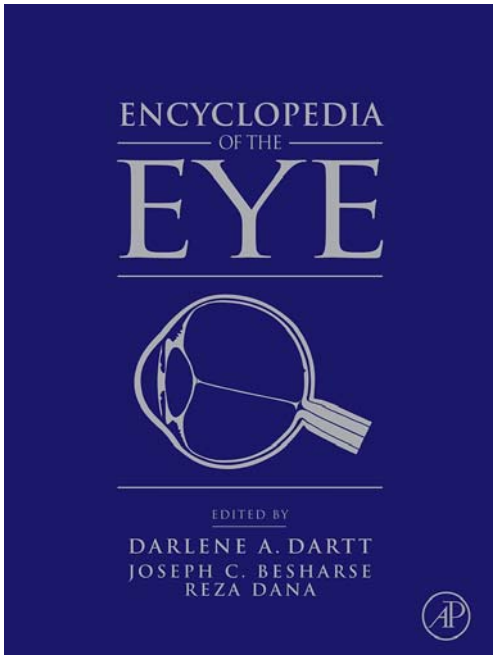

Knop E and Knop N Conjunctiva Immune Surveillance. In: Darlene A. Dartt, editor. Encyclopedia of the Eye, Vol 1. Oxford: Academic Press; 2010. pp. 356-368.

Conjunctiva Immune Surveillance

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Glossary

Conjunctiva-associated lymphoid tissue (CALT) – It is the physiological protective mucosal immune tissue of the conjunctiva. It consists of lymphoid cells and accessory cells inside the mucosal tissue and can be divided into the epithelial and underlying connective tissue (lamina propria) compartments. It is arranged as a diffuse lymphoid effector tissue along the whole extension of the conjunctiva and has interspersed organized lymphoid follicles for afferent antigen uptake and effector cell generation.

Dendric cells (DCs) – They are a special class of professional antigen-presenting cells (**APC**, together with macrophages and B-cells). They take up external antigens, degrade them into small fragments (epitopes), present them on MHC-class-II to T-helper cells, and hence, induce immune reactions. Depending on their maturation status which is influenced by the presence of inflammatory signals, they modulate between the inductions of tolerance versus inflammation. They also link the unspecific innate to the induced specific immune system and are hence key modulators of the immune reaction.

Eye-associated lymphoid tissue (EALT) – This tissue summarizes all the lymphoid tissues of the extended mucosal ocular surface, that is, of ocular surface proper (conjunctiva and cornea) along with its mucosal adnexa (the lacrimal-drainage-associated lymphoid tissue, LDALT, and the lymphoid cells inside the lacrimal gland). EALT is in line with the mucosal immune system in other parts of the body (e.g., gut-associated lymphoid tissue (GALT) in the gut and bronchus-associated lymphoid tissue (BALT) in the airways).

High endothelial venules (HEVs) – Specialized postcapillary venules that have an endothelium of bright roundish cells compared to the ordinary flat dense ones. They are located in lymphoid tissues and have tissue-specific adhesion molecules (vascular addressins) on the cell surface that specifically interact with homing receptors on circulating lymphocytes in order to maintain a regulated immigration of lymphocytes into the tissue.

Human leukocyte antigen (HLA) – A system of the major histocompatibility complex (⇒ MHC) in humans. It contains of a number of genes and their respective encoded proteins (that can act as antigens). The term HLA is frequently used to describe immunological self and nonself in the context of transplant rejection.

Intercellular adhesion molecule 1 (ICAM-1) – An adhesion molecule (CD54 according to the immunological cluster of differentiation, CD, nomenclature) mainly on vascular endothelial cells which is upregulated in inflammation and promotes the increased immigration of leukocytes, that carry corresponding integrin receptors, into the tissue.

Membraneous cells (M-cells) – Also called microfolded cells, they are a special type of cells in the modified epithelium overlying organized lymphoid follicles, the so-called follicle-associated epithelium (FAE). Their name refers to the fact that they have a different, usually smooth, surface ultrastructure compared to the ordinary epithelial cells. They form cellular pockets populated by groups of leukocytes which are separated from the lumen by a thin luminal cytoplasmic sheet. M-cells actively transcytose luminal antigens for uptake by the leukocytes and their subsequent presentation to and activation of T- and B-cells in order to generate antigen-specific effector cells.

Major histocompatibility complex (MHC) – It is differentiated mainly into class-I and class-II. Their encoded proteins on the surface of cells perform the presentation of protein antigen fragments (epitopes) to immune cells. MHC-class-I is found on all nucleated cells and presents antigens produced inside the cell (either own or viral proteins after infection) to cytotoxic CD8 lymphocytes and natural killer cells. MHC-class-II, in contrast, occurs physiologically only on specialized antigen-presenting cells and presents foreign antigens to the CD4 receptor of T-helper cells. In inflammation, it can be upregulated by other cells.

Lipopolysaccharide (LPS) – A component of the outer cell membrane of the wall of Gram-negative bacteria that acts as an endotoxine. The presence of LPS, that is detected for example, by toll-like receptors, signals the pathogenic nature of antigens to the immune system and elicits a strong inflammatory reaction.

Tolerance – Immune tolerance is a status in which the immune system is in a state of nonreactivity to an antigen in order to prevent inflammatory tissue-destructive reactions. Tolerance is actively generated and directed not only against the bodies' own cellular self-antigens but also against nonpathogenic external antigens. If tolerance fails, autoimmunological disease or allergy may occur. Tolerance is the default mode of the mucosal immune system, including CALT and EALT, in order to preserve tissue integrity.

Conjunctival Morphology and Function Are Closely Interacting for Immune Surveillance

Epithelial Defense Mechanisms

Epithelial morphology and function

The conjunctiva is a moist mucous organ that consists of a surface epithelium and an underlying loose connective tissue (lamina propria), separated by the epithelial basement membrane. The epithelium of the human conjunctiva has, in contrast to small rodents (e.g., rat and mouse), a stratified nonsquamous morphology and consists of two to three cell layers of cubical cells in most parts. It becomes multilayered and assumes prismatic morphology at the fornix whereas it tends to become squamous toward the limbus (**Figure 1(a)** and **1(b)**). Interspersed mucus-secreting goblet cells occur inside the epithelium as single cells or in small groups as well as intraepithelial lymphocytes (IELs) that reside mainly in the basal layers (**Figure 2(b)** and **Figure 3(a)**) as well as dendritic cells (DCs), that have long narrow extensions, for uptake of antigens from the surface. The conjunctival epithelial surface is covered by small cytoplasmic protrusions (microvilli and microplicae) with a well-developed surface coat of filamentous projections (glycocalyx) that form a meshwork (**Figure 1(c)** and **Figure 2(a)**).

Epithelial Immune Surveillance Takes Care of Environmental Antigens

Physical and physicochemical barriers keep antigens outside

The structure of the conjunctival epithelium already contributes to basic protective mechanisms which can be considered as part of the innate defense. Epithelial cells are mechanically connected by desmosomes and have an apical belt of intercellular junctions including tight junctions that seal the intercellular space and limit the passive para-cellular leakage of antigens in and out of the tissue (**Figure 2(a)**). This physical cellular barrier is supplemented by the physicochemical barrier of the epithelial mucins, that consist of cell membrane-spanning mucins (glycocalyx) produced by the ordinary epithelial cells and of soluble mucins secreted by the goblet cells which mix with the aqueous phase. Together they form a layer in the range of few micrometers thickness, that is, a sticky gel to which microbes adhere and can hence be cleared by the constant renewal of the preocular tear film. Soluble protective factors, including secretory immunoglobin A (SIgA), are fixed to the mucin layer in order to make it an almost impenetrable and lethal barrier to antigens and in particular to microbes.

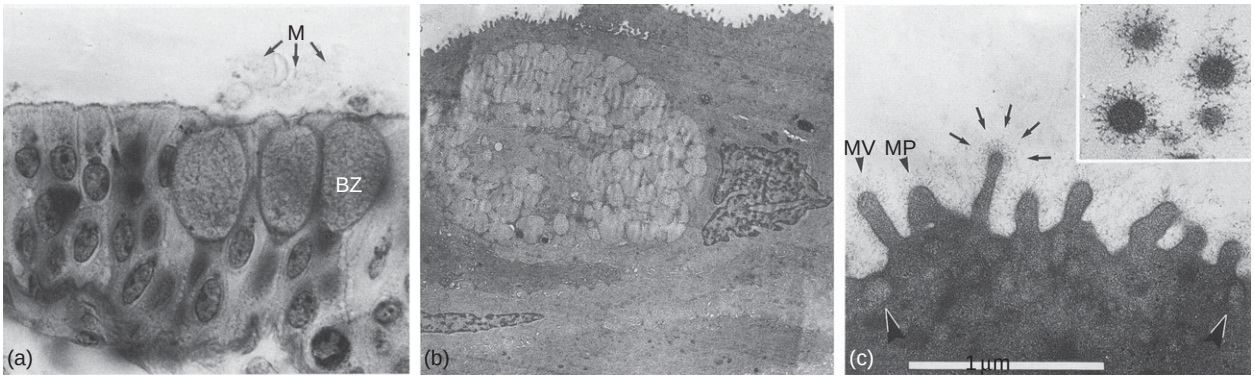

Figure 1 Structure of the human conjunctival epithelium. (a) The epithelium of the human conjunctiva is stratified cuboidal in most regions and assumes more layers with prismatic surface cells toward the fornix. Interspersed goblet cells (BZ) release mucus (M) tufts onto the surface. (b) Goblet cells contain densely packed mucin granules and a flat or triangular nucleus. They may be slightly inclined if located in the relatively flat bulbar epithelium close to the limbus. The surface of the conjunctival epithelial cells shows numerous microprotrusions that result in a rough surface in low-magnification transmission electron microscopy. (c) In higher enlargement, microvilli (MV) and microplicae (MP) are seen which have a dense glycocalyx of fine molecular antennae (arrows) that project into the lumen and form a meshwork, as better seen in cross section (inset, ×2). Reproduced from Knop, E. and Brewitt, H. (1992). *Morphology of the Conjunctival Epithelium in Spectacle and Contact Lens Wearers – A Light and Electron Microscopic Study*. Contactologia, Stuttgart: Enke Verlag.

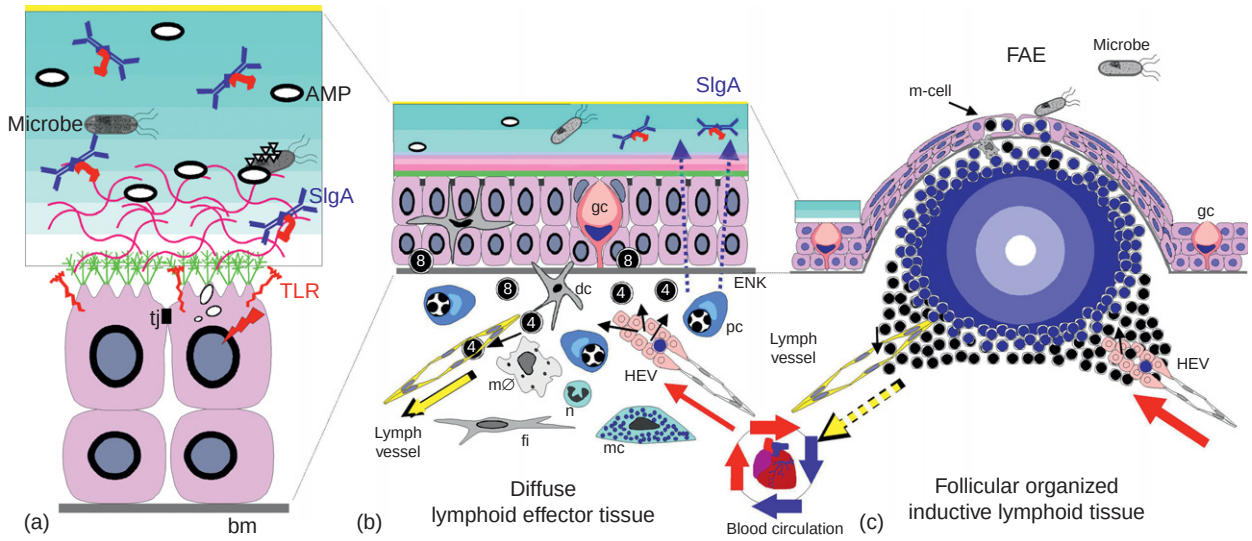

Figure 2 Defense systems of the human conjunctiva. (a) The conjunctival epithelium has an array of defense systems consisting of the integrity of the surface epithelial cells (provided with pattern recognition receptors, TLR) that are sealed by apical tight junctions (tj), of the attached mucin layer that is enforced by adhering antimicrobial proteins and peptides (AMPs) including specific secretory IgA (SIgA) and of the overlying tear film (shaded blue) that contains similar protective molecules and provides a washing effect. (b) A diffuse effector tissue is formed by lymphoid cells of the specific adaptive immune system and by innate cells such as macrophages (mø), mast cells (mc), neutrophilic granulocytes (n), and dendritic cells (dc). They functionally interact with stromal fibrocytes (fi). Lymphoid cells consist of $CD4^+$ and $CD8^+$ T-cells (black circles) that constitute intraepithelial and lamina propria lymphocytes. Differentiated B-cells (plasma cells (pc), large blue) produce dimeric IgA, which is transported through the epithelium as SIgA. (c) Interspersed solitary lymphoid follicles consist of B-cells (small blue circles), frequently have a bright germinal center due to cell proliferation, have an apical follicle-associated epithelium (FAE) with M-cells for antigen transport but without goblet cells (gc) and have para-follicular T-cell (small black circles) zones with lymph vessels (yellow) and high endothelial venules (HEVs); small arrows indicate the direction of cell migration. The mechanisms for conjunctival immune surveillance are explained topographically in this figure from the epithelium (a) over the diffusely interspersed effector cells (b) toward the organized lymphoid follicles that generate the effector cells (c). Functionally, it is reverse because the effector cells generated in lymphoid follicles after antigen uptake and presentation recirculate via the blood circulation (symbol of heart and blood flow between (c) and (b)) to and migrate into the diffuse effector sites to exert their protective function by cell contact or by soluble mediators. The drawing is not to scale.

The mechanical washing effect of the tear film wipes away antigens and detritus

The tear film is an important functional component of the ocular surface mucosal protection system. Apart from providing the necessary moisture, the constant flow of tears over the ocular surface and in particular, over the cornea, together with the wiping effect of the lid margin with every blink, provides a constant mechanical washing. This discharges antigens and removes dust and cell detritus. Other parts of the ocular surface along the retropalpebral tear film are not so rapidly cleared so that antigens can stay in longer contact with the epithelium. Therefore, the tear film contains a large number of antimicrobial factors that contribute more specifically to the innate immune defense.

Epithelial innate immune defense factors

The innate immune system uses pattern-related receptors (PRRs) that mainly detect conserved pathogen-associated molecular patterns (PAMPs) but also host antigens from destroyed cells. It reacts via effectors, which consist of soluble antimicrobial proteins and peptides (AMPs) which bind to the microbial cell wall in order to destroy it or which interfere with the microbial metabolism. The innate immune system also employs production of soluble mediators, such as inflammatory cytokines and chemotactic cytokines (chemokines) that functionally couple the innate and adaptive immune answer.

PRRs on epithelial cells provide an external alarm system

As soon as microbial antigens have breached the physicochemical barrier, they get in touch with epithelial PRRs (**Figure 2(a)**, the most prominent of which is presently the diverse family of toll-like receptors (TLRs). Binding of their ligands causes activation of the host cell via a MyD88-dependent signaling pathway that activates a nuclear transcription factor, nuclear factor kappa B (NFκB), and results in production of inflammatory cytokines such as interleukin 6 (IL-6), interferon gamma (IFN-γ), or tumor necrosis factor alpha (TNF-α). Subsequently, these induce the production of chemokines, adhesion molecules, and inducible AMP. Altogether this represents an inflammatory cascade with activation, first

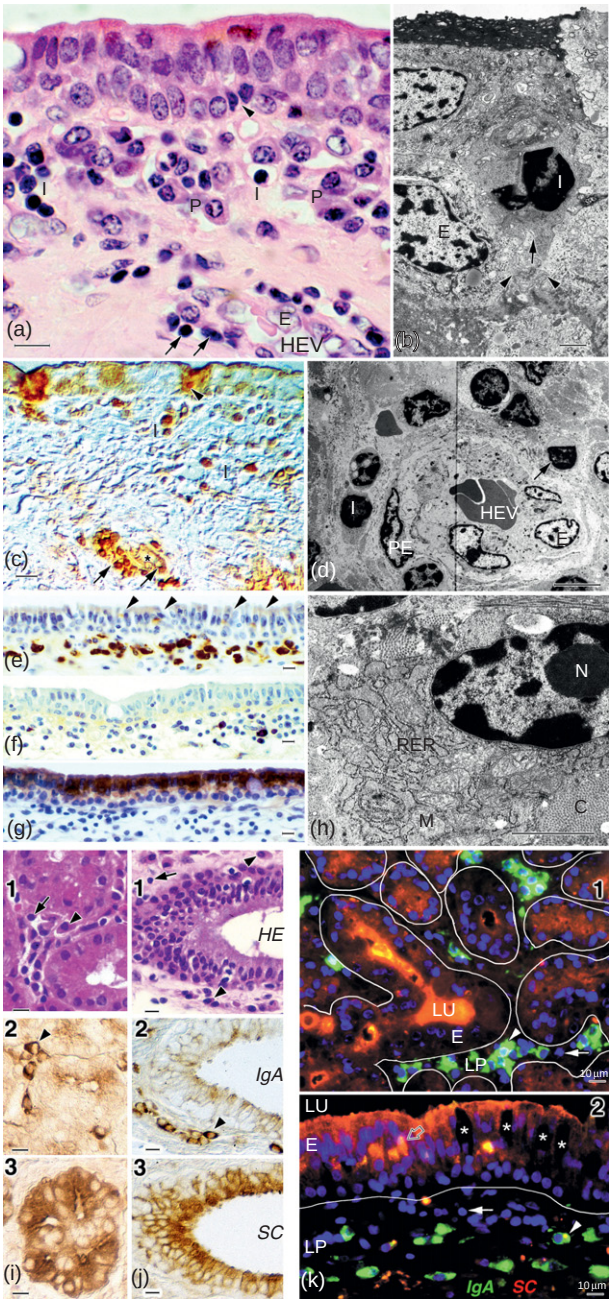

Figure 3 Characteristics of diffuse CALT. (a) Tarso-orbital conjunctiva. Plasma cells (P) and lymphocytes (l) form a diffuse lymphoid cell layer in the lamina propria covered by an epithelium with intraepithelial lymphocytes (arrowhead). A high endothelial venule (HEV) underneath has typical roundish endothelial cells (E) and contains lymphocytes within and around the wall (arrows). (b) TEM shows an intraepithelial lymphocyte (l) between epithelial cells (E) on the basement membrane (arrowheads). (c) Immunostaining indicates T-cells inside the epithelium (arrowhead), in the lymphoid layer (l) and around or in the wall (arrows) of a HEV (asterisk). (d) Ultrastructurally, a HEV shows large bright endothelial cells (E), a contractile pericyte layer (PE), and adjacent (l) or intramural (arrow) lymphocytes. (e) The vast majority of plasma cells in the lymphoid layer are IgA positive as also deposits in the epithelium (arrowheads), while IgM (f) is rare. (g) The epithelium is positive for the transporter SC. (h) A plasma cell lying in the loose collageneous (C) tissue has extended rough endoplasmic reticulum (RER), mitochondria (M), large nucleolus (N), and radial heterochromatin; (b, d, h: bar = 1 µm; a, c, e–g: bar = 10 µm). (i (1–3)) Lacrimal gland, LG, with lymphocytes (arrow), and plasma cells (arrowead) between the roundish acini. (2) IgA is found strongly in plasma cells and as weaker patchy staining in acinar epithelial cells, which more strongly express SC (3). (j (1–3)) Excretory lacrimal ducts that connect the LG to the conjunctiva have similar characteristics but in the epithelium IgA (2) and SC (3) are mainly expressed in the luminal layer (j) the duct has two cell layers but appears wider to the left due to oblique plane of section. (k (1,2)) Multiple-fluorescent staining for IgA (green), SC (red), and cell nuclei (blue) shows that the components of the secretory immune system are similarly arranged in the LG (1) and the conjunctiva (2, here orbital zone); bm level indicated by fine lines. IgA-positive plasma cells are diffusely interspersed in the LP of both tissues; in the LG frequently in groups. Epithelium (E) shows strong staining for SC;

of the epithelial cells and later also of lamina propria leukocytes and vascular endothelial cells. It induces leukocyte recruitment into the epithelium and their immigration from the blood stream into the tissue.

The normal conjunctival epithelium expresses a number of different TLRs, similar to the cornea. TLR1, 2, 3, 5, and 6 were found in all conjunctival and limbal epithelial cell samples, TLR4 and 9 only inconstantly, but not TLR7, 8, and 10. TLR2 may only occur upon stimulation by IFN-γ and bacterial cell wall extract, for example, in patients with ocular allergy. This results in upregulation of inflammatory markers, such as the intercellular adhesion molecule 1 (ICAM-1), human leukocyte antigen (HLA), TNF-α, and IL-8, in a dose-dependent manner. Bacterial-specific TLRs are of interest in ocular allergy because colonization by bacteria is common there. The activation of TLRs represents an important co-factor in ocular allergy and their blockade can significantly inhibit release of inflammatory mediators which may turn out as a promising new therapy option for ocular allergy.

The conjunctival epithelium secretes diverse AMPs

The spectrum of epithelial derived AMPs is distinct for cornea and conjunctiva but overlapping. Conjunctival epithelium produces not only the human β-defensins (hBD)-1,2,3 and further AMPs such as liver-expressed antimicrobial peptides (LEAPs) 1 and 2 and cathelicidin (LL-37) but also macrophage inflammatory protein 3alpha (MIP-3α) and thymosin beta 4 (Tβ-4). Some of the AMPs are constitutively produced, whereas others are inducible. hBD-2 is induced by inflammatory cytokines in ocular surface inflammation and by presence of bacterial LPS, while hBD3 is induced by infection and LL-37 by epithelial wounding. Conjunctival AMPs such as LL-37 are active against bacterial (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*) and viral (Herpes simplex virus 1, adenovirus) infection. They can act as multifunctional factors in wound healing and signaling pathways. Interestingly, the antimicrobial activity of some of these AMPs (e.g., hBD-1, hBD-2, and Tbeta-4) is almost completely inhibited in the presence of tear fluid. This may indicate that not all epithelial AMPs are produced in order to act as tear film factors but rather play a major role for local protection inside the conjunctival epithelium itself. Apart from AMPs, there is a plethora of other protective proteins. AMPs continue downstream in the lacrimal drainage system.

Conventional antibacterial factors are surprisingly versatile defense tools

Even the 'old fashioned' established antimicrobial proteins in the tear film, such as lysozyme and lactoferrin, have surprising newly detected functions. Apart from being bactericidal, either by lysis of components in the Gram-positive bacterial cell wall (lysozyme) or by interfering with their iron metabolism (lactoferrin), they are also antifungal and antiviral. Through the absorption of the strongly inflammatory bacterial endotoxin lipopolysaccharide (LPS), which is a surface molecule on Gram-negative bacteria, they also act anti-inflammatory. They have further anti-inflammatory functions by their influence on antigen-presenting cells (APCs) and hence appear as key elements in host defense that link innate and adaptive immunity.

Conjunctival Lamina Propria: Morphology and Function of the Diffuse Mucosal Immune System

Diffusely arranged lymphoid and innate cells contribute to conjunctival immune surveillance

The lamina propria contains bone-marrow-derived cells and vessels of different types. Apart from capillaries and lymph vessels, specialized high endothelial venules (HEVs) occur. Vessels serve for the supply with nutrition and discharge of metabolites, for hormonal regulation of the tissue, and also for the migration of immune cells. Lymphocytes, together with accessory leukocyte populations (macrophages, granulocytes, mast cells, and DCs), form a diffuse lymphoid tissue (**Figure 3(a)**) which is regarded mainly as an effector site of CALT although antigen uptake via DCs can also occur here to a certain extent. The diffuse lymphoid tissue is located in the vast majority of the surface, except for the solitary lymphoid follicles. The thickness of this cell layer depends on the location along the conjunctiva, shows a certain topographical variation, and is frequently only one to two cells wide, which may be a reason why these cells have often been overlooked in the past. It also shows a certain interindividual variation that may depend on the immune status of the person. IEL also functionally belong to the diffuse effector cells (**Figure 3(b)**).

Different subtypes of lymphocytes occur in the conjunctiva

Diffuse conjunctival lymphocytes are mainly $CD3^+$ T-cells (**Figure 3(c)**) (whereas $CD20^+$ B-cells are largely restricted to the solitary lymphoid follicles). They are activated ($CD45Ro^+$, $CD25^+$) and express the human

goblet cells (asterisks) are negative for SC. Mixed color indicating both proteins (=SIgA) is seen in the tubuloacinar lumina (LU) of the LG and frequently delineates the luminal cell surface. (a–h) Adapted from Knop, N. and Knop, E. (2000). Conjunctiva-associated lymphoid tissue in the human eye. *Investigative Ophthalmology and Visual Science* 41: 1270–1279. (i–k) Knop, E., Knop, N., and Claus, P. (2008). Local production of secretory IgA in the eye-associated lymphoid tissue (EALT) of the normal human ocular surface. *Investigative Ophthalmology and Visual Science* 49: 2322–2329.

mucosa lymphocyte antigen (HML-1, integrin αEβ7) which substantiates the integration of CALT into the mucosal immune system. In the epithelium $CD8^+$, cytotoxic/suppressor cells prevail that have been proposed to act mainly in the suppressor mode and may hence provide a component of the immune tolerance at the ocular surface. Lamina propria lymphocytes, in contrast, consist of equal or prevailing amounts of $CD4^+$ T-helper (Th) cells compared to $CD8^+$ T-cells. All known types of T-cells exist in CALT and their immune regulation is considered below (see the section titled 'Mechanisms of conjunctival immune regulation'). Plasma cells (see the section titled 'The conjunctiva contributes actively to the secretory immune system') account for about 20% of the conjunctival leukocytes in histology but their absolute number is in the range (2/3) of those in the lacrimal gland that was long regarded as the sole source of tear film SIgA. Conjunctival lymphocytes are even more abundant. This supports the concept that the conjunctiva considerably contributes to its own specific defense and it very well supports the recognition of a diffuse CALT along the whole extension of the human conjunctiva in tissue whole mounts.

The conjunctiva contributes actively to the secretory immune system

Specific soluble antigen-receptors (immunoglobulins, Ig) produced by local mucosal differentiated B-lymphocytes (plasma cells) and transported through the overlying epithelium constitute the secretory immune system. This is a major mucosal defense mechanism and is also present in the conjunctiva. Mucosal Ig mainly consist of polymeric (p)IgA that also forms the predominant Ig in the tear film, besides small amounts of the other polymeric Ig (IgM) and trace amounts of IgG. During eye closure, overnight IgA is the predominant protein in the closed eye tear film, but only recently the components of the conjunctival secretory immune system could be consistently verified by immunohistochemistry and polymerase-chain reaction.

Local conjunctival plasma cells produce mainly the anti-inflammatory IgA

The vast majority of conjunctival plasma cells produce IgA and hence stain positive for it in immunohistochemistry (**Figure 3(e)**). IgM, which performs the initial acute secretory immune answer, is rarely observed (**Figure 3(f)**) and hence indicates that the physiological conjunctival diffuse lymphoid cells do not reflect any kind of reaction to an acute insult. The epithelial transporter molecule for both of them (pIgR, represented by its extracellular domain secretory component, SC) is strongly expressed throughout the human conjunctival epithelium (**Figure 3(g)**). After transport, SC remains linked to pIgA which together constitute secretory IgA (SIgA). Conjunctival plasma cells show a typical ultrastructure in transmission electron microscopy (**Figure 3(h)**).

IgA-positive plasma cells in the lamina propria and SC in the overlying epithelium are continuously expressed from the lacrimal gland (**Figure 3(i)** 1–3) along its excretory ducts (**Figure 3(j)** 1–3) into the conjunctiva and further within the lacrimal drainage system. In multifluorescent immune staining, the secretory immune system of the lacrimal gland and the conjunctiva show the same characteristics (**Figure 3(k)** 1,2).

SIgA performs diverse protective and antiinflammatory functions at the ocular surface

SIgA is deposited onto the epithelial surface and into the tear film (**Figure 2(a)**). It contributes to the binding of specific antigens and to their immobilization and discharge. It binds to the surface of microbes and viruses and thereby limits their binding to and entrance into the tissue. It binds and thereby neutralizes bacterial toxins such as LPS. SIgA antibodies occur naturally to the physiological commensal ocular flora and are induced by the presence of pathological microbes, such as *Acanthamoeba* and *Pseudomonas*.

IgA does not only exert immune functions at the luminal ocular surface but also locally inside the tissue. IgA has a low complement-binding activity and hence acts in an antiinflammatory fashion. Bound antigens are opsonized to phagocytes which facilitates microbe uptake and destruction. IgA can bind to pathogens that have already penetrated into the tissue including intracellular viral particles. During the vectorial transport of pIgA toward the lumen, the bound pathogens are cleared from the tissue. IgA-bound antigens have an antiinflammatory effect on signaling networks and immune regulation inside the tissue by induction of the tolerogenic cytokines TGF-β and IL-10 and by limiting the activation of DC.

Lamina Propria Leukocytes Provide Immediate Innate Response against Invading Pathogens and can Orchestrate an Inflammatory Reaction

Apart from the lymphocytes, various other types of bone-marrow-derived leukocytes exist in the diffuse conjunctival effector tissue that are all not purely pathogenic, but exert important protective immune functions. Macrophages are reportedly frequent in immunohistochemistry but less obvious in conventional histology (**Figure 3(a)**). Phagocytes, such as macrophages, granulocytes, and DCs, can engulf and devour the invader but in addition, they act in different ways. Macrophages mainly destroy pathogens by internal digestion but are also capable to present epitopes of the pathogen on the MHC-class-II molecule (MHC-II) to $CD4^+$ T helper-cells for their subsequent activation. DCs digest pathogens mainly for this purpose. Granulocytes, also known as polymorphonuclear (PMN) cells, are usually the first cells that arrive at the site of acute defense reactions.

Protective functions of conjunctival lamina propria leukocytes

Neutrophile granulocytes are regularly observed in the normal human conjunctiva. They account for about 5% of the leukocytes and occur massively in the closed-eye tear film. In addition to their phagocytic capacity, they secrete several soluble factors such as antimicrobial proteins (e.g., lactoferrin, alpha-defensins, and cathelicidin) for immediate destruction of microbes. Furthermore, they secrete proteases (e.g., cathepsins, gelatinase, and neutrophile elastase) that lead to digestion of the extracellular matrix in order to provide space for accommodating the plethora of cells necessary to mount an effective inflammatory response. Eosinophile granulocytes are reportedly not observed in a normal conjunctiva but immigrate in parasitic infection. They produce chemokines and cytokines (e.g., RANTES [CCL5], TGF-β, TNF-α) for the activation and recruitment of other leukocytes including T-cells, as found in ocular allergy. Mast cells, apart from their potential physiological function, are mainly known for their inflammatory activity during IgE-mediated allergic disease where they release a variety of vasoactive mediators (e.g., histamine and heparin) and Th1 and Th2 cytokines (IL-4, IL-5, IL-6, and TNF-α) that can orchestrate an inflammatory response.

HEVs Provide the Regulated Immigration of Bone-Marrow-Derived Cells into the Tissue

The bone-marrow-derived cells that populate the conjunctiva all arrive here via the blood stream. Most of them stay here but lymphocytes, after being primed, and DCs, after antigen uptake, can also leave the tissue again via lymphatics. Although lymphocytes can emigrate through ordinary capillaries and venules, they do so with higher efficiency through conjunctival HEV via their tissue-specific adhesion molecules (lymphocyte homing molecules) that interact with vascular endothelial addressins. HEVs occur particularly in the para-follicular T-cell areas of lymphoid follicles (**Figure 2(c)**) but they are also found in the diffuse lymphoid effector tissue of the conjunctiva. Emigrated T-cells are frequently observed around HEV (**Figures 3(c)** and **3(d)**). Conjunctival HEVs are a normal component of the lymphoid tissue and they have a characteristic ultrastructure similar to that in other mucosal organs.

Conjunctival Lymphoid Follicles Have a Typical Morphology and Function

Solitary organized lymphoid follicles are interspersed into the diffuse effector tissue along the conjunctiva. They are relatively flat due to the limited space in the narrow conjunctival lamina propria but still show typical follicular characteristics. They consist of accumulations of B-cells (**Figure 4(a)**). The overlying epithelium changes its morphology toward the apex into a follicle-associated epithelium (FAE) by losing the goblet cells, by assuming a flat cell shape (**Figures 4(a)–4(c)**), and by a rarefied expression of SC (**Figure 4(c)**). Groups of lymphocytes, including $CD20^+$ B-cells (**Figure 4(a)**) and $CD3^+$ T-cells, occur inside the epithelium and are separated from the lumen just by a very narrow epithelial lining. Altogether, this is a conspicuous sign for the presence of specialized M-cells that form intraepithelial pockets populated by lymphoid cells. M-cells serve for the uptake and transport of luminal antigens toward the lymphoid cells in the pocket that can detect the antigen and present it to naive lymphocytes. The typical morphology of conjunctival M-cells and their antigen transport have been verified in a number of animal species, including guinea pig, turkey, chicken, rabbit, dog, and monkey. It has been shown that CALT is able, for example, to induce a tolerance against retinal antigens upon their topical conjunctival instillation.

The number of lymphoid follicles in elderly humans is relatively low (about 10 follicles per eye) with an average diameter of about 0.25 mm. Their small size again offers an explanation for the fact that CALT has frequently been overlooked in the past. They are more frequent in the upper conjunctiva than in the lower one (**Figure 5(a)**) and show a bilateral symmetry. In younger individuals, however, lymphoid follicles are more frequent and before puberty they occur in every person. Therefore, CALT follicles show a similar involution with age as observed for other locations of the mucosal immune system in general. Mucosal lymphoid B-cell follicles and their associated para-follicular T-cell zones (**Figure 2(c)**) serve for the generation of B- and T-effector lymphocytes, respectively (**Figure 7**).

The Topographical Distribution of CALT is in the Right Place to Assist Corneal Immune Surveillance

If the distribution of CALT is used to draw a topographical map (**Figure 5(b)**), it corresponds to the position of the cornea during eye closure. CALT in the tarso-orbital regions of the palpebral conjunctiva is then in the right position to support the immune protection of the cornea, which itself is largely devoid of lymphoid cells and other leukocytes. CALT may act during blinking as an immunological windscreen wiper and during sleep as an immunological cushion that covers the cornea (**Figure 5(c)**).

CALT can provide the cornea with innate and specific antibacterial peptides and proteins including SIgA that are not produced there. This concerns the usual daytime setting when the conjunctiva regularly glides over the cornea and wipes it clean. Even more so, CALT may be

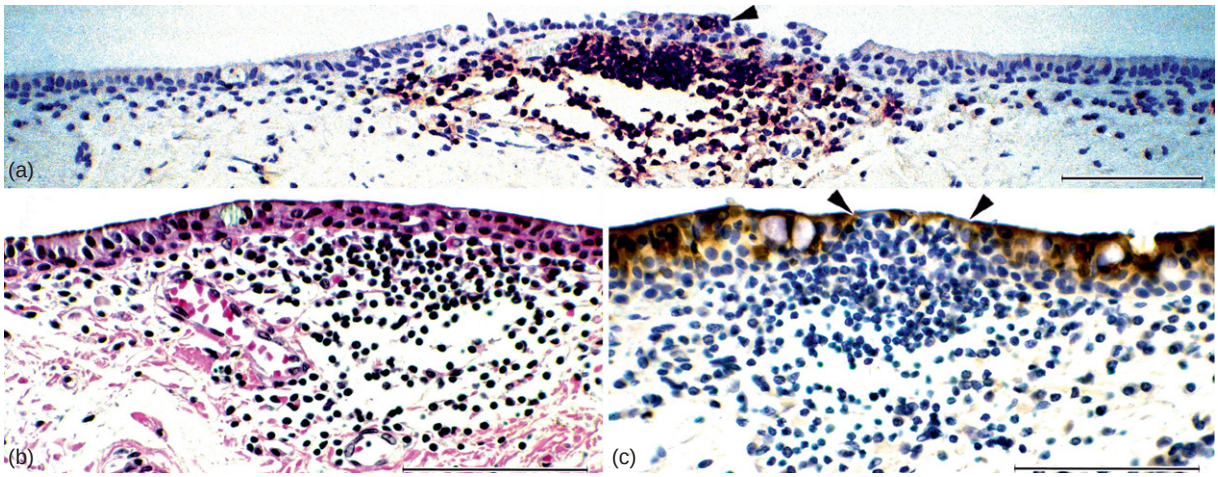

Figure 4 Characteristics of follicular CALT. (a) Even smaller lenticular lymphocyte accumulations are primarily composed of B-cells in immunostaining (brown dots). Over the apex, the overlying epithelium becomes flatter ((b), this follicle is not exactly met at the apex) and changes into a follicle-associated epithelium (FAE, between arrowheads in (c)), where goblet cells are absent, immunostaining for secretory component is rarefied (c) and numerous intraepithelial lymphocytes (IEL) are present in groups, suspicious for M-cells. The IEL are arranged in groups, including B-cells (arrowhead in (a)). Immunostaining for $CD20^+$ B-cells (a) and the IgA-transporter SC (c) of a small, almost flat, follicular accumulation that appears homogeneous, apart from disintegration at the location of the former germinal center, in HE staining (b); size bar in all figures = 100 μm. From Knop, N. and Knop, E. (2000). Conjunctiva-associated lymphoid tissue in the human eye. *Investigative Ophthalmology and Visual Science* 41: 1270–1279.

relevant during nighttime when the eye is closed. Then, an upregulated level of proinflammatory factors from PMN cells is obtained as a temporary approach in order to dampen the growth of the entrapped microorganisms that enjoy a comfortable environment without disturbance. Due to the intimate contact, CALT can also detect corneal antigens and generate respective effectors.

CALT is a Part of the Complete Eye-Associated Lymphoid Tissue

The conjunctival mucosa is, at the temporal and nasal side (**Figure 6**), anatomically continuous, through the lacrimal excretory ducts, with the lacrimal gland and through the lacrimal canaliculi with the lacrimal drainage system, respectively.

The histology clearly shows that a continuous mucosal immune system is also present from the periacinar tissue of the lacrimal gland throughout the conjunctiva into the lacrimal drainage system, that is, along the extended ocular surface. Together, this constitutes an eye-associated lymphoid tissue (EALT) and CALT is the regional part of it at the ocular surface proper (**Figure 6**).

EALT is an undividable anatomical and functional unit and its different parts support each other in function. EALT is in line with the other parts of the mucosal immune system of the body, such as gut-associated lymphoid tissue (GALT) in the gut or bronchus-associated lymphoid tissue (BALT) in the bronchi. Therefore, primed effector cells from EALT can be distributed by the regulated recirculation of lymphocytes (**Figure 2(b)** and **(c)**) within the mucosal immune system in order to repopulate the ocular surface mucosal tissues and other mucosa-associated lymphoid tissue (MALT) locations and in return, EALT can also share effector cells from other organs.

Mechanisms of Conjunctival Immune regulation

CALT Is Physiologically Biased to Tolerogenic, Anti-Inflammatory Responses

The mucosal immune regulation including CALT is maintained via the mode of antigen presentation by APC, on their MHC-class-II, to the T-cell-receptor (TCR) of naïve $CD4^+$ T-cells (To) and influenced by additional signals such as co-stimulatory molecules and the prevailing cytokine milieu within the tissue. This leads to the generation of different types of $CD4^+$ Th cells which produce characteristic cytokine patterns and have different functions (**Figure 7**). Due to the prevalence of nonpathogenic antigens and the delicate tissue construction, CALT is biased toward anti-inflammatory immune answers. Tolerance is also necessary in order to avoid autoimmune reactions against own tissue constituents by self-reactive T-cells.

Normally, CALT favors Th2 cells under the influence of cytokines such as IL-4. These interact with B-cells and produce cytokines (e.g., IL-4, IL-5, and IL-13) that promote B-cell Ig iso-type class switch to IgA and their differentiation into IgA-secreting plasma cell precursors

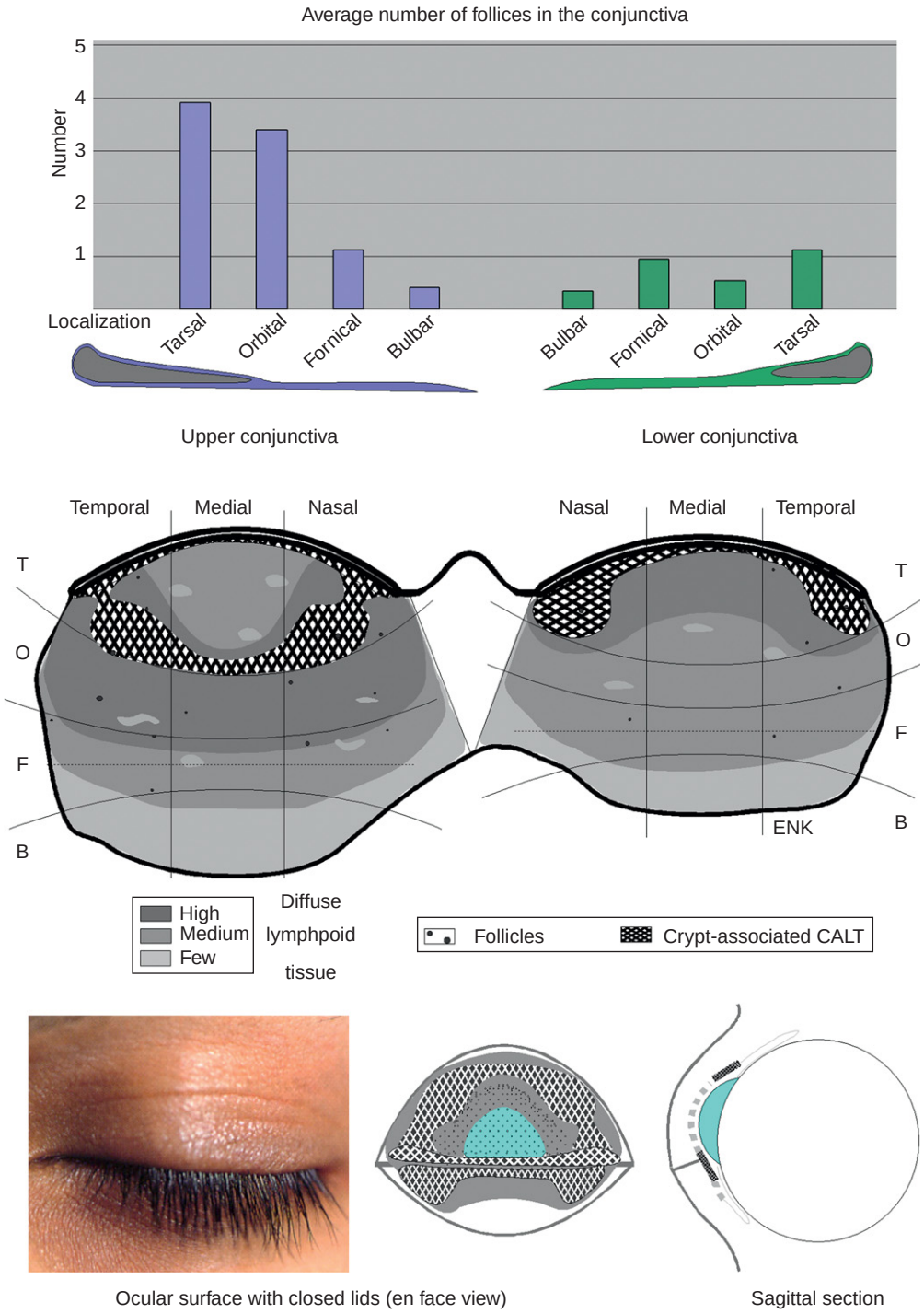

Figure 5 Topography of CALT – in the right position to assist corneal immune protection. (a) Morphometrical analysis of lymphoid follicles in the different zones (tarsal, orbital, fornical, bulbar) of upper (left) and lower (right) human conjunctiva, in a flat preparation of a tissue whole mount, shows a main expression in the tarso-orbital zones. More follicles are present in the upper lid and the average total number of CALT follicles in an elderly population is about 10 follicles per eye. (b) The topographical distribution of lymphoid follicles is the same as that of the diffuse lymphoid effector tissue in which they are interspersed, as seen in a topographical map of CALT in a complete flat whole mount of a human conjunctiva with the lid margin to the top and the nasal zone in the middle. The map differentiates the previously mentioned zones (T, O, F, B) as well as temporal, medial, and nasal locations. Increasing density of diffuse lymphoid cells is indicated as increased shades of gray. Hatched lines indicate the location of conjunctival crypts of Stieda that are associated with CALT. (c) If the topographical distribution of CALT is projected onto the bulbar surface in a closed lid situation, it is obvious that CALT covers the cornea as seen in frontal en face view (middle) and sagittal section (right). The central portion is covered by the tarsal crypts of Henle (open circles in middle figure) that are associated with CALT but not indicated in the topographical map in (b). From Knop, E. and Knop, N. (2003) *Eye-Associated Lymphoid Tissue* (*EALT*) *is Continuously Spread Throughout the Ocular Surface from the Lacrimal Gland to the Lacrimal Drainage System*. Der Ophthalmologe, Heidelberg: Springer.

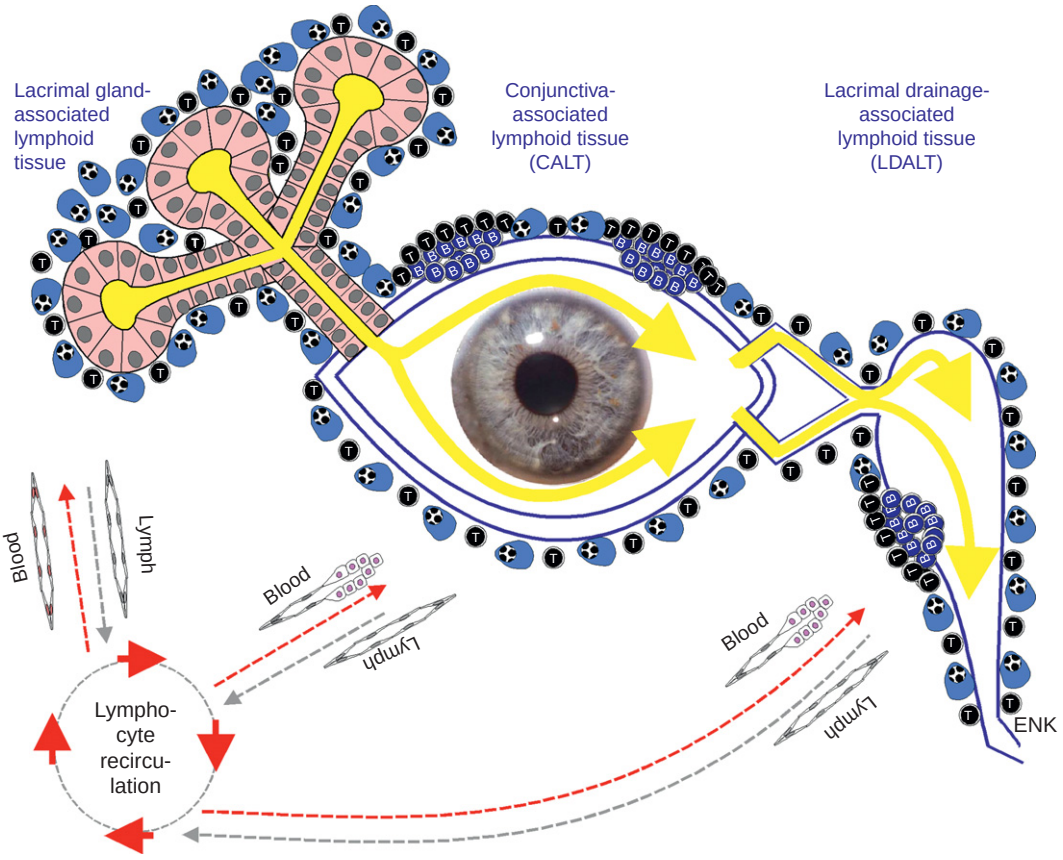

Figure 6 Eye-associated lymphoid tissue (EALT). Eye-associated lymphoid tissue integrates the continuous mucosal immune system of the conjunctiva (CALT) and its mucosal adnexa, composed of the lacrimal gland and lacrimal drainage system, which together form the extended functional ocular surface. The ocular tissues belong together embryologically and functionally. They are connected by the flow of tears (yellow arrows) which lets them share protective immune factors as well as antigens and they are furthermore connected by the regulated recirculation of lymphoid cells in the body via efferent lymph vessels and blood vessels. Lymphoid cells enter the tissues via blood vessels, including high endothelial venules (represented by roundish endothelial cells), and leave them via lymphatics. EALT is continuous throughout these organs in the form of a diffuse lymphoid effector tissue composed of T-lymphocytes (represented by black cells in the drawing) and plasma cells (represented by large blue cells) together with accessory leukocytes (not indicated here, compare **Figure 2(b)**). Inductive sites, in the form of lymphoid follicles, composed of B-cells (small blue cells) with adjacent para-follicular T-cell areas (compare **Figure 2(c)**), are present in CALT and LDALT. They serve for the uptake and presentation of antigens and for the subsequent generation of respective effector cells specific for the ocular surface relevant antigens. Not only the effector cells generated in EALT, but also those from other mucosal sites, can, after recirculation in the lymph and blood system, populate the diffuse lymphoid effector tissue that is present along the whole extended ocular surface including the large mucosa-associated gland (lacrimal gland). Adapted from Knop, E. and Knop, N. (2007). Anatomy and immunology of the ocular surface. In: Niederkorn, J. Y. and Kaplan, H. J. (eds.) *Immune Response and the Eye. Chemical Immunology and Allergy*. Basel: Karger Verlag.

and mature plasma cells as observed in the conjunctiva. Under the influence of mainly IL-6, the well-known inflammatory Th1 cells are formed that produce inflammatory cytokines such as IFN-γ and TNF-α which have the physiological function to activate cells, in particular phagocytes, to destroy intracellular pathogens. If inflammatory cytokines and other danger signals, such as bacterial LPS or components of dead cells occur in the tissue, they can bind to TLRs and mediate the secretion of excess inflammatory cytokines that skew CALT toward inflammatory immune answers. The antagonistic action of Th1 and Th2 cells led to the construction of the Th1–Th2 paradigm for explanation of immune regulation and phenomena at the ocular surface. In recent years, however, other anti-inflammatory (regulatory T-cells, Treg) and inflammatory (Th17 cells) were also observed which indicated that immune regulation is more complex (**Figure 7**) and needs further investigation.

Deregulation of EALT Is a Central Component of Inflammatory Ocular Surface Disease

Various stress mechanisms, for example, mechanical alteration, hyperosmolar tears or exposure to inflammatory cytokines can pathologically activate the ocular surface epithelium that responds by secretion of (further) inflammatory cytokines and proteases (such as matrix-metalloproteinase, MMP) and upregulates surface

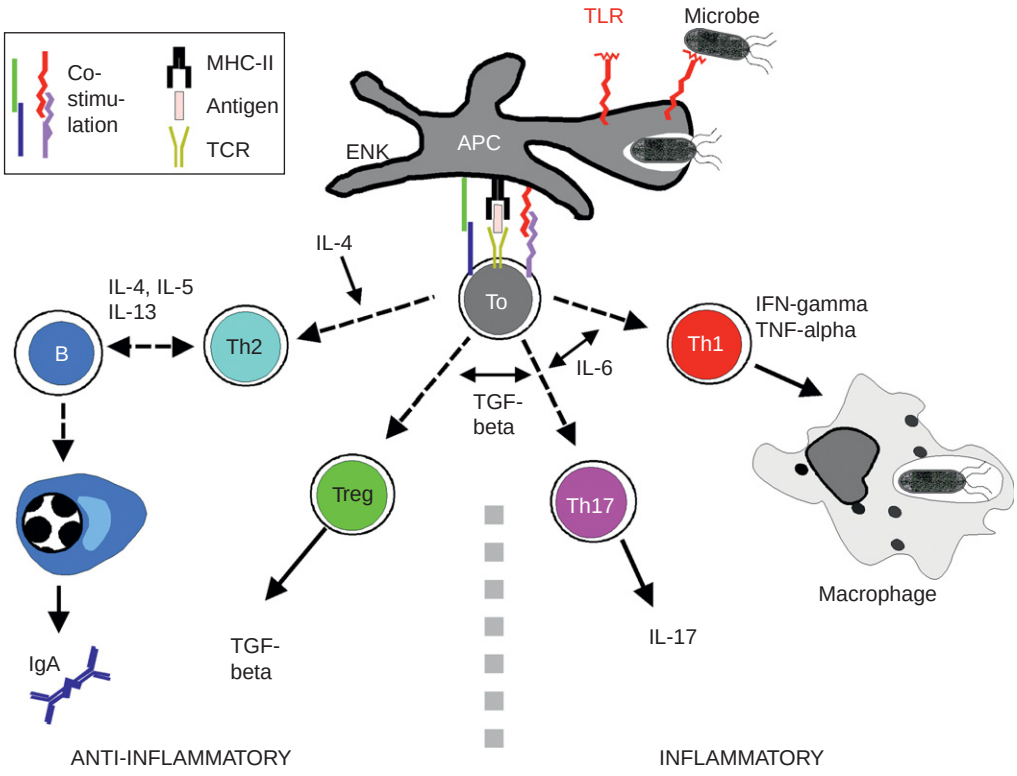

Figure 7 Generation of T- and B-effector cells in CALT. Immune regulation in CALT is governed via the presentation of antigens by antigen-presenting cells (APC) on their MHC-class-II (MHC-II) to the T-cell-receptor (TCR) of naive $CD4^+$ T-cells (To), and assisted and modulated by co-stimulatory molecules. This is influenced (indicated by uninterrupted arrows) by cytokines (interleukins, IL) and by microbial antigens that are recognized, for example, by TLR. This leads to the generation (interrupted arrows) of different types of $CD4^+$ T-helper cells (Th) that produce characteristic cytokine patterns and perform different functions. CALT is naturally biased toward anti-inflammatory immune answers. It favors the generation of Th2 that promote B-cells to differentiate into IgA secreting plasma cells. It also favors Treg that produce anti-inflammatory TGF-beta. Binding of microbial antigens to TLR and presence of IL-6 represents danger signals to the immune system and induces inflammatory immune answers via Th17 and Th1, the latter of which normally assist phagocytic cells to clear intracellular pathogens.

markers, such as ICAM-1 and MHC-class-II. This promotes an inflammatory process and through MCH-class-II the epithelial cells acquire the potential for presentation of antigens, including self-antigens, to resident conjunctival T-cells (**Figure 8(a)**) that can induce a loss of natural conjunctival immune tolerance.

Similar events are also shown for the acinar epithelial cells in inflammatory disease of the lacrimal gland where diverse perturbations result in altered intracellular protein traffic, alter the lacrimal acinar cell autoantigenic spectra, and upregulate MHC-class-II. This results in a loss of tolerance to own cell constituents, such as the M3 receptor with a subsequent autoimmune process. This again indicates that the natural tolerogenic bias can be lost in inflammatory disease and may be the underlying reason for a self-perpetuating inflammatory process at the ocular surface and its associated gland.

In fact, in inflammatory ocular surface diseases such as dry eye disease, autoreactive T-cells are generated that are specific to ocular surface tissue. They can be transferred and lead to destruction of the same ocular tissues in a naive recipient that has never experienced the pathological condition. Respective Tregs can prevent the tissue destruction and offer therapeutic potentials.

In addition, wounding can allow the entry of nonpathogenic antigens into the tissue and their presentation to T- and B-cells, as observed in ocular allergy. Downstream effects are the activation of conjunctival vascular endothelial cells that upregulate adhesion molecules (such as ICAM-1, VCAM-1, or E-selectin) with subsequent recruitment of further leukocytes from the vascular compartment and the activation of bystander cells including stromal fibroblasts. They contribute to the accumulation of MMPs that lead to tissue degradation. Altogether, this constitutes an immune-mediated conjunctival inflammatory process (**Figure 8(a)**), that can be compared with events in other mucosal organs, for example, in inflammatory bowel disease, and is based on a deregulation of the physiologically protective CALT and perpetuated by several vicious circles.

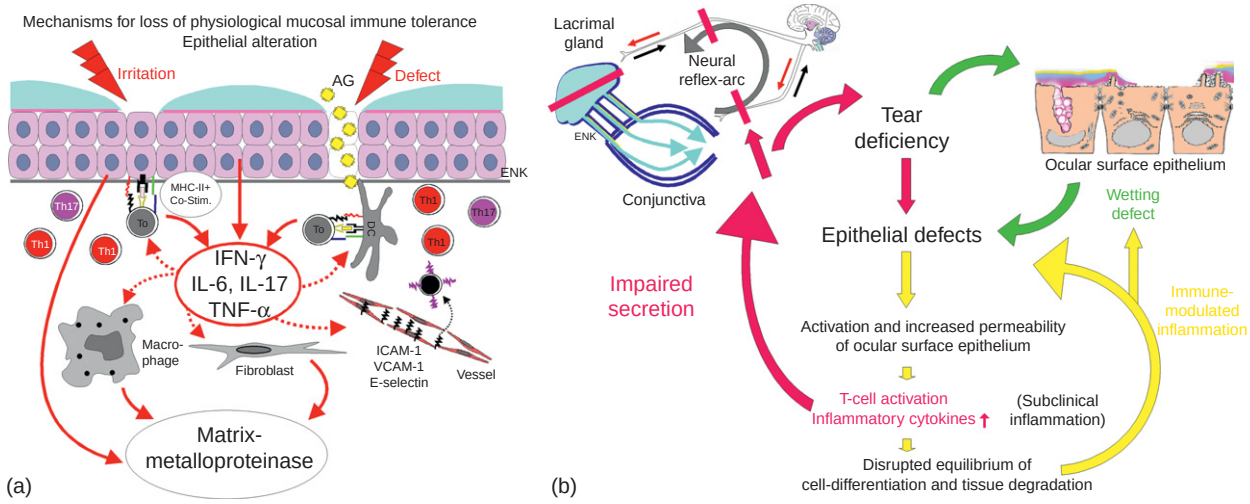

Figure 8 Deregulation of CALT determines immune-mediated inflammatory ocular surface disease. (a) Compromised integrity leads to loss of mucosal immunological tolerance and to immune-mediated inflammation. Irritation of the surface epithelium, for example, by tear film (light blue) defects, its infection or its wounding results in activation of the epithelial cells. These may respond by secretion of inflammatory cytokines and proteases (such as matrix-metalloproteinase, MMP) and by expression of the antigen-presenting molecule MHC-class-II (MHC-II) resulting in potential presentation of self-antigens to resident conjunctival T-cells. Wounding with physical defects can, in addition, allow the entry of nonpathogenic antigens into the tissue and their presentation in the context of inflammatory cytokines as observed in ocular allergy. As described for immune-regulation (**Figure 7**) this represents danger signals that contribute to a further accumulation of inflammatory cytokines in the tissue and to the generation of inflammatory, potentially autoreactive, types of T-cells. All of which is shown in inflammatory ocular surface disease. Downstream effects are the activation of vascular endothelial cells that upregulate adhesion molecules with subsequent recruitment of further leukocytes from the vascular compartment and the activation of bystander cells, including stromal cells (fibroblasts). They contribute to the accumulation of MMPs that lead to tissue degradation which all together constitutes an immune-mediated conjunctival mucosal inflammatory process that is based on a deregulation of the physiologically protective CALT. (b) Immune-mediated inflammation is a core mechanism that results in several vicious circles in the pathogenesis and propagation of ocular surface disease. An immune-mediated inflammation represents an important common factor in the vicious circles of ocular surface disease, including the dry eye syndrome and ocular allergy, which is first subclinical but tends to amplify if it is not limited. Tear film deficiency results in epithelial defects and these in turn are an important primary factor for onset of an immune-mediated inflammatory conjunctival process that tends to self-propagation via several vicious circles. These include disturbance of afferent innervation resulting in impaired secretion with further tear film deficiency and increase of epithelial damage and in impairment of mature ocular surface differentiation (leading to squamous metaplasia) that results in wetting defects and amplification of epithelial destruction. (a) From Knop E. and Knop N. (2005). *Influence of the Eye-associated Lymphoid Tissue* (*EALT*) *on Inflammatory Ocular Surface Disease*. The Ocular Surface, Ethis Communications. (b) Adapted from Knop E. et al. (2003). *Dry Eye Disease as a Complex Dysregulation of the Functional Anatomy of the Ocular Surface*. *New Impulses to Understanding Dry Eye Disease*. Der Ophthalmologe, Heidelberg: Springer.

See also: Adaptive Immune System and the Eye: Mucosal Immunity; Corneal Epithelium: Response to Infection; Defense Mechanisms of Tears and Ocular Surface; Dry Eye: An Immune-Based Inflammation; Immunopathogenesis of Onchocerciasis (River Blindness); Molecular and Cellular Mechanisms in Allergic Conjunctivitis; Pathogenesis of Fungal Keratitis; Tear Drainage.

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