

Mucosal immune tolerance at the ocular surface in health and disease

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Summary

The ocular surface is constantly exposed to environmental irritants, allergens and pathogens, against which it can mount a prompt immune response to preserve its integrity. But to avoid unnecessary inflammation, the ocular surface's mucosal immune system must also discriminate between harmless and potentially dangerous antigens, a seemingly complicated task. Despite its unique features, the ocular surface is a mucosal lining, and as such, it shares some homeostatic and pathophysiological mechanisms with other mucosal surfaces. The purpose of this review is to explore the mucosal homeostatic immune function of the ocular surface in both the healthy and diseased states, with a special focus on mucosal immunology concepts. The information discussed in this review has been retrieved by PubMed searches for literature published from January 1981 to October 2016.

Keywords: allergic conjunctivitis; conjunctival inflammation; dry eye; mucosal tolerance; ocular mucosal immune system; ocular surface.

Introduction

The ocular surface (OS), which comprises the cornea, the limbus, the conjunctiva and the tear film, contributes the largest portion of the eye's optical power, so playing a key role in the visual system. A sharp image on the retina can only be formed if light rays refract first through a clear, smooth corneal surface, and the latter depends on the protection afforded by its mucosal environment. To this aim, the surrounding limbus and the conjunctiva are armed with a fully fledged immune system because the OS is highly exposed to pathogens. Inflammation is, however, a double-edged sword, and as a proof of this, irreversible sight-threatening damage of the cornea and the conjunctiva is the end stage of many OS disorders when left untreated.¹ In addition to the inherent complexity of the cornea's immune privilege,² which is not the focus of this review, there are many unique aspects to the immune physiology of the OS, all of which have been extensively reviewed elsewhere: the tear film³ and the mucin layer/glycocalyx,⁴ the epithelial layer⁵ with its goblet cells,⁶ and

the conjunctiva-associated lymphoid tissue.⁷ But the OS could also be thought of as a specialized mucosal lining, and as such, it shares some homeostatic and pathological mechanisms with other mucosae. The purpose of this review is to explore the mucosal homeostatic immune function of the OS in both the healthy and diseased states.

Immune homeostasis in mucosal linings

From an immunological viewpoint, all mucosal linings are faced with a dilemma: whether to disregard or to mount an inflammatory response to the wide array of foreign antigens to which they are exposed. Mucosal surfaces can react vigorously because they are endowed with potent immune systems, but inflammation comes at a cost. Therefore, they strive for an uninflamed physiological state to allow for proper organ function, and to do so mucosal linings are armed with active mechanisms that keep the immune response at bay. Hence, lack of inflammation in the basal state does not imply the absence of

Abbreviations: DC, dendritic cell; DED, dry eye disease; IBD, irritable bowel disease; IL, interleukin; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B; NK, natural killer; OS, ocular surface; OVA, ovalbumin; TGF, transforming growth factor; Th2, T helper type 2; Treg, regulatory T; TSLP, thymic stromal lymphopoietin; VEGFR, vascular endothelial growth factor receptor

an immune response, but the supremacy of the aforementioned regulatory mechanisms. The latter are collectively referred to as mucosal immune tolerance, and in a strict sense they are a form of peripheral immune tolerance with both innate and adaptive immune components. Mucosal immune tolerance was first observed in the gastrointestinal tract, and it could be defined as “a state of local and systemic immune unresponsiveness that is induced after an innocuous antigen is delivered through a mucosal surface”.⁸

The mucosal epithelial lining, the actual barrier with the environment, is the main innate constituent of mucosal tolerance, as it plays a crucial role in the decision process of which type of immune response (regulatory or pro-inflammatory) to conduct.⁹ Intestinal epithelial cells are in close proximity to commensal bacteria, yet they do not initiate an inflammatory reaction aimed towards bacterial elimination.¹⁰ However, intestinal epithelial cells do keep commensal bacteria in check by secreting a thick mucus layer and antimicrobial peptides.¹⁰ Airway epithelial cells are constantly exposed to harmless airborne antigens, and the way they respond to these proteins determines a healthy state or allergic disease.¹¹ The molecular and cellular mechanisms vary greatly from organ to organ, but there is a common governing principle in mucosal immunity: the epithelial lining initiates and orchestrates the immune response. In the gut, epithelial cells secrete soluble factors such as transforming growth factor- β (TGF- β), interleukin-10 (IL-10), retinoic acid, prostaglandin E2 and thymic stromal lymphopoietin, all of which influence dendritic cells (DCs).¹² Most of these factors have also been detected in the airway epithelium, where they can be modulated directly by some allergens that trigger signalling through apical receptors.¹³

At any rate, the adaptive immune response that typifies mucosal tolerance is initiated by antigen-presenting cells, namely DCs, under the conditioning influence of epithelial cells. There are a few subtypes of epithelial and stromal DCs in each mucosal site, some of which seem more involved in pathogenic immune responses and others in mucosal tolerance.^{11,14} Recently, it has become clear that steady-state migration of DCs to the draining lymph nodes, a key step in establishing mucosal tolerance,¹⁵ is not simply a default program for these cells but a tightly regulated process in which nuclear factor- κ B (NF- κ B) signalling is deeply involved.¹⁶ However, the homeostatic upstream signals that activate this intracellular pathway in mucosal DCs remain to be identified. At least in the gut, there is evidence that luminal mucin delivers homeostatic signals to epithelial cells and DCs through its carbohydrate residues,¹⁷ and that passage of mucus-lined antigen from goblet cells to DCs leads to tolerogenic conditioning of the latter.¹⁸

Actual mucosal tolerance towards a specific antigen is carried out by regulatory T (Treg) cells, which inhibit innate immune cells, DCs and effector B and T lymphocytes that ultimately would drive mucosal inflammation.¹⁹ There are several subtypes of Treg cells, some of which originate in the thymus as a result of T-cell ontogeny (central or natural Treg cells), and others that develop in the peripheral lymphoid organs from naive precursors after contacting tolerogenic DCs (peripheral or inducible Treg cells). Natural and most inducible Treg cells are CD4⁺ Foxp3⁺ CD25⁺, and other inducible Treg cells are CD4⁺ Foxp3⁻ (Tr1 cells). There are also CD8⁺ Treg cells. The contribution of these subtypes to mucosal homeostasis varies from organ to organ,²⁰ and they also differ in how they suppress inflammation.¹⁹

The OS immune system

The OS comprises both innate and adaptive immune mechanisms that aid in maintaining its integrity (Fig. 1). Tear film clearance and regular blinking continuously remove antigens and microbes away from the OS,³ whereas the glycocalyx and the tight junctions in the apical cell layers of the conjunctival and corneal epithelia serve as a formidable physical barrier with immunomodulatory properties.^{4,21,22} In addition, tears favour the protective, tonic activation of stress response-associated transcription factors NF- κ B and activator protein 1 (AP-1) in these cells,²³ which influence mucosal immune responses.^{24,25} The epithelial layer itself is not a mere barricade, but an active component of the immune system: corneal and conjunctival epithelial cells secrete microbicidal and immunomodulatory peptides and cytokines and can respond to pathogen- and danger-associated molecular patterns through their functional Toll- and NOD-like receptor signalling system.^{26–29} Moreover, the OS epithelium expresses membrane receptors that modulate DC and lymphocyte function.^{28,30–32} On the one hand, corneal epithelial cells constitutively express programmed death-ligand 1, which deters lymphocytic infiltration by reducing chemokine secretion,³³ and also secrete vascular endothelial growth factor receptor 3 and pigment epithelium derived factor, which prevent neovessel growth and may also have an immunomodulatory role.³⁴ In addition, conjunctival goblet cells specifically modulate DCs through secretion and extracellular activation of TGF- β ₂ in a thrombospondin-1-dependent fashion.³² On the other hand, in the context of allergic disease, corneal and conjunctival keratinocytes produce thymic stromal lymphopoietin and favour a T helper type 2 (Th2) response.^{30,31} By contrast, these epithelial cells, when exposed to desiccating conditions, secrete chemokines CCL20, CXCL9, CXCL10 and CXCL11, which preferentially recruit Th1/Th17 effector T cells from the

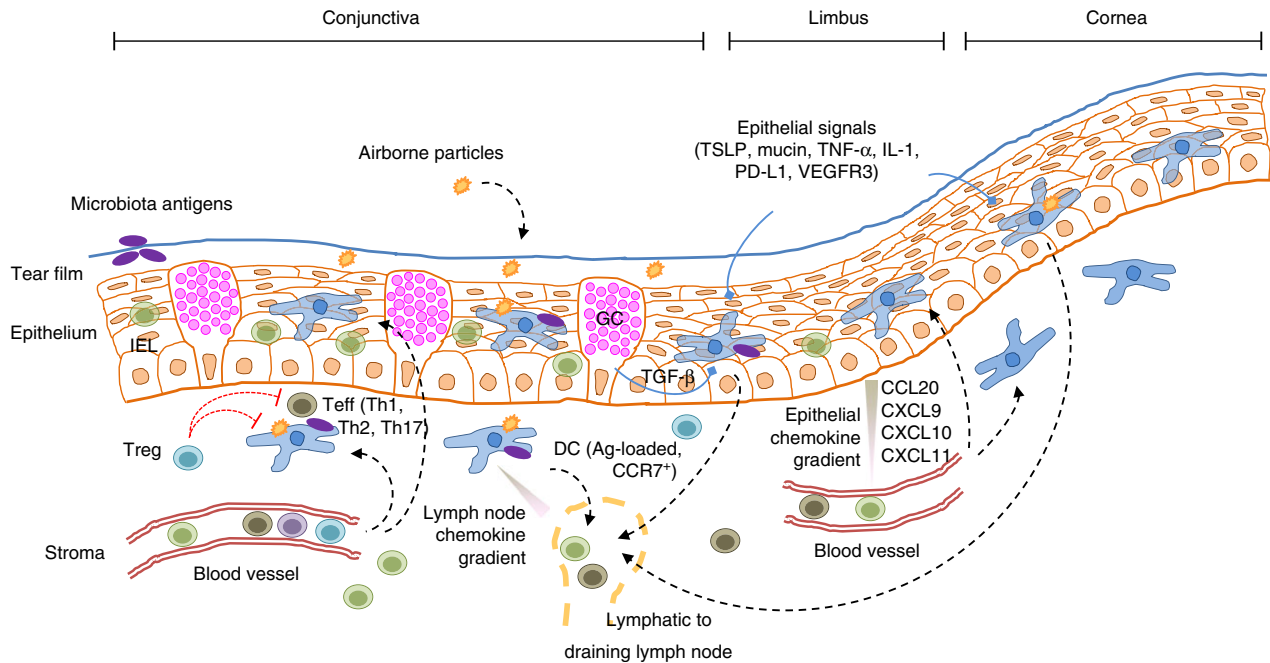


Figure 1. Some of the most relevant cell types and interactions in the ocular surface mucosal system. Airborne and microbiota-derived antigens (Ag) reach the tear film, the outermost layer of the ocular surface. Conjunctival and corneal keratinocytes and conjunctival goblet cells (GC) make up the epithelial barrier, and within their confines also reside epithelial dendritic cells (DC) and intraepithelial lymphocytes (IEL). There is extensive crosstalk between all these cell types through soluble factors and membrane receptors: transforming growth factor- β (TGF- β), thymic stromal lymphoectin (TSLP), tumour necrosis factor- α (TNF- α), interleukin-1 (IL-1), programmed cell death-ligand 1 (PD-L1) and vascular endothelial growth factor receptor 3, among others. Keratinocytes also secrete chemokines CCL20 and CXCL9-11, which attract blood-borne lymphocytes and DCs. Among lymphocytes, T cells play a pivotal role in mucosal tolerance, and there are regulatory (Treg) and effector (Teff) T cells. The former suppress inflammation, whereas the latter favour it.

circulation, and express membrane ligands that activate resident natural killer (NK) cells and favour interferon- γ release.^{35,36} Hence, differential 'sensing' of environmental challenges by the OS epithelium sets the stage for rather dissimilar immune responses.

In a strict sense, OS DCs initiate adaptive immune responses, either by priming naive T cells in the draining lymph node or by activating effector T cells *in situ*.³⁷ DCs are most abundant in the conjunctiva of humans and mice,^{38,39} and their density decreases from the limbus towards the central cornea under normal conditions.⁴⁰⁻⁴² All major non-lymphoid tissue-resident DC populations (CD11b⁺, CD103⁺ and plasmacytoid) have been identified in the normal murine conjunctiva, and these populations change under inflammatory conditions.^{41,42} The DC subtypes play different roles in maintaining homeostasis and inducing inflammation in other mucosal sites, and this also applies to the OS.⁴¹ Conjunctival CD11b⁺ DCs are more abundant, increase after allergic challenge and apparently induce more potent secondary allergic inflammation than CD103⁺ DCs.⁴¹ The CD103⁺ and plasmacytoid DCs might be more important for immune homeostasis, as observed in other mucosal sites,⁴³ but specific studies for conjunctival DCs are lacking. The

cornea, on the other hand, is also endowed with most, if not all, major DC subtypes.^{40,44,45} However, some of these DCs display features not found in other mucosal sites, such as no MHC II expression in the basal state.⁴⁶ As in other mucosal sites, the normal conjunctiva bears a sizeable lymphocyte population. Intraepithelial T cells are mostly CD8⁺, but NK and $\gamma\delta$ T cells are also abundant.⁴⁷ By contrast, CD4⁺ and CD8⁺ T-cell numbers are more balanced in the lamina propria.⁴⁸ Remarkably, the DC and T-cell subtypes that carry out immune tolerance at other mucosal sites are well characterized, but little is known about tolerogenic DCs and Treg cells in the OS. There is at least evidence for a homeostatic role of CD8⁺ Treg cells and CD4⁺ CD25⁺ Foxp3⁺ Treg cells in dry eye disease (DED),^{49,50} but it remains to be established whether these are natural or inducible Treg cells.

Finally, it should be noted that the OS is subject to a highly contrasting circadian rhythm because sleep-associated eyelid closure markedly reduces oxygen exchange and the secretion and clearance of tears.⁵¹ There is increased complement activation in the tear film during the first hours of sleep, which is later accompanied by a significant influx of neutrophils.⁵² Among other changes,⁵¹ hypoxia increases Toll-like receptor expression

in conjunctival epithelial cells,²⁷ and blinking induces physiological corneal epithelial cell exfoliation, which drops to a minimum during sleep.⁵³ In other words, a physiological but pro-inflammatory shift in the OS takes place daily in the closed eye.^{51,54,55}

Evidence for mucosal immune tolerance at the OS

Mucosal immune tolerance can be operationally defined and characterized by the immune system's ability to actively suppress systemic immunization against a specific antigen if such an antigen is administered beforehand through a mucosal surface.⁸ In other words, it is a form of mucosally induced peripheral tolerance that goes beyond the apparent local unresponsiveness to a given antigen, as it implicates an active, antigen-specific regulatory immune response with systemic implications. The existence of mucosal tolerance at the OS was first reported in 1994,⁵⁶ and the underlying immune mechanisms were addressed a few years later by Egan *et al.*,⁵⁷ who developed a murine model that involved the ocular instillation of a harmless antigen [ovalbumin (OVA)] and allowed tracking of the specific immune response. They showed that repeated ocular instillation of a low dose of OVA or single administration of a higher dose was sufficient to prevent subsequent systemic immunization with the same antigen and a strong adjuvant. Consistently, a similar experimental set up had been employed more than a decade earlier to characterize oral tolerance.⁵⁸

Moreover, Egan *et al.*⁵⁷ observed that after ocular instillation, OVA peptide-bearing antigen-presenting cells could be detected in the submandibular lymph node, but not in the conjunctiva or in other lymph nodes, so determining the anatomical site for T-cell priming during tolerance induction in the OS. The submandibular lymph node is also crucial for tolerance induction in the closely linked nasal mucosa.⁵⁹ In fact, both mucosal surfaces are connected through the nasolacrimal ducts, through which tears secreted onto the OS drain into the nasal cavity. Therefore, it could be argued that systemic tolerance after ocular instillation is an artefact induced by passive antigen drainage to the nasal mucosa and subsequent nasal tolerance induction. However, Chentoufi *et al.* later showed that both mucosal surfaces can independently respond to antigen after surgical nasolacrimal duct closure in rabbits.⁶⁰ More importantly, immune responses that originated in either site differed in the T-cell migration pattern, highlighting that each mucosal site imprints its own phenotype to the immune response despite their common draining lymph node.⁶⁰

Migration of antigen-loaded DCs from the mucosal lining is a prerequisite for tolerance induction, and these cells rely on CCR7 expression to follow a chemokine gradient stemming from the lymph nodes.^{15,61} CCR7 also

plays a key role in immunogenic DC migration from the OS,⁶² and it is likely to be involved in tolerance induction as well. Although there is no conclusive experimental evidence, antigens in the tear film are most likely sampled in the conjunctiva. The conjunctival epithelium is permeable to large molecules,⁶³ and its population of goblet cells might aid the delivery of antigen to lamina propria DCs, as shown in the gut.¹⁸ Moreover, healthy corneal epithelium seems to be quite impermeable to soluble proteins.⁶⁴ At any rate, migration of antigen-loaded DCs to the submandibular lymph node in mice peaks at 24 hr after single ocular instillation of antigen, and there it induces vigorous T-cell proliferation that peaks on day 3.⁵⁷ Remarkably, the OVA-specific T-cell adoptive transfer system that was used in those experiments suggested that some ocular antigen-loaded DCs might be migrating and activating T cells in distant lymph nodes. Consistently, other reports around that time suggested that antigen-loaded DCs could reach distal secondary lymphoid organs, such as the spleen, and activate T cells.^{65,66} More recent work, however, has apparently settled this issue in favour of initial T-cell priming at the local lymph node and rapid migration of activated T cells to other lymphoid organs, where they continue to proliferate.^{61,67} Of note, if the corneal epithelial barrier is mechanically removed in mice, topically applied antigen rapidly reaches the lymphatic system and even the spleen, but this probably represents a pathological situation and not the physiological setting.⁶⁴

Regarding the T cells responsible for conjunctival tolerance, Egan *et al.*⁵⁷ suggested that ocular instillation of antigen in mice leads to the development of anergic T cells. However, they did not perform the required experiments to properly identify Treg cell function,⁶⁸ and others have shown that oral tolerance induction after administration of a high antigen dose is indeed due to T-cell anergy,⁶⁹ but low antigen dose is conducive to specific T-cell suppression. More recently, we corroborated that low doses of antigen instilled onto the OS lead to the generation of antigen-specific Treg cells.⁷⁰ These Treg cells can be found in the lymph nodes and spleen after tolerance induction and readily suppress antigen-specific effector T-cell proliferation after transfer to naive recipients.^{71,72} Some of these cells are CD4⁺ ICOS⁺ Foxp3⁻ and secrete IL-10,⁷⁰ so resembling the Tr1 Treg cells that are known to mediate mucosal tolerance in the nasal and bronchial surfaces.^{20,73}

In summary, there is evidence from mouse studies that a mucosal adaptive immune response continuously takes place at the OS (Fig. 2). This process begins with corneal and conjunctival DCs picking up local antigens, which could originate from the environment, from the microbiota or even ocular autoantigens. In the basal state, the OS microenvironment imprints a tolerogenic profile on the DCs that migrate to the lymph nodes. Once there,

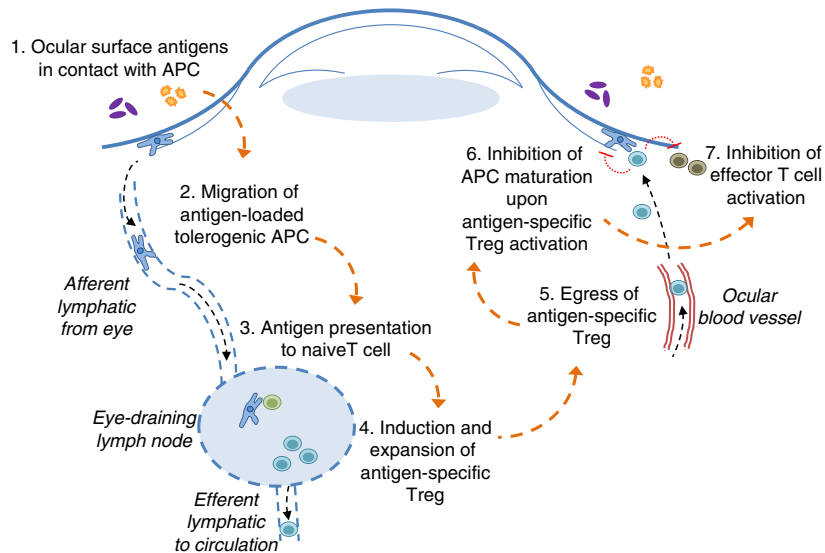


Figure 2. Ocular surface mucosal immune tolerance at work. (1) Airborne and microbiota-derived antigens (Ag) reach the ocular surface, where they are eventually taken up by antigen-presenting cells (APC). (2) In the basal state, still uncharacterized factors condition these APC to a tolerogenic profile, and also trigger their lymphatic migration to the draining lymph node. (3) Once there, APC present ocular surface-derived antigens to circulating naive T cells, (4) which upon specific recognition expand and become regulatory T (Treg) cells because of the tolerogenic profile that was previously imprinted on the APC at the ocular surface. Some Treg cells leave the lymph node and eventually reach the bloodstream, where they circulate indefinitely until they detect specific signals during their transit through ocular surface blood vessels. (5) After traversing the vessel wall, Treg cells come in contact with ocular surface APC that present the same antigen that they first encountered in the lymph node. (6) After this activation step, Treg cells deliver inhibitory signals to APC and to effector T cells, (7) which effectively suppress the local inflammatory response, thus promoting further tolerance induction to new antigens.

DCs encounter naive T cells to which they present antigens and induce a Treg cell phenotype. These Treg cells can then home to the OS, where they become specifically activated by their cognate antigen and exert their regulatory effect, so contributing to the non-inflammatory milieu and local homeostasis.

Mucosal immune tolerance in OS disease

For immune-mediated OS disease to develop, the mucosal homeostatic mechanisms must be overcome by the inflammatory stimuli that elicit the disease in the first place. Depending on the disorder, inflammation may be initiated by chemical or physical agents that damage the OS, by danger- and pathogen-associated molecular patterns that drive the innate response, by self and/or non-self antigens targeted by an adaptive immune response, or by a combination of them.

Dry eye disease

A few years ago, Stern *et al.* proposed that DED could be thought of as an autoimmune mucosal disease of the OS,⁷⁴ or in other words, as a localized autoimmune process that arises after OS immune tolerance is disrupted, probably by tear hyperosmolarity and/or microbial stimuli. The concept was initially supported by mouse studies

showing that the disease phenotype could be transferred to T-cell-deficient recipients by the CD4⁺ T cells from affected donors.^{75,76} These pathogenic CD4⁺ T cells readily migrate to the cornea, conjunctiva and local lymph nodes and are of a Th1/Th17 profile.^{47,75–77} As administration of exogenous antigen was not required to induce disease, it followed that the CD4⁺ T cells involved must be specific for some OS autoantigen. In fact, DED-specific autoreactive antibodies have been shown to contribute to OS damage in mice.⁷⁸ However, it should be noted that the OS is also exposed to potential antigens from the environment and the local microbiota, and so the specificity of the pathogenic CD4⁺ T cells could also include non-self antigens from these sources.

From a pathophysiology perspective, DED and inflammatory bowel disease (IBD) have striking common points. IBD is characterized by a Th1/Th17 T-cell response localized to the intestinal mucosa and can also be modelled in mice by adoptive transfer of naive CD4⁺ T cells to T-cell-deficient recipients.⁷⁹ Moreover, autoantibodies are readily detected in patients with IBD,⁸⁰ and therefore a strictly autoimmune aetiology also seemed likely at first. However, it was later found that germ-free mice do not develop intestinal inflammation in the adoptive transfer and other colitis models, which revealed the crucial role that the gut microbiota plays in IBD pathogenesis.⁸¹ In addition, among the many IBD susceptibility

genes isolated so far, those related to bacterial recognition and mucosal barrier function have the strongest association with disease.⁸² Because of all these findings, IBD is currently thought of as a disruption of mucosal tolerance towards the gut microbiota, which is elicited by still unidentified environmental factors in genetically susceptible individuals.⁸³

We have recently shown that mucosal tolerance to an exogenous antigen is indeed disrupted in two different murine models of DED, but this phenomenon is detectable only after continuous desiccating stress is exerted on the OS for 3 days.^{71,72} Remarkably, prevention of this change in the mucosal immune response by topical NF- κ B inhibitors was associated with reduced corneal damage under the same desiccating stress conditions, a finding that highlights its pathogenic role.^{71,72} The role of challenging environmental conditions initiating mucosal inflammation at the OS is well established. Tear hyperosmolarity resulting from increased evaporation is frequently observed in patients with DED,⁸⁴ and desiccating stress readily induces OS inflammation after 90 min in healthy subjects.⁸⁵ The aforementioned timing of mucosal tolerance breakdown in murine DED models is in line with the early events that take place at the OS epithelium under desiccating stress: expression of activating NK cell receptor ligands and secretion of Th1 chemokines³⁶ in the first 6 hr and the consequent burst of IFN- γ released by conjunctival NK cells that peaks in the first 3 days.⁸⁶ Within OS epithelial cells, hyperosmolar stress readily activates the mitogen-activated protein kinase (MAPK) pathway, which in turn up-regulates many

pro-inflammatory genes and leads to increased expression of several pro-inflammatory cytokines⁸⁷ and matrix metalloproteinases.⁸⁸ Matrix metalloproteinase-9 plays a crucial role in the disruption of the OS epithelial barrier, a hallmark finding of DED, and its secretion is further promoted by the IL-17 stemming from the adaptive immune response that later ensues.^{4,89} Moreover, interferon- γ secreted by NK and T cells favours goblet cell loss,⁹⁰ another key feature of DED, and these cells contribute significantly to the protective mucin layer of the OS and exert an immunomodulatory influence on DCs.³² Loss of the epithelial barrier probably accounts for the exacerbated inflammatory response to bacterial lipopolysaccharide that has been reported in DED.²⁸ The ocular microbiota that could serve as a source of these microbial by-products, however, still remains uncharacterized and subject to much debate.^{91,92}

All of these reports integrate into an alternative, non-autoimmune hypothesis for DED pathogenesis (Fig. 3): that harsh environmental conditions on the cornea and conjunctiva of susceptible individuals lead to a series of pro-inflammatory changes in the epithelial lining, which eventually disrupt the epithelial barrier and mucosal tolerance to the abundant exogenous antigens available at the OS, so setting the stage for the pathogenic Th1/Th17 adaptive immune response that potentiates corneal damage.

Ocular allergy

The four clinical forms of ocular allergy (allergic conjunctivitis, vernal keratoconjunctivitis, atopic keratoconjunctivitis

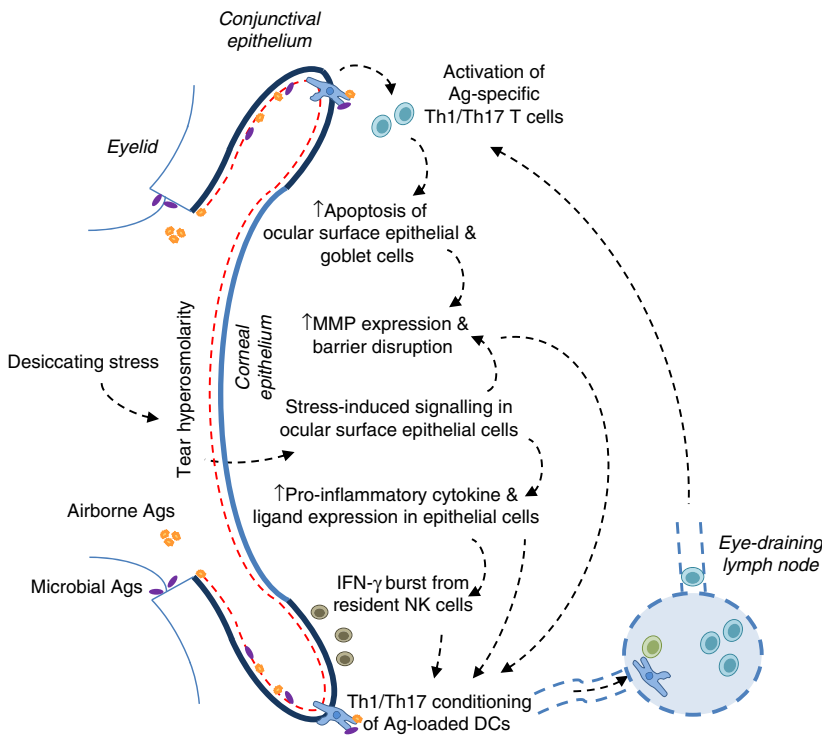


Figure 3. Alternative non-autoimmune hypothesis to dry eye disease pathogenesis. Desiccating stress (a common denomination for harsh environmental conditions) and tear film abnormalities lead to tear hyperosmolarity acting on ocular surface epithelial cells, which in response activate specific signalling pathways. As a result, there is an increase in pro-inflammatory mediators and subsequent epithelial barrier disruption, which in turn promotes the T helper type 1 (Th1)/Th17 conditioning of dendritic cells that capture microbial and airborne antigens that reach the ocular surface. Upon migration to the lymph node, these dendritic cells initiate an effector Th1/Th17 T-cell response that further fuels this vicious cycle at the ocular surface by contributing to epithelial cell damage.

and giant papillary conjunctivitis) differ in the contribution of IgE-mediated and non-IgE-mediated immune reactions in their pathogenesis,⁹³ but they share the hallmark Th2 adaptive immune response to an otherwise harmless antigen that typifies allergic disease. In other words, an allergic reaction at a mucosal surface is in fact a clinical example of the breakdown of mucosal tolerance to one or more specific antigens. The breach of tolerance that a mucosal allergic response actually represents has been studied most extensively in the context of oral tolerance and food allergy,⁸ but nonetheless still applies to other mucosal sites. In the context of ocular allergy, the most relevant aspects of mucosal tolerance are the pathogenic mechanisms that cause its disruption and the therapeutic opportunities offered by its manipulation.

The incidence of ocular allergy has been on the rise for the past two decades in both developed and developing nations, with some reports suggesting a prevalence of up to 20% of the population.⁹⁴ Increased exposure to air pollution is partially responsible for this phenomenon,⁹⁵ and several pathogenic mechanisms have been outlined.⁹⁶ Diesel exhaust particles increase oxidative stress and induce a pro-inflammatory response in corneal and conjunctival epithelial cells, with release of IL-6 and changes in mucin expression.^{97–99} Cigarette smoke has similar effects on the OS epithelium,¹⁰⁰ and at least for the airways, it prevents the induction of mucosal tolerance to aerosolized protein.¹⁰¹ DCs migrating from the lungs of smoke-exposed mice exhibit an immunogenic phenotype,¹⁰¹ which facilitates Th2 sensitization.¹⁰² The OS mucosal response has not been analysed under comparable conditions, but given the evidence that a pro-inflammatory response does take place at the OS epithelium,^{97–100} it is plausible to consider a similar disruptive effect on the local mucosal response. In this regard, it is interesting to consider the evidence of increased DED incidence and severity in smokers,^{103,104} an OS disease with an entirely different immunopathology but that shares the kick-start of mucosal tolerance disruption in its pathogenesis.^{71,72}

The therapeutic aspect of OS mucosal tolerance has been explored for vaccination purposes¹⁰⁵ and for treating uveitis,⁵⁶ but not specifically for ocular allergy. Another form of mucosal tolerance, oral tolerance (generation of inducible Treg cells after antigen ingestion), was shown to be effective in mice with allergic conjunctivitis. Such an approach involved transgenic rice seeds engineered to express pollen allergens,¹⁰⁶ an ingenious way of obtaining the large amounts of antigen required for oral tolerance induction. As Treg cells induced at a particular mucosa are expected to preferentially home to that mucosal site, it would be expected that Treg cells induced after ocular instillation readily suppressed antigen-specific ocular allergy. This is indeed the case for OVA-induced allergic conjunctivitis in mice,¹⁰⁷ and similar strategies might be successful for treating ocular and extraocular allergy in patients.^{108,109}

Eyedrop preservative toxicity

Toxicity from eye drop preservative toxicity is frequently observed in glaucoma patients under long-term medical treatment.¹¹⁰ Benzalkonium chloride, a commonly used preservative, induces epithelial cell death, pro-inflammatory cytokine secretion and inflammatory infiltration of the OS.^{111,112} In addition, this preservative readily disrupts conjunctival immune tolerance in mice, in part by conditioning DCs migrating from the OS to the lymph nodes, which in turn induce effector T cells instead of tolerogenic Treg cells.⁷⁰ This observation could explain the increased incidence of allergic reactions and DED in preservative-exposed patients, and the concept was demonstrated in a murine model of allergic conjunctivitis.¹⁰⁷ In brief, physiological OS immune tolerance to harmless antigen protects from subsequent allergic conjunctivitis even if mice are actively immunized with a potent adjuvant such as alum. However, if tolerance is affected by simultaneous instillation of the antigen and the preservative, mice develop full-blown allergic reactions. Remarkably, benzalkonium chloride-induced NF- κ B activation in the OS epithelium appears to be a key event in the pathophysiology of this model, as topical co-delivery of NF- κ B inhibitors completely prevents the subsequent allergic reaction. These findings are consistent with the widely described role of the epithelial NF- κ B pathway in mucosal tolerance.^{9,25} It is still unclear how the preservative leads to increased NF- κ B activation in ocular epithelial cells, although its reported effect on the Wnt/ β -catenin intracellular pathway in corneal epithelial cells could be a link.¹¹³ In any case, the iatrogenic nature of eyedrop preservative toxicity provides a unique therapeutic opportunity, for the event that incites the breakdown in mucosal homeostasis is known.

Therapeutic manipulation of ocular mucosal tolerance

In the same way that current models of extraocular mucosal immunology can improve our understanding of OS pathophysiology, therapeutic strategies for extraocular mucosal disorders might apply to the eye. As was mentioned earlier for ocular allergy, antigen-specific mucosal immunotherapy looks promising.^{108,109} In the context of ocular mucosal tolerance, there is also the prospect of modulating epithelial activation and specifically targeting DC conditioning, effector T cells and Treg cells.

Within corneal and conjunctival cells, there are two major signalling pathways that have been studied extensively: MAPK^{87,114–117} and NF- κ B.^{70–72,107,118} Both have significant impact on the mucosal immune outcome, as they control the extent of epithelial cell activation and the subsequent programming of local DCs. Activation of MAPK in OS epithelial cells leads to secretion of pro-inflammatory factors IL-1 β , IL-8, tumour necrosis

factor- α and metalloproteinase-9,¹¹⁷ and the activation of the NF- κ B pathway induces a comparable array of pro-inflammatory mediators in corneal epithelial cells.¹¹⁹ Remarkably, two antibiotics that are in clinical use for DED and meibomian gland dysfunction, doxycycline and azithromycin, are known to inhibit the activation of MAPK¹¹⁶ and NF- κ B,¹¹⁹ respectively, in these cells, and these intracellular effects might account for their clinical efficacy at improving DED signs.¹²⁰ In line with this, NF- κ B inhibitors delivered topically to the OS can improve disease outcome measures in animal models of DED, allergy, preservative toxicity and corneal burn.^{70–72,107,121}

Imprinting of OS DCs with a tolerogenic profile is probably an additional effect of topical NF- κ B inhibitors,^{70–72,107,121} as this pathway plays a pivotal role in DC maturation as well.¹⁶ There are additional molecular targets specific for DCs, such as topical blockade of CCR7, which prevents their migration to the lymph node in DED.⁶² However, CCR7 is also responsible for DC migration under homeostatic conditions in other mucosal surfaces,¹⁵ so there might be disadvantages to such an approach. Given the importance that retinoic acid and TGF- β have for the induction of tolerogenic DCs and Treg cells in the gut, it is readily apparent how the local delivery of either of these mediators improved disease score in mouse models of IBD.¹²² However, these findings might not translate to OS disease, where TGF- β seems to exert both anti- and pro-inflammatory effects.^{32,116,123} On the other hand, Treg-based immunotherapy is being explored for several mucosal disorders,^{124,125} and *in vitro*-expanded CD25⁺ Foxp3⁺ Treg cells can suppress OS inflammation in murine DED.⁵⁰ Regarding effector T cells, they are known to rely on their CCR6 receptor, which binds the CCL20 chemokine produced by corneal and conjunctival epithelial cells, to home to the OS.^{35,126} Hence, the strategy of blocking CCL20 by topical instillation of anti-CCL20 antibody to ameliorate murine DED is intriguing,³⁵ and is akin to successful approaches explored in other mucosal surfaces.¹²⁷

Finally, the ocular microbiome has become a hot research topic. Whether there is a stable microbiota in every OS is still subject to debate, but evidence is accumulating on the changes in microbial diversity in the context of eye disease.^{91,92,128,129} As the microbiota in other mucosal surfaces is known to have potent immunoregulatory functions, its therapeutic manipulation could be of benefit for OS disease. Tear levels of secretory IgA are reduced in germ-free mice, and this protein is known to promote mucosal tolerance by promoting IL-10 production and modulating DCs.¹²⁸ However, it is unclear whether this effect on IgA levels is exerted by the ocular microbiome or by commensals elsewhere. Intriguingly, intestinal microbial imbalance worsens DED in mice, and consistently, intestinal bacterial diversity is reduced in patients with DED.¹²⁹ Moreover, increased prevalence of a single bacterial species

on the ocular surface and increased specific systemic IgG titres are associated with the development of chronic OS inflammation in another mouse model of DED,¹²⁸ a finding that supports the non-autoimmune hypothesis for DED detailed above.

Conclusions

The ocular mucosal immune system shares many features with other mucosal surfaces, among others, the ability to mount an immunomodulatory adaptive response to the diverse harmless antigens that reach its confines. Hence, ocular mucosal tolerance is a crucial homeostatic mechanism, and at the same time, its disruption is perhaps the tipping point that skews the balance towards disease. Here we summarized the published evidence on such mechanism (or lack thereof) in the basal state and in several clinical entities. By doing so from a general mucosal immunology viewpoint, we established similarities and differences with the gut and the airways. As there is still much to learn about ocular mucosal immune pathophysiology, it should be of advantage to apply to its study the immunological models that have already been established for other mucosal sites.

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Disclosures

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