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REVIEW

Pattern recognition receptors in microbial keratitis

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Abstract

Microbial keratitis is a significant cause of global visual impairment and blindness. Corneal infection can be caused by a wide variety of pathogens, each of which exhibits a range of mechanisms by which the immune system is activated. The complexity of the immune response to corneal infection is only now beginning to be elucidated. Crucial to the cornea's defences are the pattern-recognition receptors: Toll-like and Nod-like receptors and the subsequent activation of inflammatory pathways. These inflammatory pathways include the inflammasome and can lead to significant tissue destruction and corneal damage, with the potential for resultant blindness. Understanding the immune mechanisms behind this tissue destruction may enable improved identification of therapeutic targets to aid development of more specific therapies for reducing corneal damage in infectious keratitis. This review summarises current knowledge of pattern-recognition receptors and their downstream pathways in response to the major keratitis-causing organisms and alludes to potential therapeutic approaches that could alleviate corneal blindness.

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Introduction

Corneal disease is a significant and often underreported problem. The most recent World Health Organization (WHO) update estimates that 1% of global visual impairment is due to corneal infection or inflammation.[1] Of those reported as blind, corneal opacities contribute to 4%, making it the joint fourth most common cause of blindness worldwide; the main aetiological factor is infectious keratitis. In some parts of the world, corneal blindness can be caused by keratitis in as many as 20% of adults and 40% of children.[2] Trauma caused by agrarian activity is a major factor in its development. Infectious keratitis in the UK results in ~4000 people annually needing hospital treatment[3] and studies in the US have shown a rise in cases associated with increasing contact lens wear.[4,5] Particularly important is the fact that in both higher and lower income countries corneal disease frequently affects people of working age, causing significant associated morbidity and visual impairment.[2,6] In most parts of the world bacteria are the leading pathogens and despite the use of antibiotics, irreversible corneal damage still occurs.

Visual impairment in infectious keratitis results as a consequence of (i) the interactions of the pathogen with the host tissue, (ii) the host innate inflammatory response, and (iii) the therapeutic drugs used to treat the infection. The strength of this inflammatory response and its associated damage can vary depending on the pathogen and the severity of infection. Attempts are being made to better understand the mechanisms behind pathogen recognition and host innate immune responses with the aim of identifying future targets for immunomodulatory therapies. If targeted therapies can be achieved, the inflammatory damage and loss of vision currently caused by corneal infection and its treatment could be reduced, with an associated improvement in visual outcomes and reduction in morbidity.

Pattern recognition of pathogens in the cornea

Corneal barriers against infection

The intact corneal epithelium is a formidable barrier to bacterial penetration into deeper layers.[7] This is partly due to the tight junctions between superficial cells and also partly due to antibacterial peptides and innate immune signalling.[8–12] In addition, bathing tear fluid contains mucins, secretory immunoglobulin A (sIgA), and surfactant protein D, all

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antimicrobial factors that can bind microbes and potentially alter their interactions with corneal epithelial cells.[13–15] Epithelial cells also express several antimicrobial peptides, including human β-defensin 2 (hBD-2), cathelicidin LL-37,[11,12,16,17] and cytokeratin 6A.[18] In addition, superficial epithelial cells can internalise bacteria and then desquamate, thereby reducing the infective load.[19] Despite these defences, if microorganisms succeed in traversing the multilayered corneal epithelium, the epithelial basement membrane still presents a formidable barrier to further penetration. This is due to physical filtration via its pores that are smaller than the size of most bacteria,[20] or by improving the barrier function of the epithelial cells growing on top of them.[21]

Penetration of bacteria to the stroma typically requires a breach in the corneal epithelium, either by mechanical trauma such as epithelial abrasions, or by intrastromal inoculation, as happens in animal models of microbial keratitis, in which pathogens are either inoculated onto a scarified corneal surface or injected intrastromally.[22] Similar mechanisms may also be involved in fungal and protozoan infections. In some cases of extended contact lens wear however, infection and penetration can happen without an epithelial breach. This might be due to biofilm formation on the contact lens surfaces where residing bacteria are exposed only to sublethal concentrations of host antimicrobials and other defence factors, thereby enabling bacteria to adapt and overcome them.[7]

Corneal epithelial barriers to infection therefore consist of defences against adhesion and traversal. This likely involves junctional complexes, secreted and internal antimicrobial peptides, mucins, and the basal lamina foundation that provides a physical barrier while also supporting epithelial homeostasis. During and after microbial challenge, corneal epithelial defences are enhanced and regulated by epithelial derived cytokines and chemokines that can facilitate communication with cells of the immune system to further enhance corneal defences.

Innate immune cells in the mammalian cornea

A wide range of pathogenic organisms can infect the cornea, with varying mechanisms of infection and virulence (see Table 1). Although under normal circumstances the eye exhibits a certain amount of immune privilege, the cornea nonetheless must have a means of detecting and combating these pathogens.

Recent work has identified that immune cells including macrophages and dendritic cells have key roles in detecting and initiating the innate immune response in the cornea, expressing pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (nucleotide-binding oligomerisation domains; NLRs). PRRs recognise invariant pathogen structures known as pathogen-associated molecular patterns (PAMPs). TLRs were discovered in human immune cell lines in the late 1990s[38] and it was noted that activation of these receptors in humans produced a cascade of inflammatory cytokines such as IL-1, IL-6, and IL-8 via NF-kB. To date, 10 of these TLR molecules have been identified in humans, each of them recognising specifically conserved regions of pathogens or their products. Recognition of these conserved domains induces a signalling pathway resulting in a pro-inflammatory response, with resultant tissue damage and the potential for sight loss. Table 2 summarises the major domains that are recognised by each member of the TLR family. NLRs are discussed in the context of inflammasomes below.

Early attempts to identify the presence of myeloid-derived cells in the cornea concluded that Langerhans cells (LCs) were present in the peripheral third of the human corneal epithelium[43–45] but that antigen-presenting cells and other bone marrow-derived lineages were largely absent from the central cornea. This led to the belief that the cornea was an immune-privileged structure, such that when its regulatory mechanisms appeared to break down it was thought to be due to external factors such as corneal grafting.[46–48] However, resident corneal macrophage populations have been observed in murine and human cornea.[47,49,50] A picture now emerges of a stratified resident myeloid population in the human cornea, with major histocompatibility complex (MHC) $II^+/CD45^+$ bone marrow-derived cells present throughout all layers of the peripheral stroma, as well as in the anterior stroma of the central cornea.[51] Classification of cell surface markers has enabled cell lineage identification: myeloid-derived dendritic cells (DCs) are considered $CD11c^+$, while monocyte-derived lineages, including LCs, are $CD14^+$.[52]

Hamrah *et al*[53] observed that some murine DCs in the periphery of the anterior stroma also express MHC II, CD80, and CD86, but the DCs located in the centre present an immature, MHC $II^-CD80^-CD86^-$ phenotype until inflammation is induced. Work by Knickelbein *et al*[49] in humans has identified that $CD11c^+$ DCs are present in the basal epithelium and anterior peripheral stroma. LCs appear to be predominantly confined to the basal epithelial layer of the peripheral cornea with a very few cells present in the peripheral stroma. Macrophages have been found to be largely present in the anterior stroma. The presence of these bone marrow-derived cells in the cornea has proved crucial to elucidating the immune response of the cornea to infection.

Table 1 Pathogens causing corneal disease. Pathogens within each class of microorganism are listed in descending order of prevalence[23–37]

Bacterial	Gram-positive	Gram-negative
	Staphylococcus spp (*aureus*, *epidermidis*)	*Pseudomonas aeruginosa*
	Streptococcus pneumoniae	*Moraxella* spp
	Streptococcus pyogenes	*Klebsiella pneumoniae*
	Streptococcus viridans	*Enterobacter aerogenes*
	Corynbacterium diphtheroides	*Serratia* spp (*marcescens*, *liquefaciens*)
	Nocardia spp	*Acinetobacter* spp
	Propionibacteria acnes	*Enterococcus* spp
	Bacillus spp	*Burkholderia cepacia*
	Mycobacterium spp	*Escherichia coli*
	Micrococcus spp	*Stenotrophomonas maltophilia*
		Neisseria gonorrhoeae
		Haemophilus spp
		Kingella kingae
		Pseudomonas spp (non-*aeruginosa*)
		Citrobacter spp
		Aeromonas spp
Chlamydial	*C trachomatis*, *psittaci*	
Fungal	*Fusarium* spp	
	Aspergillus spp (*flavus*, *fumigatus*, *niger*, *terreus* and *nidulans*)	
	Alternaria spp	
	Cuvularia spp	
	Lasiodiplodia theobromae	
	Paecilomyces spp	
	Penicillium spp	
	Scedosporium apiospermum	
	Cephaliophora irregularis	
	Cladosporium cladosporoides	
	Cylindrocarpon spp	
	Exserohilum rostratum	
	Bipolaris spp	
	Candida spp	
	Pythium insidiosum	
Viral	Herpes simplex virus type 1	
	Varicella zoster virus	
	Human adenovirus (serotypes 8, 19, and 37)	
	Enterovirus type 70	
	Coxsackievirus type 24	
	Echovirus type 13	
	Poliovirus type 3	
	Cytomegalovirus	
Parasitic	*Acanthamoeba* spp (*castellani*, *polyphaga* and *culbertsoni*)	
	Microsporidia spp	
	Hartmannella spp	
	Vahlkampfia spp	
	Dictyostelium polycephalum	
	Phthriasis palpebrarum	
	Oestrus ovis	
Nematodal	*Loa loa*	
	Onchocerca volvulus	

Table 2 Summary of human TLRs and their major ligands.[39–42] For full general details of TLRs and their ligands please refer to reviews [39–41]

Toll-like receptor	*Receptor ligand(s)*	*Pathogen association*
TLR1 associates with TLR2	Triacyl lipopeptides	Bacteria
TLR2	Lipoprotein	Predominantly Gram – ve bacteria, fungi
	Zymosan	Fungi
	Peptidoglycan	Gram +ve bacteria
	Lipoteichoic acid	Gram +ve bacteria
	Porins	*Neisseria* spp.
TLR3	Double-stranded RNA	Viruses
TLR4	LPS	Gram – ve bacteria
TLR5	Flagellin	Bacteria
TLR6 associates with TLR2 to aid discrimination between diacyl and triacyl lipopeptides	Diacyl lipopeptides	*Mycoplasma* spp.
TLR7	Single-stranded RNA	Viruses may have a role in immune response to cancer
TLR8	Poly-G oligonucleotides	Viruses
TLR9	Single-stranded RNA Unmethylated CpG-DNA	Bacteria
TLR10	Currently remains unknown	

Intracellular Nod-like receptors and inflammasome activation after pathogen recognition

The concept of the inflammasome was coined in 2002[54] and rapidly generated intense study, adding a new dimension to scientific understanding of inflammation and innate immunity. NLRs are intracellular PRRs expressed within immune cells that sense invading microorganisms and initiate the innate immune response. Phylogenetic studies propose three NLR subfamilies based on a structural nucleotide-binding domain present in these receptors: (1) the NOD (nucleotide-binding oligomerisation domain); (2) the NLRP (pyrin domain; PYD); and (3) the NLRC (caspase-associated recruitment domain, CARD), also known as IPAF.[55]

In response to intracellular PAMPs, some NLRs induce the assembly of multiprotein complexes named inflammasomes that serve as platforms for caspase activation and, consequently, maturation of the pro-inflammatory mediators IL-1ß and IL-18. This inflammatory response is called pyroptosis. The multiprotein scaffold is commonly described as either a 'canonical' or a 'non-canonical' inflammasome. The canonical inflammasome describes the inflammasome platform containing NLRP3, the ASC adaptor protein required to recruit the pro-caspase-1 (CASP1), and the caspases 1 or 11. The canonical inflammasome is the most studied and well characterized. A non-canonical inflammasome is used to refer to any other inflammasome complex containing molecules other than those mentioned above.[55–57]

To date, two inflammasomes have been identified as assembling on the corneal surface in response to an infectious agent: the NLRP3 and NLRC4 inflammasomes. In contrast to NLRC4, the canonical NLRP3 is not constitutively expressed within the cell and requires TLR activation to induce its transcription via the NF-kB pathway.[58] The stimulation of NLRP3 and NLRC4 inflammasomes leads to the activation of caspase-1, a cysteine protease responsible for the cleavage and release of pro-IL-18 and pro-IL-1β. NLRC4 requires accessory proteins called Naips for its stimulation, but does not require recruitment of the ASC molecule for caspase-1 activation. However, association with ASC enhances the assembly of the inflammasome and maturation of the pro-inflammatory cytokines.[59,60] NLRC4 is also able to activate caspase-11 for pro-IL-18 and pro-IL-1β processing.[61] In addition, Meunier *et al*[62] recently described the requirement for small GTPases known as guanylate-binding proteins to be present for the complete stimulation of caspase-11. Figure 1. illustrates the major components of the inflammasome pathway.

Aspergillus fumigatus, a fungal mould, is known to stimulate the NLRP3 inflammasome and to induce maturation of pro-IL-1β to IL-1β,[63] and both NLRP3 and IL-1β expression were found to be increased in humans suffering from fungal keratitis infection.[64] *Pseudomonas aeruginosa* in mice is able to activate the NLRC4 inflammasome via immune cell internalisation of its bacterial flagellin, as well as via components of its type 3 secretion system.[65] *Streptococcus pneumoniae*

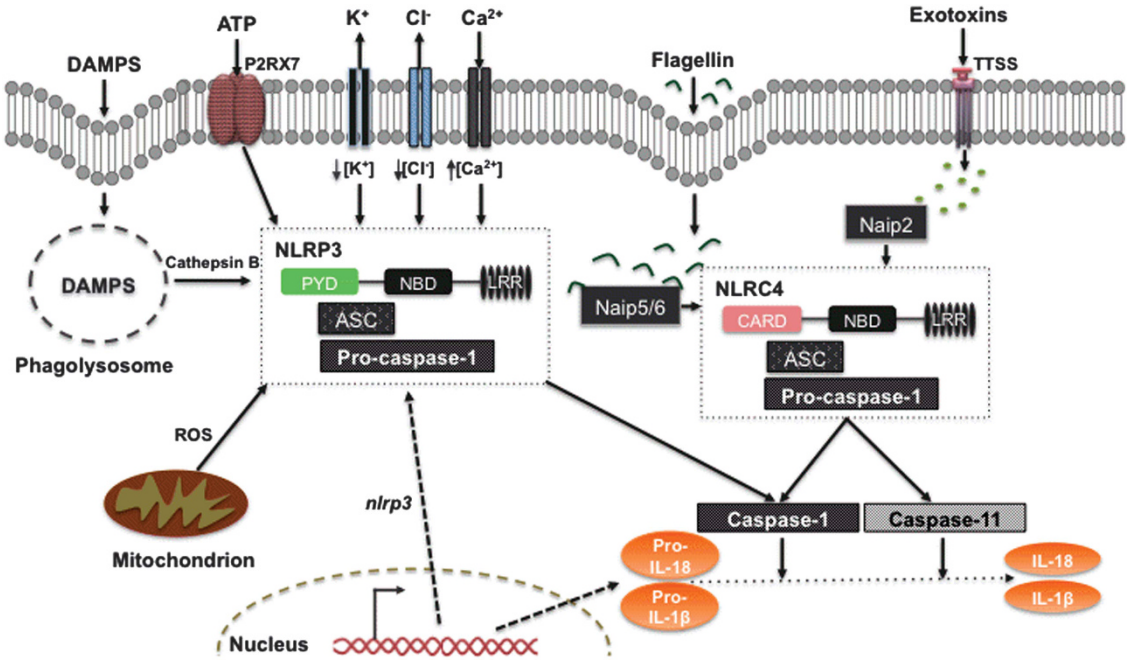

Figure 1 Assembly and activation of the inflammasome. NLRP3 and NLRC4 are the two inflammasomes described to date that assemble at the corneal surface in response to infectious agents. TLR4 stimulation induces *Nlrp3* gene expression and NLRP3 transcription; in contrast, NLRC4 is constitutively expressed in the cell.[58] Both inflammasomes share common structural domains (nucleotide-binding domain and LRR) and the ability to recruit the adaptor molecule ASC, which facilitates the association of pro-caspase-1.[56] Different mechanisms of activation have been described for each inflammasome: extracellular ATP activates NLRP3 via P2RX7 receptor stimulation that provokes a decrease of the intracellular K^+ levels.[68] Alteration of Cl^- or Ca^{2+} fluxes or production of reactive oxygen species by mitochondria (a consequence of cell damage), are also able to stimulate the NLRP3 inflammasome. Furthermore, phagocytosis of damage-associated molecular pattern molecules promotes lysosome destabilization and the release of cathepsin B protein into the cytosol, activating the canonical inflammasome.[69,70] NLRC4 is activated via internalisation of flagellin and components of the *Pseudomonas* bacterial virulence factor type 3 secretion system.[65] NLRC4 needs a protein known as Naip for its stimulation: Naip5-6 proteins are responsible for binding the internalised flagellin and Naip2 binds the components of the type 3 secretion system.[56,57,59] These inflammasomes lead to the activation of Caspase-1 protease. Caspase-1 protease is required to cleave the pro-interleukins necessary to generate the mature forms of IL-18 and IL-1*β*. The cysteine protease Caspase-11 can be also activated by NLRC4 and promote the maturation of these pro-interleukins.[61]

pneumolysin has been shown to activate NLRP3 in mice[66] and both *S. pneumoniae* and *P. aeruginosa* corneal infection caused upregulated expression of NLRP3 and NLRC4 in human keratitis.[67] The significance of the inflammasome and stimulation of pyroptosis in corneal infection appears therefore to be a considerable contributory factor to tissue destruction and impairment of vision.

Pathogens causing corneal disease

Pattern recognition mechanisms activated by microbial pathogens in the cornea

Pseudomonas aeruginosa The healthy human cornea is inherently resistant to infection. This is attributable to multiple integrated factors that have evolved across species to protect the eye against a broad range of microbial pathogens, including bacteria, fungi, viruses and protozoa.[7]

P. aeruginosa is the most common cause of Gram-negative bacterial keratitis[29,71] with a more severe clinical course than other bacterial pathogens if left untreated.[72] The healthy eye is generally protected against *P. aeruginosa* infection. Although cultured corneal epithelial cells are easily invaded and killed by most clinical and laboratory isolates of *P. aeruginosa*,[19,73] extremely large inoculates of invasive and cytotoxic *P. aeruginosa* onto intact mouse or rat corneas *in vivo* results in rapid bacterial clearance from the ocular surface without pathology.[74] This suggests that certain defence mechanisms against infection exist in the healthy eye that are absent from laboratory culture conditions. Although many *P. aeruginosa* strains grow readily in undiluted tear fluid despite its antimicrobial components such as lactoferrin and lysozyme,[75,76] tear fluid can still protect corneal epithelial cells against them.[77,78] This is thought to be due to the tear fluid acting directly upon corneal epithelial cells to make them more resistant to *P. aeruginosa* invasion and cytotoxicity.[74]

Should conditions enable *P. aeruginosa* to breach the corneal epithelium, its lipopolysaccharide (LPS) and

flagellin molecules are recognised by TLR4 and TLR5 on macrophages of the corneal stroma,[79] initiating a pro-inflammatory pathway via the well-characterised adaptor molecule MyD88. MyD88 appears to be essential to all TLR signalling except TLR3.[80]

Another adaptor molecule used by TLR4 is TIRAP (also known as Mal); although this is activated in *P. aeruginosa* infection, it does not appear to mediate *in vivo* keratitis.[79] TLR4 must be associated with co-stimulatory molecule MD-2 to recognise LPS;[81] accessory proteins lipopolysaccharide-binding protein and CD14 transfer the LPS molecules from the bacteria to the TLR4/MD-2 complex.[82] Resident myeloid cells are capable of conferring LPS responsiveness,[83] but although TLR4 is also expressed on human corneal epithelial cells, MD-2 is not detectable. It is thought that MD-2 expression is enabled by the infiltration of immune cells such as natural killer cells, which activate the JAK2/STAT1 transcription pathway and produce IFN-γ.[84,85] In a murine model of corneal inflammation, Chinnery *et al*[83] demonstrated that TLR4 activation on resident corneal macrophages and DCs stimulated macrophage and neutrophil recruitment and induced corneal haze.

On activation, numerous adaptor proteins are recruited to initiate the inflammatory response (see Figure 2). In addition to the MyD88 pathway, a non-MyD88 pathway via TRIF exists which has also been found to be present in *P. aeruginosa* keratitis.[83] MyD88/TRIF pathways induce production of a chemokine known as CXCL1/KC which recruits neutrophils to the cornea from limbal blood vessels. MyD88/TRIF pathways also promote the production of IL-1α and IL-1β, which creates a positive feedback loop via the interleukin 1 receptor IL-1R1 interaction with MyD88.[79] The presence of TLR4 in murine corneal *P. aeruginosa* infection has also been associated with the release of antimicrobial factors such as nitric oxide (NO), the product of inducible nitric oxide synthase (iNOS) and β-defensin 2.[86]

Staphylococcus aureus Although studies consistently report *Staphylococcus* spp. as some of the commonest causes of bacterial keratitis,[90–92] contact with the organisms infrequently results in infection. A study by Moreau *et al*,[93] in which *S. aureus* was topically applied to scarified rabbit eyes resulted in bacterial killing and identified a role for phospholipase A2 in the tear film as

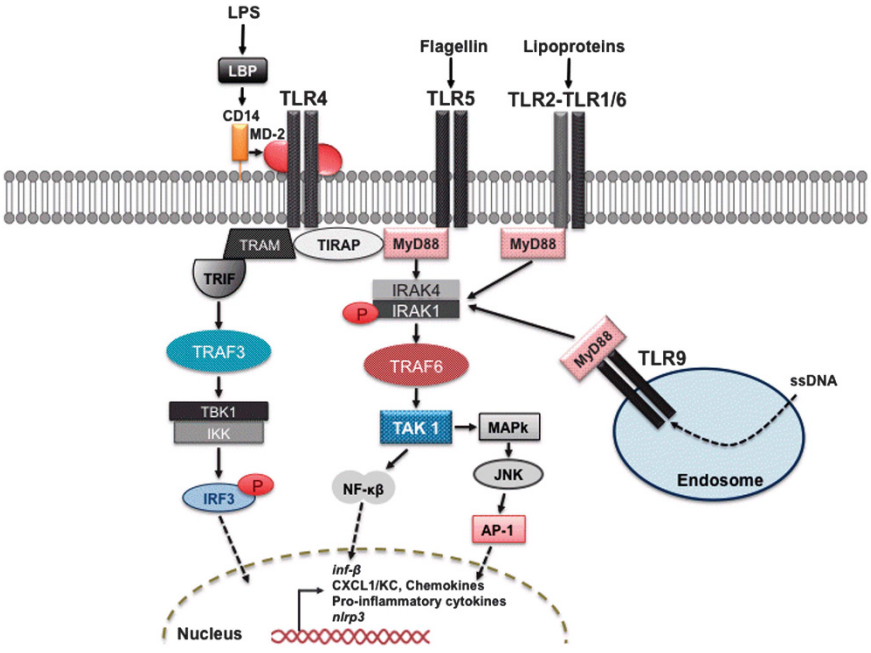

Figure 2 Immune recognition of *Pseudomonas aeruginosa* molecular patterns. LPS-binding protein binds the LPS from the *P. aeruginosa* cell wall and the complex is recognised by the CD14 receptor. CD14 transfers the LPS to the MD-2 molecule that is associated with TLR4. MD-2 undergoes changes in its conformation and triggers the stimulation of TLR4. TLR4 dimerises and recruits TIR-domain-containing adaptor proteins (TRIF and TIRAP) to start the signal transduction. TIRAP requires MyD88 protein for its activation, which also recruits IL-1 receptor-associated kinases IRAK4 and IRAK1. Phosphorylation of IRAK1 by IRAK4 activates the E3 ubiquitin protein ligase TNFR-associated factor 6 (TRAF6). This activates TGF-β associated kinase (TAK)1 which results in transcription of pro-inflammatory cytokines via the NF-kB and mitogen-activated (MAP) kinase/JNK pathways. TLR3 can also signal via TRIF.[87] Activation of TLR3/4 recruits TRIF via TRAM which signals through RIP1 and TRAF6 to the NF-kB and JNK pathways. TRIF also activates TRAF3, which through TBK1 and IKK phosphorylates IRF3 and stimulates the production of type I interferons.[80,88,89] ssDNA containing unmethylated CpG motifs is sensed by TLR9. In contrast to the other TLRs mentioned above, TLR9 is localised in the endosome. It also activates the MyD88-dependent pathway.[39,41]

a crucial bactericidal agent. Interestingly, work by Heimer *et al*[94] suggests that on infection of human corneal epithelial cells *in vitro* the principal difference between toxigenic and non-toxigenic strains of *S. aureus* appears to be an increased activation of stress response molecules such as heat shock proteins on exposure to a toxigenic strain of *S. aureus*.

Recognition of *S. aureus* at the corneal surface has been shown to be mediated by TLR2.[95,96] At least three different PAMPs have been identified as binding to TLR2 and activating the immune response in *S. aureus* keratitis: bacterial lipoproteins,[95] peptidoglycan molecules,[97] and *S. aureus* protein A (SpA).[98] TLR2 then activates the MyD88 pathway;[95] however, Kumar *et al*[98] demonstrated that SpA also activated other pathways (p38 and ERK) via an unknown receptor, citing TNF-α receptor 1 (TNFR1) as a potential candidate. The work of Heimer *et al*[94] lends further support to this theory: a large increase in the dendritic cell chemokine CCL20 was identified when human corneal epithelial cells were stimulated with both toxigenic and non-toxigenic strains of *S. aureus*, which appeared to be independent of TLR2 stimulation. Bacterial lipoproteins have also been shown to activate alternative pathways to MyD88, including p38 and ERK but also JNK. This results in release of pro-inflammatory mediators and chemotactic cytokines such as IL-6, IL-8, and ICAM-1 as well as antibacterial molecules such as NO.[95] Peptidoglycan was shown to activate all four of the pathways described above;[97] this study also identified release of human β-defensin 2 by the epithelial cells, the same group later confirming its release via pathways downstream of TLR2.[99] A study by Adhikary *et al*[100] described the JNK pathway as predominant in TLR2 activation.

Onchocerca volvulus (onchocerciasis) Onchocerciasis is due to infection with the microfilarial nematode worm *Onchocerca volvulus*, which can produce a blinding keratitis. Systemic response to *O. volvulus* has been shown to vary based on differing immunological profiles, with some infected individuals displaying no symptoms, while others develop a severe dermatitis known as sowda.[101,102] Studies in animals suggest that previous exposure to *O. volvulus* antigens is necessary to develop systemic symptoms, indicating a role for the adaptive immune response.[103,104] A strong Th2 response has been implicated, with release of IL-4, IL-5, IFN-γ, IgG_1, and IgE.[105–107]

More recently, attention has focused on the fact that *O. volvulus* maintains an endosymbiotic relationship with *Wolbachia* bacteria and that microfilaria release bacterial products which activate the immune response in human embryonic kidney cells and mice.[108,109] Microfilaria can invade the eye and stimulate both innate and adaptive immunity. Recognition of *Wolbachia* products on macrophages and DCs is mediated by TLR2/TLR6, leading to MyD88/TIRAP activation and the release of pro-inflammatory cytokines via NF-kB.[110] Previous work suggests that Th2 immune responses predominate (see Pearlman and Hall[111] for review), but Turner *et al*[112] showed that activated $CD11c^+$ DCs upregulate CD80 and CD86 adhesion molecules and can stimulate a $CD4^+$ T-cell activation with a predominantly Th1 response. Interestingly, this study also demonstrated that *Wolbachia* depletion resulted in a switch to a Th2-driven inflammatory response.

Once activated, macrophages release further Th1-driving cytokines such as IL-12 and TNF-α.[109,111] Neutrophils are recruited from the limbal vessels via release of CXC chemokines such as CXCL1 and CXCL2 and upregulation of vascular adhesion molecules such as PECAM-1.[113,114] Eosinophil infiltration occurs later in the disease but aggravates the inflammatory response.[106,114–116] However, neutrophil involvement is thought to be the driving factor behind the progressive corneal haze seen in onchocerciasis.[116]

Fungi The most common agents in fungal infection are *Fusarium* spp. and *Aspergillus* spp.[117] Compared with bacterial keratitis, fungal keratitis is frequently associated with poorer outcomes[118] and is a recurrent problem in many parts of USA with a recent increase in contact lens wearers.[119–121] In developing countries, ocular trauma is a major risk factor.[31,122,123] Fungal spores are able to penetrate compromised epithelium where they germinate, expressing β-glucan and α-mannan molecules on their surface. These are recognised by the receptors Dectin-1 and Dectin-2 on macrophages, which then signal via spleen tyrosine kinase (Syk) and CARD 9 proteins to activate the NF-kB pro-inflammatory pathway. This produces IL-1β and chemokines such as CXCL1 and CXCL2.[124,125] IL-1β is recognised by the IL-1R1 receptor and via MyD88 activates further pro-chemotactic cytokines and upregulation of vascular adhesion molecules to promote neutrophil recruitment. Leal *et al* have also demonstrated a role for TLR4 in fungal killing.[125] Analysis of infected human corneas has revealed high levels of IL-1β, IL-8, IL-17, and TNFα in the early stages of fungal infection. Later stages identified $CD3^+$ and $CD4^+$ T-cells, with associated high levels of IL-17 and interferon-γ.[126] A study by Taylor *et al*[127] identified IL-17 as a cytokine involved in fungal killing; infiltration of Th1 and Th17 cells and a subpopulation of IL-17 producing neutrophils enabled optimal immune protection.

Herpes simplex virus Herpes simplex virus (HSV) is thought to be the primary global cause of infectious blindness. HSV1 is more commonly associated with oral

and ocular pathology, while HSV2 is frequently transmitted through sexual contact.[128] In addition, HSV infection presents the particular problem of recurrent infection.

HSV1 binds to host cells via membrane glycoproteins. To date, five glycoproteins have been described that mediate binding and fusion with the host cell: glycoprotein C (gC), gB, gD, gH, and gL. Each of these has a role in the binding and adhesion process to enable the viral capsid to enter the cell.[129–131] Several host surface receptors have been identified, including herpes virus entry mediator, nectin-1, 3-O-sulphated heparan sulphate and paired immunoglobulin-like type 2 receptor α (PILR-α).[132–135] However, a study by Shukla *et al*[136] identified nectin-1 as being the crucial mediator in a murine model of corneal infection. Of note when considering the mechanism of HSV1 infection is the fact that phylogenetic analysis suggests the evolution of at least six distinct HSV1 genogroups, with multiple recombination events detected in the genome coding for glycoproteins.[137,138] The high mutation rate of such viral proteins provides an additional challenge when aiming to identify targeted and/or personalised therapeutic options.

HSV1 activates multiple immune pathways on binding with a cell. TLR2$^{-/-}$ mice have been shown to have a decreased inflammatory response to infection,[139,140] whereas CpG sequences of HSV1 DNA are recognised stimulators of TLR9.[141,142] TLR3 recognises double-stranded RNA and has been identified as an important mediator of HSV1 infection.[143,144] Human corneal epithelial cells have been shown to respond to HSV1 via TLR3 and also to express TLR7 as a result of infection.[145] Additionally, TLR4 is activated in mice by endogenous heat shock protein 70 (Hsp70) and β-defensin-3 expressed by corneal cells in response to the virus.[140] Interestingly, MyD88$^{-/-}$ mice had reduced corneal inflammation, but were unable to control viral spread, with 70% dying of presumed HSV encephalitis.

TLR9 is known to activate the NF-kB, p38 and JNK pathways.[40] In addition, the transcription factors cAMP response element (CRE) and CCAAT/enhancer binding protein (C/EBP) are activated in the mouse corneal endothelium, leading to release of numerous pro-inflammatory cytokines including IL-6, RANTES/CCL5 and CXCL10.[146] Ligand binding to TLR3 activates TRIF, which in turn activates NF-kB and interferon regulatory factor 3 (IRF3), an interferon regulatory factor. In addition to cytokines such as IL-6, IL-8, and TNF-α produced by NF-kB transcription, activation of IRF3 results in production of anti-viral cytokines such as type 1 interferons (IFN) -α and -β.[145,147–151] TLR7 signals both via MyD88 and NF-kB but also via IRF7, which causes type 1 IFN to be produced; in addition, IRF7 can interact with the MyD88 pathway via TRAF6 and IRAK4.[152,153]

Activation of these pathways recruits DCs, macrophages, neutrophils and NK cells to aid in clearance of the virus (see Rowe *et al*[154] for a review of HSV keratitis, including innate immune cells and their ligands). Latency of the virus occurs due to a switch in the viral genome that suppresses lytic genes and maintains an equilibrium between latency associated transcripts (LATs) and micro RNAs that silence the active genome.[155] Immune control of the virus during the latency phase is thought to exist, mediated by HSV1-specific CD8$^+$ T-cells capable of releasing IFN-γ.[156] Although the causes and mechanisms for reactivation are beyond the scope of this review, several differences between primary infection and recurrent disease are known to exist. Despite the fact that IL-6 is an important pro-inflammatory cytokine in primary disease, it has been shown to be of little significance in reactivation, in contrast to CXCL1 which acts as a chemokine to attract neutrophils to the site of infection.[157] CD4$^+$ and CD8$^+$ T-cells are important mediators in recurrent disease,[158] while other factors such as the chemokines CCL2 and CCL3 appear to have their roles reversed in reactivation, with CCL2 increasing disease severity and CCL3 appearing to offer some protection.[159] However, full details of the differences between primary and recurrent infection remain to be elucidated.

Acanthamoeba *Acanthamoeba* is a ubiquitous protozoan pathogen capable of causing severe and persistent corneal infection. It exists in active trophozoite form but can also encyst, creating the potential for reactivation of disease after treatment is ended.[160] Several studies have concluded that infection with *Acanthamoeba* does not protect against reinfection, suggesting that immunological defence is predominantly mediated by the innate immune system.[161–164]

Patients presenting with *Acanthamoeba* infection have been found to have low levels of anti-*Acanthamoeba* sIgA in their tearfilms,[165] raising the suggestion that IgA is an important protective factor against infection at the corneal surface. Various protective mechanisms of IgA have been identified, including inhibition of *Acanthamoeba* binding to epithelial cells,[161,166] complement activation and opsonisation[167,168] and augmentation of neutrophilic killing.[169]

More recently, the involvement of TLRs in pathogenesis of *Acanthamoeba* keratitis has been characterised. Under favourable conditions, *Acanthamoeba* is able to attach to the cell surface via mannose-binding protein, which interacts with mannose-containing glycoproteins on the corneal surface.[170,171] The trophozoite then releases a mannose-induced serine protease (MIP-133) which causes cytolysis of corneal epithelial cells and enables the *Acanthamoeba* to infiltrate the deeper layers of corneal

tissue.[172,173] *Acanthamoeba* is recognised by TLR4, which initiates the MyD88/NF-kB pathway and also the MAPK/ERK pathway, leading to release of pro-inflammatory and chemotactic mediators such as IL-8, CXCL2, TNF-α, and IFN-β.[174,175]

Macrophage activation in the presence of anti-*Acanthamoeba* antibody and IFN-γ has been shown to demonstrate trophozoite killing activity.[167,176] Macrophages are thought to be a crucial mediator in early infection: depletion of macrophages in a Chinese hamster model of *Acanthamoeba* keratitis produced a notable worsening of disease.[177] Neutrophils are also capable of killing trophozoites,[169,178] and both neutrophils and macrophages are able to kill cysts; however, no chemotactic stimulus is recognised when cysts are dormant in the cornea.[179] Steroid use in treatment of *Acanthamoeba* has been shown to increase the virulence of the disease, with proliferation of trophozoites and stimulation of excystment.[180]

An additional factor in the pathology of *Acanthamoeba* keratitis is its frequent co-existence in symbiotic relationships with potentially pathogenic species of bacteria. A study by Iovieno *et al*[181] identified strains of *Legionella, Pseudomonas, Chlamydia* and *Mycobacterium* present in confirmed cases of *Acanthamoeba* keratitis. Where an endosymbiotic relationship was found, an increase in corneal toxicity was also noted. Moreover, the authors suggest that bacterial co-infection could delay or confound the diagnosis of *Acanthamoeba*, further damaging the cornea before correct treatment is initiated.

The relevance of animal models of keratitis

Much of the work that has been carried out in understanding the pathogenesis of infectious keratitis has used animal models. These provide a valuable insight into immune function in the mammalian cornea, but require complementary approaches when regarded from a clinical perspective. Animal models are limited by the fact that they are anatomically and physiologically distinct from human tissues. Examples include the fact that the blink rate in both mice and rabbits is lower than that in humans;[182,183] humans also have a much higher concentration of lysozyme in their tears.[184] Rabbits possess a nictitating membrane, and the structure of a human cornea is different in several ways to that of mice and rabbits: Bowman's layer and Descemet's membrane are thicker,[185] the orientation of the collagen is different[186] and the depth of each of the layers varies depending on species.[187]

On a molecular level, examples of species differences include the fact that mice are known to use several NAIP accessory proteins to activate the NLRP4 inflammasome, whereas in humans only one type of NAIP has been found.[59,188] Caspase-11 is known to be present in mice, but its analogue in humans is caspase-4.[189] Differences in cytokines also exist, such as MIP-2 in mice which is equivalent to human IL-8.[190] In addition, the surface mucins of humans are structurally different to those of other mammals.[191] Moreover, the methods used to cause keratitis in animal models do not always mimic the natural infective process. Intrastromal injection is frequently used that bypasses the cell surface stage of infection;[192–195] other methods include removal of the corneal epithelium,[196] corneal scratch[197] or applying contact lenses coated in bacteria to wounded corneas.[198]

Generally, the differences between animal and human anatomy and immunology suggest that a cautious approach is necessary when interpreting findings from animal studies in a clinical perspective. The development of primary human cell/explant models would bring us closer to revealing the true nature of pathogen–host cell interactions in microbial keratitis.

Potential targeted molecular therapies

Studying immune pathways and mechanisms of infectious keratitis could identify new therapeutic targets to ameliorate disease and reduce tissue damage. At present, clinical use of topical steroids is frequently relied upon, but this is a non-specific attempt to reduce inflammation and can be associated with complications such as corneal thinning and perforation, increased intra-ocular pressure and poorer outcomes in infections such as *Nocardia*, HSV1, and *Acanthamoeba* keratitis.[180,199,200] The Steroids for Corneal Ulcers Trial (SCUT) has shown in large multicentre trials that steroids have no significant benefit or harm if used adjunctively alongside treatment of bacterial keratitis in the short term, but appear to improve visual outcomes at 12 months post treatment. Those with the most significant improvement in visual outcomes were those whose baseline vision was poorest, with most of the improvement occurring within the first 3 months for all patients.[199,201,202]

In the search for more specific, tailored therapies, molecular targets are being investigated as a means of reducing tissue damage and restoring sight. Various avenues are being explored according to specific pathogen characteristics and cellular pathways. Work is continuous, but it appears that promising opportunities are being identified. Alekseev *et al*[203] have recently identified a kinase, ataxia telangiectasia mutated (ATM) kinase, whose inhibition reduces the severity of keratitis in a mouse model of HSV1 keratitis and additionally slows viral replication in rabbit and human cultured corneas. A high-affinity human monoclonal antibody to S. *aureus* α-toxin has been found to be effective at reducing corneal damage[204] and a new antibiotic, targocil,

has had some preliminary success at inhibiting the severity of staphylococcal infections.[205] Several potential therapies have been identified in *Pseudomonas* infection, including lithium chloride[206] and a caspase-1 inhibitor that reduces the amount of IL-1β produced.[207] Factors that improve corneal inflammation include the apoptotic Fas pathway which regulates the production of pro-inflammatory cytokines[208] and a phospho-inositol-3-kinase (PI3K)/Akt pathway that activates a receptor known as triggering receptor expressed on myeloid cells 2 (TREM-2).[209] All of these reduce inflammation and tissue damage and may be avenues to explore further.

A novel approach has been investigated in fungal keratitis, with some success: using small interfering RNA targeted against TLR2, Guo *et al*[210] were able to improve the outcome of *A. fumigatus* disease in a rat model, with a decreased fungal burden and reduced production of pro-inflammatory cytokines leading to clearer corneas and fewer instances of perforation. A pathway has also been identified in sterile corneal inflammation to inhibit neutrophil infiltration and macrophage activation: heat shock protein HSPB4 appears to act as a damage-associated molecular pattern, activating TLR2 and the NF-kB pathway.[211] The authors showed that inhibition of this pathway via HSPB4 antibodies or TNF-α stimulated gene/protein 6 (TSG6) suppressed macrophage activation and resultant neutrophil infiltration, leading to an improvement in corneal clarity. Meanwhile the endosymbiotic nature of *O. volvulus* with *Wolbachia* bacteria has led to trials of doxycycline being used to deplete the bacteria and facilitate treatment of the worms.[212–214]

Corneal infiltrates in non-keratitic diseases show some promise and offer another avenue of exploration: a case report of cryopyrin associated periodic fever syndrome, in which a mutation in the *NLRP3* gene causes overexpression of IL-1β, demonstrated successful treatment outcomes with IL-1β monoclonal antibody kanakinumab,[215] and anakira, an IL-1 antagonist, has been used successfully in corneal infiltrates.[216]

Currently, there is strong interest in the use of collagen cross-linking (CXL) using ultraviolet-A (UV-A) light and riboflavin is a treatment of infectious keratitis. In this procedure riboflavin is applied to the affected corneal surface and the agent acts as a photosensitizer, which generates reactive oxygen species which are activated by UV-A light. The resulting photochemical reactions are thought to result in covalent bonds to cross-link collagen fibres in the corneal stroma and thus increase the strength of the cornea. Alio *et al*[217] performed a meta-analysis of 12 studies that used this approach against a variety of types of microbial keratitis. They analysed a total 104 eyes that were treated using a range of treatment protocols with varying levels of clinical presentation. In the majority of cases cross-linked patients were reported to have halting of the corneal melting process. Few patients developed complications from CXL therapy. The treatment appeared to show promise in in bacterial and *Acanthamoeba* infections but less so in fungal infections. There were concerns that CXL could halt drug penetration for patients with fungal keratitis. Additionally, in rabbit studies of *Acanthamoeba* keratitis CXL showed no beneficial effect. A major difficulty with the data from the studies so far is the wide variation in the type of infectious keratitis cases, with patients at different stages of clinical presentation treated with varying therapeutic regimens. Until more controlled studies are undertaken and reported, it is difficult to recommend CXL routinely. However, CXL in microbial keratitis does offer a reasonable rationale to halt corneal melting in selected cases. It is likely that a combined approach using PAMP inhibitor-based anti-inflammatory agents with newer antimicrobials/CXL/anti-matrix metallo-proteineases will show the strongest effect in treating corneal tissue damage.

In the context of infectious keratitis the goal of individualised, targeted therapy appears to be moving closer. However, in addition to the range of molecular pathways activated, the characteristics of a bacterial keratitis may depend upon such features as bacterial virulence and toxigenicity whereas a viral keratitis may depend upon the viral genotype and rate of mutation. Factors such as these may continue to present challenges that must be taken into account when designing future therapies.

Conclusion

The importance of PAMPs has significantly increased over the past decade. We have reached an exciting point in the study of infectious keratitis, with so many molecular pathways being identified. This provides several opportunities to develop therapies targeted to modifying disease outcomes. However, more work on the various potential therapeutic targets that exist within the molecular pathways we are beginning to define must be done. Ultimately, these therapies will need to balance adequate treatment of disease with minimising tissue damage in such a thin tissue as the highly specialised cornea. This presents an ongoing challenge for both scientists and clinicians.[218] Nonetheless, we may now be closer than previously imagined to effective, targeted therapies to treat corneal tissue damage and subsequent blindness as a result of the global disease that is infectious keratitis.

Conflict of interest

The authors declare no conflict of interest.

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