Published in final edited form as:

Cytokine. 2012 April ; 58(1): 107–111. doi:10.1016/j.cyto.2011.12.022.

The Role of Cytokines and Pathogen Recognition Molecules in Fungal Keratitis – insights from human disease and animal models

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Abstract

Fungal infections of the cornea are an important cause of blindness and visual impairment worldwide, with contact lens wear being the main risk factor in the USA and other industrialized countries, and traumatic injury being the main risk factor in developing countries. In this review, we highlight recent advances in the understanding of the host response to *Aspergillus* and *Fusarium* species in infected human corneal tissue and in mouse models of fungal keratitis.

Keywords

Aspergillus; *Fusarium*; cornea; keratitis; blindness; innate immunity; Toll Like Receptors; Dectin-1

1.1 Impact of Fungal Keratitis on Global Visual Health

Fungal keratitis (infection and inflammation of the cornea) is an important cause of blindness and visual impairment in the USA and other industrialized countries, where contact lens wear is the primary risk factor. This was illustrated by several hundred cases in the USA, Western Europe and Singapore during the 2005/2006 outbreak of contact lens associated *Fusarium* keratitis (1–3), and contact-lens and trauma associated fungal keratitis are continually reported in the USA (4). However, in developing countries, trauma to the ocular surface, most commonly in relation to agricultural work, is the major risk factor for fungal keratitis. (5). On a global scale, fungal keratitis accounts for ~65% of all corneal ulcers (6–13). In India, it is estimated that 80,000 total cases and 10,000 corneal transplants are performed each year due to fungal corneal infections (6, 8, 13).

Aspergillus (*A. flavus*, *A. fumigatus*,) and *Fusarium* (*F. solani*, *F. oxysporum*) species are the main etiologic agents of fungal keratitis (14). However, other species and genera of filamentous fungi that cause keratitis include: *Curvularia*, *Alternaria*, and *Penicillium* (2, 15–18). Most infections with these organisms are initiated in an agricultural environment as a result of ocular surface trauma caused by plant material, insects or branches contaminated with fungal spores (7, 8, 19). In the corneal stroma, conidia germinate into hyphae, which

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then penetrate throughout the stroma and the basement membrane, where they also infect the anterior chamber, causing severe pain, photophobia and vision loss. Topical natamycin or voriconazole are effective if given very early, but fungal keratitis is notoriously difficult to treat, especially after the hyphae penetrate deeper stromal layers. Depending on the inoculum, the time until treatment, infected individuals will require corneal transplantation (10% of cases). Following transplantation, the rejection or reinfection of transplanted cornea tissue can occur, and in severe cases the only recourse is enucleation of the affected eye (5). In milder cases, resolution of infection is accompanied by fibrosis, resulting in visual impairment. In contrast to trauma – induced fungal keratitis, contact lens associated fungal keratitis is likely due to the hyphal stage. Airborne conidia settle in a lens case, germinate and form a biofilm on the lenses and the case (20, 21). Following contact with the ocular surface, hyphae penetrate into the cornea stroma through minor epithelial abrasions where they establish infection (22).

2.1 Anti-Microbial Defenses at the Ocular Surface

Figure 1A illustrates a cross-sectional diagram of the human eye and cornea. The transparent cornea provides most of the refractive index that is essential for the accurate transmission of light through the pupil and to the retina, where photoreceptor cells transmit images to the visual cortex of the brain. Infection or inflammation disrupts the role of the cornea, as inflammation is associated with edema and change in refractive index, resulting in impaired vision. The avascularity of the cornea is also important in maintaining corneal clarity.

The cornea and ocular surface are protected from trauma and infection by physical and molecular defenses. Perhaps the simplest and most effective defense involves eyelid closure and blinking, which protects the cornea from physical trauma, and removes microbes from the ocular surface. In addition, ocular surface mucins and the tear film restrict pathogen interaction with the corneal epithelium (23). The tear film also contains β -defensins, calprotectin and, lysozyme (24, 25) (23, 26, 27). Tears also contain high levels of the iron-chelating protein lactoferrin (28) and the siderophore-binding protein lipocalin (29). Though their role in tears is not fully understood, it is likely that cation (Fe^{2+} Zn^{2+}) sequestration in the ocular surface inhibits fungal germination and growth, which requires these essential metals (28).

Figure 1B is a histological section of the normal human cornea, showing the main layers of cornea, which are the corneal epithelium, stroma, endothelium, in addition to the underlying anterior chamber. The major physical barrier against bacterial and fungal infection is the non-keratinized, stratified corneal epithelium, comprising three layers of epithelial cells with tight junctions that form a physical barrier preventing microbial access to the corneal stroma (30). Murine studies of fungal keratitis or *Pseudomonas aeruginosa* have shown that an intact corneal epithelium will restrict access of millions of live organisms to the corneal stroma even under stressful conditions such as long term contact lens wear (22, 31–34). However, the main site of infection is the corneal stroma, which comprises 90% of the tissue, and is a dense, highly organized matrix, with anti-parallel layers of collagen separated by keratan sulfate proteoglycans that are essential for corneal transparency (35).

When the integrity of the corneal epithelium is breached due to trauma, fungal conidia or hyphae can penetrate into enter the corneal stroma, where growth is uninhibited in the absence of leukocyte recruitment (22, 33, 34). During infection, hyphae penetrate through this dense matrix and also the basement membrane of the corneal endothelium. In the absence of an effective host response, hyphae enter the underlying anterior chamber and in some cases can also invade the posterior eye vitreous causing blinding endophthalmitis (Inflammation in the posterior eye), at which point enucleation of the infected eye is often indicated. Figure 1C, shows heavy fungal burden and penetration throughout the corneal

stroma and towards the anterior chamber in a post-transplant infected cornea from a patient with fungal keratitis (*A. flavus*) who underwent corneal transplantation. The cornea has resident macrophages and dendritic cells (36–41), and following infection, infiltrating macrophages express TLRs (Toll-like receptors), and Dectin-1 (Dendritic Cell Associated C-type lectin 1), and Dectin-2 and can respond to fungal cell wall components by producing pro-inflammatory cytokines that mediate neutrophil recruitment from the peripheral, limbal blood vessels, and kill fungal hyphae (33, 42).

3.1 The Host Response in Infected Human Corneas

Susceptibility to fungal infections is associated with polymorphisms in genes associated with innate immunity, including the Dectin-1/CARD9 and TLR4 pathways (43–45). Our recent study in collaboration with investigators at the Aravind Eye Hospital in southern India characterized innate and adaptive immunity in infected corneal tissue at early and late stages of fungal keratitis (46). In the early stages of infection when infected individuals were examined within days after experiencing ocular trauma, a scraping was taken from the corneal ulcer, RNA was extracted, and quantitative PCR analysis showed that Dectin-1, TLR2, TLR4, TLR9, NLRP3 (NOD-like receptor pyrin domain containing 3), and ASC (Apoptosis-associated speck-like protein) transcripts were elevated >1,000-fold compared with normal donor corneas, whereas Dectin-2 was constitutively expressed in normal corneas. Further, IL-1 β expression was elevated >1,000 fold, whereas IL-1 α expression was not increased. IL-8, IL-17 and TNF- α expression was also elevated. Interestingly, > 95% of the cells from the corneal scrapings were neutrophils, suggesting that these cells are a source of pro-inflammatory and chemotactic cytokines expressed at this time point.

To examine later stages of corneal infection (> two weeks post infection), infected corneas recovered after transplant surgery were examined by qPCR and immunohistochemistry. These studies showed that β -glucan ligand was expressed on the hyphal cell wall during infection and estimated that the cellular infiltrates during corneal infection constituted: 70% neutrophils, 25% macrophages, and 5% CD3⁺ CD4⁺ T cells. qPCR analysis also revealed elevated IFN- γ and IL-17, but not IL-4 expression, which is consistent with the presence of Th1 and Th17 cells in fungal infected tissues. Interestingly, there were no significant differences in the host response between *Aspergillus* and *Fusarium*-infected corneas at any time point.

3.2 The Host Response in Animal Models of Fungal Corneal Infection

Earlier models of fungal keratitis in rats, rabbits and mice implicated a role for the host response by showing that generalized immunosuppression resulted in robust fungal growth in the cornea and destruction of the tissue (47–51). These observations are consistent with the poor clinical outcome found when corticosteroids or other immunosuppressants are given prematurely. We developed a murine model of trauma induced *Fusarium* and *Aspergillus* keratitis in which conidia are injected directly into the corneal stroma (33, 34). Following germination, hyphae spread through the corneal stroma, and neutrophils infiltrate, causing pronounced corneal opacification. Figure 1D shows an example of this model using RFP expressing *A. fumigatus* conidia and GFP expressing neutrophils. At 48h post-infection, corneal opacity and neutrophil infiltration increase further, while RFP expressing fungi decreases. Neutrophils are detected at higher magnification in the live cornea (Fig 1D, **central panels**). Subsequent experiments found that systemic neutrophil depletion resulted in uncontrolled fungal growth (Leal, data not shown), thereby implicating neutrophils as having an essential role in fungal killing.

In murine models of traumatic injury related fungal keratitis in which *Fusarium* or *Aspergillus* conidia are inoculated into the corneal stroma, the conidia germinate within

hours exposing β -glucan on their surface. (33, 46, 52). The β -glucan is recognized by Dectin-1 on the surface of resident macrophages leading to phosphorylation of spleen tyrosine kinase (Syk) and CARD9-dependent NF κ B translocation to the nucleus and transcription of IL- β and CXCL1/KC mRNA (33, 53). Data from our *Aspergillus* study demonstrated that macrophage depletion using transgenic mice that conditionally undergo apoptosis (MAFIA; Macrophage Fas Induced Apoptosis) resulted in impaired neutrophil infiltration and fungal killing, and is consistent with a report by Hu et al showing that macrophage depletion using clodronate beads has a similar effect in *Fusarium* and *Candida* keratitis (33, 54).

The pro-form of IL- β protein is cleaved into the mature, active form primarily by inflammasome dependent caspase activity. Although not yet shown in keratitis, this pathway is important in mucosal candidiasis, and involves the NALP3 and NLRC4/IPAF inflammasomes (55, 56). IL-1 β is produced in *A. fumigatus* corneal infection, and is dependent on Dectin-1 expression and macrophages (33, 34). Mice deficient in IL-1R1, which signals through MyD88, exhibit minimal cellular infiltration into infected corneas (33, 34). IL-1R1 also mediates the host response in trauma-induced and biofilm-associated *Fusarium* keratitis (34, 57). Consistent with the role for IL-1R1, MyD88^{-/-} mice have impaired CXC chemokine production and neutrophil recruitment to *Aspergillus* or *Fusarium*-infected corneas (22, 33, 54). Similarly, *A. fumigatus* can activate the NLRP3 inflammasome, leading to IL-1 β processing by macrophages (58), further demonstrating an important role for IL-1 β in the host response to fungal pathogens. In the cornea, our data support the concept that mature IL-1 β secretion by macrophages activates IL-1R1 on macrophages, fibroblasts and epithelial cells, inducing production of the CXC chemokine CXCL1/KC, which binds to CXCR2 on neutrophils and mediates recruitment of neutrophils from peripheral limbal vessels to the corneal stroma.

IL-1R1 is expressed not only by macrophages and dendritic cells, but also by resident keratocytes in the corneal stroma and corneal epithelial cells (23). Both IL-1R1 and TLR activation lead to the production of the neutrophil chemoattractant ELR⁺ CXC (N-terminal Glutamate-Leucine-Arginine-Cysteine-X-Cysteine) chemokines: CXCL8/IL-8 (humans) and CXCL1 (mice, humans) (59–61). CXCL8/IL-8 is elevated in fungal-infected human corneas, suggesting that IL-8 through CXCR1 mediates neutrophil recruitment into fungal-infected human corneas (46). IL-1R1 is also expressed on limbal vascular endothelial cells and activation increases expression of ICAM-1 on these cells (23), which binds the leukocyte-expressed CD11a/CD18 (LFA1) allowing extravasation into inflamed tissues (62). Our preliminary studies using a mouse model of infection indicate that both CXCR2 and CD18 are required for neutrophil infiltration into the corneal stroma during fungal infection (data not shown).

In addition to Dectin-1 expression, several studies have implicated TLR2 and TLR4 in fungal keratitis. Although *A. fumigatus*-infected rat corneas treated with TLR2 siRNA had decreased corneal opacity (51), and TLR2^{-/-} mice infected with *C. albicans* had less cellular infiltration to the cornea and decreased fungal killing (63), we found that TLR2^{-/-} mice infected with either *Aspergillus* or *Fusarium* had similar responses to C57BL/6 mice (22, 33, 34). Although we found no role for TLR2, our studies clearly demonstrated that TLR4^{-/-} mice infected with either *Aspergillus* or *Fusarium* exhibited impaired fungal clearance, indicating a role for TLR4 in fungal killing (22, 33, 34). The mechanism of TLR4 regulation of fungal survival has yet to be determined; however it likely involves production of reactive oxygen species by infiltrating neutrophils. We also found a similar role for TLR4, but not TLR2 in an epithelial model of fungal keratitis in which the epithelium is abraded, and exposed to a contact lens on which *Fusarium* had formed a biofilm (22, 50). These findings are also similar to a model of pulmonary aspergillosis, in which TLR4^{-/-}

mice show impaired killing of *Aspergillus* (64, 65), and further supports a role for TLR4 mediated killing. Interestingly, the absence of the TLR4 co-receptor MD-2, which is the receptor for lipid A, has no effect on corneal infection, indicating that the *Aspergillus* ligand binds TLR4 rather than MD-2. In conclusion, our data are consistent with a role for β -glucan expression on hyphae binding to Dectin-1 on neutrophils, and with a role for TLR4, which has been shown to bind O-linked mannosyl residues of *Candida* cell wall (66). We propose that TLR4 and Dectin1- signaling activates NADPH-oxidase, leading to production of reactive oxygen species, which likely mediates killing of fungal hyphae and eventual resolution of corneal opacity.

4. Conclusions

Studies using infected human corneas and murine models of *Fusarium* and *Aspergillus* keratitis are consistent in implicating Dectin-1, TLR4, and neutrophils, in addition to inflammasomes and IL-1 β in the pathogenesis of fungal keratitis. Results from these studies led us to propose the sequence of events illustrated in Figure 2. **1)** Trauma to the eye caused by dirt or plant material with adherent conidiophores containing multiple conidia. **2)** Once in the corneal stroma, conidia germination results in shedding the hydrophobin layer that coats resting conidia, and exposing cell wall β -glucan on the surface. β -glucan binds to Dectin-1 on resident corneal macrophages. **3)** Dectin-1 signals through syk and CARD9 to activate NF κ B and transcription of CXC chemokines and IL-1 β , which is cleaved by inflammasomes and caspase 1, and activates neighboring cells to produce CXCL chemokines and upregulates ICAM-1 expression on vascular endothelial cells in the peripheral vessels (as the cornea is avascular). **4)** Elevated ICAM-1 and CXC chemokines mediate recruitment of neutrophils to the corneal stroma, and **5)** Cell surface β -glucan and mannosyl residues on hyphae in the cornea activate Dectin-1 and TLR4 on neutrophils, stimulating production of ROS and fungal killing, although corneal inflammation also results in corneal opacification and loss of vision.

Targeting any of these mediators, including Dectin-1, IL-1R1 and CXCR2, could restrict excessive cellular infiltration into infected corneas in an effort to minimize host-mediated tissue damage that would be administered together with antifungal therapy such as topical natamycin or voriconazole, which are often unsuccessful when given with steroids as up to 60% of patients undergo corneal transplantation (5). Currently, IL-1R antagonists, CXCR2 and CXCR1 antagonists (67) are clinically available, though their efficacy to treat fungal keratitis has not been addressed. It is possible that targeting these receptors and proteins during corneal infection will help minimize host-mediated tissue damage while effectively killing fungal hyphae during corneal infection.

Acknowledgments

We gratefully acknowledge our collaborators on the work presented, including Drs Prajna, Lalitha Dharmalingam and Karthikeyan at the Aravind Eye Hospital in south India, and Dr Michelle Momany (University of Athens, GA). This work was supported by NIH grants F31 EY019841 (SML), RO1 EY018612 (EP), P30 EY011373 (EP), and funding from the Alcon Research Institute (EP, Awardee) the Research to Prevent Blindness Foundation (SML/EP), and the Ohio Lions Eye Research Foundation (EP).

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Highlights

This review will discuss:

1. The global impact of fungal keratitis as a worldwide cause of blindness and visual impairment
2. Anti-microbial defense at the ocular surface
3. The current understanding of innate and adaptive immune responses during fungal keratitis
4. Potential targets for immunomodulatory therapy to limit host-cell mediated tissue damage during fungal keratitis

Figure 1. Eye anatomy, Cornea Histology, Human and Experimental Murine Fungal Keratitis
A. Diagram of human eye. **B.** Normal H&E stained human cornea histology section. **C.** GMS-stained cornea of a patient with *A. flavus* keratitis requiring corneal transplantation. **D.** Murine model of fungal keratitis in which 50,000 RFP-expressing *A. fumigatus* conidia are injected into the corneal stroma of mice whose neutrophils express eGFP (LysM eGFP). Cornea opacity (BF), fungal growth (RFP), and neutrophil infiltration (GFP) are tracked at 0h, 24h, and 48h post-infection. Also shown is a magnified image of fungal hyphae (RFP) and neutrophil (GFP) interaction in the live murine cornea during infection. Epi-epithelium, Endo-endothelium, RFP- red fluorescent protein, GFP- green fluorescent protein **E.** The corneas of *Aspergillus*-infected mice were sectioned and stained with Periodic-acid schiff and hematoxylin or with neutrophil specific antibody, NIMPR-14.

Figure 2. Proposed Sequence of Events During Fungal Keratitis
(See text for details)