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## Ocular Surface Immunity: Homeostatic Mechanisms and Their Disruption in Dry Eye Disease

Stefano Barabino<sup>a</sup>, Yihe Chen<sup>b</sup>, Sunil Chauhan<sup>b</sup>, and Reza Dana<sup>b,\*</sup>

<sup>a</sup>Clinica Oculistica, Department of Neurosciences, Ophthalmology and Genetics, University of Genoa, Viale Benedetto XV 5, 16132 Genoa, Italy

<sup>b</sup>Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Department of Ophthalmology, 20 Staniford St., Boston, MA, 02114 USA

### Abstract

The tear film, lacrimal glands, corneal and conjunctival epithelia and Meibomian glands work together as a lacrimal functional unit (LFU) to preserve the integrity and function of the ocular surface. The integrity of this unit is necessary for the health and normal function of the eye and visual system. Nervous connections and systemic hormones are well known factors that maintain the homeostasis of the ocular surface. They control the response to internal and external stimuli. Our and others' studies show that immunological mechanisms also play a pivotal role in regulating the ocular surface environment. Our studies demonstrate how anti-inflammatory factors such as the expression of vascular endothelial growth factor receptor-3 (VEGFR-3) in corneal cells, immature corneal resident antigen-presenting cells, and regulatory T cells play an active role in protecting the ocular surface.

Dry eye disease (DED) affects millions of people worldwide and negatively influences the quality of life for patients. In its most severe forms, DED may lead to blindness. The etiology and pathogenesis of DED remain largely unclear. Nonetheless, in this review we summarize the role of the disruption of afferent and efferent immunoregulatory mechanisms that are responsible for the chronicity of the disease, its symptoms, and its clinical signs. We illustrate current anti-inflammatory treatments for DED and propose that prevention of the disruption of immunoregulatory mechanisms may represent a promising therapeutic strategy towards controlling ocular surface inflammation.

### Keywords

ocular surface; dry eye disease; autoimmunity; inflammation; anti-inflammatory agents

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\*Corresponding author: Reza Dana, MD, MSc, MPH, Schepens Eye Research Institute, 20 Staniford St., Boston, MA 02114, USA, reza.dana@schepens.harvard.edu.

S.B. and Y.C. contributed equally to this work and share primary authorship.

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## 1. Introduction

### 1.1 Impact of dry eye disease

Dry eye disease (DED) is now defined as “a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface, accompanied by increased osmolarity of the tear film and inflammation of the ocular surface” (Dry Eye Workshop, 2007). There is no definitive therapy for DED and it remains one of the leading causes of patient visits to ophthalmologists and optometrists (Schaumberg et al., 2002). Based on recent DED studies, an estimated 3.2 million women (Schaumberg et al., 2003) and 1.7 million men (Schaumberg, 2009), nearly 5 million Americans 50 years and older, suffer from dry eye. Tens of millions more have less severe forms of the disease. In most cases, patients endure a more episodic manifestation of their condition that is notable only in adverse conditions, such as a low humidity environment or contact lens wear. DED significantly affects the quality of life due to symptoms of pain and irritation. Severe forms of the disease are comparable to reported cases of moderate to severe angina (Buchholz et al., 2006), which limits and degrades performance of common vision-related daily activities, such as reading and driving (Miljanovic et al., 2007). Considering the significant impact of DED on the quality of life, the need for a thorough understanding of the pathogenic mechanisms, which determine the chronicity of the disease, is pressing for the development of new effective treatments.

### 1.2 Classification and risk factors for DED

The traditional notion for the cause of DED was principally held as an inadequate quantity or quality of the tear film. DED is now recognized as a disease of the Lacrimal Functional Unit (LFU); LFU is an integrated system comprising the ocular surface (tear film, corneal and conjunctival epithelia, and Meibomian glands), lacrimal glands, and nerves that connect them (Stern et al., 1998). Based on etiological factors that can influence this system, DED has been divided into aqueous tear-deficient dry eye and evaporative dry eye (Dry Eye Workshop, 2007).

*Aqueous tear-deficient dry eye* (ADDE) is characterized by reduced lacrimal tear secretion and volume due to a failure of lacrimal gland function; ADDE has two major subclasses: Sjögren’s syndrome dry eye and non-Sjögren’s syndrome dry eye. Sjögren’s syndrome is an exocrinopathy in which the lacrimal, salivary, and potentially other exocrine glands are targeted by an autoimmune process that possibly involves other organs in conjunction with other systemic diseases such as rheumatoid arthritis. The cause of apoptosis of the glandular epithelial cells (Kong et al., 1998) and infiltration of CD4<sup>+</sup> T cells in the lacrimal gland of Sjögren’s syndrome is now attributed to viral infections such as Epstein-Barr virus, hepatitis C virus and human T-cell leukaemia virus type 1. The causative role of these viruses remains uncertain.

Non-Sjögren DED is a form of ADDE due to lacrimal dysfunction without apparent signs of systemic autoimmunity. The most common form is age-related dry eye due to decreased tear volume and flow, increased osmolarity (Mathers et al., 1996), decreased tear film stability (Patel and Farrell, 1989), and alterations in the composition of the Meibomian lipids (Sullivan et al., 2006). Other common causes of DED that could trigger the pathogenic cycle of chronicity are systemic drugs that inhibit tear production (Moss et al., 2000), sex hormones (with the generalization that low levels of androgen facilitate ocular surface inflammation), low humidity, a constant air flow environment that causes increased tear evaporation (Barabino and Dana, 2007), chronic use of preserved drop (Baudouin et al.,

2010), contact lens wear (Poggio and Abelson, 1993), and refractive surgery (Battat et al., 2001).

*Evaporative dry eye* (EDE) is due to an excessive evaporation rate of the tear film from the ocular surface while tear secretion is in the normal range. The most common cause is Meibomian gland dysfunction because it determines a significant quantitative or qualitative alteration of the tear film lipids; these have the role of limiting evaporation of the aqueous layer. Other possible causes of EDE include poor lid congruity, low blink rate, and vitamin A deficiency (Dry Eye Workshop, 2007).

## 2. Immunoregulation of the ocular surface

In 1977 Thoft and Friend introduced the term “ocular surface” in order to describe the regeneration of corneal epithelium and to highlight the importance of the tear film, corneal and conjunctival epithelium connection (Thoft and Friend, 1977). Recent studies have demonstrated that the ocular surface can be considered not only as a part of ‘visual functional unit’, but also an ‘immunological’ unit with the ability to respond to external and internal stimuli. More importantly, the ocular surface can modulate the immunological response in order to avoid possible negative consequences on its components due to an “exaggerated” response or chronic activation of the immune system (Table 1).

### 2.1 Angiogenic privilege of cornea

The normal transparent cornea is devoid of both lymphatic and blood vessels, a characteristic referred as corneal “angiogenic privilege” (Cursiefen, 2007). This alymphatic and avascular characteristic of the cornea holds important implications for the tissue’s “immune privileged” status for it retards both trafficking of antigen-presenting cells (APCs) to the lymphoid compartment (due to its lack of lymphatics) as well as raising the threshold for effector cell access to the cornea (by virtue of its lack of blood vessels); this rationale was previously applied in order to explain the high success rate of corneal transplantation (Küchle et al., 2002), which has also been attributed to the immune privilege of the anterior chamber, recognized as anterior chamber associated immune deviation (ACAID) mechanisms (Streilein, 2003). Recent studies suggest maintenance of this privileged status is not a passive, but an active process that involves a balance between angiogenic and anti-angiogenic factors in the corneal epithelium (Ellenberg et al., 2010). The normal cornea constitutively expresses soluble vascular endothelial growth factor receptor-1 (sVEGFR-1 or sflt-1), which functions as an endogenous vascular endothelial growth factor (VEGF)-A trap; the latter is a potent stimulator of angiogenesis (Ambati et al., 2006; Ambati et al., 2007). The corneal epithelium constitutively expresses VEGFR-3, which binds to angiogenic VEGF-C and VEGF-D. As a result, it inhibits both hemangiogenesis and lymphangiogenesis, thereby contributing to the regulation of ocular surface immunity (Cursiefen et al., 2006). Another important anti-angiogenic factor constitutively expressed by cornea is thrombospondin (TSP)-1 (Hiscott et al., 1997), which helps to suppress inflammation-induced corneal angiogenesis (Cursiefen et al., 2004; Cursiefen et al., 2011). Endogenous IL-1 receptor antagonist (IL-1 Ra), expressed by cornea (Kennedy et al., 1995; Heur et al., 2009), is a potent anti-angiogenic factor in corneal neovascularisation (Lu et al., 2009). Tissue inhibitor of metalloproteinases (TIMPs)-1 and -2, contained in the tear film (Sack et al., 2005), are also able to suppress corneal neovascularization (Ma and Li, 2005). In addition to this unique innate mechanism of cornea, the ocular surface also uses an array of other endogenous mechanisms to modulate and suppress the immuno-inflammatory responses that comprise regulation of induction of the immune response (afferent loop) (Fig. 1) as well as effector cells and molecules (efferent loop) (Fig. 2).

## 2.2 Corneal resident APC

APCs specialize in capturing and processing antigens, displaying them to T lymphocytes, and providing costimulatory signals that stimulate the differentiation and proliferation of T lymphocytes. Studies in mice have shown that a normal healthy cornea harbors several populations of immature APCs (Fig. 1); these include CD11b<sup>+</sup> CD11c<sup>-</sup> macrophages/monocytes in the deep stroma and CD11c<sup>+</sup> CD11b<sup>lo/-</sup> dendritic/Langerhans cells in the epithelium. There is also a third and much smaller subpopulation of CD11b<sup>+</sup> CD11c<sup>variable</sup> dendritic cells in the anterior stroma (Hamrah et al., 2003a). A proportion of CD11c<sup>+</sup> CD11b<sup>lo/-</sup> cells in the epithelium are further defined as CD11c<sup>+</sup> CD11b<sup>lo/-</sup> Langerin<sup>+</sup> Langerhans cells (LCs); the dendritic cells in the stroma are CD11c<sup>+</sup> CD11b<sup>+</sup> Langerin<sup>+</sup> (non-LC) dendritic cells (Hattori et al., 2011). The preponderance of APCs (epithelial and stromal) reside in the corneal periphery and limbal areas, with numbers of APCs tapering rapidly toward the corneal center (Hamrah et al., 2002). These immature APCs are characterized by a very low surface expression of MHC class II (which provides antigenic peptide to cognate naïve T cells) along with absence of B7 and CD40 costimulatory molecules (which function together with antigen to stimulate T cells). Such “immature” APCs can contribute to T cell tolerance (Lutz and Schuler, 2002). On the other hand, following a challenge/insult to the ocular surface, these immature APCs are able to undergo acquisition of MHC class II and costimulatory molecules that are induced by pro-inflammatory cytokines such as IL-1 and TNF- $\alpha$  (Hamrah et al., 2003b). However, several anti-inflammatory factors that are present on the ocular surface are able to regulate this process by antagonizing the effects of pro-inflammatory cytokines. One of such factors is transforming growth factor (TGF)- $\beta$ , which is found in human tears (Gupta et al., 1996) and holds a critical suppressive effect on APC maturation in the cornea (Shen et al., 2007a). IL-1 Ra can attenuate the effects of IL-1 by binding to IL-1 receptor (Hannum et al., 1990). Vasoactive intestinal peptide (VIP), a neuropeptide, is constitutively secreted by nerve endings in the cornea (Mottlerle et al., 2006). VIP can down-regulate pro-inflammatory cytokines (IL-1, TNF- $\alpha$ ) while up-regulating anti-inflammatory cytokines (TGF- $\beta$ , IL-10) in the cornea, which is mediated largely via VIP receptors on macrophages (Szliter et al., 2007).

## 2.3 Regulation of T cell response

Ocular surface APCs migrate toward the draining lymphoid compartments and activate naïve T cells, which subsequently peripheralize and home to the ocular surface. A growing body of evidence suggests regulatory T cells (Tregs) are positioned in the center of the modulation loop for limiting immune damages incurred by autoreactive T cells (Fig. 2). Studies show that several different types of T cells bear a suppressive/regulatory activity (Shevach, 2006), but transcription factor forkhead box P3 (Foxp3)-expressing Tregs, most of which are CD4<sup>+</sup> T cells that express CD25 (the interleukin-2 (IL-2) receptor  $\alpha$ -chain), are widely accepted as a distinct “professional” Treg population with a committed suppressive function (Sakaguchi et al., 2010). CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs consist of two indistinguishable subsets developed in the thymus (natural Treg, nTreg) or induced in the periphery in the presence of IL-2 and TGF- $\beta$  or after encounters with foreign antigens (induced Treg, iTreg) (Piccirillo and Shevach, 2004; Kretschmer et al., 2005). CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs express different chemokine receptors and adhesion molecules enabling them to home to a secondary lymphoid compartment (directed by CCR7) (Szanya et al., 2002) where they dampen naïve T cell priming or sites of inflammation (directed by  $\alpha$ E $\beta$ 7 (CD103)) (Huehn and Hamann, 2005) where they attenuate effector T cell function. Previous studies in ocular surface inflammation (Niederhorn et al., 2006; Siemasko et al., 2008) show that mice depleted of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs develop exacerbated DED. Reconstitution with CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs conferred resistance to the development of DED in mice and muted ocular surface inflammation in a pathogenic CD4<sup>+</sup> T cell adoptive

transfer DED model. Recent studies reveal Tregs dysfunction in suppressing Th17 response in DED mice (Chauhan et al., 2009). Although it is known that Foxp3 is essential to Treg function, the exact mechanisms of Treg-mediated suppression lacks full elucidation. Two proposed mechanisms include secretion of anti-inflammatory cytokines (such as TGF- $\beta$  and IL-10) by Treg cells and cell-contact-dependent Treg/APC or Treg/T cell interactions (Levings et al., 2006). In regard to ocular surface inflammation, it was shown that APC is crucial to the function of Treg in a corneal transplantation model (Jin et al., 2010).

CD8<sup>+</sup> regulatory T cells were the object of intense study in 1980s. These cells were found to exert a regulatory function in ACAID (Streilein and Niederkorn, 1985; Wilbanks and Streilein, 1990). Although CD8<sup>+</sup> T cells are the predominant T cell population in the human conjunctival epithelium (Sacks et al., 1986) and a recent study showed that treatment with topical dexamethasone in DED patients increased conjunctival CD8<sup>+</sup> T cells (Reinoso et al., 2011), the functional role of intraepithelial CD8<sup>+</sup> regulatory T cells on the ocular surface remains largely unknown. Nevertheless, research on CD8<sup>+</sup> regulatory T cells has received renewed attention. Since then, several subsets of CD8<sup>+</sup> regulatory T cells using different suppressive mechanisms have been described. One subset of CD8<sup>+</sup> regulatory T cells recognizes peptides in the context of MHC class I complex, which induces direct killing of targeted cells (Smith and Kumar, 2008). A second subset of CD8<sup>+</sup> regulatory T cells expressing CD122 secretes immunosuppressive cytokines such as IL-10 to inhibit APC functions (Endarthy et al., 2005). The third subset has a CD8<sup>+</sup>CD28<sup>-</sup> phenotype that downregulates APC function by cell-cell contact (Chang et al., 2002). Unlike other CD8<sup>+</sup> regulatory T cells that are cytotoxic and as the counterpart of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs, CD8<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells were discovered mediating suppression through cell-cell contact mechanisms (Cosmi et al., 2003; Correale and Villa, 2010).

In addition to regulatory T cells, there are other mechanisms that limit homing and effector functions of autoreactive T cells on the ocular surface. A new member of the B7 family, programmed death-ligand (PD-L)1 is constitutively expressed by corneal epithelial cells (Shen et al., 2007b) and endothelial cells (Sugita et al., 2009). Ligation of PD-L1 with receptor programmed death (PD)-1 on activated T cells inhibits T cell proliferation and cytokine production (Sugita et al., 2009; Freeman et al., 2000; Mazanet and Hughes, 2002). Moreover, PD-L1 is relevant to T cell chemotaxis in dry eye-associated inflammation on the ocular surface (El Annan et al., 2010). Fas ligand (FasL) is also amply expressed on corneal epithelium and endothelium. The interaction of FasL with Fas expressed on activated T cells leads to the death of T cells by apoptosis (Griffith et al., 1995), thereby diminishing ocular surface inflammation.

### 3. Failure of ocular surface immunohomeostasis

In DED, the ocular surface loses its immunohomeostasis and presents variable degrees of inflammation characterized by an enhanced expression of pro-inflammatory cytokines and chemokines along with the infiltration of autoreactive T cells (Stern et al., 2010) (Table 1). Clinically, inflammation of the ocular surface may appear as conjunctival hyperemia and epithelial disturbance (Fig. 3); however, in some cases it requires laboratory examination to be diagnosed.

#### 3.1 Early activation of natural killer (NK) cells and ocular surface epithelium

The precise immunopathogenic mechanisms of DED are not firmly established, but the first step could be an activation of innate immune components (Fig. 1). In several autoimmune diseases, innate immune responses (such as NK cell activation) play an important role not only by direct actions, but also by shaping subsequent adaptive immune responses (Winkler-Pickett et al., 2008; Shi et al., 2000). Our study demonstrates early activation of NK cells in

DED mice. These IFN- $\gamma$ -secreting NK cells promote induction of DED via direct damage to ocular surface and facilitating maturation of APC in secondary lymphoid compartment (Chen et al., 2011). Another study on DED patients (Barabino et al., 2010) did not show a significant increase in NK cells in the conjunctival epithelium. The subjects in this study were in the chronic disease stage instead of the induction stage; the functional status of NK cells in this study could not be investigated. As discussed later, stressed ocular surface epithelium is a major source of innate cytokines and chemokines, which in turn cause damage to epithelial cells in an autocrine manner and activate other immune cells such as APC.

### 3.2 Activation of toll-like receptors (TLR)

A family of innate immune proteins called TLR is involved in the ocular surface inflammation of DED. TLR is one of the primary innate immune mechanisms that can be activated not only by pathogen associated molecular patterns (PAMPs) on pathogens, but also by a number of endogenous ligands such as intracellular components of dead cells. In particular, apoptosis increase on the ocular surface in DED (Yeh et al., 2003) could provide chromatin and small ribonuclear particles (snRNPs) to activate TLRs. One of the most common and important TLR signaling pathways is through adaptor molecule myeloid differentiation protein 88 (MyD88), which activates IL-1R-associated kinase (IRAK) and leads to the activation of several transcription factors such as activating protein (AP)-1, nuclear factor  $\kappa$ B (NF $\kappa$ B), and interferon regulatory factor (IRF)-5 (Kawai and Akira, 2007). This pathway ultimately stimulates the expression of multiple pro-inflammatory cytokine, chemokine, and adhesion molecule genes. On the human ocular surface, all ten known functional human TLRs (TLRs 1–10) were identified at mRNA level. Of these ten, TLR2, 3, 4, 5, and 7 were confirmed at the protein level (Redfern and McDermott, 2010). However, no significant changes on the transcriptional levels of TLRs 1–10 were found in corneal and conjunctival impression cytology samples from DED patients (Mohammed et al., 2011). Our unpublished data on a murine DED model showed no significant change of TLR4 mRNA level, but increased cell surface expression of TLR4 protein on corneal epithelium. It is probably due to the translocation of cytoplasmic TLR4 to the cell surface. In addition, increased expression of TLR4 were found in the corneal stroma at both transcriptional and protein levels. Much is yet to be understood about the role of TLR in immunopathogenesis of DED, but it is believed that activation of TLR-related innate immune mechanisms will facilitate the induction of autoimmunity in DED.

### 3.3 Increased pro-inflammatory cytokines and chemokines

Pro-inflammatory cytokines IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-23, IFN- $\gamma$ , and IL17 are elevated in DED patients (Table 2) (Solomon et al., 2001; Massingale et al., 2009; Kang et al., 2011; De Paiva et al., 2009) as well as in experimental DED models (Rashid et al., 2008; Goyal et al., 2009; De Paiva et al., 2009). IL-1 and TNF- $\alpha$  are capable of stimulating the recruitment of leukocytes to inflamed ocular surface and activating these cells by promoting expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) on the ocular surface epithelium (Gao et al., 2004; Pisella et al., 2000) and expression of MHC class II and co-stimulator B7 on APCs (Hamrah et al., 2003). IL-6 synergizes with TGF- $\beta$  to enhance expression of transcription factor ROR $\gamma$ t to promote development of Th17 cells (Mills, 2008). IL-6 concentration in tears has a strong correlation with the severity of ocular surface epithelial disease, including clinical symptoms (pain and irritation) and signs (corneal fluorescein staining and conjunctival lissamine staining) (Lam et al., 2009; Enríquez-de-Salamanca et al., 2010). IL-23 is essential to the survival and proliferation of Th17 cells (Langrish et al., 2005; Weaver et al., 2007). In addition to ICAM-1, increased expression of other trafficking molecules was detected on the ocular surface of DED patients and animal models. Chemokines, discovered on the basis of their activities as leukocyte

chemoattractants, serve an important function in regulating the activation and traffic of lymphocytes to the ocular surface through peripheral lymphoid tissues. Increased expression of chemokine ligands and receptors noted in murine DED include CCL3 (macrophage inflammatory protein 1 $\alpha$ , MIP-1 $\alpha$ ), CCL4 (MIP-1 $\beta$ ), CCL5 (regulated on activation, normal T-cell expressed and secreted, RANTES), and their receptor CCR5 as well as chemokines CXCL9 (monokine induced by interferon- $\gamma$ , MIG), CXCL10 (interferon- $\gamma$  inducible protein 10, IP-10), and their receptor CXCR3 (Yoon et al., 2007). In dry eye patients, especially those with Sjögren's syndrome, expression of CXCL9, 10, 11, CXCR3, and CCR5 were found increased on the ocular surface (Yoon et al., 2010; Gulati et al., 2006; Enríquez-de-Salamanca et al., 2010). These chemokine ligands and receptors primarily mediate Th1 cell homing and migration (Fig. 4). Chemokine CCL20 is also involved in the Th17 cell homing and was reported to increase in DED (De Paiva et al., 2009). In addition, Enríquez-de-Salamanca et al. (2010) detected increased level of fractalkine/CX3CL1 in evaporative-type dry eye patient tears, which is a potent chemoattractant for CX3CR1<sup>+</sup> leukocytes, including monocytes, NK cells, and some T cells.

### 3.4 Acquisition of MHC class II by ocular surface epithelium

In a cytokine rich ocular surface environment of dry eye, conjunctival epithelium overexpressed MHC class II (Pisella et al., 2000; Viau et al., 2009; Barabino et al., 2010). Corneal epithelial cells were able to express MHC class II in vitro under the stimulation of IFN- $\gamma$  (Iwata et al., 1992; Dreizen et al., 1988). Reports show that the infected upper reproductive tract epithelial cells present virus antigen via MHC class II to CD4<sup>+</sup> T cells and activate T cells in vitro (Jayarapu et al., 2009). In a case of DED, the percentage of goblet cells in conjunctiva demonstrated a significant negative correlation with up-regulation of MHC class II (Pisella et al., 2000). The contribution of MHC class II expression by ocular surface epithelia to the pathogenesis of DED needs to be functionally characterized.

### 3.5 Infiltration, maturation and efflux of corneal APCs

There is strong evidence showing the critical involvement of autoreactive T cells in sustained ocular surface inflammation in DED (Stern et al., 2002; De Paiva et al., 2009; Niederkorn et al., 2006; El Annan et al., 2009; Chauhan et al., 2009). The most fundamental initial element in promoting such adaptive immune responses, that is, antigen presentation by APCs, lacks elucidation. As described above, both healthy corneal epithelium and stroma are endowed with several CD11b<sup>+</sup> and CD11c<sup>+</sup> subpopulations of resident immature APCs. Although the contribution of these resident corneal APCs in the induction of immunity is well defined in corneal transplantation (Liu et al., 2002), the same important question remains poorly answered in DED. In an experimental model of DED, increased corneal infiltration of CD11b<sup>+</sup> cells (Fig. 5) and acquisition of MHC class II expression by some of these cells were observed (Rashid et al., 2008; Goyal et al., 2009; Goyal et al., 2010). This model of DED suggested that desiccating stress could induce mobilization and maturation of ocular surface APCs. In vivo confocal microscopy studies of the cornea confirm the presence and increased number of dendritic-like cells in patients with Sjögren's syndrome dry eye (Fig. 6) (Villani et al., 2007). Significantly more evaluation on the phenotypic alterations (such as B7, CD40) of APCs and factors affecting APC maturation need future examination. Another question worth examining is how activated corneal APCs migrate to secondary lymphoid compartments where they prime cognate naïve T cells to putative ocular surface antigens. In this regard, studies in corneal transplantation suggest that chemokine receptor switching (e.g. from CCR1 and CCR5 to CCR7) is critical for trafficking of corneal APCs to the draining lymph nodes (Yamagami et al., 2005; Hamrah et al., 2007; Jin et al., 2007). Although similar mechanisms cannot be simply assumed in DED, further investigations on this area are necessary. We recently demonstrated that there is considerable and exclusive growth of lymphatic, not blood, vessels in murine dry eye



corneas (Goyal et al., 2010), which are primarily induced by IL-17 through VEGFR3-dependent pathway (Chauhan et al., 2011) (Fig. 7). These newly formed lymphatics increase both in caliber and area while advancing toward the corneal center with progression of dry eye. This may serve as potential conduits for migration of corneal APCs to lymphoid tissues where they generate autoreactive T cells. Although some autoantigens from the lacrimal and salivary glands have been implicated (Rose et al., 2005; Jiang et al., 2009), another question remains unresolved; which endogenous ocular surface antigen provides “danger signals” and is captured by resident immature APCs?

### 3.6 Autoreactive Th1 and Th17 responses in draining lymph nodes and ocular surface

Now, it is clear that the activation and expansion of CD4<sup>+</sup> T cells occurs in the secondary lymphoid compartment in DED. Evidence shows that IFN- $\gamma$ -secreting CD4<sup>+</sup> T (Th1) and IL-17-secreting CD4<sup>+</sup> T (Th17) cells are two well-defined critical subsets generated in the draining lymph nodes of murine DED (Fig. 8) (El Annan et al., 2009; Chauhan et al., 2009). The differentiation and proliferation of Th1 and Th17 lineages is influenced by different cytokine milieu with IL-12 and IFN- $\gamma$  promoting polarization of Th1 cells and IL-6, TGF- $\beta$ , and IL-23 skewing CD4<sup>+</sup> T cells toward Th17 cells (Mills, 2008). Elevated IL-6 expression in the draining lymph nodes from murine DED was observed (Chauhan et al., 2009). Moreover, recent appreciation for the importance of dysfunctional CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in the pathogenesis of DED was established in murine DED. Findings indicated that it is the Th17, not Th1, subset that is resistant and functionally antagonistic to Treg activity. Interestingly, the in vivo blockade of IL-17 significantly decreases disease severity along with the restoration of Treg function in an experimental model of DED (Chauhan et al., 2009). Following the demonstration of T cell infiltration to dry eye ocular surface (Stern et al., 2002), increased expression of IFN- $\gamma$  and IL-17 on human and murine ocular surface has recently been reported by independent studies (De Paiva et al., 2009; Chauhan et al., 2009; De Paiva et al., 2007). These findings indicate that ocular surface infiltrating T cells in DED are Th1 and Th17 effectors, which are generated in the regional draining lymph nodes. Both IFN- $\gamma$  and IL-17 contribute to the corneal barrier disruption, but IFN- $\gamma$  is associated with decreased conjunctival goblet cell density (De Paiva et al., 2009; De Paiva et al., 2007). Aside from causing corneal damage in DED, IL-17 induces corneal lymphangiogenesis via a VEGFD/C-VEGFR3 signaling pathway, thereby promoting the progression and amplification of autoimmune responses by facilitating the trafficking of immune cells (Chauhan et al., 2011). With respect to the homing of these effector T cells from draining lymph nodes to the ocular surface, very limited data is available on the homing mechanisms for different CD4<sup>+</sup> T cell subsets. Our studies have confirmed increased frequency of CCR5- and CXCR3-expressing Th1 cells in the draining lymph nodes of dry eye mice. CCR6-expressing Th17 was recruited to inflamed sites via CCL20 in rheumatoid arthritis (Hirota et al., 2007), which is yet to be addressed in DED.

### 3.7 Sex hormones

In a clinical experience similar to many other immune-mediated conditions, significantly more female patients with dry eye are observed. The female sex is regarded as a risk factor for DED (Schaumberg et al., 2003; Schaumberg et al., 2009; Gayton, 2009; Jie et al., 2009), which indicates that sex hormones likely play a key role in the development and course of the disease. Hormonal studies suggest that androgens suppress and estrogens may promote DED (Krenzer et al., 2000; Schaumberg et al., 2001; Uncu et al., 2006). Lacrimal and meibomian glands seem to be the main target organs for both androgens and estrogens. Androgen can stimulate meibomian gland genes related to lipid metabolic pathways. Its deficiency in human may promote meibomian gland dysfunction and evaporative dry eye (Sullivan et al., 2009; Sullivan et al., 2002) while estrogen may up-regulate metalloproteinase-2 and -9 expression by rabbit lacrimal glands (Zylberberg et al., 2007).

The effect of sex hormones on the immuno-inflammatory responses in DED is not widely investigated. It was reported that estrogen increases the expression of inflammatory genes such as IL-1 $\beta$ , IL-6, and IL-8 in human corneal epithelial cells (Suzuki and Sullivan, 2005). Androgen may exert anti-inflammatory effects by reducing macrophage TNF- $\alpha$  and IL-1 $\beta$  expression (Corcoran et al., 2010). However, some clinical reports indicate that estrogen may ameliorate dry eye severity (Lang et al., 2002; Guaschino et al., 2003; Scott et al., 2005). Given the widespread expression of sex hormone receptors in various ocular and adnexal tissues, further research is necessary to establish the precise role of sex hormones in the pathogenesis of DED.

#### 4. Strategies for controlling ocular surface inflammation

Recent advances in the comprehension of the pathogenesis of DED have led to significant changes in the therapeutic management of the disease. The traditional approach based on a tear substitute demonstrated some limitations. Tear replacement is certainly important to decrease tear evaporation and osmolarity and to restore a physiological tear clearance and barrier to protect the ocular surface. In severe cases of DED, it should be administered together with an anti-inflammatory therapy. The aim of this approach is to break the vicious cycle of lid margin inflammation/MGD – dry eye – ocular surface inflammation, which is the cause that leads to ocular surface epithelial damage and to symptoms and signs experienced by patients with DED.

Topical and systemic anti-inflammatory agents such as cyclosporine, corticosteroids, tetracyclines, omega-3 and -6 fatty acids and monoclonal antibodies are now directed to specific factors of the inflammatory cascade of the ocular surface. As discussed in the following sections, these anti-inflammatory agents were reported in both clinical trials and animal models as effective in treating DED.

##### 4.1 Cyclosporine A

Cyclosporine is a natural occurring fungal metabolite that is extensively studied due to its widespread use as an immunosuppressant to control the rejection of solid organ transplants and to treat autoimmune diseases. Topical cyclosporine received FDA approval in December 2002 as Restasis™ (Cyclosporine ophthalmic solution 0.05%, Allergan, Inc. Irvine, CA) for treating underlying inflammation in DED. Restasis is a sterile, preservative-free emulsion that appears white opaque to slightly translucent.

Cyclosporine was shown to relieve the signs and symptoms of DED in two phase III randomized multicentre, double-blinded, 6-months clinical trials establishing the efficacy, safety, and anti-inflammatory activity of cyclosporine ophthalmic emulsion in patients with moderate to severe DED (Sall et al., 2000). Cyclosporine can reduce the need for artificial tear palliative treatment. Cyclosporine improved subjective symptoms like blurred vision and improved global response to treatment in many patients. It may also improve the results of objective tests of DED (corneal staining, Schirmer test) (Kunert et al., 2000). The benefits of restoring the ocular surface as manifested by improved corneal staining, included enhanced vision, normalized lacrimal gland response to blinking and other stimuli, and reduction of concomitant artificial tear instillation. The use of cyclosporine for the treatment of dry eye was also tested in a variety of situations that correlated with the syndrome in patients who underwent LASIK (Salib et al., 2006) and in patients with graft-versus-host disease after stem cells transplantation (Rao and Rao 2006); both cases produced improvement in subjective and objective signs of dry eye. Oral and intravenous cyclosporine administration is associated with serious side effects including hypertension and nephrotoxicity. However, due to low systemic absorption, these are not reported with topical cyclosporine treatment in DED (Sall et al., 2000). The most common adverse reaction on

instillation is a burning sensation that does not necessarily necessitate therapy discontinuation. Still, this bothersome side effect affects patient compliance in a sizeable minority of patients.

Cyclosporine exerts its immunomodulatory effect by inhibiting T cell activation. The T cell receptor on the cell surface is bound by an appropriate ligand that determines a cascade of events. Events include the release of calcium, stimulation of phosphatase calcineurin, and the activation and migration of the nuclear factor for T cell activation (NF-AT) in the nucleus along with the transcription of the gene for IL-2 and other pro-inflammatory factors. Secreted IL-2 binds to its receptors on the T cell surface, stimulating cell division and activation. Cyclosporine acts in the cytoplasm by forming a complex with cyclophilin A. It then binds to calcineurin, inhibiting its dephosphorylating activity and preventing translocation of NF-AT to the nucleus, resulting in cytokines production (Donnenfeld and Pflugfelder, 2009). Biopsies from dry eye patients treated with topical cyclosporine for 6 months showed a significant decrease in IL-6 mRNA relative to pre-treatment biopsies (Turner et al., 2000). Kunert et al. (2000) demonstrated significant reduction of HLA-DR and a marker of activated T cells, CD11a, in conjunctival biopsies from patients with DED after following a 6 month cyclosporine ophthalmic solution treatment. The increase of goblet cells number in the conjunctiva after 6 months of therapy is an indicator of improved ocular surface conditions, which was determined by reduced inflammatory environment (Yuksel et al., 2010).

Another mechanism of cyclosporine is inhibition of apoptosis. This is determined by forming a complex with cyclophilin D, which prevents the opening of the mitochondrial permeability transition (MPT) pore (Donnenfeld and Pflugfelder, 2009). The opening of this pore is in response to cellular stress damage; it is an early step in the apoptosis cascade. In an experimental mouse model of DED, cyclosporine significantly reduced apoptosis of conjunctival epithelial cells. These results demonstrated a decreased level of DNA fragmentation and activated caspase-3 (Strong et al., 2005). Topical application of cyclosporine on patients with DED determined significant reduction of molecular markers of apoptosis such as CD40, CD40 ligand, and Fas in conjunctival epithelium. In a canine model of DED, cyclosporine determined a significant reduction in lacrimal cells apoptosis (Kaswan et al., 1989).

Despite these promises, it is important to emphasize the limitations of topical cyclosporine. Many patients with DED have incomplete responses to cyclosporine. Opinion varies significantly over ranges that result from 10% to over 50% of patients who may not experience or report significant improvement. Cyclosporine requires several months of application in most patients before demonstrable efficacy; this complicates compliance with the drug regimen for many patients who may prematurely terminate treatment. Many (likely 15–20%) of patients using topical cyclosporine experience drug tolerability issues, which include burning and irritation upon drug instillation. This is an issue that anecdotally was linked to some patients who ceased therapy shortly after initiating use.

#### 4.2 Topical corticosteroids

Topical, preferably non-preserved, corticosteroid therapy, like methylprednisolone, demonstrated reduction of inflammation in patients with DED (Marsh and Pflugfelder, 1999; Prabhasawat and Tseng, 1998); this effect was due to traditional glucocorticoid receptor mediated pathways that directly regulate gene expression and potent inhibition of many inflammatory pathways mediated by the NF- $\kappa$ B signal transduction pathway. Some of these include inhibition of inflammatory cytokine and chemokine production, decreased expression of cell adhesion molecules (e.g., ICAM-1), stimulation of lymphocyte apoptosis,

decreased synthesis of matrix metalloproteinases and lipid mediators of inflammation (e.g., prostaglandins) (Dursun et al., 2001; Liden et al., 2000; Yoshida et al., 1999).

Applied for two weeks, three to four times a day, topical methylprednisolone therapy provided significant relief of moderate to severe irritation symptoms in Sjögren's syndrome DED patients resistant to maximum aqueous enhancement therapies (Marsh and Pflugfelder, 1999). A concomitant decrease in corneal fluorescein staining and complete resolution of filamentary keratitis was also demonstrated. In another randomized clinical trial, the severity of ocular irritation symptoms and corneal fluorescein staining was significantly reduced in a group of patients treated with topical non-preserved methylprednisolone for 2 weeks followed by punctal occlusion as compared to a group that received punctal occlusion alone (Sainz de la Maza Serra et al., 2000). In a group of 70 patients with delayed tear clearance, Prabhasawat and Tseng (1998) reported improvement of irritation symptoms, ocular surface dye staining, and fluorescein tear clearance after a three week treatment with 1% methylprednisolone that was applied one drop to each eye three times a day. Symptomatic relief was reported to extend for months after steroid application ended.

The positive effect of steroids on the ocular surface of patients with DED was determined by their ability to reduce inflammation and therefore MMP-9 expression (De Paiva et al., 2006a) and to decrease desquamation of apical corneal epithelial cells and maintain the integrity of corneal epithelial tight junctions (De Paiva et al., 2006b). However, long-term use of steroids is associated with severe side effects such as ocular hypertension, cataract formation, glaucoma, and infection. In most cases, this restricts the use of topical steroids for severe symptoms and/or only for acute treatment of dry eye exacerbations. Since there is no data showing that higher potency corticosteroids are preferred to lower potency "soft" steroids, and the latter have a more acceptable safety profile, weaker and/or dilute corticosteroids are recommended for use. Recently, loteprednol etabonate, an ester corticosteroid with anti-inflammatory efficacy and improved safety compared with other corticosteroids (Loteprednol Etabonate US Uveitis Study Group, 1999) was used in dry eye patients. Pflugfelder et al. (2004) demonstrated that loteprednol etabonate-treated patients had significant improvement in inferior tarsal and nasal bulbar conjunctival hyperemia without the clinically significant increase in intraocular pressure over the placebo-treated patients. One point that requires emphasis is that response to corticosteroid therapy is far quicker than response to cyclosporine therapy; thus, 'pulse therapy' with corticosteroids would be expected to show results in a short time frame.

A promising novel therapeutic approach is based on selective glucocorticoid receptor agonists (SEGRAs). SEGRAs represent a novel class of compounds that regulate glucocorticoid receptor-mediated gene expression via repression carrying out anti-inflammatory activities with reportedly reduced side effects as compared to classical steroids (Rosen and Miner, 2005). In vitro data suggest that mapracorat, a SEGRA compound, inhibits hyperosmolarity-induced pro-inflammatory cytokines IL-6, IL-8, and MCP-1 in human corneal epithelial cells with comparable efficacy and potency as dexamethasone (Cavet et al., 2010); however, the clinical utility of these SEGRA agents need to be definitively demonstrated in prospective randomized trials.

### 4.3 Tetracyclines

Tetracyclines are antibiotics that interfere with protein synthesis at the ribosomal level of many gram-positive and gram-negative bacteria, mycoplasmas, chlamydiae, and spirochetes. Among these, tetracycline is a cost-effective agent. However, due to its short half-life (8.5 hours), tetracycline requires a regimen of four times a day. In contrast, doxycycline and minocycline have a much longer half-life (15–17 hours), which permits a daily dosage of one tablet. Tetracyclines are excreted in the urine except for doxycycline, which is excreted

primarily in the feces. Therefore, doxycycline is considered the tetracycline of choice for patients with renal failure.

Recently, tetracyclines were found in possession of numerous anti-inflammatory properties, including inhibition of matrix metalloproteinase (MMP) activity (Ryan et al., 2001; Smith et al., 1999) and synthesis (Hanemaaijer et al. 1997), nitric oxide synthesis (Amin et al., 1996), collagenases activity (Shlopov et al., 1999), and B cell activation (Kuzin et al., 2001). Orally administered, doxycycline is able to inhibit experimental choroidal neovascularization (Samtani et al., 2009). On the ocular surface, findings demonstrated that doxycycline suppresses expression of stimulated MMP-1, -13, and -10 at the mRNA and protein levels (Li et al. 2003), MMP-9 production (Li et al. 2001), and IL-1 expression and activity (Solomon et al. 2000) by human corneal epithelial cells. In an experimental model of dry eye, topical treatment with 0.025% doxycycline reduced expression and activity of MMP-9, decreased levels of IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$  RNA transcripts, and activity of MAPK in the corneal epithelium (De Paiva et al., 2006a). Similarly to methylprednisolone, doxycycline demonstrated an ability to preserve the integrity of corneal epithelium (De Paiva et al., 2006b). Unfortunately, in the animal model of dry eye the effect of doxycycline on clinical parameters such as tear secretion and corneal fluorescein staining remains unstudied.

Studies on the effect of systemic tetracyclines on DED in humans are related primarily to treatment of ocular rosacea. Since 1966, several tetracyclines analogues, including tetracycline, oxytetracycline, doxycycline, and minocycline, have been proven to be effective treatments for patients with acne rosacea (Frucht-Pery et al., 1993; Sneddon, 1966). However, a recent review of the published literature on the use of tetracyclines as therapy for ocular rosacea, pointed out that the studies performed with tetracyclines and doxycycline were not placebo controlled. The dose and schedule of administration were not evaluated and oxytetracycline, which is not available in the United States, showed only modest benefits when compared to a placebo (Stone and Chodosh, 2004). The effect of tetracyclines may be related to a decrease or elimination of bacterial flora from the eyelids or to the reduction of its lypolytic function (Shine et al., 2003) and not to its direct anti-inflammatory activity (Ta et al., 2003). In our opinion, further masked and placebo-controlled prospective studies are necessary to clarify the potential role of tetracyclines in treatment of ocular rosacea and other forms of dry eye when administered orally or topically.

#### 4.4 Macrolides

Azythromycin ophthalmic solution 1% (AzaSite, Inspire Pharmaceuticals) is a topical macrolide broad-spectrum antibiotic approved for the treatment of bacterial conjunctivitis (Abelson et al., 2008). Researchers have demonstrated that in addition to their antimicrobial activity, macrolides have anti-inflammatory properties. The mechanisms for this potential activity are not completely understood, but recently Li and co-workers (2010) demonstrated that azythromycin suppresses zymosan-induced production of pro-inflammatory mediators by human corneal epithelial cells via blocking NF- $\kappa$ B activation. Topical azythromycin significantly reduced leukocyte infiltration into the cornea and the expression of mRNA of IL-1 $\beta$ , TNF- $\alpha$ , and ICAM-1 in a murine model of corneal inflammation (Sadrai et al., 2011). In a clinical study measuring the effect of topical azythromycin in patients with blepharitis, Haque et al. (2010) demonstrated a significant improvement in signs and symptoms after 4 weeks of treatment; however, they were unable to show significant changes in pro-inflammatory cytokines in tears.

#### 4.5 Omega-3 and -6 fatty acids

Omega-3 and -6 are polyunsaturated essential fatty acids (EFAs) critical for optimum ocular surface homeostasis. The human body cannot synthesize EFAs. Therefore, EFAs must be obtained from the diet. Omega-3's, once consumed, are elongated by enzymes in order to produce largely anti-inflammatory prostaglandin E<sub>3</sub> (PGE<sub>3</sub>), anti-inflammatory leukotriene B<sub>5</sub> (LTB<sub>5</sub>), thromboxane (which reduces vascular permeability), and resolvins (Rosenberg and Asbell, 2010). The latter is a family of locally acting compounds that inhibit the production of inflammatory mediators, cytokine expression and leukocyte infiltration, thereby controlling the inflammatory response (Jin et al., 2009) by promoting its resolution. The omega-6 fatty acids, linoleic acid and  $\gamma$ -linolenic acid, are precursors of PGE<sub>1</sub>, a potent anti-inflammatory agent successfully used in animal models of ocular inflammation (Hoyng et al., 1986).

Horrobin (1986) argued for a possible role of EFAs supplements in patients with Sjögren's syndrome and DED. Anecdotal case reports in non-peer reviewed literature described improvement in symptoms and signs of DED. Nonetheless, there are few randomized placebo-controlled studies on the effects of systemic fatty acid in DED. Systemic linoleic acid (28.5 mg) and  $\gamma$ -linolenic acid (15 mg) were proven to reduce ocular surface inflammation (in particular HLA-DR expression on conjunctival epithelial cells) and improve DED symptoms when administered twice daily for 45 days in conjunction with artificial tears (Barabino et al., 2003). Aragona et al. (2005) showed that omega-6 EFAs are effective in ameliorating symptoms and ocular surface signs in patients with Sjögren's syndrome by increasing PGE<sub>1</sub> in tears after 1 month of treatment.

For the first time, Rashid et al. (2008) used topical EFAs in a mouse model of dry eye, which demonstrated significant changes in corneal fluorescein staining and inflammation of the ocular surface after treatment with alpha-linolenic acid (ALA). It was demonstrated that the infiltration of CD11b<sup>+</sup> cells in the central cornea and the expression of corneal IL-1 $\alpha$ , TNF- $\alpha$  and conjunctival TNF- $\alpha$  were significantly reduced. In our opinion, these results are encouraging, but further studies on the use of EFAs in treatment of DED and its effects on the ocular surface are needed before considering systematic use of these drugs in patients with DED.

#### 4.6 Novel directions in anti-inflammatory and immunomodulatory treatments

Several novel treatment strategies are in development due to the research advances in understanding the immunopathogenic mechanisms of DED. The anti-CD4 monoclonal antibody was found to suppress the local activation of CD4<sup>+</sup> T cells reducing the expansion of pathologic CD4<sup>+</sup> T cells against  $\alpha$ -fodrin in a mouse model of Sjögren's syndrome (Hayashi et al., 2005). In another controlled-environment chamber-induced dry eye mouse model (Barabino et al., 2005), the control of the migration of leukocytes by inhibition of adhesion molecules with the topical application of very late antigen 4 small-molecule antagonists (anti-VLA-4 sm) was associated with a significant decrease in corneal damage, conjunctival T cell numbers, and TNF- $\alpha$  levels in the cornea and conjunctiva (Ecoiffier et al., 2008). Using the same animal model of DED, Goyal et al. (2009) evaluated the efficacy of a topical antagonist to the chemokine receptor 2 (CCR2) on the clinical signs and markers of inflammation of the ocular surface. Interestingly, mice treated with CCR2 antagonist showed a significant decrease in corneal fluorescein staining and decreased infiltration of CD11b<sup>+</sup> cells and conjunctival T cells compared with the vehicle-treated and untreated dry eye groups. The CCR2 antagonist significantly decreased the level of expression of mRNA of IL-1 $\alpha$  and IL-1 $\beta$  in the cornea and TNF- $\alpha$  and IL-1 $\beta$  in the conjunctiva.

Other possible new treatments include DA-6034 which showed therapeutic efficacy by restoring tear function and inhibiting inflammatory response in a rabbit lacrimal gland inflammation model of dry eye (Seo et al., 2010), and phosphodiesterase 4 (PDE4) inhibitors which have the potential to control ocular surface inflammation by increasing cAMP levels (Govek et al., 2010). Recently, the demonstration of corneal lymphangiogenesis in DED, associated with significant increases in expression of pro-lymphangiogenic factors VEGF-C, VEGF-D, and VEGFR-3, and the detection of increased level of VEGF in tears of patients with dry eye (Enríquez-de-Salamanca et al., 2010), have opened the potential for new therapeutic approaches. One promising approach is the blockade of IL-17, a cytokine which in addition to its role in Th17-mediated ocular surface damage (Chauhan et al., 2009) has shown to be a chief regulator of VEGF-D expression and lymphatic endothelial proliferation (Chauhan et al., 2011). Alternatively, use of anti-VEGF-C antibody resulted in a significant reduction of lymphatic vessel caliber and area along with concomitant reduction in the expression of inflammatory cytokines in the conjunctiva and lymph nodes in dry eye mice (Goyal et al., 2011). Nonetheless, IL-1 was implicated as a potential target in DED (Okanobo et al., 2012). Furthermore, a recently completed randomized double-masked phase I/II clinical trial at our institution, Massachusetts Eye and Ear Infirmary, revealed significant reduction in both signs and symptoms of DED in response to topical administration of an IL-1 antagonist (unpublished data).

## 5. Conclusions and future directions

The current concept of the ocular surface as part of lacrimal functional unit (LFU) has changed the clinical approach to anterior segment diseases of the eye. Traditionally, corneal, conjunctival and lacrimal gland diseases were considered single entities and treated separately. It is now clear that diverse, yet functionally related, endocrine and neural and immune factors are essential for the functional maintenance of this unit in order to preserve the integrity of the epithelial barrier and normal visual function through corneal transparency. In fact, the role of the immune system in the induction and amplification of DED cannot be overemphasized. Significant and confirmatory data from independent laboratories and research groups, as referenced here, along with ample clinical experience with the use of anti-inflammatories, suggest that modulation of the ocular surface immune response can be a highly potent strategy for the treatment of DED.

What are not so well understood are factors that regulate the chronic host response in DED. Much work still needs to be done to precisely delineate the interactions between the immune, nervous, and epithelial systems that perpetuate chronic disease in dry eye. Areas that require further investigation call for a better understanding of the regional lymphoid tissue responses in DED and alterations in the function of regulatory T cells that may exacerbate dry eye disease severity. Optimization of cell (impression cytology) and fluid (tear film) procurement techniques that permit quantitative assessment of soluble or membrane-associated proteins and gene transcription (mRNA) are facilitating pathophysiologic studies. Advances in in-vivo imaging permit careful and prospective evaluation of the interaction of corneal nerves, epithelial cells, leukocytes, and lymphatics (Mantopoulos, 2010) in patients with ocular surface disease. This in turn will aid not only in a better understanding of pathophysiologic mechanisms, but also potentially lead to the development of more precise outcomes measures in clinical trials.

In summary, we have come a long way from the past decade in understanding the immunopathogenic mechanisms of dry eye and related ocular surface diseases. Whether a cause or consequence of dry eye, clinical and experimental studies suggest that inflammation plays a crucial role in the development of clinical disease in dry eye. Its regulation holds significant promise in therapeutic strategies. Given the significant attention

that ocular surface inflammation is now receiving in the R&D efforts of numerous academic and industry concerns, there is good reason to anticipate that in the near future several novel strategies will transform our approach toward DED.

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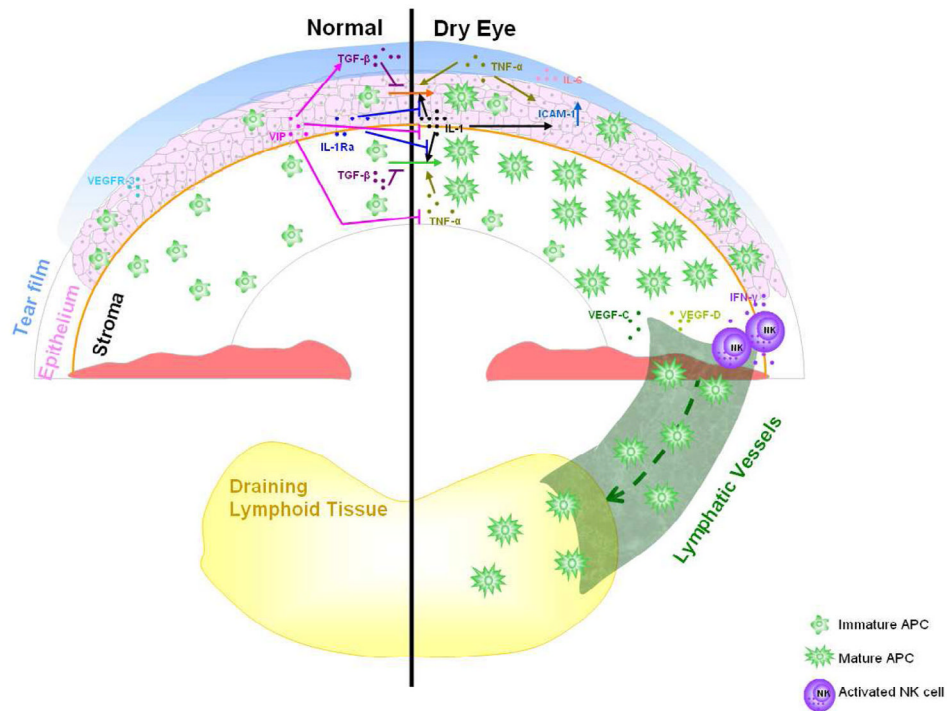
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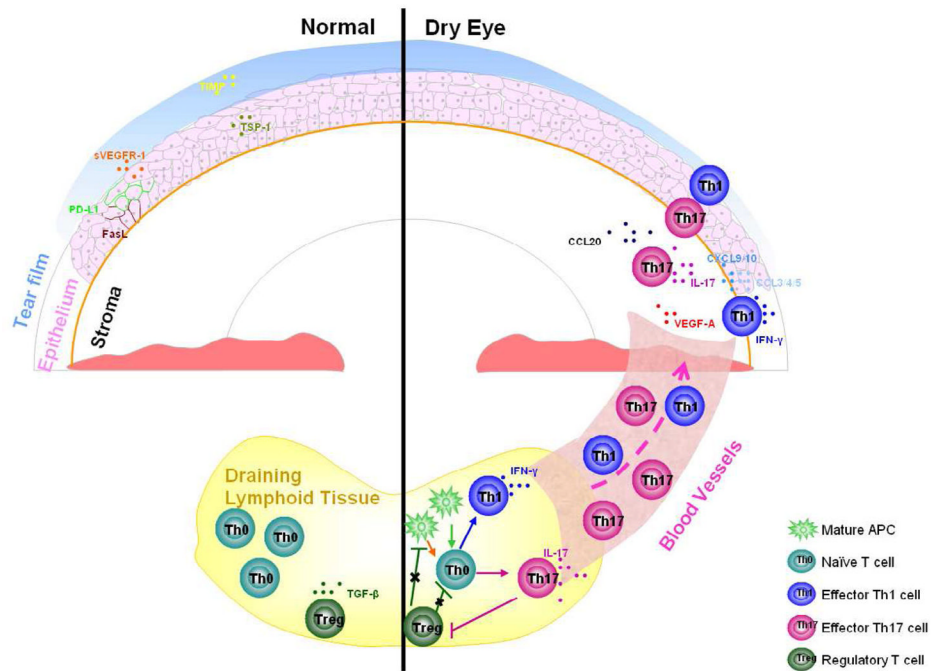


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**Figure 1. Major afferent immunoregulatory mechanisms on normal ocular surface and their disruption in dry eye disease**

The normal immunohomeostatic environment (left) is characterized by expression of vascular endothelial growth factor receptor-3 (VEGFR-3) by cornea epithelium, which binds and thus inhibits angiogenic VEGF-C and VEGF-D. Immature resident antigen-presenting cells (APCs) are present in normal cornea and are consisted of several populations, including  $CD11c^+ CD11b^-$  dendritic / Langerhans cells in the epithelium,  $CD11b^+ CD11c^{variable}$  dendritic cells in the anterior stroma, and  $CD11b^+ CD11c^-$  macrophages / monocytes in the deep stroma. The preponderance of APCs reside in the corneal periphery and limbal areas, with numbers tapering rapidly toward the center. In addition, anti-inflammatory factors such as TGF- $\beta$  and IL-1 receptor antagonist (IL-1 Ra) present on the normal ocular surface can regulate APC maturation by antagonizing the effects of pro-inflammatory cytokines. Vasoactive intestinal peptide (VIP) secreted by nerve endings in the cornea can down-regulate pro-inflammatory cytokines while up-regulate anti-inflammatory cytokines such as TGF- $\beta$ . Spreading on the cornea and conjunctiva, tear film is not only as a physical barrier but contains soluble mucins and several immunoregulatory factors. In dry eye (right), activation of innate NK response not only damages target tissues but promotes APC maturation through IFN- $\gamma$ . Pro-inflammatory cytokines IL-1, TNF- $\alpha$  and IL-6 released from stressed ocular surface epithelium causes epithelium damage, activates APC, and promotes expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) on ocular surface epithelium. Additionally, increased mature APCs on ocular surface migrate to draining lymph nodes (LNs) via newly-formed lymphatic vessels (facilitated by VEGF-C and VEGF-D).

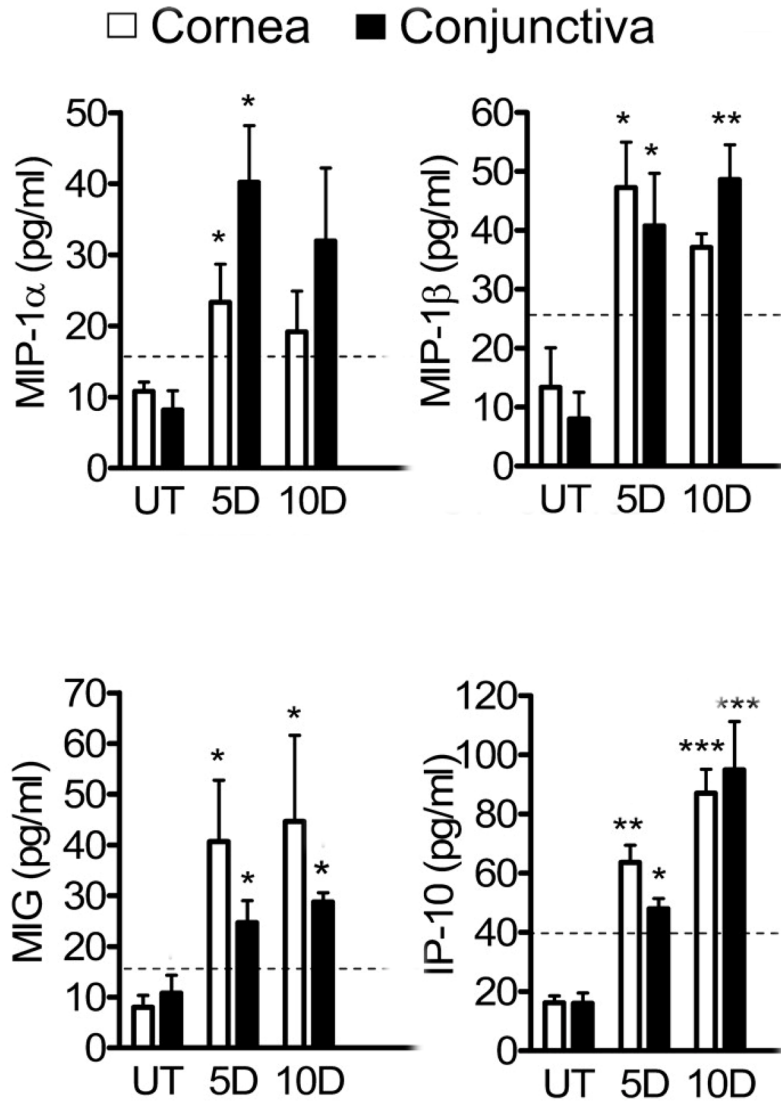


**Figure 2. Major efferent immunoregulatory mechanisms on normal ocular surface and their disruption in dry eye disease**

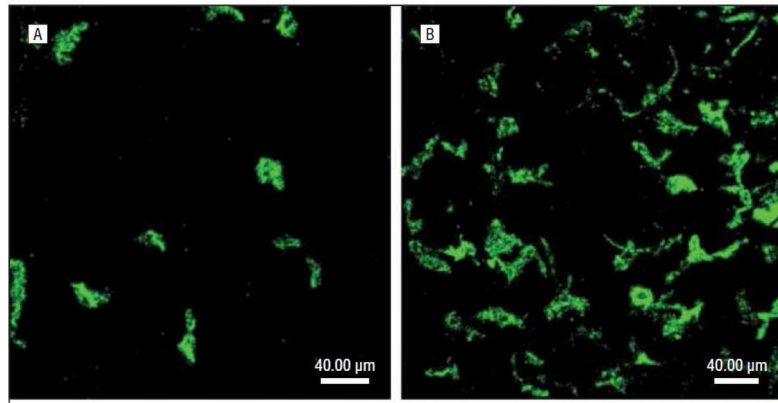
The normal immunohomeostatic environment (left) is characterized by expression of soluble vascular endothelial growth factor receptor-1 (sVEGFR-1) by cornea, which functions as endogenous VEGF-A trap. In addition, thrombospondin (TSP)-1 expressed by cornea, as well as tissue inhibitor of metalloproteinases (TIMPs)-1 and -2 contained in the tear film, are able to suppress corneal neovascularisation. Regulatory T cells (Tregs)-mediated suppression of naïve T cell priming in draining LNs could be through secretion of TGF- $\beta$  by Treg cells and cell-contact-dependent Treg / APC interactions. Programmed death-ligand (PD-L)1 and Fas ligand (FasL) are constitutively expressed by cornea, and their ligation with respective receptors PD-1 and Fas on activated T cells leads to the death of T cells. In dry eye (right), activation and expansion of IFN- $\gamma$ -secreting CD4<sup>+</sup> T (Th1) and IL-17-secreting CD4<sup>+</sup> T (Th17) cells occur in draining LNs with the help of mature APCs. Furthermore, dysfunctional Tregs can not regulate effector T cells, especially Th17 cells. These unrestrained effector T cells migrate from draining LNs to ocular surface via blood vessels (facilitated by VEGF-A) under the influence of increased levels of chemokines on ocular surface, including CCL3/4/5, CXCR9/10 (for Th1 influx), and CCL20 (for Th17 influx). Increased levels of IFN- $\gamma$  and IL-17 from activated T cells on ocular surface lead to corneal barrier disruption and decreased conjunctival goblet cell density.



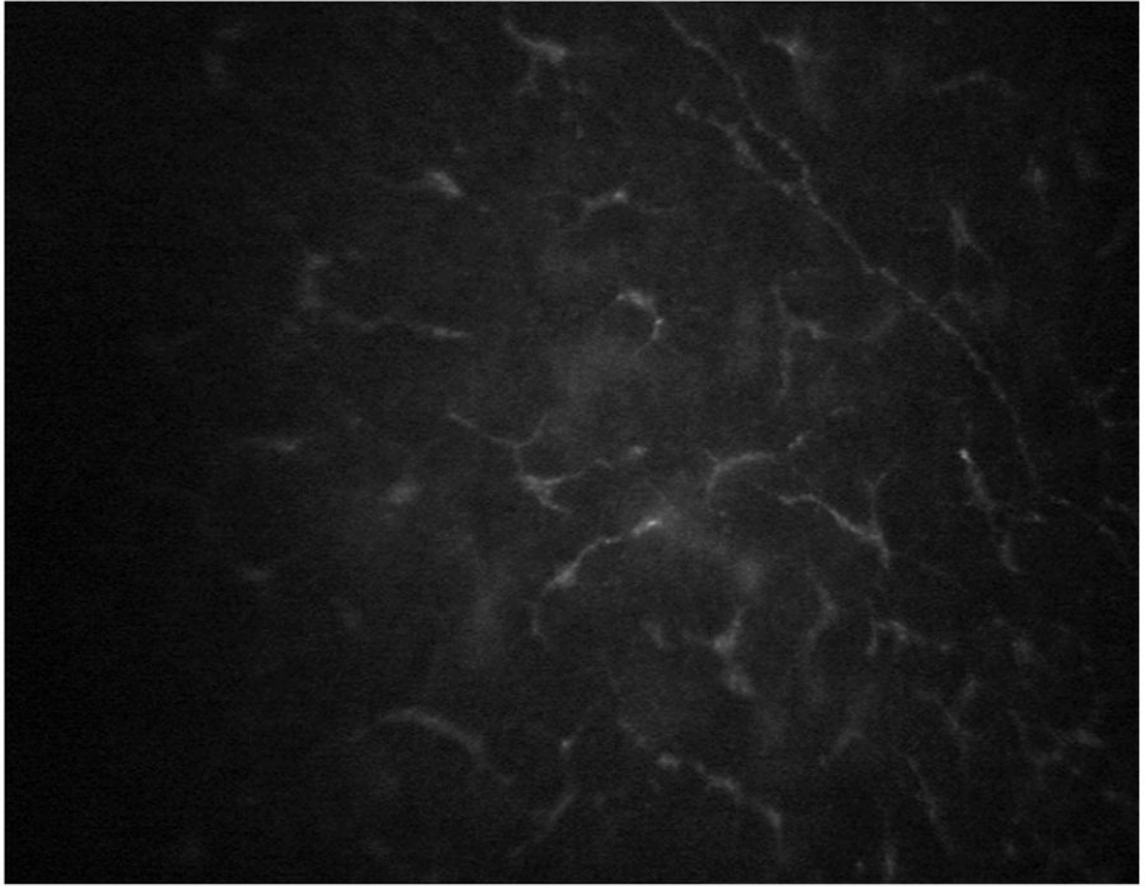
**Figure 3. Representative image of ocular surface damage in a patient with DED**  
Conjunctival hyperemia and positive lissamine green staining of conjunctival and corneal epithelia are clinical signs indicative for ocular surface inflammation.



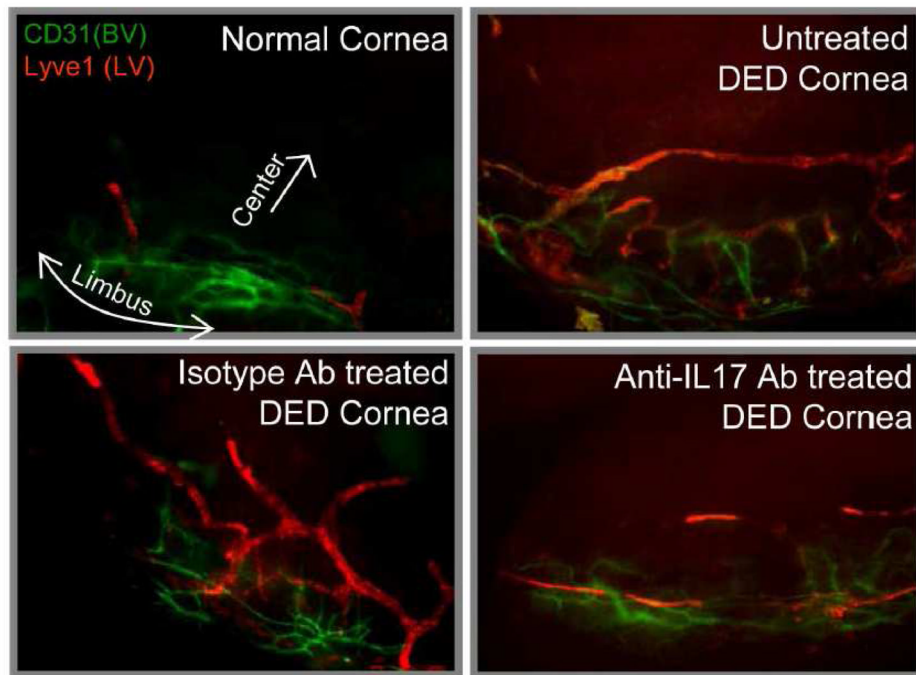
**Figure 4.** Concentrations of MIP-1α, MIP-1β, MIG, and IP-10 in the corneal epithelium and conjunctiva of untreated (UT) mice and mice with experimental dry eye for 5 (5D) and 10 (10D) days, as determined by immunobead assay. Data are expressed as the mean ± SEM. Dotted line: lowest value in the linear portion of the curve generated from the observed mean fluorescence intensities versus the observed concentrations. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs. UT. (Adapted and modified from Yoon et al., 2007).



**Figure 5.** Representative confocal images of center of whole-mount corneas showing CD11b<sup>+</sup> cells (green) in normal (A) and experimental dry eyes (B). (Adapted from Rashid et al., 2008).

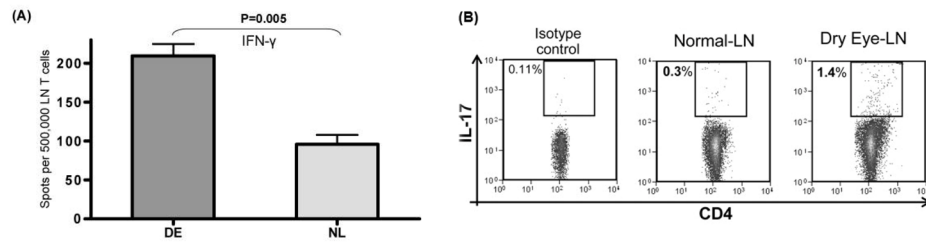


**Figure 6. In vivo confocal microscopy representative image (Confoscan 4, Nidek Technologies, Italy) of the cornea in a patient with DED**  
Numerous dendritic-like cells are demonstrated in the epithelial cell basal layer, whereas in normal age-matched controls very few of these cells are detected.



**Figure 7. Analysis of corneal lymphangiogenesis in normal and dry eye mice**  
Corneas were immunostained with CD31 (green) and Lyve1 (red) antibodies. Newly-formed lymphatic vessels (CD31<sup>lo</sup>/LYVE-1<sup>+</sup>) were seen in dry eye disease (DED) corneas. IL-17 blockade significantly reduced the lymphangiogenesis compared to untreated and isotype-Ab-treated DED corneas. (Adapted from Chauhan et al., 2011).





**Figure 8. Detection of IFN- $\gamma$ -secreting Th1 and IL-17-secreting Th17 cells in draining lymph node (LN) of normal (NL) and dry eye (DE) mice**

(A) ELISPOT assay for IFN- $\gamma$  secretion of T cells. The results are depicted as the mean number of spots per 0.5 million responder T cells loaded  $\pm$  SEM. (Adapted and modified from El Annan et al., 2009). (B) Flow cytometry analysis of Th17 cells ( $p = 0.026$ ). (Adapted and modified from Chauhan et al., 2009).

**Table 1**

Alterations in the cellular and molecular 'microenvironment' in dry eye disease

<b>Mechanisms</b>	<b>Normal ocular surface</b>	<b>Dry eye disease</b>
<i>Molecular level</i>		
Cytokines	TGF- $\beta$	IFN- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-17
Chemokines		CCL3, CCL4, CCL5, CXCL9, CXCL10, CCL20, CX3CL1
Other factors	sVEGFR-1/sflt-1, VEGFR-3, TSP-1, IL-1 Ra, TIMP, VIP, PD-L1, FasL	TLR, ICAM-1, VEGF-C, VEGF-D
<i>Cellular level</i>		
Immune cells	Immature antigen-presenting cell, Treg	NK cells, Mature antigen-presenting cell, Th1, Th17, Dysfunctional Treg
Corneal ingrowth of blood and lymphatic vessels	None	Lymphatic vessels

Table 2

Cytokine protein levels in tears and mRNA levels in the conjunctiva of patients with dry eye disease (DED) and normal controls

Cytokines	Tears		Conjunctiva		References
	DED mean $\pm$ SD (pg/mL)	Normal mean $\pm$ SD (pg/mL)	<i>P</i> value	mRNA fold increase	
IL-1 $\alpha$	253.7 $\pm$ 90	43.1 $\pm$ 24	<0.05	–	Solomon et al., 2001
IL-1 $\beta$	664.3 $\pm$ 148.8	436.3 $\pm$ 116.7	0.01	1.72	Massingale et al., 2009
TNF- $\alpha$	435.7 $\pm$ 145.6	250.6 $\pm$ 63.2	0.01	1.32	Massingale et al., 2009
IL-6	1625.7 $\pm$ 430.9	632.3 $\pm$ 167.9	0.001	2.48	Massingale et al., 2009
IL-23	–	–	–	~ 8	De Paiva et al., 2009
IFN- $\gamma$	458.3 $\pm$ 95.7	279.1 $\pm$ 45.4	0.001	~ 4	Massingale et al., 2009; De Paiva et al., 2009
IL-17A	423.0 $\pm$ 389.8	~ 845.2 $\pm$ 391.1	0.001	~ 3	De Paiva et al., 2009; Kang et al., 2011