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Immunity to pathogenic fungi in the eye

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Abstract

Fusarium, Aspergillus and *Candida* are important fungal pathogens that cause visual impairment and blindness in the USA and worldwide. This review will summarize the epidemiology and clinical features of corneal infections and discuss the immune and inflammatory responses that play an important role in clinical disease. In addition, we describe fungal virulence factors that are required for survival in infected corneas, and the activities of neutrophils in fungal killing, tissue damage and cytokine production.

Keywords

Fungal infection; Aspergillus; Fusarium; Candida; Innate immunity; Neutrophil; Cornea; Keratitis; Ocular infection; Reactive oxygen species; NADPH oxidase; Nutritional immunity; Cytokines; IL-1 alpha; IL-1 beta

1. Introduction

The mammalian cornea is exquisitely designed to allow focused light passage through the lens to photoreceptors in the retina. Light is then converted to electrical signals that go through the retinal ganglia to the optic nerve and is interpreted in the visual cortex of the brain. Corneal transparency is achieved by tight junctions in the corneal epithelium and by the activity of the single layer of corneal endothelial cells that pump water from the corneal stroma into the anterior chamber. Together, these cells preserve the corneal stroma at ~80% hydration, which is essential for optimal spacing between the anti-parallel rows of collagen

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fibrils in the stroma. Corneal transparency is also dependent on the avascular state of the cornea and the relative paucity of resident immune cells (mostly $CX3CR1^{+}$ macrophages) in the healthy corneal stroma. Disruption of this tightly regulated process results in loss of corneal transparency and visual impairment.

Trauma and contact lens usage are the main predisposing risk factors for infection of the cornea. Therefore, when *Aspergillus* or *Fusarium* conidia penetrate the corneal epithelium, they enter the stroma and germinate, forming hyphae that spread in the cornea. These induce a profound inflammatory response that leads to loss of corneal transparency, loss of visual acuity, and in severe cases corneal blindness. Further, if not rapidly controlled, hyphae will invade the posterior eye, causing endophthalmitis that may require removal of the globe. An example of hyphal growth in an infected compared with a healthy cornea is shown in Fig. 1.

2. Epidemiology of fungal keratitis

Keratitis caused by bacteria and fungi is a major cause of visual impairment and blindness worldwide, especially in southern and eastern Asia, where cases of fungal keratitis can exceed 2 million per year [1] (Fig. 2). Fungal keratitis mostly affects poor, rural agricultural areas where individuals are exposed to plant material that can penetrate the cornea following blunt injury. As *Aspergillus and Fusarium* molds thrive in these hot and humid environments, they are the major causes of fungal keratitis. In addition to these two major genera, *Curvularia, Alternaria*, *Paecelomyces*, *Scedosporium* and *Purpureocilium* infect the cornea following traumatic inoculation with the relative incidence of each dependent upon geographic distribution [2,3]. Larger population studies conducted at the Aravind Eye Hospitals in southern India from 2006 to 2009 found that 63% of culture-positive microbial keratitis cases were caused by pathogenic fungi [4]. *Fusarium* spp. were the predominant causes of fungal keratitis (42.3%), and the number of cases of fungal keratitis peaked in July and January, which corresponds to harvest season when there is increased exposure to mold spores from crops.

Another study of 25,000 keratitis patients from the same region, seen between 2002 and 2012, also found that *Fusarium* was the major cause of corneal ulcers [5]. Similarly, in northern and central China, *Fusarium* spp. were the major cause of corneal ulcers [6,7]. Studies in India and China concluded that corneal injury was the predominant underlying cause, especially in males in agricultural regions, where the incidence was greatly elevated during the harvest season [6,7]. Further, studies from smaller cohorts in Africa, South America, and regions of southern and East Asia reported similar findings regarding prevalence and risk factors [8]. Overall, the major risk factor is agricultural work, where ocular injury and exposure to *Fusarium, Aspergillus*, and other filamentous fungi in soil and plant matter is common. The relative predominance of *Fusarium* compared with *Aspergillus* species as a cause of disease is likely a reflection of these species as the predominant fungi infecting crops although as discussed below, fungal virulence factors provide a selective advantage during corneal infection and thereby contribute to disease severity.

In the USA and industrialized countries, the incidence of *Fusarium* and *Aspergillus* keratitis is moderate compared with bacterial causes (Fig. 2), and the major risk factor for both

is poor contact lens hygiene. Within the US, fungal keratitis occurs more commonly in hot and humid regions, such as the Southeastern United States and is likely significantly underreported within agricultural migrant worker communities at highest risk . Keratitis caused by *Fusarium* came to the attention of health care workers in the USA and Europe in 2005 – 2006 with 164 confirmed cases associated with contact lens wear and a specific lens care product that was unable to control the growth of fungi introduced from the environment. The ability of the organisms to form biofilm on contact lenses and on lens cases was also a major factor. *Fusarium* forms biofilm on silicone hydrogel lenses, and the lens care solution was less effective on biofilm-forming clinical isolates than the ATCC 36031 reference isolate, which does not form a biofilm on soft contact lenses [9]. One consequence of these and other findings is that the US Food and Drug Administration now utilizes *Fusarium* clinical isolates instead of ATCC 36031 to test lens care solutions.

Corneal infection with yeasts, primarily *Candida* spp., is more commonly seen in industrialized countries and is associated with contact lens wear, prior ocular surface disease and ocular surgery as described in a recent 10-year survey in England [10]. *Candida* spp. are also an important cause of fungal endophthalmitis, either as a result of hematogenous spreading of systemic infection (endogenous) or following ocular infection (exogenous, reviewed in [11]). The major risk factors associated with endogenous endophthalmitis include untreated fungemia due to use of catheters and other medical devices, intravenous drug use, increased gastrointestinal permeability and immune suppression, whereas exogenous endophthalmitis is mainly caused by failed treatment of *Candida* keratitis resulting in hyphal invasion into the posterior eye [11].

3. Clinical features, diagnosis, and treatment of fungal keratitis

Microbial keratitis patients typically present with discrete lesions in the cornea, photophobia, and impaired vision, and corneal ulcers caused by fungi differ from bacterial ulcers by having a peripheral feathery appearance, often with satellite lesions [10] (Fig. 3A–C). Given that the cornea is highly innervated, these lesions are also extremely painful, and infected individuals rapidly seek treatment. Since bacterial keratitis is much more common, frequent misdiagnosis as a bacterial etiology, results in inappropriate use of antibacterial agents and infection progression prior to initiation of antifungal therapy.

In vivo confocal microscopy and anterior segment optical coherence tomography (OCT) are also used in the clinic to detect and characterize the microbial corneal infection and can be used as a follow up for treatment responsiveness. Confocal microscopy enables visualization of conidia, yeast and hyphae in the cornea [3,12], whereas OCT, a non-contact *in vivo* ocular imaging technology, captures high-resolution, cross-sectional images that show corneal architecture and thickness (Fig. 3C, inset, D). Both approaches can also visualize the cellular infiltrate.

Current treatment options for fungal keratitis patients were recently reviewed by Hoffman and Burton, drawing mostly from studies in southern and eastern Asia [13]. Treatment typically involves topical application of anti-fungal agents followed by topical steroids to control the inflammatory response and prevent corneal scarring. The first line of treatment

is application of topical polyenes, most commonly natamycin (5%) or Amphotericin B (0.15%) at hourly or two-hourly intervals. Both are effective against *Aspergillus* and *Fusarium* infections. Topical drug concentrations reach higher concentrations in the cornea than is achievable by systemic administration. However, the effect of topical anti-fungal agents in the cornea may be limited, particularly given the delay in diagnosis and treatment initiation, because growing hyphae can invade the deeper, more densely packed stromal lamellae, and that polyenes have relatively poor stromal penetration. The second-line treatment is systemic administration of azoles including imidazole, and voriconazole. Voriconazole 1% has better penetration into the eye, and it can also be injected directly into the cornea to target the site of the infection. The third line of treatment is combined oral and subconjunctival, or intravitreal injection of azoles or amphotericin B. If these approaches are not effective, patients undergo full thickness corneal transplantation, which is considered high risk because of underlying inflammation and the potential for any remaining hyphae to infect the newly transplanted cornea.

Following antimycotic treatment, the inflammatory response is managed using topical corticosteroids; however, if there are any remaining viable fungi, hyphae can rapidly grow in the absence of an immune response, resulting in severe corneal disease and an increased risk for endophthalmitis.

4. Immune response in patients with *Fusarium* and *Aspergillus* infected corneas

In contrast to *Fusarium* and *Aspergillus* systemic infections which occur mostly in immune compromised patients, corneal infections are seen in otherwise healthy individuals with a fully functioning immune system. To characterize the immune response in infected corneas at the site of infection, corneal ulcer patients in south India with confirmed fungal infections were examined for early and late-stage immune responses. Scrapings from acute corneal infections, and later stage infections were examined in corneas obtained following corneal transplantation. Another clinical feature of infection is a cellular infiltrate in the anterior chamber, termed a hypopyon, which is comprised primarily of neutrophils (Fig. 4A). Neutrophils also comprised > 90% of the cells in the corneal ulcers (Fig. 4B,C). Even at later stages of disease following treatment failure and corneal transplantation, histology and immunohistochemistry of post-transplant corneas revealed that neutrophils were also the predominant cell types, although macrophages and T cells were also present [14].

These studies also characterized gene expression of pathogen recognition molecules and cytokines. RNA was extracted from corneal ulcers and relative gene expression was compared to uninfected (donor) corneas. These experiments found elevated expression of Toll Like Receptor (TLR)2, TLR4, Dectin-1 (*Clec7a*), IL1β, TNFα, IL8 and IL17a in corneal ulcers caused by fungi [14]. Similar studies were also performed with bacterial ulcers [15]. Given the preponderance of neutrophils in infected corneas, they are likely the major source of cytokines. Studies described below show support the notion that neutrophils are a major source of pro-inflammatory cytokines.

5. The eye as an immune privileged site

There is an extensive literature describing the concept of immune privilege and immune deviation in the eye, including the observation that antigens injected into the anterior chamber (underlying the cornea, Fig. 1) induce immune suppression characterized by an impaired delayed type hypersensitivity to the same antigens (reviewed in [16–18]). The anterior chamber contains TGF-β and immunosuppressive neuropeptides such as alpha-melanocyte stimulating hormone that contribute to the ability to perform allogeneic corneal transplants without having to administer immunosuppressive therapy. There is considerable teleological justification for preserving vision [18]; however, there is arguably more teleological justification for not succumbing to infection, and active infection in the cornea or in the posterior eye (endophthalmitis) overwhelms the immunosuppressive environment and induces a robust host response that can cause severe tissue damage, visual impairment and blindness. While perhaps stretching the definition of immune privilege, the avascular nature of our corneas gives bacteria an advantage of being able to reproduce and for fungi to germinate and form hyphae before immune cells can be recruited to the site of infection from peripheral, limbal vessels. As described below, the criteria that govern the outcome of corneal infections include those that regulate the timing and ability of host cells, primarily neutrophils, to reach the site of infection. It is also important to note that while the human cornea is 12 mm diameter, which requires extensive migration of immune cells following central corneal infection, most of the studies cited in this review examine infection in mouse corneas (2.5 mm diameter).

In conclusion, while the eye has unique features that regulate immune responsiveness, including avascularity and a dense stomal collagen matrix, when met with the challenge of microbial infection, it responds similarly to other tissues by facilitating recruitment of inflammatory and immune cells. The predominance of neutrophils to infected tissues in acute infection does not just reflect the relative number of these cells in peripheral blood, but also their unique ability to penetrate dense extracellular matrix. This in turn is at least partly due to the tri-lobed nucleus that allows the cell to rapidly migrate through tight junctures and between tightly packed collagen fibrils.

6. Innate immunity in murine models of fungal keratitis

Fungal infections of the corneal are almost exclusively opportunistic and are initiated following a breach in the corneal epithelial cell barrier from ocular trauma that allows conidia to enter the corneal stroma. As the outcome of infection depends on an immediate response, it is the innate arm of the immune response that mediates fungal killing and disease severity. Murine infections in healthy immune competent mice are mostly self-limiting due to innate immune responses, and mice given cyclophosphamide exhibit uncontrolled hyphal growth and corneal perforation [19]. *These two scenarios represent the major phenotypes in these models and likely define the outcome in infected patients - i) rapid influx of neutrophils, development of corneal opacification and microbial killing – in this case, the corneas do not perforate; and ii) delayed recruitment of neutrophils, unrestricted hyphal growth, and corneal perforation.*

Using gene knockout mice, cell depletion studies and antibody neutralization, the Pearlman lab and others have identified cells and mediators of innate immunity that have non-redundant roles in regulating hyphal killing and corneal disease severity in *Fusarium* and *Aspergillus* keratitis.

6.1. The role of pathogen recognition molecules and the Rod A hydrophobin in fungal keratitis

Patient studies showed elevated Dectin-1 and TLRs transcripts in corneal ulcers caused by *Aspergillus and Fusarium* species [14], which are likely expressed by infiltrating cells, primarily neutrophils. Our earlier studies used gene knockout mice to examine the role of TLR2, TLR4 and the TLR adapter protein MyD88 in fungal keratitis. These experiments revealed an absolute requirement for MyD88 in controlling *A. fumigatus* and *F. oxysporum* hyphal growth and development of severe corneal opacification that was associated with impaired cellular infiltration to the corneal stroma and subsequent corneal perforation [19,20]. However, we found that TLR2$^{-/-}$ mice had the same phenotype as control, C57BL/6 mice whereas *Tlr4*$^{-/-}$ mice exhibited elevated hyphal growth, but minimal effect on corneal disease. Instead, we found that for both pathogens, only *Il1r1*$^{-/-}$ mice phenocopied MyD88$^{-/-}$ mice in being completely unable to regulate hyphal growth and corneal disease severity [19,20]. Thus, although there is a partial phenotypic change in *Tlr4*$^{-/-}$ mice, the findings with *Il1r1*$^{-/-}$ mice revealed that it is primarily the IL-1R1/MyD88 rather than the TLR/MyD88 axis that determines the outcome of corneal infection. We subsequently reported that *Il1β*$^{-/-}$ mice also failed to clear *A. fumigatus* [21], whereas there was no role for IL-1α (Ratitong, unpublished observations). IL-1β production in infected corneas is discussed in detail below.

Our earlier studies also showed that β-glucan is expressed on hyphae in infected human and murine corneas, and that Dectin-1 is required for clearance of *A. fumigatus* from infected corneas. Although Dectin-1 played a more important role during infection with a virulent clinical isolate than with the Af293 lab-adapted strain [19], Latgé and co-workers reported that dormant *Aspergillus* and *Fusarium* conidia have an outer hydrophobic layer incorporating a family of RodA proteins [22], and subsequently showed that these block host recognition of the underlying cell wall carbohydrates β-1,3 glucan and α-mannan [23]. Given the number of airborne conidia (spores) in the environment, it is likely that not only do these proteins protect the conidia, but they are not recognized by immune cells following infection. In support of this concept, Latgé and co-workers generated ΔrodA mutants and demonstrated that dormant conidia have exposed cell wall carbohydrates that induce increased cytokine production in the lungs [23].

Carrion, Latgé et. al. took a similar approach to determine if there is a role for the RodA protein in *F. solani* and *F. oxysporum* where they found that removal the RodA protein using hydrofluoric acid resulted in increased β-1,3 glucan and α-mannan on the cell wall of conidia, which induced cytokine production by macrophages; further, in the absence of RodA (Δ*rodA* mutants), *A. fumigatus* induced a more rapid and robust host response in infected corneas than the parent strain, resulting in neutrophil infiltration and more rapid fungal killing, and which was dependent on Dectin-1 and Dectin-2 [24] (Fig. 5).

6.2. A pivotal role for neutrophils in fungal keratitis

To determine if neutrophils are indeed a source of pro-inflammatory cytokines in fungal keratitis, murine models of *A. fumigatus* and *Fusarium oxysporum* were developed in which viable conidia are injected directly into the corneal stroma. Following germination and hyphal growth, resident macrophages, corneal epithelial cells and keratocytes secrete IL-1α and CXC chemokines that initiate neutrophil recruitment. Neutrophils activated by these cytokines and by microbial products then secrete CXCL1 and CXCL2 that leads to a positive feedback loop and a pronounced infiltration of neutrophils. Using LysM GFP mice (where myeloid cells are GFP^+) and RFP-expressing *A fumigatus* strain *Af293,* we found that the presence of neutrophils in infected corneas correlates with areas of hyphal growth. We also showed that neutrophils are absolutely required for hyphal killing in infected corneas by depleting neutrophils using the Ly6G antibody NIMP-R14 [25] (Fig. 6A, B). Subsequent studies examined specific fungal killing mechanisms of neutrophils and identified essential fungal 'virulence factors' that fungi use to evade neutrophil killing. Although there are several approaches to characterize neutrophil activities, we consider this in terms of oxidative and non-oxidative mechanisms, including 'nutritional immunity' that limits availability of metals that are essential for hyphal growth.

6.3. Oxidative anti-fungal response in infected corneas – the role of NADPH oxidase

The requirement for NADPH oxidase to control microbial infections has been well documented in individuals with chronic granulomatous disease (CGD). These individuals have well defined mutations in NADPH oxidase complex genes and are highly susceptible to systemic fungal infections [26,27]. Similarly, mice lacking a functional NADPH oxidase complex ($GP91^{PHOX-/-}$, $Cybb^{-/-}$, CGD mice) cannot generate ROS, and Leal et. al. used these mice to show that *A. fumigatus* and *F. oxysporum* are not killed in infected corneas [25]. The phenotype is distinct from neutrophil depleted mice as neutrophils continue to infiltrate the cornea, resulting in exacerbated corneal opacification (Fig. 6C). While we speculated at the time that this is a consequence of 'frustrated neutrophils' that are unable to kill the hyphae, more recent studies from Dinauer and colleagues reported that neutrophils from CGD mice and patients produce higher levels of pro-inflammatory and chemotactic cytokines that is associated with increased neutrophil recruitment and activation [28]. That study also showed increased CD11b expression on CGD neutrophils, which may also exxacerbate the infection as CD11b/CD18 (CR3) contains a lectin receptor that recognizes β-glucan [29].

Leal also tested *A. fumigatus, F. oxysporum* and *F. solani* with mutations related to antioxidant activity, and reported that proteins regulated by the transcription factor Yap1, including superoxide dismutase genes *Sod1, 2 and 3* were important while there was no role for catalases or secondary metabolites, including gliotoxin; there was also no role for iNOS [25]. In collaboration with Latge et al., we also showed that phosphatase Z is required to combat oxidative stress and cause corneal disease [30].

6.4. Non-oxidative neutrophil antimicrobial activity in infected corneas

6.4.1. Nutritional immunity: iron and zinc chelation and sequestration—Skaar and colleagues were among the first investigators to introduce the concept of nutritional

immunity where host cells prevent bacteria from acquiring transition metals such as iron and zinc for growth, especially in infected tissues [31]. Fe^{++} acquisition involves release of small molecules called siderophores that are then incorporated into the organism [31]. Microbes also regulate the concentration of these metals to limit their toxicity. In collaboration with Hubertus Haas and Antonio di Pietro, Leal examined *A. fumigatus* and *F. oxysporum* siderophores and identified a key role for secreted rather than intracellular siderophores and for the *HapX* transcription factor in Fe^{++} acquisition and growth in infected corneas [32]. Consistent with this finding, systemic application of Fe^{++} or the xenosiderophore deferoxamine enhanced fungal growth, whereas iron depletion in the cornea using topical lactoferrin or deferiprone limited hyphal growth. Further, blockade of the mevalonate pathway required for extracellular siderophores using statins also inhibited hyphal growth in infected corneas [32]. In a subsequent study, Haas et al. reported that histidine biosynthesis regulates iron and zinc cytotoxicity in *A. fumigatus* and that *HisB* mutants were avirulent in murine models of pulmonary and corneal infection [33].

In addition to iron, *Aspergillus* hyphae require zinc for growth, which is acquired by the zinc transporters ZrfA-C that are regulated by the ZafA transcription factor [34]. Clark et al. showed that *A. fumigatus* ZafA mutants were limited in their ability to grow in infected corneas although there was no difference in mutants with single zinc transporter mutations [35]. Calprotectin (S100A8 / S100A9) is a heterodimeric member of the S100 family of proteins that binds and sequesters essential metals [31,36]. Calprotectin has two sites for binding metals - one that binds Zn^{++} only and a second that binds Zn^{++} and Mn^{++}. Although multiple cell types produce calprotectin, neutrophils are the primary source as it comprises ~40% of total cytoplasmic proteins . Using calprotectin proteins with mutations in either the Mn^{++} or the Zn^{++} / Mn^{++} Zn^{++} binding domains (generated by Chazin), Clark showed that the Zn^{++} binding site was essential for limiting *A. fumigatus* hyphal growth *in vitro*, although the Mn^{++} region also contributed [35]. These findings were supported by experiments where Mn^{++} and Zn^{++} were added exogenously to overcome the inhibitory effect of parent calprotectin (Fig. 7A,B). Following corneal infection with *A. fumigatus* Af293, we found that neutrophils were the major source of calprotectin, and that $S100A9^{-/-}$ mice exhibit an impaired ability to regulate *Aspergillus* hyphal growth in infected corneas (Fig. 7C,D), although this phenotype was reversed by injection of recombinant calprotectin.

In collaboration with Tobias Hohl, we found that unlike corneal infections, there was no significant role for calprotectin in a murine model of pulmonary aspergillosis [35]. We attributed this difference to a selective role for calprotectin in limiting hyphal growth (in the cornea) compared with conidia that are predominant in infected lungs. However, the differences may also be due to the murine model of fungal keratitis where neutrophils comprise > 80% total cells infiltrating the tissue. A follow up study by Clark et. al. showed that the ubiquinone analog Atovaquone inhibited growth of *Aspergillus* and *Fusarium in vitro* by disrupting mitochondrial function and zinc uptake, and that topical application of atovaquone blocked hyphal growth in infected corneas [37].

6.5. Non-oxidative mechanisms of fungal killing by neutrophils in fungal keratitis - acidic mammalian chitinase (AMCase)

Although mammals do not produce chitin, macrophages and eosinophils produce enzymatically active AMCase and chitotriosidase that contribute to lung pathology in asthma and to protective immunity in intestinal helminth infections [38,39]. Carrion, Abbondante et al. found that neutrophils are the major source of AMCase in *A. fumigatus* infected corneas, and that AMCase$^{-/-}$ mice showed an impaired ability to clear the infection even though there was no difference in total neutrophils recruited to infected corneas [40]. These findings were supported by experiments showing impaired hyphal killing by AMCase$^{-/-}$ neutrophils and by human neutrophils treated with the AMCase inhibitor Bisdionin F or the general chitinase inhibitor Bisdionin C [40]. Conversely, hyphal growth in infected corneas was inhibited by the chitin synthase inhibitor Nikkomycin C. In collaboration with Latge and colleagues, we also found that chitin synthase Family 2 mutants *ChsE/Eb/D/F* exhibited impaired hyphal growth compared with Family 1 mutants *ChsA/B/C/G* [40]. However, using a similar genetics approach, Mouyna and Latge found no role for chitin deacetylases in *Aspergillus fumigatus* keratitis or pulmonary disease [41].

6.6. Neutrophil extracellular traps (NETs) are generated in A. fumigatus infected corneas, but are not required for hyphal killing or disease severity

Zychlinsky and colleagues first identified NETs following phorbol myristate acetate (PMA) stimulation of human neutrophils, and subsequently characterized the stages of NETosis from neutrophil elastase entering the nucleus , nuclear swelling, and release of chromatin into the cytoplasm, finally resulting in rupture of the cells and release of DNA, chromatin and cytosolic proteins into the extracellular environment (recently reviewed by Sollberger and by Dubyak [42,43]). Subsequent studies showed that NETs are released in response to bacteria and yeast, and Urban et. al. identified multiple proteins (including calprotectin) in isolated NETs from neutrophils infected with *Candida albicans* [44]. While the underlying mechanisms of NETosis remain under investigation, an important advance in the field was the observation that protein deiminase 4 (PAD4) in the nucleus plays an important role in changing positively charged arginine residues to neutral citrullines, thereby contributing to chromatin decondensation. This process can be detected using antibodies to citrullinated histone-3 (H3Cit).

Clark, Abbondante et. al. found that *A. fumigatus* extracts and curdlan induced NETosis quantified using Sytox Green™, and by histone citrullination detected by antibodies to H3Cit (Fig. 7).(Fig. 8).

NETosis was also dependent on CR3 rather than Dectin-1, and on production of ROS [45]. Although H3Cit was clearly detected in infected corneas, there was no difference in infection with *A. fumigatus Af293* in PAD4$^{-/-}$ compared with C57BL/6 mice, indicating that PAD4- dependent NETs are not required in fungal keratitis. A recent paper showed that while counterintuitive, PAD4$^{-/-}$ mice more efficiently cleared *A. fumigatus* from infected lungs and exhibited less inflammation than control mice, possible because of a pro-inflammatory environment regulated by PAD4 or because hyphae predominate in the cornea compared with conidia in the lungs [46].

6.7. Conclusions on neutrophil regulation of hyphal growth

Collectively this series of studies identified multiple non-redundant mechanisms by which neutrophils regulate hyphal growth *in vitro* and in infected corneas. These findings are remarkable given that all these mechanisms are presumably active at the same time, and we would expect to see redundancy. However, it is likely that as with ROS production and NETosis where ROS is required for NET formation, many of these pathways are interactive and interdependent. Future studies will examine additional potential interactions.

7. Virulence of *Aspergillus* and *Fusarium* in corneal infections – *Fusarium* extrachromosomal elements encoding novel virulence factors

Studies described above used mutants strains of Fusarium and Aspergillus to identify specific virulence factors associated with neutrophil anti-fungal activities, including the RodA protein family, mutants in fungal antioxidant proteins such as superoxide dismutase, and mutants deficient in siderophore production, zinc transport or chitin production. However, these are primarily (housekeeping) genes that are required for normal activities of these filamentous organisms. Mutations generated in the *A. fumigatus* apoptosis pathway (AfBIR1) results in ROS resistance and exacerbated virulence in infected animals [47]; Virulence can be defined as the *inherent* capacity of microbes to survive in mammalian hosts, and increased virulence can be defined as their ability to cause disease. To this end, Cramer and Obar described three strains of *A. fumigatus* that are more virulent than the Af293 lab strain – CEA10 that was isolated from a patient with pulmonary aspergillosis, EVOL20 that was derived following multiple passages of Af293 *in vivo*, and a clinical isolate from severe allergic disease (W72310) that persists and causes severe disease in murine lungs [48,49]. CEA10 virulence derives from its ability to germinate rapidly in an immunocompetent pulmonary inhalation model of *A. fumigatus*.

Much less is known about *Fusarium* species, which as noted are the major causes of fungal keratitis worldwide, and *Fusarium* genetics has mostly been characterized by plant pathogen investigators. *F. oxysporum* was listed among the top five most important plant pathogens by the American Phytopathology Society [50]. Host specific pathogenicity has been attributed to horizontally transmitted pathogenicity chromosomes, first discovered in 2010 in a tomato pathogen, and were subsequently confirmed in other pathotypes [51–53]. These findings identified a role for horizontal transfer, and an association with increased plant pathogenicity.

Pathogenicity chromosomes are highly variable and are distinct from core chromosomes that are conserved and are vertically transmitted among all *F. oxysporum* genomes [51]. These pathogenicity chromosomes are also termed accessory chromosomes or lineage-specific chromosomes, and are characterized by low gene density and a high content of transposable elements. They also lack genes for essential housekeeping functions.

A recent study by Ma et. al. identified pathogenicity chromosomes in a clinical isolate of human systemic Fusariosis strain NRRL 32931 and in a clinical isolate from infected corneas NRRL 47514 [54]. The latter strain is *F. oxysporum* strain MRL8996, which was

isolated during the contact lens outbreak in 2005 and has been used in many of the murine infection studies described above. A comparison of the human clinical isolate strain NRRL 32931 and Fol4287, a tomato wilt pathogen found that although the core genome comprising 11 homologous chromosomes (gray) is conserved between these strains, there are four unique and smaller lineage-specific chromosomes of human-infecting strain NRRL that are clearly distinct from the tomato pathogen (Fig. 9). Genes shared between two human pathogenic strains include a homolog of ceruloplasmin and the genes that contribute to the expansion of the alkaline pH-responsive transcription factor PacC/Rim1p, which are associated with increased virulence in humans.

8. Neutrophils as a source of cytokines: IL-1α and IL-1β

As noted above, *Fusarium and Aspergillus* keratitis is tightly regulated by the IL-1R1 / MyD88 pathway as in their absence mice are unable to control hyphal growth in the cornea, resulting in severe corneal disease and migration of hyphae through the cornea to the anterior chamber [19,20]. A similar role for IL-1R1 was reported by Obar and colleagues in a murine model of pulmonary aspergillosis [55], and the underlying mechanism was found to be delayed recruitment of neutrophils to the sites of infection. Although both IL-1α and IL-1β activate IL-1R1, we initially focused on the role of IL-1β. Two signals are generally required for processing and secretion of IL-1β by murine macrophages, including activation of TLRs or C-type lectin receptors that induces transcription of biologically inactive pro-IL-1β (signal 1). Signal 2 requires assembly of inflammasomes that facilitate caspase-1 activation and IL-1β processing to the bioactive form. Secretion of IL-1β from macrophages requires caspase-1 or caspase-11 cleavage of the pore forming protein Gasdermin D (GSDMD) that causes a lytic form of cell death termed pyroptosis (reviewed in [56,57]).

Using intracellular and imaging flow cytometry, we reported earlier that neutrophils are the predominant source of bioactive IL-1β in corneas that corneal infections with *Pseudomonas aeruginosa* or *Streptococcus* pneumoniae [58,59]. More recently, Sun, Abbondante et. al. reported that neutrophils are also the major source of this cytokine *A. fumigatus* keratitis [21] (Fig. 10A,B). Neutrophil secretion of IL-1β in that study showed a role for the canonical NLRP3/caspase-1 pathway; however, they also showed a requirement for caspase-11 (Fig. 10C,D). In these experiments, caspase-$11^{-/-}$ mice were susceptible to infection. While the signaling pathway has yet to be determined, our findings indicate that caspase-11 production is dependent on the Type I IFN receptor (IFNAR-1), which is known to induce caspase-11 gene expression, and also that caspase-11 was required for caspase-1 activation [21,60].

In contrast to macrophages, we and others reported that neutrophils do not undergo inflammatory pyroptotic cell death following activation by the canonical NLRP3 activator ATP (which signals through the P2X7 receptor), or by *S. pneumoniae*, *Salmonella typhimurium* or *A. fumigatus* [21,58,61,62]. We recently demonstrated that although pro-GSDMD is cleaved in LPS/ATP stimulated neutrophils, and is required for IL-1β secretion, N-GSDMD localizes to primary granules, which appear to act as a ‘sink’ for GSDMD [REF #63]. Given that there is a limited pool of pro-GSDMD at any time, we assume that there is relatively little remaining N-GSDMD that reaches the plasma membrane [63]. We and

others reported that pro-GSDMD is also cleaved by serine proteases, including neutrophil elastase [63–65]. Mechanisms of neutrophil cell death, including pyroptosis and NETosis are complex and far from completely understood (recently reviewed in [43]). Che and colleagues used GSDMD siRNA or disulfiram as a GSDMD inhibitor to treat *A. fumigatus* keratitis in a murine model, and reported that these treatments result in less severe corneal disease [66,67]. Similarly, Lian et. al. reported that sub-conjunctival injection of shRNA for NLRP3 inhibited corneal disease caused by *C. albicans* [68]. Although the siRNA and shRNA treatment had only partial activity on gene expression, these studies support the possibility of targeting inflammasomes or GSDMD for therapeutic intervention.

In addition to IL-1β, Ratitong et. al. recently reported that neutrophils also produce IL-1α *in vivo,* and following incubation with *A. fumigatus* conidia or curdlan . However, while IL-1α and IL-1β secretion by macrophages and dendritic cells was GSDMD-dependent, IL-1α secretion by neutrophils was GSDMD independent [69]. Instead, IL-1α co-localized with CD63, which is a tetraspanin marker for exosomes (Fig. 11). Further, IL-1α release was blocked following incubation with the exosome inhibitor GW4869, which is distinct from IL-1β secretion by neutrophils [69,70].

Surprisingly, *Il1a*$^{-/-}$ mice more rapidly cleared *P. aeruginosa* from infected corneas, whereas *Il1b*$^{-/-}$ mice exhibited an impaired ability to kill the bacteria [71]. While the underlying mechanism has yet to be identified, RNA sequencing showed increased expression of the complement component C1q in neutrophils from infected *Il1a*$^{-/-}$ corneas. Given that the biology of IL-1α differs from IL-1β as it is constitutively present in the nucleus, and bioactivity does not require enzymatic (calpain) processing, these differences may contribute to the distinct phenotype in these mice. However, there was no significant difference in fungal killing between IL-1α$^{-/-}$ and C57BL/6 corneas infected with *A. fumigatus* strain Af293 (unpublished data). As Cramer and Obar showed that IL-1α rapidly clears virulent (rapidly germinating) *A. fumigatus* isolates compared with relatively avirulent Af293 [49,55], there may be a role for IL-1α in corneas infected with more virulent *Aspergillus* and *Fusarium* strains.

9. Conclusions

In this review, we presented epidemiological findings that fungal keratitis primarily caused by filamentous organisms represent major public health problems worldwide. Fungal infections cause severe pain, vision loss, and corneal blindness, and hyphae can invade the posterior eye, leading to blinding endophthalmitis. The avascular nature of the cornea and the densely packed collagen fibrils present a distinct challenge to immune cells migrating to the site of infection. Conversely, while neutrophils play an essential role in killing hyphae and limiting hyphal growth in infected corneas, they are also a major cause of visual impairment due to degranulation and release of serine and matrix metalloproteinases, including collagenases such as MMP8. Given that antifungal agents are limited in their activity and in their ability to penetrate to deeper layers of the corneal stroma, and have problems of efficacy and toxicity, there is an unmet need to identify novel approaches to limit hyphal growth in the cornea. Similarly, anti-inflammatory treatments using corticosteroids are non-specific and are highly immunosuppressive. Findings presented in

this review show an increased understanding of *Aspergillus and Fusarium* virulence factors and highlight multiple functions of neutrophils that regulate hyphal growth and corneal disease severity, including production of pro-inflammatory cytokines. While multiple studies focused on the pathogenesis of this disease, these insights also identify potential therapeutic targets for development of novel anti-fungal and anti-inflammatory agents.

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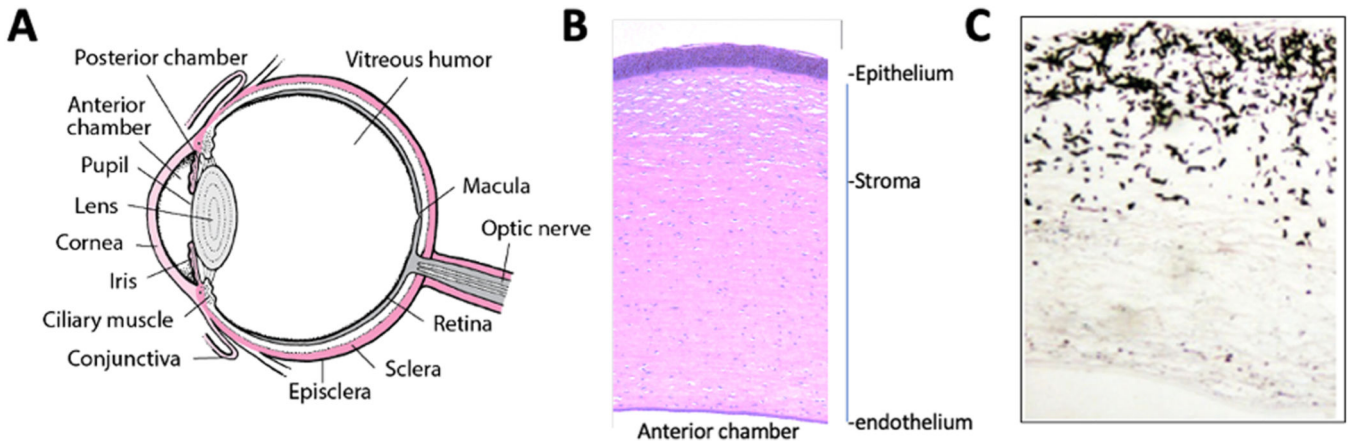

Fig. 1.

(A) Diagram of the human eye; (B) H&E stain of a human corneal section showing the major layers. (C) *Aspergillus flavus* infected human cornea (GMS stain).

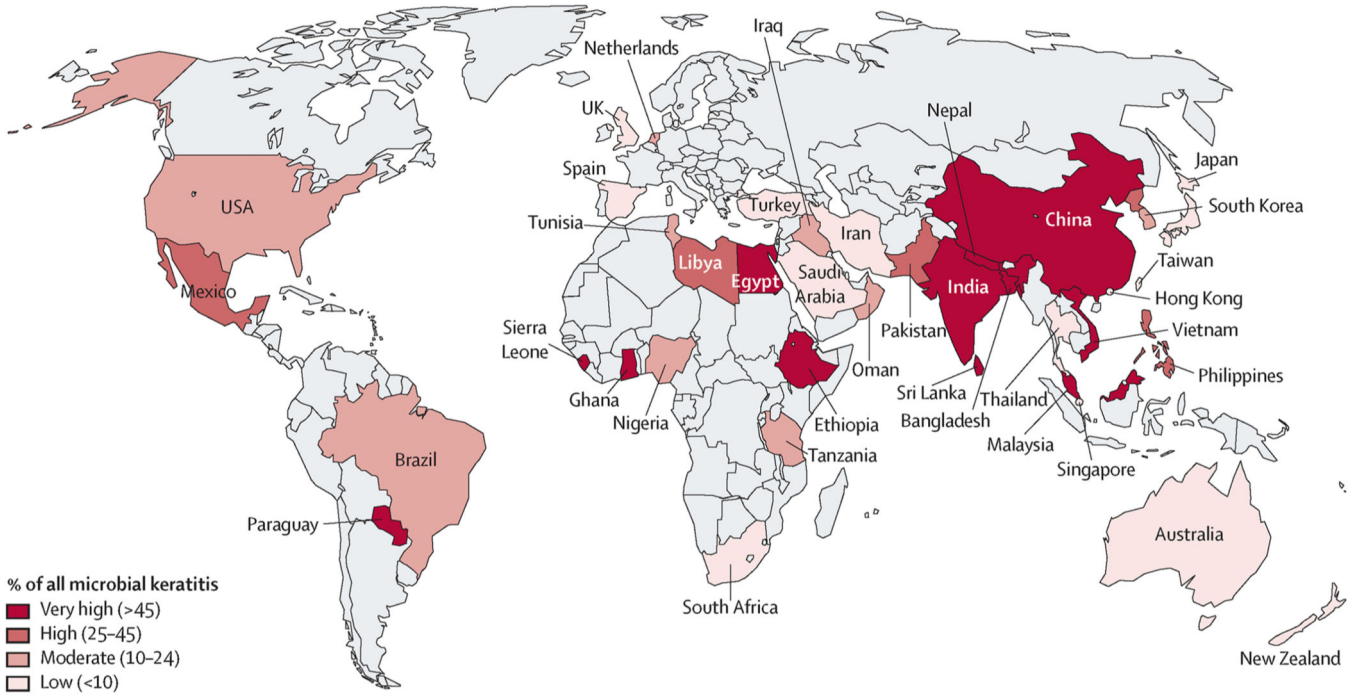

Fig. 2.

Percent of cases of microbial keratitis caused by fungi.

Reproduced with permission from Brown et al. [1].

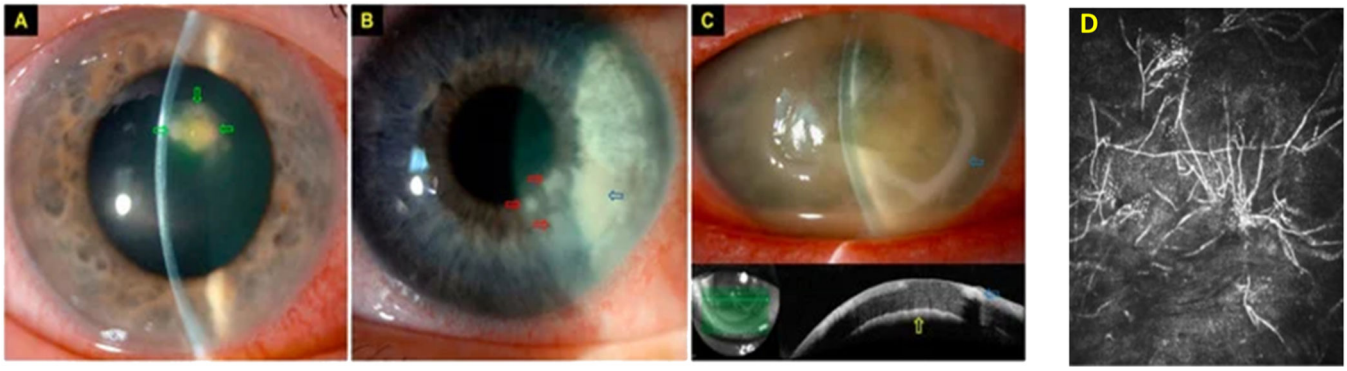

Fig. 3.

Clinical features of fungal keratitis in England. (A,B) contact lens related Fusarium keratitis (A) with multifocal infiltrates (green and red arrows); (B) Later stage fungal keratitis with hypopyon (neutrophil infiltrate to the anterior chamber, yellow arrow). (A–C) slit lamp images; C (inset)-optical coherence tomography of the eye in C showing endothelial plaque (yellow arrow). (D) In vivo confocal microscopy of a fungal infected cornea showing hyphae in the stroma.

(A–C) From Ting et al. [10]. (d) From Wang et al. [12].

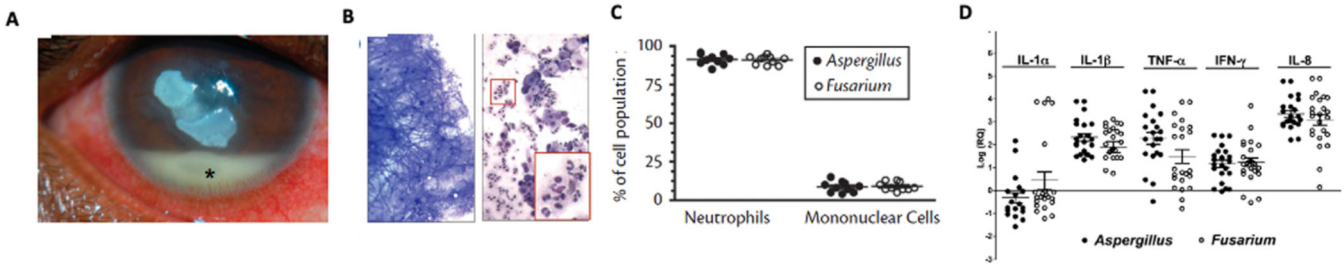

Fig. 4.

Immune response in patients with corneal ulcers caused by *Aspergillus and Fusarium.* (A) Fungal keratitis caused by *Aspergillus flavus* (* neutrophil rich hypopyon in the anterior chamber). (B,C) *A. flavus* hyphae and cellular infiltrate in a corneal ulcer showing >90% of the cells were neutrophils. (D) Gene expression of cytokines in corneal ulcers caused by *Aspergillus* and *Fusarium* compared with uninfected (donor) cornea. Data points represent individual patients.

From Karthikeyan et al. [14].

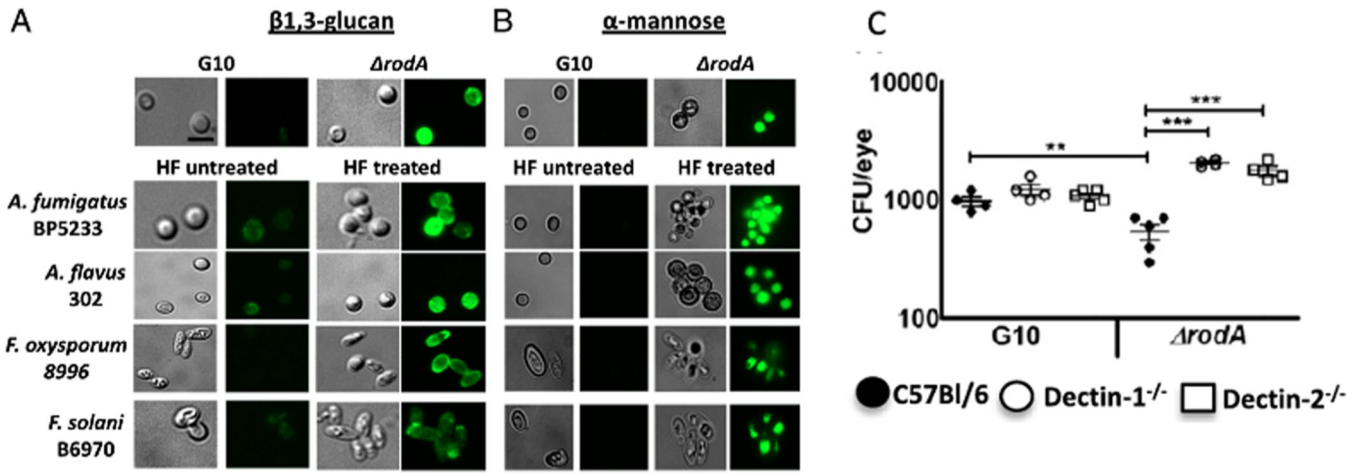

Fig. 5.

RodA hydrophobin proteins mask conidia cell wall carbohydrates and impair *A. fumigatus* hyphal killing in infected corneas. (A,B) β-glucan and α-mannose on parent G10 *A. fumigatus,* Δ*rodA* mutants and hydrofluoric acid (HF) treated *Aspergillus* and *Fusarium* conidia. (C) CFU 24 h post-corneal infection with G10 or Δ*rodA* conidia. Note that mutants are more rapidly cleared, which is dependent on Dectin-1 and Dectin-2. Data points represent corneas from infected mice.

From Carrion Sde et al. [24].

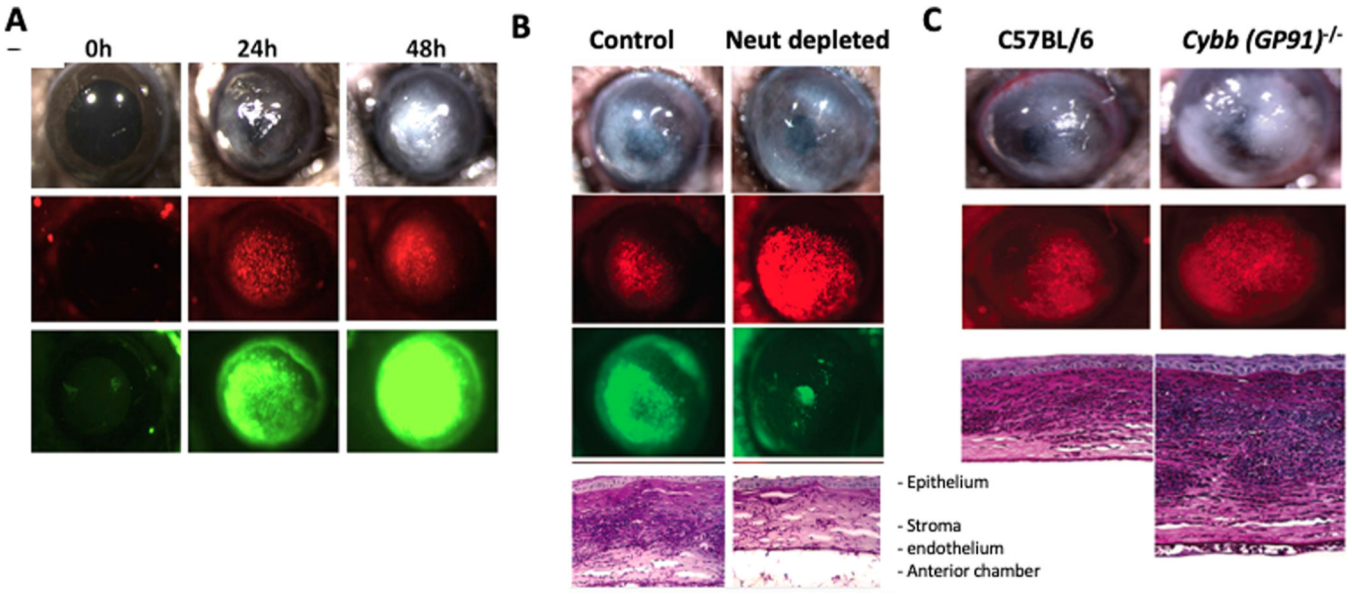

Fig. 6.

Neutrophil ROS mediated regulation of *Aspergillus fumigatus* corneal infection (keratitis). (A,B) Red fluorescent (RFP) expressing *A. fumigatus* spores were injected into the corneal stroma of mice expressing GFP neutrophils. Brightfield: Corneal opacification, RFP and GFP were examined by fluorescence microscopy. Neutrophils were depleted by intra-peritoneal injection of Ly6G antibody NIMP-R14 (corneas were examined 48 h post infection). (C) Infection of mice with a deletion in the NADPH oxidase subunit $GP91^{PHOX}$ (original magnification: corneas x20; histology (PASH) x 200).

From Leal et al. [25].

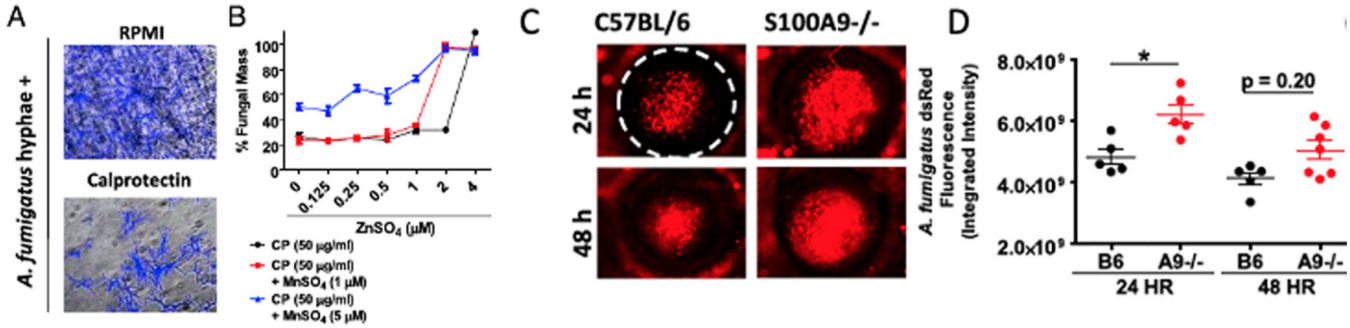

Fig. 7.

Calprotectin (CP) is required for *Aspergillus fumigatus* hyphal growth in infected corneas. (A, B) Exogenous Zn^{++} and Mn^{++} rescue hyphal CP inhibition of hyphal growth *in vitro*. (C, D) Increased hyphal growth in corneas of calprotectin deficient $S100A9^{-/-}$ mice infected with RFP expressing *A. fumigatus* AF293. Representative images (C) and quantification by imaging analysis.

From Clark et al. [35].

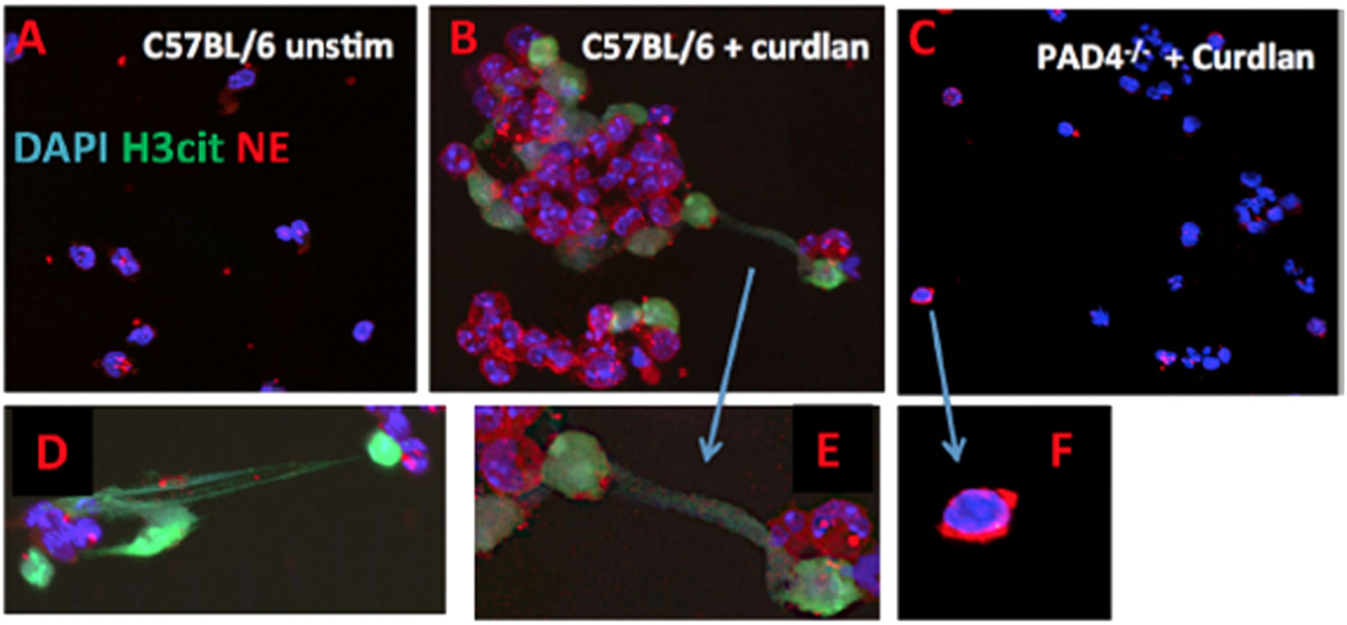

Fig. 8.

NETosis induced by curdlan is dependent on PAD4. (A–C) Neutrophil elastase (NE) and citrullinated histone 3 (H3cit) in bone marrow neutrophils from C57BL/6 or PAD4$^{-/-}$ mice. (D–F) highlighted areas showing NE in the nucleus of neutrophils from C57BL/6 but not PAD4$^{-/-}$ mice and extracellular H3cit associated with NETs. Original magnification for A-C: x200, for D-F x400.

From Clark et al. [45].

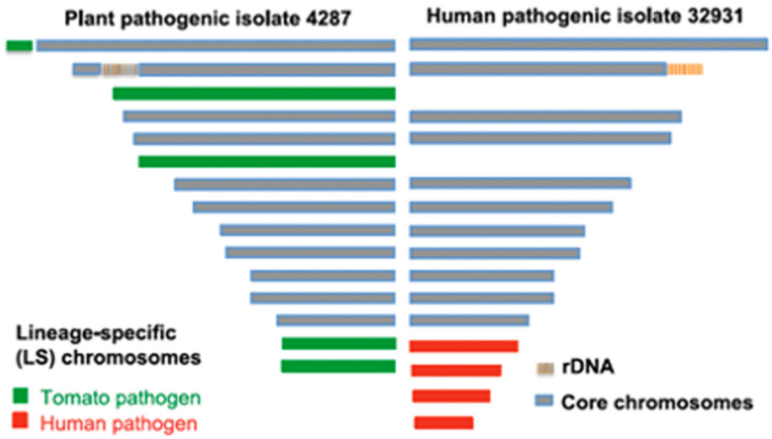

Fig. 9.

Optical maps of the human systemic fusariosis strain NRRL 32931 and the tomato wilt pathogen Fol4287. The core genome comprises 11 homologous chromosomes (gray) that are conserved within the *Fusarium oxysporum* complex. However, the four unique and smaller lineage-specific chromosomes of human-infecting strain NRRL 32931 in red are clearly distinct from the tomato pathogen.

From Zhang et al. [54].

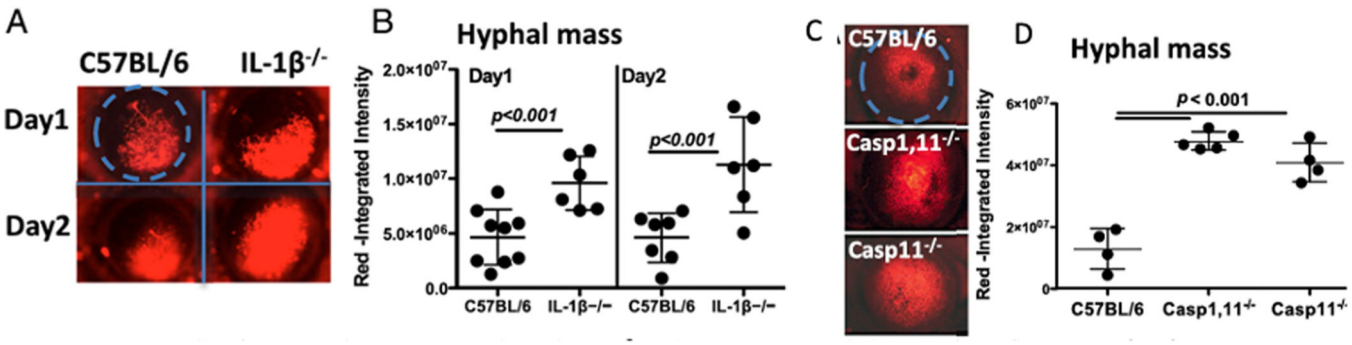

Fig. 10.

Aspergillus fumigatus keratitis is regulated by IL-1β and caspase 11. Hyphal mass of Red fluorescent (RFP) expressing *A. fumigatus* in the corneal stroma of C57BL/6 and IL-1β$^{-/-}$ mice at d1 and d2 post infection (A, B) or caspase1/11$^{-/-}$ or caspase-11$^{-/-}$ mice at d2 post-infection. RFP was examined. (A,C) representative RFP expressing corneas; (B,D) RFP quantification by image analysis. Data points represent individual infected corneas. From Sun et al. [21].

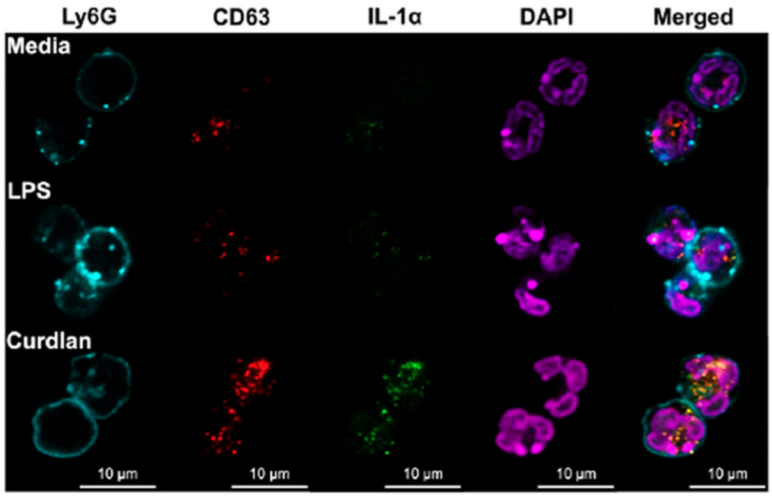

Fig. 11.

Co-localization of IL-1α with the tetraspanin CD63 marker for exosomes. Neutrophils from the peritoneal cavity of C57BL/6 mice (following induction of sterile inflammation) incubated 6 h with LPS or curdlan, and immunostained with antibodies to CD63 and IL-1α and examined by confocal microscopy.

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