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The Open Ophthalmology Journal, 2015, *9*, 49-55 49

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Ocular Surface as Barrier of Innate Immunity

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Abstract: Sight is one of the most important senses that human beings possess. The ocular system is a complex structure equipped with mechanisms that prevent or limit damage caused by physical, chemical, infectious and environmental factors. These mechanisms include a series of anatomical, cellular and humoral factors that have been a matter of study. The cornea is not only the most powerful and important lens of the optical system, but also, it has been involved in many other physiological and pathological processes apart from its refractive nature; the morphological and histological properties of the cornea have been thoroughly studied for the last fifty years; drawing attention in its molecular characteristics of immune response. This paper will review the anatomical and physiological aspects of the cornea, conjunctiva and lacrimal apparatus, as well as the innate immunity at the ocular surface.

Keywords: Cornea, innate immunity, mucins, neuropeptides, ocular surface, pattern recognition receptors.

INTRODUCTION

In order to eliminate a pathogen, the immune system must recognize it and develop and adequate response to destroy it. The immune system has two types of mechanisms: innate and adaptive immunity. The innate immunity's strategy consists of recognizing a group of molecular patterns common to an entire family of pathogens.[1] Thus, all individuals within one species are born with innate ability to recognize and destroy numerous microorganisms immediately. Innate immunity is, therefore, able to fight infections from the beginning and during early stages with high efficiency (about 0-5 days). If these mechanisms fail to eliminate the infection, they at least at least they keep it under control until other mechanisms take place [1]. The first and most basic defense mechanism against infection are epithelia and mucous membranes, which are mechanical, chemical (defensins, lysozyme) and biological barriers against pathogens [2]. If microorganisms are able to cross these barriers and establish an infection, there are two pre-existing innate mechanisms that act immediately: humoral and cell - mediated immunity. Humoral immunity is mediated by macromolecules (as opposed to cell-mediated immunity) such as antibodies, complement proteins and certain antimicrobial peptides. Cell –mediated immuntity involves the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen [3]. All cells of the immune system need to be closely related to each other to develop a coordinated immune response that culminates in the elimination of the pathogen. Cells use two main ways to communicate: by direct contact with membrane molecules, and through cytokines synthesis. Many of the cytokines are produced in the first moment of cell activation, alerting cells with cytokine membrane receptors. Cytokines are molecules that often have only a local effect, taking part in antigen recognition and distance response, cytokines participate in regulation of the length and intensity of the innate and specific immune response, recruiting cells towards the conflict zone and inducing the generation of new cells from hematopoietic precursors [4]. Cytokines share functions with the so called host defense peptides (HDPs), proteins that act as effector molecules of innate immunity [5]. The cornea, as the outmost part of the eye, is constantly exposed to various agents potentially harmful to its surface and the internal structures of the eye, the reason why is endowed with various elements of innate and adaptive immunity, and is

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1874-3641/15

sheltered by anatomical structures, conjunctiva, and lacrimal system. The following will describe this in greater detail.

OCULAR SURFACE ANATOMY

Cornea

The epithelium is the external layer of the cornea with approximately 50µm thickness, underneath is Bowman's layer which is 10µm thick, stroma 480 µm thick in the center and 900 µm thick in the periphery, Dua´s layer (recognized by some researchers) which is hypothetically 15µm thick, Descemet's membrane 12 µm thick and at the most inner level the endothelial cells with a 4-6 µm thickness [6,7].

The cornea has the highest dioptric power of the optical complex due to its prolate shape where the central 4mm tends to be spherical, but then gradually flattens toward the periphery [6,8]; The rigidity of the stroma contributes to keep the shape and its refractive properties [9]. In order to be transparent the cornea needs to be avascular, receiving its nutrients through diffusion from the tear film and through aqueous humor [10,11]. The cornea is one of the body structures most densely innervated, the innervations comes from axons of the sympathetic ganglion and trigeminal ganglion [10]. Corneal nerve fibers and associated neurotrophins, influences on the corneal epithelium and contribute to the maintenance of a healthy ocular surface.

The corneal epithelium is a stratified, non-keratinized squamous layer [12]. It has three types of cells: The most external type of cells are the superficial epithelial cells, in the middle are the wing cells located on top of the inner layer which are the basal epithelial cells [6]. Due to its histological nature, the epithelium has the primary function of providing a barrier to the cornea and the rest of the eyeball [7]. Also important, the tear film contributes to the refractive and barrier function. An important characteristic of this epithelium is waterproof property, because the epithelium provides additional protection against water-soluble substances that could alter the refractive properties and/or intraocular environment [13]. Interestingly, the corneal epithelium has also an anti-angiogenic function, preventing blood vessels from proliferation and subsequent loss of corneal transparency [14]. Basal cells are in charge of regenerating loss of integrity of superficial layers [15]. They detach from the basal lamina and progressively replace the winged and superficial epithelial cells, which are displaced anteriorly and are eventually desquamated. They are tightly attached to the underlying basement membrane, which allows the epithelium to remain bound to the underneath cells.

Underneath the epithelial basal membrane layer of the cornea, lies the Bowman's layer. This layer has no structures in it. It is composed of thin, type I, III, V and VI collagen microfibrils. It is not an independent membrane, but a modification of the most superficial portion of the stroma of the cornea [7]. The stroma represents the main support of the corneal structure and comprises up to 90% of its volume. This compartment is about 450 µm thick and it contains nerves of variable size, stromal keratocytes with different morphology and type I and V collagen fibers [16,17]. The nerves are located in the anterior stroma, just below the Bowman's layer, where they form the subepithelial nerve plexus [18]. Keratan sulfate is part of the most abundant proteoglycans in the cornea, there are also chondroitin sulfate and dermatan sufate. Proteoglycans contribute to the structural function of the stroma.

These molecules are synthesized by the stromal keratocytes, which also produce matrix metalloproteases to maintain the homeostasis of the stroma [19]. As neighboring layers, there are a series of interactions between the stroma and the epithelium, where the keratocytes from one layer produce cytokines to modulate the functions of keratocytes of the other layer [20]. Dua´s layer is a strong acellular layer in the pre-Descemet's cornea, made of 5 to 8 thin lamellae of tightly packed type I, VI, and VI collagen bundles running in longitudinal, transverse, and oblique directions [21]. This structure is capable of supporting up to 2 bars of pressure. The Descemet´s membrane, like the Bowman´s layer, has no structures in it. It represents the basal membrane of the posterior epithelium and is secreted by them. It is formed by very thin filaments of type IV collagen, which are arranged in a very regular pattern. The endothelium is a monolayer of cells that aids in keeping the corneal transparency not only by its barrier function, but also by its ionic pump function [22]. Primarily in the basolateral membrane of endothelial cell, there are sites for the Na+/K+- ATPase pump, contributing to corneal transparency [23-25]. It is important to consider that contrary to the epithelium, endothelial cells are not replaced with ageing or severe damage. Endotelial cells decrease their barrier and pump function over time, sometimes resulting in corneal edema and visual loss [26]. Additionally the endothelium has the function of providing the upper layers with nutrients coming from the aqueous humor, thus serving as a semipermeable membrane that selectively allows passage of glucose and other small molecules to the stroma [27]. This semipermeable property is also used to maintain the water balance of the stroma [28]. For this function, the integrity of the tight and adherent junctions between the endothelial cells is required [29]. For there to be fluid movement from one chamber to another, as in the rest of the body, there must be an osmotic gradient. Compared with the aqueous humor, which is hypertonic, the stroma is hypotonic. This difference in osmolarity is generated by Na+/K+-ATPase pumps, and it is maintained by the semipermeability of the endothelium to ion flux, to set up the osmotic gradient [30]. It has also been recently demostrated that aquaporin water channels, more specifically AQP1, are expressed in the corneal endothelial cells to contribute to transcelular fluid flow [31].

THE TEAR FILM AND BIOCHEMISTRY

The tear film covers the ocular surface, and provides major refractive power of the visual system [32]. Other functions of the tear film include nutrition of the ocular surface, lubrication and a chemical barrier [33]. It forms a thin film layer of 8 µm thick; Although is typically said that is formed of three layers; the external or lipid layer, the central or aqueous layer and the inner or mucin layer, It is now recognized that the tear film is more a lipid boundary layer with aqueous phases incorporating differing concentrations of mucins throughout. Meibomian and Moll glands produce the lipid component [34], mainly wax esters,

triglycerides, free fatty acids, as well as neutral diesters [35]. Lacrimal glands produce the aqueous component and goblet cells which are located in the conjunctiva, secrets the mucin and contains membrane associated glycoproteins [36]. Other components of the tear film are metabolites and electrolytes [37]. Interestingly, the proteins contained in the tear film take part in other processes, for instance, they work as antimicrobials, anti-inflammatories and also help in healing processes after trauma, as well as mechanical protection to the surface of the cornea [38]. Tears also transport carbon dioxide and oxygen, taking part the in ocular surface metabolism [39]. The meibomian glands are present in both the upper and lower lids in the tarsal plate and they are composed of many acini, formed by cells which secrete unique meibomian gland lipids, such as cholesterol, wax and cholesterol esters, which all the latter form part of the non-polar lipids, and account for 65% of the tear film lipids, as well as polar lipids (phospholipids and glycolipids) [40]. These substances are then excreted onto the ocular surface on the lid margin, where the mucocutaneous junction is located [41]. As mentioned, lacrimal glands are responsible of synthesizing the aqueous portion of the tear film. The main gland (orbital and palpebral portion) produces the reflex tear secretion onto the supero-lateral portion of the fornix, as well as accessory lacrimal glands produce the basal portion of the lacrimal film on the upper fornix [42]. The aqueous component accounts for 60% of the tear and it mainly contains water, electrolytes and proteins [43]. There have been identified dozens of different proteins contained in the tear, but the three main ones are: lysozyme, lactoferrin and tear-specific prealbumin (lipocalin) [44]. The lysozyme is an enzyme with antimicrobial function, as it destroys the glycosidic bonds between peptidoglycans in bacteria cell wall [45]. Lactoferrin protects the ocular surface from the effects of free radicals. The role of the lipocalins has not been clearly demonstrated, but they may have more than one function in the tear film; depurating the ocular surface from hazardous substances or aiding to spread the lipids on the tear film [46].

Mucins are hydrophilic glycoproteins which carbohydrate mass is of approximately 50-80%, Residues that are O-linked to serine and threonine, have in their protein backbone tandem amino acid repeats [47]. On the ocular surface, mucins can be associated with glycocalyx of conjunctival and corneal epithelium or with a mucous layer produced by the goblet cells [36]. The glycocalyx is a carbohydrate- based cover found on the surface of epithelia, to where mucous layers can get attached [48]. Mucins in the eye have multiple functions; by themselves, mucins lubricate the eye, indirectly they protect the epithelium from drying as they prevent tears from evaporating, they serve as another protective barrier against the hostile environment, they secure the aqueous layer of the tear film to the ocular surface, they protect from microbial invasion of the epithelium as they serve as a platform to hold other anti-microbial proteins to the eye [49,50]. Depending on the structure of their molecule, mucins can be of two different kinds; secreted or cell- surface associated mucins. Goblet cells are in charge of the synthesis of secreted mucins and of the properties of the mucous as well, while the membrane-spanning mucins have more to do with structural function [52]. Within the secreted mucins, two types can be identified, large gel-forming mucins and smaller nonpolymeric mucins. The main gel-forming mucin at the ocular surface is MUC5AC, which is localized in the goblet cells of the conjunctiva, whereas the nonpolymeric mucin MUC7 can be found in acinar cells of the lacrimal gland [51].

MUC1, MUC4 and MUC16 are examples of mucins expressed in the corneal and conjunctival epithelia. Other mucins such as MUC1, MUC2, MUC4, MUC5AC and MUC16, have been found in the tear film [47].

OCULAR SURFACE INNATE IMMUNITY

The term ocular surface is often employed in order to describe the assembly conformed by the cornea, the conjunctiva and the tear film which help maintaining visual acuity and also present interesting biochemical interactions that allow them to work as an immunological unit capable to respond to internal and external stimuli as well as modulating those responses in order to avoid exaggerated or chronic reactions [52]. Innate immunity provides a nonspecific surveillance system against corneal infection and is able to be the first form of protection, during the first minutes or hours, when the infection presents.

The ocular surface is formed by a variety of anatomical and functional components that include the bony orbit, eyelids, tears, corneal nerves, the corneal epithelium, leukocytes and cytokines [53,54]. The bony orbit and eyelids comprise the main anatomical structures that provide protection to the ocular surface, specially against traumatic events. Tears are secretions with the main function of lubricating and preventing the cornea from drying, flushing away foreign particles from the ocular surface, as well as distributing immunoglobulins (IgA and IgG) and antimicrobial proteins (lactoferrin, lysozyme, lipocalin and beta-lysin) to the ocular surface in order to prevent infections [55]. Cell-cell junctions of corneal and conjunctival epithelia complete the first line of anatomical barriers in the eye [56]. The corneal' s innate immune system is comprised by many types of cells including epithelial cells, fibroblasts and Langerhan's cells. Epithelial cells are in charge of the secretion of TNF-α, IL-1, IL-6 and IL-8.

Fibroblasts in the stroma may be in charge of the production of IL-1, IL-6, IL-8, TNF-α, and α-defensin as a way of after microbial infections; in addition, Langerhans cells in the cornea and conjunctiva are in charge of the adjustment of the B and T lymphocyte activity of the adaptive immunological response [53,57]. Toll- like receptors (TLR) are a group of fundamental molecules for the development of the innate immune response; so far 13 types of TLR have been recognized, TLRs 1 to 10 are present in humans, while TLRs 11 to 13 are unique to mice [58-60]. TLRs have extracellular and intracellular domains. The extracellular domain contains repeats that are rich in leucine (LRR: leucine-rich plates) while the intracellular domain is equivalent to the receptor of IL-1 (IL-1R: IL-1 receptor), and it may be named as the TIR: toll/il-1R domain [61].

Through the recognition of pathogen-associated molecular patterns (PAMPs), Toll like receptors are able to unleash immune responses. Structures that are preserved

across the microorganisms of a given class and are vital for their survival, have become a great place of detection by the innate immune system since they enable a quick way to differentiate between non-self and self [60]. TLRs can be broadly divided by their location (intra or extracellular), or by the type of ligands they recognize (cell wall or genetic material) [54].

TLR2's main function is to form heterodimers with TLR6 or TLR1 in order to recognize ligands from bacteria; TLR1/TLR2 heterodimers are able to recognize bacterial lipopeptides, including mycobacterial lipoprotein, meningococcal lipoproteins and triacylated lipoproteins [62-65]; TLR2/TLR6 heterodimer are able to recognize mycoplasma lipoproteins and peptidoglycan [66]; TLR2 can also act as an homodimer for the recognition of Gram-positive cell walls, lipotheicoic acid, mycobacterial lipoarabinomannan, zymosan and heat-shock protein [60,54,67,68]. TLR2 has also been suggested to be a Staphylococcus aureus innate receptor, functioning as a detector of Gram-positive bacterias in the cornea [64].

TLR4 is expressed in corneal and conjunctival cells, fibroblasts and dendritic cells; it is able to form complexes with CD14 and MD2 for the recognition of lipopolysaccaride (LPS) from Gram- negative bacteria cell walls [69]; its function requires several co- receptors, like MD2 and CD14 [65]. Unnecesary proinflammatory reactions due to the presence of commensal bacteria on the corneal surface are avoided by expressing minimal levels of TLR4 on the apical cells and the absence of MD2, one of the main co-receptors of TLR4 [66]. TLR5 is able to recognize flagellin, a major protein present in bacterial flagella [70]; it is a major sensor for the detection of bacteria that is Gram- negative and activates signalign pathways that liberate NF-kB and activate pro-inflammatory genes [71,72].

TLR5 are expressed on the surface of wing and basal corneal cells, different from superficial epithelial cells that do not express TLR5. Therefore, its activation only occurs when the barrier function of the cornea is compromised [70]. TLR3 is expressed by surface epithelial cells, where it acts as a receptor for viral double-strained RNA (dsRNA) [73], poly I:C and an analog of dsRNA [74]. Upon TLR3 stimulation NF-kB is activated, with the secretion of IL-6, IL-8, indicating that TLR3 is capable of unleashing an antiviral response.73 TLR7 and TLR8 have been implicated in the recognition of viral PAMPs, specifically single-stranded RNA (ssRNA), causing the virus infected macrophages to produce IFN-α and dendritic cells [54,75]. TLR9 is located in intracellular endosomal compartments, and specializes in the recognition of DNA with unmethylated CpG motifs from bacterial and viral genomes [76]. Activation of TLR9 induces a Th1 response with secretion of IL- 1277 IL-18 [54, 76, 77]. TLR activation leads to immune cell recruitment, cytokine release and the modulation of corneal healing. Activation of TLR pathways induces the specific expression of cytokines, and MHC molecules, helping an immune adaptive response to take place [78]. TLRs have several pathways: common adaptor molecule that is the primary-response protein 88 of myeloid differentiation (MyD88), TIR domain that comprises adaptor protein (this latter may also be known as TIRAP, MyD88-adaptor-like protein, or MAL), TIR-domain that has an adaptor that induces interferon β (also known as TIR-domain-containing-adaptor molecule 1, TICAM-1, or TRIF), and the molecule that is associated to the TRIF-related-adaptor (also known as TIR- domain-containing-adaptor molecule 2, TRAM or TICAM-2) [79].

The above have been described as the adaptor molecules, after this, the downstring signaling pathways are divided into MyD88-dependent and MyD88-non dependent. In order to produce signaling by TLR2 and TLR4, MyD88- dependent signaling is needed. This latter subsequently activates NF-kB [80,81]; For the induction of IFN-ß, TRIF works through the *via* of TLR3 and TLR4 MyD88-independent pathway [82].

The signaling pathway known as MyD88-independent/TRIF-dependent, is associated with activation by TRAM, and this takes places with the help of TLR4 that subsequently causes genes that are induced by IFN to express [83]. In order to have an adequate ligation of their PAMPs, receptors that are not related to TLR, for example MD2 and CD14 are used by TLRs. After the binding of this ligand, conformational adjustments take place with the purpose of enrolling downstream molecules for signaling.

The domain that is present in the C-terminus of MyD88, interacts with a TIR-domain and this causes the TLRs to recruit MyD88 which is the adapter molecule, and all of this as part of the MyD88- dependent pathway [84,85].

Through the death domain communication, the TLR complex receives through recruitment by MyD88, the IL-1R associated kinase (IRAK) and IRAK-4. Association of MyD88 to IRAK and IRAK, causes their phosphorylation and subsequent activation. Once IRAK is phosphorylated, it then separates from MyD88, and binds with the tumor necrosis factor receptor- associated factor 6 (TRAF6) [86]. Then, the TRAF6-IRAK complex binds to the TGF-β-activated protein kinase (TAK-1), which is associated with the TAK-1 binding proteins: TAB-1 and TAB-2. All these pathways trigger secondarily phosphorylation and activation of TAK1 [85].

As a result, the NF-kB-dependent transcription factor is activated after TAK1 once activated as well, phosphorylates the inhibitor of the NF-kB (IkB)- kinase (IKK) complex. This latter consists of IKKa, IKKb and NF-kB essential modulator (NEMO)/IKKg. TAK 1 can also causes the mitogen-activated protein and the stress associated protein to activate kinases (MAP and SAP respectively), for example: extracellular signal-related kinase (ERK), Jun N-terminal kinase (JNK) and p38. Pro inflammatory molecules such as IL-6, IL-8, IL-18, MIP-1 and TNF-α are produced secondarily after NF-kB is activated.

The transcription factor IFN regulatory factor 3 (IRF3) is phosphorylated due to activity of the MyD88-independent signaling. IFN-β type I is formed when phosphorylated IRF3 is translocated to the nucleus and activates its production. The production of IFN-β type I induces more production of IFN-α, IFN-β and the expression of IRF7 [85,87].

Despite its capability to produce and release proinflammatory cytokines, the ocular surface is also in constant contact with its comensal bacteria, mainly Staphilococcus epidermidis and Propionibacterium acnes, 88 which guides us to believe there must be negatively

regulatory pathways for innate immunity, since a chronic inflammatory response in the cornea would lead to scarring and loss of visual acuity. Several molecules have been proposed to act as counter regulators, including MyD88 short, SIGIRR, Tollip, and ST2 [54]. These molecules confer the ocular surface unique characteristics that may contribute to the coexistence of comensal bacteria and TLRs [88]. Complete elimination of the offending microorganisms can only be achieved by the induction of adaptive immunity. TLRs initiate the inflammatory cascade by recruiting innate inflammatory cells to the sites of infection, like PMNs and NK lymphocytes; afterwards the arrival of monocytes and dendritic cells will initiate the adaptive immune response by capturing foreign antigens and presenting them to naive T lymphocytes by expressing MHC molecules [89, 90]. TLR agonists have proven to be effective for the treatment of some allergic, immunologic and infectious diseases in the ocular surface, TLRs are increasingly becoming therapeutic targets for the modulation of inflammation in the ocular surface, avoiding or becoming alternatives to broadly employed anti- inflammatories like corticosteroids [57]. The cornea can be considered an immune privileged tissue, a term developed in order to explain the high success rate of corneal transplantation [91]. This situation was at first explained by the lack of vasculature and lymphatic drainage in the cornea, resulting in a delay in the traffic of antigen presenting cells (APC) to lymphatic nodes, and the barrier the lack of vasculature represents for effector cells. However, recent research has revealed the process to be more complex and active, rather than passive; diverse endocrine, neural and immunological factors are essential for the preservation of the ocular surface and normal visual function [52]. Local and systemical modulation is necessary to maintain immunological homeostasis. Intraocular regulation is achieved in part due to the pigmented epithelial cells of the iris by producing immunosuppresive molecules like CD86, PD-L1 and CTLA-2α, that are capable of inducing Tregs. Pigmented cells also release TGFß, an immunoregulatory cytokine, antigen presenting cells in the ocular tissue are exposed to it and generate antigen-specific Tregs that migrate to the spleen and establish immune tolerance to those antigens, this process is known as anterior chamber immune deviation (ACAID) [92]. Neural pathways also have an important paper in inducing inflammatory response. Presence of neuropeptides inside the aqueous humor possesses immunoregulatory characteristics, while the presence of sensory and sympathetic nerve fibers in the cornea allows the maintenance of ocular immune privilege by increasing the le vels of TGFß [93]. Some authors have proposed that parasympathetic nerve fibers could also have an important paper on the ocular surface immunoregulation by increasing the levels of VIP and somatostatin [94].

CONCLUSION

There are many studies indicating that a myriad of molecules such as neuropeptides, mucins, pattern recognition receptors, among others are involved in the very complex immune system of the ocular surface just described. Taken together these results, reinforce the idea that the ocular surface is a versatile epithelial barrier.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Declared none.

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Received: November 18, 2014 Revised: April 6, 2015 Accepted: April 13, 2015