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Perturbations of the ocular surface microbiome and their effect on host immune function

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Structured Abstract

Purpose of review: Current literature describing the ocular surface microbiome and host immunity are reviewed alongside experiments studying perturbations of the microbiome to explore the hypothesis that disruption of a healthy microbiome may predispose the ocular surface to inflammation and infection.

Recent findings: The ocular surface of healthy subjects is colonized by stable, pauci-microbial communities that are tolerant to the host immune response and are dominated by the genera *Corynebacterium, Propionibacterium*, and *Staphylococcus*. In animal studies, commensal microbes on the ocular surface interact with toll-like receptors to regulate the immune system through immune cell and inflammatory cytokine production, promoting homeostasis and protecting against infection. Contact lens wear, lens wash solutions, and preserved topical medications can disrupt the native microbiome and alter the relative diversity and composition of microbes on the ocular surface.

Summary: The ocular surface microbiome confers protection against pathogenic colonization and immune dysregulation. Disruption of this microbiome by exogenous factors may alter the resistance of the ocular surface to infection. Further study of the relationships between human ocular surface microbiome and the local immune response are needed.

Keywords

Ocular surface microbiome; host immunity; immune response and regulation; corneal infection

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Introduction to the Ocular Surface Microbiome

The Human Microbiome Project was launched in 2007 by the National Institutes of Health, leading to the discovery that bacteria, viruses, and fungi live in dynamic, diverse, and interdependent commensal microbial community niches within the human body.[1] The term microbiota is defined as the 10–100 trillion symbiotic microbial cells that inhabit biologic niches including the gastrointestinal tract, skin, urogenital tract, and ocular surface, with the genetic material of these microbes characterized as a microbiome.[2] Microbiome research has vastly improved the understanding of disease and pathology especially in the domains of inflammation and immunity. The first articles about the ocular surface microbiome (OSM) date back to the 1970's. However, most research has occurred within the last decade due to next-generation sequencing techniques (16S rRNA, shotgun metagenomics, RNA-based metagenomic deep sequencing) that replaced previous culture-based methodologies, allowing for better characterization of this unique mucosal environment. In next-generation sequencing, operational taxonomic units (OTUs) function as proxies for species and are defined as genetic sequences that can be clustered together based on similarity. OTUs in early 16s rRNA studies generally were defined by having 97% sequence similarity, although more recent studies frequently use 100% similarity.

In culture-based studies, the OSM was reported to be primarily composed of Gram-positive bacteria, including *Staphylococcus, Streptococcus, Corynebacterium*, and *Propionibacterium* (now classified as *Cutibacterium)*. Gram-negative microbes were reported in lower abundances, consisting of primarily *Haemophilus* and *Neisseria*.[3] Culture-based studies underestimated the diversity of organisms on the ocular surface, although not to the extent initially thought. The first 16S rRNA sequencing of the OSM of four human subjects revealed an average of 221 OTUs per subject, classified into five phyla and 59 genera. At the phyla level, *Proteobacteria, Actinobacteria*, and *Firmicutes* comprised greater than 87 percent of the sequences with *Cyanobacteria* and *Bacteroides* present in much lower quantities.[4] The five most common genera were *Pseudomonas (18%), Bradyrhizobium (12%) Propionibacterium (11%), Acinetobacter (9%), and Corynebacterium (8%)*, comprising more than 58% of all sequence reads. *These along with Brevundimonas (4%), Staphylococci (2%), Aquabacterium (2%), Streptococcus (0.5%), Sphingomonas, Streptophyta, and Methylobacterium* were detected in each of the subjects.[4] However, 31% of the sequence reads belonged to unclassified or novel bacteria. Another study of 31 healthy subjects found similar results with *Corynebacterium* (28.22%), *Pseudomonas* (26.75%), *Staphylococcus* (5.28%), *Acinetobacter* (4.74%), *Streptococcus* (2.85%), *Millisia* (2.16%), *Anaerococcus* (1.86%), *Finegoldia* (1.68%), *Simonsiella* (1.48%) and *Veillonella* (1.00%) accounting for over 76% of the microbial communities.[5] The number of distinct OTU per sample at 97% similarity ranged from 159 to 2042, suggesting a highly diverse OSM.

Doan and colleagues performed an elegant set of experiments comparing culture to 16S rRNA sequencing to biome representational in silico karyotyping (BRiSK), a metagenomic detection method for DNA-based life forms, of ocular surface and buccal mucosa. On the ocular surface, *Corynebacterium, Propionibacterium, Staphylococcus*, and *Streptococcus* were isolated in greatest abundance, along with *Elizabethkingia and Delftia*. The OSM was distinct from that of buccal mucosa and harbored 150-fold fewer bacteria, with only

1 bacterium per 20 conjunctiva epithelial cells.[6] Based on the amount of DNA recovered per sample, they estimated that only 40 individual bacteria were on any given conjunctival sample swab. However, when these samples underwent 16s rRNA sequencing, each was associated with an estimated 460 OTU, suggesting significant artifact.

Artifact in 16S rRNA sequencing may arise from contamination of reagents, sequencing errors, misattribution errors, and PCR amplification errors. This is especially true for paucibacterial samples like the ocular surface. Using 20 copies each of four pure bacterial 16s sequences, Doan[6] demonstrated that after 25 PCR cycles with typical error rates of 1×10^{-6} for heat stable polymerases, 36 new OTUs were generated, suggesting that the high number of genera found by Dong[4] and Huang[5] may be largely artifactual, even with the use of negative controls.

Studies of healthy human subjects have demonstrated that males harbor increased abundance of *Propionibacterium acnes* and *Staphylococcus epidermidis* while females have relatively more *Escherichia coli* in their ocular surface microbiome.[7] There are also age-related differences between older (age 47–84 years) and younger (age 23–44 years) individuals, with differential abundances of *Propionibacterium, Escherichia, Micrococcus, Staphylococcus*, and *Mycoplasma* species[7] as well as significant colonization of *Corynebacterium* in the elderly.[8] However, there appear to be no differences in microbial composition between right and left ocular surfaces.[7]

Despite the ocular surface being a paucibacterial niche with constant turnover of tear film and exposure to the environment, the presence of a stable microbial community has been demonstrated. Over the course of three months, 26 taxa were identified that were present in at least one or more subjects, with the most prevalent phyla being *Proteobacteria, Firmicutes*, and *Acinetobacteria* and the most prevalent genera being *Corynebacterium* and *Streptococcus*, confirming the findings from Doan *et al*.[6,9]

Following the characterization of the ocular surface microbiome of healthy subjects, subsequent studies have sought to identify the relationship between these microbes and immune pathways on the ocular surface to understand the manifestations, mechanisms, and possible therapeutics for complex ophthalmic conditions. In this review, we further explore the relationships between the microbiome and ocular surface immunity, focusing on what may occur when the microbiome is disrupted.

Ocular Immunology and the Microbiome

The ocular surface is composed of epithelial cells that detect both commensal and pathogenic bacteria through recognition of pathogen-associated molecular patterns (PAMPs). PAMPs, lipoteichoic acid, lipoproteins, and additional bacterial metabolic products are detected by the innate immune system through toll-like receptors (TLRs), a family of transmembrane receptors present on the ocular surface. Upon binding of the ligand, TLRs become activated, recruiting adaptor proteins and promoting gene transcription of nuclear factor-kappa B (NFKB) and mitogen-activated protein kinases (MAPK)[10,11] resulting in downstream activation of pro-inflammatory cytokines (TNF-α,

IL-6, IL-1β), chemokines, growth factors, and cell-adhesion molecules that communicate with and recruit dendritic cells, helper T cells, and B lymphocytes.[3] (Figure 1.) Studies have provided preliminary evidence that *Staphylococcus, Streptococcus, Corynebacterium, Propionibacterium, Haemophilus, and Neisseria* on the ocular surface can prime epithelial immune cells to regulate the composition and production of mucin, while increasing crosstalk with pattern recognition receptors and TLRs to strengthen mucosal immunity.[3] Toll-like receptors 1, 2, 5 and 6 exist on the cell surface whereas toll-like receptor 3, 7, 8, and 9 function intracellularly within endosomes. TLR4 is unique in that it can function both at the cell surface and within endosomes, responding to different substrates and utilizing separate pathways depending on location. On the cell surface TLR4 responds to lipopolysaccharide, the major virulence factor of Gram-negative bacteria, whereas TLRs 1/2 and 2/6 respond to lipopeptides associated predominantly with gram positive bacteria and fungi. TLR5 expression is induced by flagellated pathogenic species such as *Pseudomonas aeruginosa* and not by benign ocular commensals.[12]

TLR4 is expressed mainly by wing and basal, but not superficial, corneal epithelial cells.[13] (Figure 2.) In a mouse model, LPS activation of TLR4 increased IL-1β, CXCL10, IL-12a, and IFN-γ in the conjunctiva, and IL-1β and CXCL10 in the cornea.[14] LPS significantly increased IL-12a in conjunctiva and IL-1β in cornea in a dry eye mice when compared to LPS-treated control mice,[14] suggesting that bacterial endotoxin activation of TLR4 is enhanced with breakdown of the epithelial barrier function and exposure of the deeper wing and basal cells.

The tear film contains secretory IgA, which produces anti-inflammatory regulatory factors such as IL-10 to induce tolerance at mucosal sites[15] by responding to fluctuations in commensal bacteria without disrupting mucosal homeostasis.[16] Studies of germ-free mice have demonstrated diminished lymphoid infiltrates, decreased levels of secretory IgA heavy chain transcripts, and decreased IgA expression at the ocular surface.[15] Specific IgAs have been identified for *Staphylococcus intermedius*, a member of the healthy ocular microbiota, that plays a role in cross-reacting with *Staphylococcus aureus*, potentially limiting colonization by harmful Gram-positive species.[17] While it is unknown if specific microbial colonizers of the ocular surface can directly influence local secretory IgA production, mucosal surfaces on the lung and gut have pathways suggesting TLR signaling by commensal or pathogenic microbes can activate dendritic cells, release B-cell activating factors, and promote T-cell mediated production of IgA.[18] Alteration of gut microbiome with antibiotics has been shown to reduce both secretory IgA and IgA transcripts at the ocular surface in mice, with these levels subsequently increased by supplementation with *Bacteroides*, inducing an IL-1β response.[19] Interestingly, in the absence of IgA, the normally non-inflammatory gut bacterium *Bacteroides* starts to express high levels of gene products that produce nitric oxide and elicit downstream cytokine signaling that promote subclinical inflammation.[20]

The balance of T helper cell type 1 and 2 (Th1 and Th2) expression has been implicated in autoimmune, allergy, and inflammatory response. *Acinetobacteria Iwoffi* F78, a strain found in the ocular microbiota, has been shown to activate human monocyte derived dendritic cells via lipopolysaccharide (LPS), inducing Th1 differentiation through the TBet/

GATA3 pathway and shifting the balance away from Th2 expression to decrease allergic symptoms.[21]

The conjunctiva on the ocular surface contains eye-associated lymphoid tissue (EALT) and immune follicles where antigen presenting cells, B cells, and T cells can generate a local inflammatory response to TLR signals.[22] (Figure 2.) Approximately half of the T cells in the EALT are γδ T cells. These γδ T cells were found to be stimulated in a murine model by *Corynebacterium mastitidis*, a known skin commensal, and an organism of interest due to the prevalence of *Corynebacterium spp*. In the ocular microbiota.[23] The γδ T cells produced greater concentrations of IL-17 in the presence of *C. mastitidis*, and the immune response was observed to prevent colonization by pathogenic organisms such as *Candida albicans* and *Pseudomonas aeruginosa*. (Figure 3). Depletion of *C. mastitidis* using topical antibiotic ointments reduced IL-17 production significantly while inoculation of *C. mastitidis* to ocular tissues induced stable colonization and associated immune response.[24] Thus, species normally found on the ocular surface such as *Corynebacterium spp*. May have significant effects on the local function of T cells and expression of cytokines.

Kugadas and colleagues demonstrated that both the gut and ocular surface microbiotas are important for protection against infection by pathologic bacteria in mice.[25] Specific pathogen free (SPF) mice pretreated with topical gentamycin ophthalmic ointment for 4 days before infectious exposure to *Pseudomonas aeruginosa* had significantly higher corneal bacterial burden of *P. aeruginosa* and worse corneal pathology scores than mice not pretreated with gentamycin. Pretreatment with gentamycin was associated with reduced levels of IL-1β, which is known to be important for protection against *P. aeruginosa*. In a second experiment, germ free (GF) mice and SPF mice pretreated with an oral antibiotic cocktail that reduced gut bacteria but did not alter the ocular surface microbiota also had significantly higher corneal bacterial burden of *P. aeruginosa* and worse corneal pathology scores after infectious challenge than did untreated SPF mice. Neutrophils derived from mice that received oral antibiotics had reduced bactericidal capacity against *P. aeruginosa*, suggesting that microbial-derived signals promote neutrophil function. Reconstituting the gut microbiome with either human gut microbiome or mouse gut microbiome reversed the susceptibility to infection. In fact, mono-colonization of the ocular surface with coagulase-negative *Staphylococcus* (CNS) alone was enough to increase neutrophil bactericidal capacity, increase IL-1β, and reverse the susceptibility to *P. aeruginosa* keratitis. (Figure 3.)

The depletion of gut commensals appears to alter the corneal response to bacterial endotoxins. Wang and colleagues found that mice pretreated with oral antibiotics for 14 days demonstrated an altered gut microbiome and increased systemic levels of LPS.[26] When compared to control mice, these antibiotic treated mice demonstrated increased expression of corneal inflammatory cytokines TNF-α, CXCL10, and IL-12 after topical application of LPS to the ocular surface.[26]

It is unclear what role ocular surface microbial metabolites have in modulating local immunity, but studies from other microbial niches may suggest possibilities. Indole is a microbial metabolite that promotes epithelial cell barrier function in the gut via the pregnane

X receptor and increases secretion of glucagon-like peptide 1, exhibiting systemic effects on host metabolism.[27] Short-chain fatty acids produced by gut microbes also serve as an energy source for intestinal surface epithelial cells, influencing oxygen consumption, cellular metabolism, and hypoxia-inducible factor.[28] Thus, additional insight into the metabolic profile of commensals on the ocular surface can provide insight into potential ocular probiotic therapies and variations in treatment response.

Perturbations of the Ocular Surface Microbiome

In a study of the OSM in contact lens wearers, lens wearers exhibited a microbiota that was significantly greater in similarity to the skin microbiota compared to non-lens wearers. The microbiota of lens wearers had higher abundance of *Pseudomonas*, *Acinetobacter, Methylobacterium*, and *Lactobacillus*, while having a decreased abundance of *Haemophilus, Streptococcus, Staphylococcus*, and *Corynebacterium* compared to non-lens wearers.[29] Contact lenses also alter the proteome of the conjunctiva, with significant changes in antimicrobial proteins, lipid metabolism, and inflammatory cytokines,[30] resulting in changes to the lipid and mucin composition of the tear film. Contact lens storage solution may also impact the ocular surface microbiome as it contains peroxide-based preservatives, decreasing the abundance of *Corynebacterium, Haemophilus*, and *Streptococcus*, strains at times considered to be pathogenic colonizers.[31]

Exposure to topical medications containing the preservative benzalkonium chloride (BAK) appears to have a significant effect on the ocular surface microbiome. BAK prevents the growth of pathogenic bacteria in ophthalmic preparations by acting as a detergent, disrupting cell walls and releasing cytoplasmic contents.[32] At lower concentrations, BAK primarily inhibits Gram-positive organisms including *Staphylococcus*[33] with increased activity against Gram-negative organisms such as *E. coli* and *Pseudomonas aeruginosa* at higher concentrations.[34] The half-life of BAK is 20 hours in corneal epithelial tissues and 11 hours in conjunctival layers,[35] with detectable concentrations up to 1 week.[36] Culture-based studies found that topical glaucoma eyedrops increased the abundance of Gram-negative organisms compared to healthy controls[37] and increased the abundance of antibiotic-resistant *Staphylococcus epidermidis* compared to preservative-free formulations.[38] Chang and colleagues utilized 16S rRNA sequencing to characterize the ocular surface microbiome of patients with unilateral or asymmetric glaucoma treated with preserved topical ophthalmic medications. The ocular surface microbiome of patient eyes receiving topical medications was dominated by *Akkermansia* (11.8%), *Faecalibacterium* (6.3%), *Lachnospiraceae* (5.9%), *Komagataeibacter* (4.8%), *Finegoldia* (4.6%), *Corynebacterium* (3.9%), and *Blautia* (3.6%). This was different from the more typical commensals of the healthy control eye samples, *Corynebacterium* (71.7%), *Cutibacterium* (5.4%), and *Blautia* (4.4%).[39] Metagenome inference analysis suggested that the ocular surface microbes of patients using drops were more capable of lipopolysaccharide synthesis whereas the microbes of healthy controls had capacity for reducing inflammation. There is evidence that biannual oral antibiotics can also alter the ocular surface microbiome by increasing the biodiversity and richness of the microbial community.[40]

Synthesis

The ocular surface harbors an innate host immunity characterized by a complex network of immune cells that release inflammatory cytokines in response to pathogenic signals. The surface also harbors a commensal and stable microbiome tolerant to the native immune system that is important for protection against infection. A review of the literature suggests that perturbations that alter, reduce, or eliminate the normal commensal microbes such as the use of topical antibiotics, preserved ophthalmic preparations, or contact lenses may disrupt homeostasis and the normal immune response at the ocular surface.

Contact lens wear is an example where perturbation of the ocular microbiome may contribute towards increased susceptibility of infection. While increased microbial burden on the lens and overgrowth of pathogenic colonizers have long been implicated in increasing infection risk, loss of ocular surface commensals that protect against infection, e.g., coagulase-negative *Staphylococcus* and certain *Corynebacterium* species, may also contribute. It is not fully understood why contact lens wearers have an altered microbiome; however, chronic exposure to peroxide-based preservative solutions that bathe the lenses when not in use may play role. In patients using preserved topical glaucoma medications,[39] the ocular surface microbiome was likely altered by chronic daily exposure to the detergent BAK, with a resultant microbial composition capable of increased synthesis of lipopolysaccharide, a pro-inflammatory endotoxin. The effect of topical medications and contact lenses on the ocular surface microbiome warrants further study to determine how local immune function may be affected by these disrupted microbiomes. Future research on this paucibacterial niche should employ methods to reduce contamination and artifactual sequencing results, including the use of positive and negative controls, as well as utilize sequencing methods and other biological assays to better understand microbial function and interactions with the host.[41]

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40*. Doan T, Hinterwirth A, Worden L, et al. Post-antibiotic ocular surface microbiome in children: A cluster-randomized trial. Ophthalmology 2020;127(8):1127–1130. [PubMed: 32197915] This article reports the use of RNA-seq based ocular surface microbiome identification in a large prospective clinical trial of children receiving four doses of oral azithromycin 6 months apart versus placebo. Conjunctival swabs were collected at baseline and at 24 months. The authors found significant ocular surface microbial composition and diversity differences as well as differences in the ocular surface resident virome in the azithromycin treated group at 24 months, indicating that oral antibiotics can have lasting effects on the ocular surface microbiome and virome.

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contaminant reduction, methods of characterizing the ocular surface microbiome, and approaches for validating microbes.

Key points

- The ocular surface harbors a low-burden microbiome composed of primarily *Proteobacteria, Firmicutes*, and *Acinetobacteria* at the phylum level and genera *Corynebacterium, Streptococcus, Propionibacterium*, and *Staphylococcus*.
- Pathogenic and commensal microbes interact with the ocular surface through direct activation of toll-like receptors or production of intermediate metabolites to modulate down-stream effects on immune cell production and inflammatory cytokine production.
- Certain microbes, including coagulase-negative *Staphylococcus* and *Corynebacterium mastidis*, protect against infection by pathologic organisms on the ocular surface. Loss of these microbes increases infection risk and severity.
- Contact lens wear and chronic exposure to preservatives in contact lens wash solutions and topical eyedrop medications are associated with changes to the ocular surface microbiome, including the loss of microbes that protect against infection in animal studies.
- Future studies are needed to understand how disruption of the ocular surface microbiome by common external factors such as contact lenses and preserved eye drops affects local host immune function.

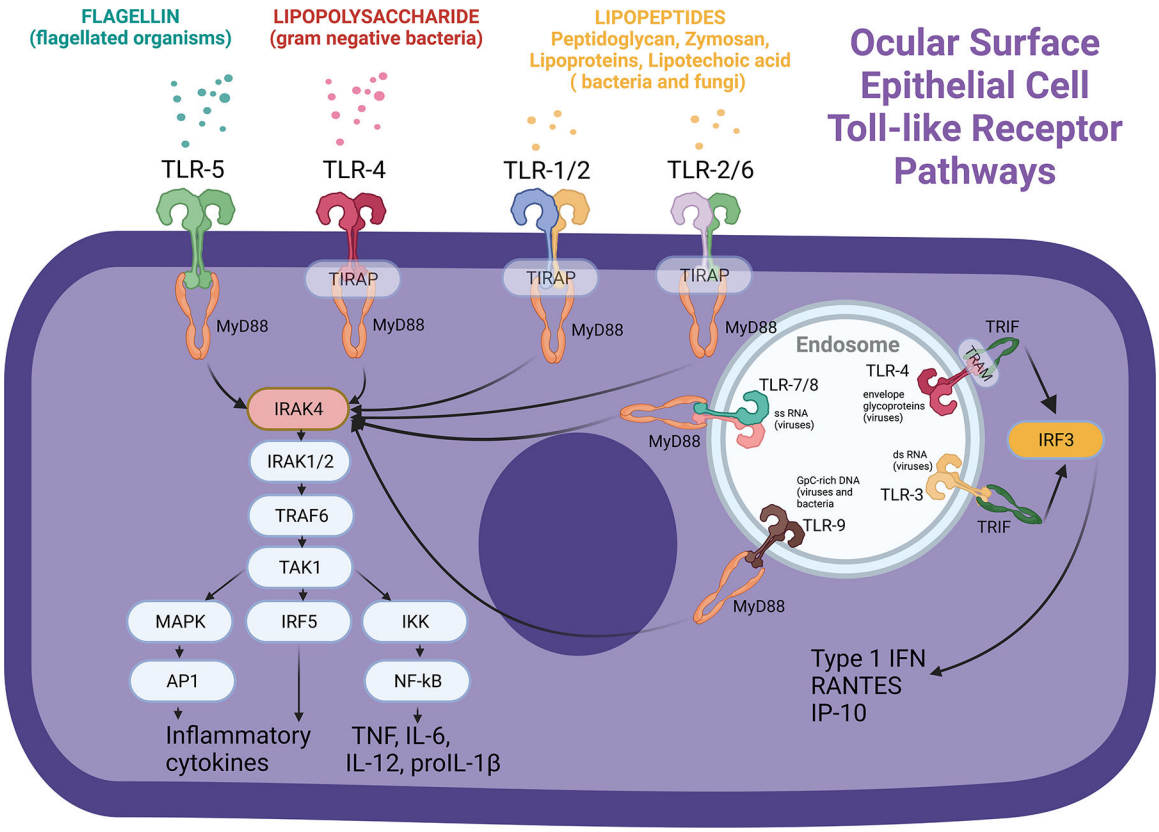

Figure 1.

Toll-like receptor (TLR) pathways on ocular surface epithelial cells. TLRs recognize specific pathogen-associated molecular patterns (PAMPs) on the ocular surface and in endosomes. Once activated, they regulate the production of inflammatory cytokines, chemokines, and adhesion molecules via either Myeloid Differentiation Primary Response Gene 88 (MyD88)-dependent or MyD88-independent pathways. TAK1 = Transforming growth factor-β-Activated Kinase 1. TIR = toll/interleukin-1 receptor. TIRAP = TIR Domain-Containing Adaptor Protein. MAPK = Mitogen-Activated Protein Kinase. IKK = IκB Kinase. IRAK = IL-1 Receptor-Associated Kinases. TRAF = TNF Receptor-Associated Factor. TRIF = TIR-domain-containing adaptor inducing interferon-β. TRAM = TRIF-related Adaptor Molecule. IRF = Interferon Regulatory Factor.

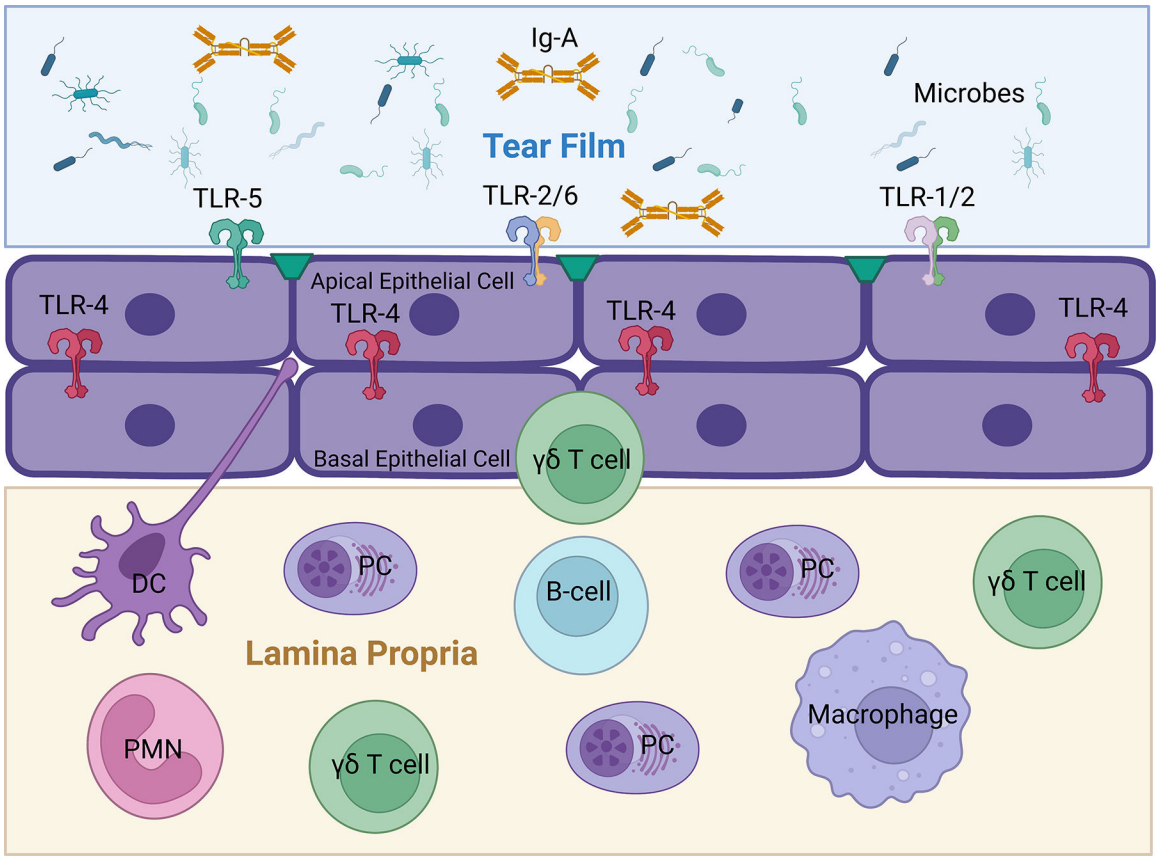

Figure 2.

The eye-associated lymphoid tissue (EALT). The bulk of the inflammatory cells lie deep to the epithelial cell layer, in the lamina propria, although T cells and dendritic cells (DC) can be found within the epithelial cell layers as well. Toll-like receptors (TLR-4) on epithelial cells recognize pathogen-associated molecular patterns (PAMPs). TLR-4, which recognizes lipopolysaccharide, is found on basal and wing epithelial cells, but not on apical epithelial cells. PC = plasma cell. PMN = polymorphonuclear leukocytes.

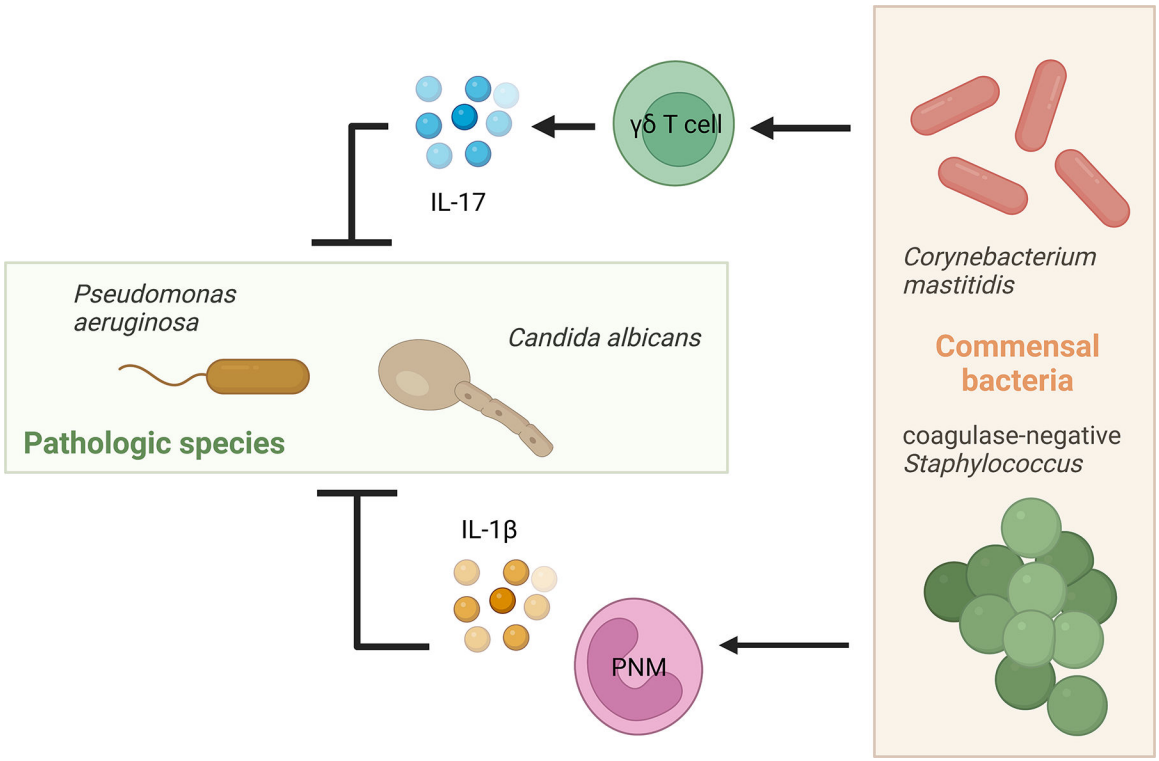

Figure 3.

Commensal bacterial protect against pathologic organisms on the ocular surface. γδ T cells produce increased IL-17 in the presence of *Corynebacterium mastitidis*, which prevents colonization by pathogenic organisms *Candida albicans* and *Pseudomonas aeruginosa*. Coagulase-negative *Staphylococcus* (CNS) increases neutrophil bactericidal capacity, increases IL-1β, and reverses susceptibility to *P. aeruginosa*.