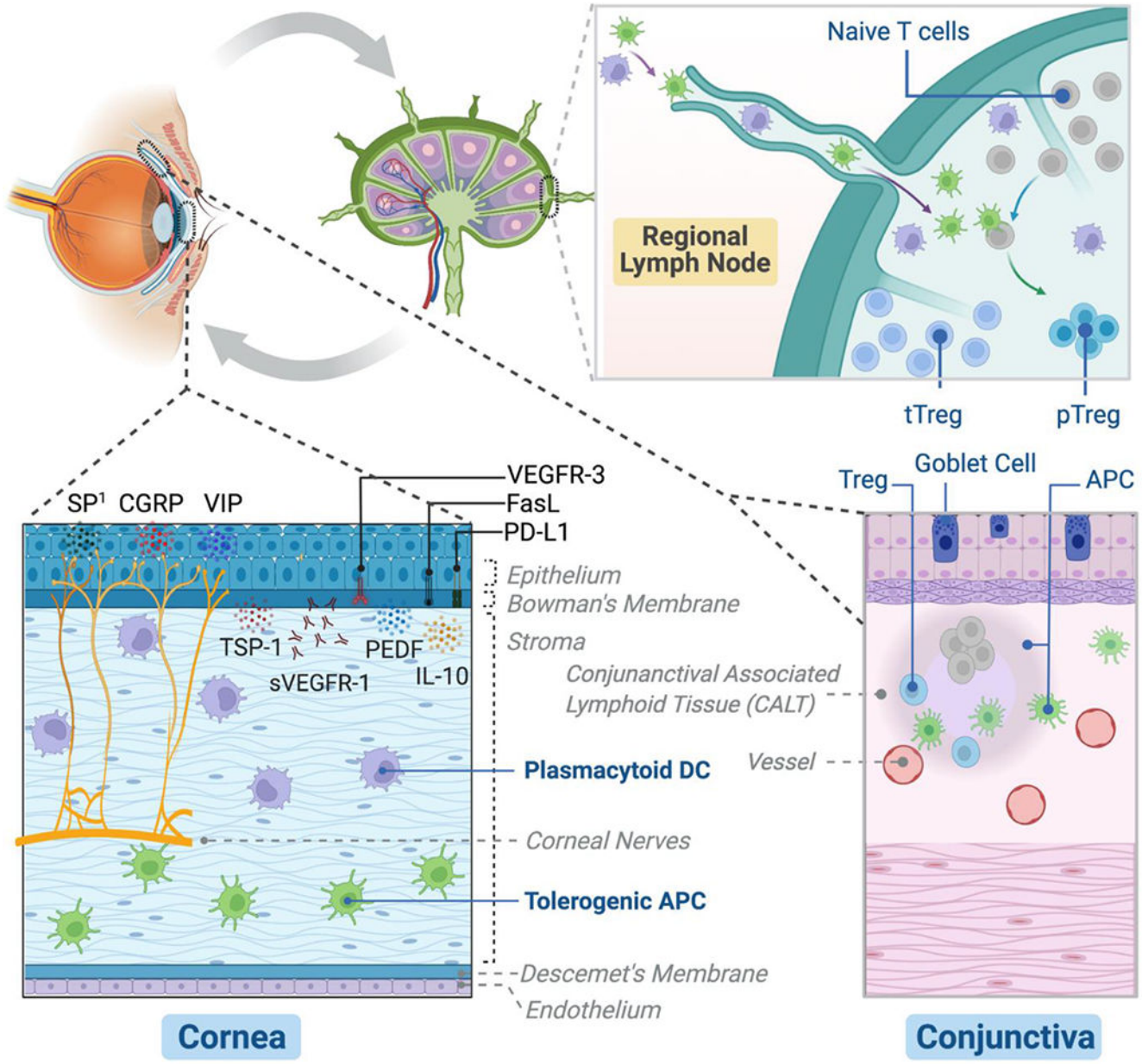

Figure 1. The major cells and molecules promoting ocular surface immune homeostasis.

(1) Cornea: Epithelium constitutively expresses an array of immunoregulatory factors to suppress inflammatory cells and inhibit angiogenesis, including membrane-bound programmed death ligand-1 (PD-L1), Fas ligand (FasL), and vascular endothelial growth factor receptor-3 (VEGFR-3), as well as secreted pigment epithelial-derived factor (PEDF), thrombospondin-1 (TSP-1), soluble VEGFR-1 (sVEFGR-1), and IL-10. The tolerogenic antigen-presenting cells (APC) and plasmacytoid dendritic cells (DC) primarily residing in the stroma are critically involved in the induction of immune tolerance at both ocular surface and regional lymph nodes, and plasmacytoid DC additionally contribute to corneal nerve integrity and avascularity. The intense innervation of the cornea is essential for ocular

surface barrier function and epithelial health with the support from the physiological level of substance P (SP) expressed by the nerves ([1]Excessive SP, on the contrary, promotes ocular surface inflammation and tissue damage). Other cornea nerve-derived neuropeptides, including vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) exert important anti-inflammatory functions. (2) Conjunctiva: The specialized goblet cells in the epithelium are critical to maintaining tear film stability by secreting soluble mucins. It promotes ocular surface immune tolerance by inducing tolerogenic APC. In addition, conjunctiva-associated lymphoid tissue (CALT), which harbors regulatory T cells (Treg) is involved in the protective mucosal immune regulation. (3) Regional lymph nodes: Ocular surface adaptive immunity is primarily regulated in the local eye-draining lymph nodes by the specialized immunoregulatory cells – Treg, which include the larger thymus-derived Treg (tTreg) population and the smaller but more potent peripheral induced Treg (pTreg) population after antigen stimulation by tolerogenic APC or plasmacytoid DC.