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Host-Microbe Interactions in Cornea

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Abstract

Corneal infections result through interaction between microbes and host innate immune receptors. Damage to the cornea occurs as a result of microbial virulence factors and is often exacerbated by lack of a controlled host immune response; the latter contributing to bystander damage to corneal structure. Understanding mechanisms involved in host microbial interactions is critical to development of novel therapeutic targets, ultimate control of microbial pathogenesis, and restoration of tissue homeostasis. Studies on these interactions continue to provide exciting findings directly related to this ultimate goal.

1. Introduction

Host microbe interactions at the corneal surface are facilitated by epithelial defects or injuries that predispose the eye to pathogens. Among bacterial pathogens, *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*), are most often encountered. Fungi, including both filamentous and yeast (e.g., *Fusarium*, and *Candida* species), also induce destructive corneal disease. However, the leading cause of corneal ulcers and blindness worldwide is viral induced and follows recurrent infection of the cornea by herpes simplex virus (HSV-1). This virus is most commonly transmitted through contact with HSV in herpes lesions, mucosal surfaces, genital secretions, or oral secretions. This review will focus on several of these pathogens, their virulence factors and how the host responds.

2. *Staphylococcus aureus* (*S. aureus*)

S. aureus is a leading cause of keratitis globally and is the most virulent of the *Staphylococcus* species [1]. It possesses many virulence factors which allow for host adhesion, cytolytic activity, and evading the innate immune system. The bacterium uses collagen and fibronectin to adhere to corneal epithelial cells, initiating keratitis. A rabbit model, in which contact lenses soaked in *S. aureus* strains containing the collagen-binding adhesin (Cna+) or its isogenic mutant lacking the adhesin (Cna–), were placed on the

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injured cornea and outcome showed that Cna significantly contributed to bacterial adherence and corneal colonization [2]. The proteome of *S. aureus* isolates from keratitis [3] was analyzed and four adhesin virulence factors identified: fibronectin binding protein A, staphopain, glyceraldehyde-3-phosphate dehydrogenase 2 and extracellular adhesin proteins. When compared with a less virulent lab strain, the clinical isolate produced more potentially important virulence factors than the less virulent lab strain, accounting for ability of the clinical *S. aureus* isolate to cause more severe keratitis [4]. Fibronectin-binding proteins (FnBP) contribute to adherence in vitro (cultured human corneal epithelial cells, HCEC). Evidence suggests that FnBP promotes binding to the corneal epithelium, and ligation of $\alpha 5\beta 1$ integrin (receptor) which allows actin polymerization, endocytic vesicle formation and pathogen invasion [3]. Recently, studies with HCEC showed that staphopain A, an extracellular protease, increased adhesion and cell invasion by increasing fibronectin binding; its inhibition decreased pathogenesis [5]. Further studies of staphopain A will be required to determine its potential as a target of treatment.

2.1 Toxins

The role of alpha (α)-toxin in corneal infection has been established by comparing virulence in animal models using α -toxin-deficient isogenic mutants with their wild-type parental strains [6]. α -toxin lyses erythrocytes and leukocytes, but not the predominant one, the neutrophil (PMN) [7]. The toxin binds to disintegrin and metalloproteinase 10 (ADAM10), resulting in binding to eukaryotic cells. Following binding, disruption of focal adhesions [8, 9], such as E-cadherin in epithelial cells, loss of epithelial barrier function and pore formation ensues [10]. The importance of other toxins: β -toxin, γ -toxin, and Pantone–Valentine leukocidin, have been analyzed to a lesser degree and their roles in eye infections are less clear [11]. However, it has been shown that γ -toxin can cause corneal pathology and induce disease when injected intrastromally [12]. Other toxins such as the phenol-soluble modulins have yet to be examined in any animal models for their virulence in the eye [11].

Emergence of methicillin-resistant *S. aureus* (MRSA strains), resistant to the most commonly used fluoroquinolones necessitates development of new antimicrobials for bacterial keratitis. Besifloxacin, the first topical chlorofluoroquinolone was developed solely for ophthalmic use. It inhibits DNA gyrase and topoisomerase IV (ubiquitous in bacteria) and is essential for bacterial survival [13]. The drug has been shown effective in a rabbit model of keratitis and reduced MRSA colony-forming units more effectively than gatifloxacin or moxifloxacin [14]. A retrospective study showed that besifloxacin treatment of human cases of bacterial keratitis yielded no serious adverse effects and had low incidences of corneal scarring or neovascularization similar to that seen with moxifloxacin treatment [15]. The use of already existing agents to treat MRSA is also being studied. Lysostaphin is a zinc metalloproteinase extracted from *S. simulans* that lyses *S. aureus* by disrupting its peptidoglycan layer. It is effective in MRSA keratitis and endophthalmitis [16]. Las A protease (staphylolysin) targets the peptidoglycan of *S. aureus* and was comparable to vancomycin when treatment was begun early. It was more effective when treatment was begun in the late phase of infection, suggesting that Las A can lyse bacterial cell walls during the stationary growth phase [16]. Use of animal models of keratitis and endophthalmitis continue to provide insights into *S. aureus* virulence and host factors that

are active in limiting these infections. It is hopeful that data from the animal studies will translate into potential human treatment.

3. *Pseudomonas aeruginosa* (*P. aeruginosa*)

P. aeruginosa, (a gram-negative bacterium), is widely dispersed in the environment [17] and causes acute and chronic infections in humans. *P. aeruginosa* can cause acute infection, including: keratitis, otitis externa, bacteremia, burn wound infections, and pneumonia associated with cystic fibrosis. It also can cause severe chronic infections [18]. The bacteria causes severe infections of the cornea, particularly in contact lens wearing patients [19] in industrialized countries, such as the USA, and it is one of the leading causes of corneal ulcers [20]. If untreated, or non-responsive to topical treatment, corneal perforation may occur, which necessitates a transplant; the annual cost to treat keratitis (approximately 175 million) is a financial, as well as a health burden [19,21].

3.1 Virulence factors and host response

P. aeruginosa expresses numerous virulence factors, including pili and non-pili adhesins, flagella, proteases, hemolysins, exoenzymes and exotoxins, that play a role in *P. aeruginosa* invasion and infection of the cornea. Antibacterial agents in tears and a stratified epithelium protect the cornea, but trauma, contact lens wear and other adverse events can compromise the tissue and allow attachment to host receptors, initiating disease. The glycocalyx prevents phagocytosis of invading *P. aeruginosa* by host cells [18]; while proteases (elastase and alkaline protease) and toxins (exotoxin A), compromise host defenses by degrading proteins (immunoglobulin A and surfactant protein D) [18]. Exotoxins, released by the type III secretion system (T3SS), which secretes ExoS, ExoT and ExoU, promote bacterial survival and spreading by inducing apoptosis of infiltrating PMN [22]. ExoU also has phospholipase activity and promotes stromal necrosis and inflammation [22]. Other studies have shown a novel virulence strategy in *P. aeruginosa* involving bleb niche formation (a niche within the plasma membrane of epithelial cells) in which bacteria survive, replicate and swim. Their intracellular survival involves ExoS ADP ribosyl activity [24] and implicates a connection between bleb niche formation and the known role of Exo-S mediated apoptosis and Rab GTPase activation [25].

P. aeruginosa lipopolysaccharide (LPS) and flagella bind to host cell surface receptors including TLR2, TLR4 and TLR5 [23]. Interactions initiate a signaling cascade in resident macrophages and corneal epithelial cells to produce inflammatory cytokines and chemokines, which promote PMN and other inflammatory cell recruitment to the cornea. PMN are essential for eradicating bacteria, but their persistence contributes to the severity of keratitis [20,23]. For instance, elevated expression of macrophage inflammatory protein-2 (MIP-2) and IL-1 β , known chemoattractants for PMN [20], is consistent with increased disease severity, PMN infiltrate, persistence and bacterial load. This scenario is played out in *P. aeruginosa* infected susceptible C57BL/6 (cornea perforates) compared to resistant BALB/c mice (cornea heals) [20]. However, antibody depletion of PMN, the major inflammatory cell seen in keratitis, results in unhindered bacterial growth, and corneal destruction in both groups of mice (unpublished data, Fig.1), and in C57BL/6

mice, septicemia and death. In this regard, inhibition of IL-1 β improves disease outcome in C57BL/6 mice by reducing, but not eliminating, PMN in the infected cornea. Besides PMN, CD4⁺ T cells and Langerhans cells, the latter especially in contact lens wear, migrate to the site of infection and contribute to the host immune response against *P. aeruginosa* [20]. It also was demonstrated that depleting CD4⁺ T cells in infected C57BL/6 mice prevented corneal perforation, while in controls, perforation occurred at 5–7 days after infection [20]. Suryawanshi et al. [26] added to this body of information and found that infection in C57BL/6 mice also induced Th17 cells (later in disease), which mediated corneal pathology, and that treatment with galectin-1 was efficacious. In addition to phagocytosis, PMN recruited to the corneal stroma produce reactive oxygen species (ROS) and matrix metalloproteinases (MMPs), including collagenases, to prevent microbial dissemination [23]. Specifically, recent confirmatory studies have shown that ROS and MMPs, including MMP13 [27] cause bystander damage to the cornea by disrupting the collagen matrix which causes corneal scarring, loss of corneal clarity and impaired visual acuity. Other studies [28] showed that HIF-1 α , a transcription factor, is essential for effective PMN bacterial killing, antimicrobial peptide production and PMN apoptosis. PMN recruitment to the infected cornea were shown to limit biofilm production to prevent bacterial dissemination. During this process, PMN were found to move to the base of the forming biofilm where they underwent PMN extracellular trap formation (NETosis) in response to the bacterial T3SS. Further, blocking Psl exopolysaccharide and type III secretion, together with antibiotic treatment, broke the biofilm and reversed keratitis [29]. LasR (quorum sensing) defective mutants failed to induce robust NET formation compared to LAS-R sufficient strains [30], confirming the importance of this virulence factor in disease. In the host, other factors, such as epithelium produced thymic stromal lymphopoietin also is protective in keratitis by targeting dendritic cells and in IL-23/IL-17 signaling [31]. Other groups using the C57BL/6 mouse model and previously documented approaches, including antibody neutralization and recombinant protein injection, showed downregulation of IL-24 reduced severity of keratitis. They also showed that recombinant IL-24 protein induced SOCS3, IL-24, IL-1 β and IL-6 in primary human corneal epithelial cells [32]. This lab also reported that IL-1Ra and IL-36Ra have opposing effects and that IL-36 agonists could be used as an alternate therapeutic to IL-1 β neutralization [33]; the latter confirming work originally shown before by Rudner et al [34]. In some of these studies, effects were measured at very early times (1 and 2–16 h) after infection and although important mechanistically, it is difficult to understand how these studies will have patient applicability due to the narrow treatment window of these targets in the eye. IL-10 is important in the resistance response of BALB/c mice, as recombinant IL-10 treatment rescued rapamycin treated mice that had decreased IL-10 levels, improving disease outcome [35]. It has also been shown that IL-33 promotes a Th2 type immune response in recombinant protein injected C57BL/6 mice and reduced inflammation by polarizing macrophage production of anti-inflammatory mediators in cornea [36]. Immunostaining showed that recombinant injection shifted macrophage polarization from NO synthase 2 to arginase production and that peritoneally elicited cells from C57BL/6 mice treated with LPS and recombinant IL-33 exhibited elevated ST2 levels and a shift from IL-12 to IL-10 (protective) production [36]. More recent work has shown that the NLR4 vs NLRP3 inflammasome contributes to resistance through regulation of caspase-1, IL-1 β and IL-18 in a CD11b^{low} Ly6G^{low} cell

population [37]. Others [38] found that NLRP12 decreased the severity of keratitis by reducing bacterial burden and inflammation through downregulating NF κ B signaling and thereby cytokine production. Collectively, these studies provide much detail about disease pathogenesis, but treatment clinically remains elusive. In that regard, a silicone-gel contact lens model using the C57BL/6 mouse may prove useful, as studies revealed that without deliberate inoculation with *P. aeruginosa*, contact lens wear did not result in bacterial infection. Other immune cells in cornea were detected, however which may prime the cornea to respond to insult more quickly and vigorously than in the absence of a contact lens [39]. *P. aeruginosa* has extreme ability to trigger antibiotic resistance mechanisms, making them difficult to eradicate even using current treatments [40]. For instance, inefficient porins in the *P. aeruginosa* outer membrane limit the rate of antibiotic penetration to the bacterial cell, resulting in efflux pump overexpression and enzymatic antibiotic modification [40,41]. In addition, *P. aeruginosa* acquires spontaneous genetic mutations based on the antibiotic therapy and the environment and transfers those mutated genetic elements horizontally between cells, further reducing its susceptibility to antibiotics [40]. Inappropriate use of antibiotics along with other risk factors cause a breakthrough resistance in *P. aeruginosa*, which results in emergence of multi-drug resistant (MDR), extensively drug resistant (EXD) and pan-drug resistant (PDR) *P. aeruginosa* strains, with no or minimum effective antibiotic therapy [40,42]. Alternate approaches that will enhance treatment are needed, as the number of new antibiotics in the pipeline are not keeping pace.

3.2 Antimicrobial peptides (AMPs) and proteins

β -defensins are natural peptides produced by corneal and conjunctival epithelial cells. These peptides are cationic and interact with bacterial membranes, composed of negatively charged LPS. Such interaction enhances bacterial cell membrane permeability and cell lysis [43]. Using siRNA knockdown of mouse beta defensins (mBD) mBD2 and mBD3 in C57BL/6 mice, it was reported that both mBD2 and 3 enhanced resistance to disease [44]. Studies also revealed that mBD2 and mBD3 function together to regulate the expression of pro- and anti-inflammatory mediators, PMN infiltrate and bacterial load in the infected cornea. AMPs also have a range of non-microbicidal functions including signaling, immunomodulation, and influence vascularization and wound healing, all important for resolution of ocular surface disease in humans [45]. Vasoactive intestinal peptide (VIP) is an anti-inflammatory neuropeptide with multiple functions that was shown to balance pro- and anti-inflammatory cytokines in the *P. aeruginosa* infected cornea and protect C57BL/6 mice against corneal perforation [46]. Li and coworkers [47] reported that OH-CATH30, a natural peptide in snakes, is protective alone or as adjunctive therapy with levofloxacin, and reduced the expression of pro-inflammatory cytokines (TNF- α , IL-1 β), leukocyte infiltration, bacterial load and improved disease. Despite demonstrating various protective effects of AMPs and proteins, there are challenges to clinical use. These small peptides and proteins are effective in their native α -helical or β -sheet conformation. However, extraction or synthetic conditions, as well as enrichment, (to facilitate delivery to the cell). may lead to loss of a native conformation which would retard the activity of these molecules. In addition, the ability of these molecules to penetrate the cornea, the residence time and the stability in the ocular environment in the presence of a wide variety of proteases and other inhibitors, are other issues of concern, all of which slow progress.

High mobility group box-1 (HMGB1), a small 215 amino acid protein is an alarmin, belonging to the family of danger associated molecular patterns (DAMPs), which amplify inflammatory reactions. It is a ubiquitous molecule seen in the normal cornea and is released extracellularly after infection (Fig. 2 A,B). HMGB1 is an extracellular mediator of pro-inflammatory responses, and strategies to inhibit the molecule in various diseases are being tested. Silencing HMGB1, using small interfering RNA (siHMGB1), significantly improved disease outcome of pseudomonas keratitis in susceptible C57BL/6 mice [48]. HMGB1 knockdown downregulated pro-inflammatory cytokine expression, while upregulating anti-inflammatory cytokines. Treatment also downregulated IL-1 β and MIP-2, reducing the PMN infiltrate. Since knockdown of HMGB1 would not be desirable clinically, pursuing use of other molecules such as glycyrrhizin (GLY), a natural anti-inflammatory and antiviral triterpene, to reduce HMGB1, appear promising and sensible. Experiments in mice using invasive (clinical isolate) and cytotoxic (ATCC strain 19660) pseudomonas strains, revealed that GLY treatment reduced HMGB1 levels, bacterial plate count, and inflammatory consequences and enhanced antibiotic therapy [49]. GLY was also effective against three ocular clinical isolates, reducing viability, biofilm formation and adherence [50]. It was also effective against multi-drug resistant isolates (systemic and ocular), of *P. aeruginosa*, even when treatment was delayed to 18h after infection. GLY is antimicrobial, and was found able to alter bacterial virulence factors (efflux pump activity), viability and increased the effects of ciprofloxacin to reduce plate count, and MPO activity [41]. The challenge for all of these approaches will be to bring successful candidates from animal models to the clinic for human testing/clinical trials.

4. Fungal keratitis

Fungal keratitis is a common, severe eye infection, particularly common in tropical and subtropical areas of the world. Ocular trauma and contact with plant material, insects or other vehicles contaminated with fungal spores [51–53], are causative. In China and India, for example, agricultural work is the major risk factor; fungal keratitis increases during the harvest season, because of increased airborne soil and contaminated plant materials [51–55]. However, in industrialized countries use of contact lenses remains a key risk factor [56–58]. In either case, if the integrity of the corneal epithelium is breached due to trauma, fungi such as *Aspergillus* and *Fusarium* conidia or hyphae penetrate into the corneal stroma, and germinate causing ulceration, extreme pain, and visual impairment. Fungal keratitis patients are immunocompetent compared with systemically infected patients [59–61]. They are able to mount a vigorous immune response to *Aspergillus* and *Fusarium* hyphae characterized by infiltration of host innate immune cells and secretion of inflammatory, chemotactic, and regulatory cytokines. Other work has shown [62], in both mouse and human PMN, production of NADPH oxidase to control fungal growth. The antifungal activity depended on CD18, and not the β -glucan receptor dectin-1. Others, [63] studied the effects of calprotectin, (PMN source), and showed that zinc and manganese binding contribute to its anti-hyphal activity. However, calprotectin was not important in killing intracellular fungal conidia. It was also shown that dectin-1 dependent IL-6 production regulated expression of heme and siderophore binding proteins in infected mice and that human PMN synthesize lipocalin-1 that restricts fungal growth. Mice that were immunized with fungal conidia

before infection had improved fungal killing and less stromal opacity versus unimmunized animals [62]. PMN, innate immune cells, that produced IL-17, as well as T cells producing IL-17, but not IFN- γ , contributed to improved fungal killing. IL-17 production in PMN from fungal keratitis patients, as well as uninfected people, were studied [64] and IL-17 levels were detected in all groups, but were significantly higher in PMN from keratitis patients. Toll-like receptors (TLR) and lectin-like-receptors also are important in the host innate immune response to fungi (e.g., *Candida albicans* (*C. albicans*) [65], leading to cytokine production. A main fungal recognition receptor, dectin-1 deficiency results in enhanced susceptibility to fungal infection in mice [66,67]. TLR2- and TLR4 amplification by dectin 1 leads to production of tumor necrosis factor (TNF) [68], and alone enhances IL-17, IL-6 and IL-10 [69]. In a clinical study, mutations in dectin-1 led to defective production of several cytokines after β -glucan challenge, but fungal phagocytosis and killing were unimpaired [70]. Analysis of fungal vs bacterial keratitis (human late stage) was done using comparative transcriptomics confirmed by RT-PCR [71]. The study revealed overlap in gene expression profiles, but showed about 50 unique genes to fungal disease that highlighted a corneal epithelial wound healing response. Those genes could provide new therapeutic targets and will require further investigation to determine their usefulness in treatment of fungal disease.

3.3 Microbiome

The gut microbiome is a complex ecosystem and plays a pivotal role in maintaining ocular health and affects immunity of the eye [72]. In mice, both genetics and environment play a role in its composition [73], which we have recently confirmed for two inbred strains (C57BL/6 and BALB/c), often used in eye studies. It is tempting to hypothesize that differences between the two, one susceptible and the other resistant to *P. aeruginosa* keratitis, may reflect the disparate gut microbiome (Fig. 3). This is an attractive hypothesis we believe, as bacteria that reside in the gut play a key role in the regulation of mucosal immunity, particularly in balancing Th17/Treg cells [72]. In fact, gut dysbiosis has been implicated in infectious keratitis [72]. A recent study in patients with bacterial keratitis showed dysbiosis in the gut bacterial and fungal communities when compared to healthy human subjects [74]. The study implicated that increased abundance of pro-inflammatory and pathogenic bacteria in bacterial keratitis subjects may contribute to the disease phenotype [74]. Another study, evaluating patients with fungal keratitis linked gut dysbiosis to disease phenotype. A decrease in abundance of beneficial bacteria and increase in abundance of pro-inflammatory and pathogenic bacteria was observed in these patients [75]. A study by Zaidi et al [76] evaluated the link between the gut microbiome and *S. aureus* infection in the eye. The results showed that the gut microbiota was important in immune cell maturation. Further, along with PMN recruitment mediated by ICAM-1, mature CD4⁺ T cells producing IL-17 and IL-22 were required for antibody-mediated adaptive immunity to *S. aureus* in the anterior eye compartment [76]. These studies indicate the presence of a gut-eye axis and provide a strong link between changes in the microbiota of the gut and eye infections. Future studies focused toward microbiome modulation can potentially lead to therapeutic interventions, both at the ocular surface and by modulating the gut microbiome.

Due to the lack of extensive studies, the role of the ocular microbiota in regulating ocular immunity is not well understood [72]. A classic study [77] however revealed that in mice an

ocular commensal protected against corneal infection (*Candida albicans* and *P. aeruginosa*) by driving mucosal $\gamma\delta$ T cells and IL-17. A few studies have investigated contact lens wear, as it is the leading cause of corneal ulcers, with *P. aeruginosa*, accounting for nearly 50% of all infections [72]. Increased levels of opportunistic pathogens and differences in the distribution of gram-negative bacteria were reported in the conjunctiva of contact lens vs non-lens wearers [72]. A pioneering study by Kugadas et al [78] provided the first insight in the role of ocular microbiota in regulating ocular immunity in *P. aeruginosa*-induced keratitis. This study revealed that in healthy mice, the ocular innate immune barrier was strengthened by the presence of ocular microbiota. A significant increase in the levels of immune effectors such as secretory IgA and complement proteins in the tear film were observed. Future studies dedicated to identifying the different species of ocular microbiota and microbiota-induced signaling that regulate PMN and bactericidal activities may be an avenue for development of new therapeutic interventions, including fecal transplant.

5. Herpes stromal keratitis (HSK)

Herpes simplex virus-1 (HSV-1) is a leading cause of corneal blindness worldwide [79] and reactivation of latent virus from trigeminal ganglion (TG) sensory neurons is a deleterious outcome. Upon reactivation, infectious virus and/or viral proteins travel through axons of the ophthalmic branch of the TG to penetrate the corneal epithelium through sensory nerve terminals [80]. In the epithelium, virus causes development of herpes epithelial keratitis (HEK) [81]. These lesions tend to be self-limiting, but heal more rapidly when treated. For example, topical ganciclovir (formulated as an ophthalmic gel), has low toxicity, and is well tolerated when treating acute herpetic keratitis [82]. Overall, HSV-1 can infect the corneal epithelium, and the stroma, with or without ulceration; it also can infect the corneal endothelium, manifesting as keratitis in each case [83]. Clinical manifestations of HSK include: stromal opacity, swelling, and neovascularization. Unfortunately, once corneal scarring has occurred, penetrating keratoplasty may be required and points to the need to develop new approaches to treatment [84–87]. Disruption of viral entry into cells, is amongst a number of novel means to treat this disease [88].

5.1 Innate and adaptive immunity

Studies in mice have confirmed participation of the immune system in viral clearance and in development of HSK pathogenesis following experimental infection with HSV-1. These studies have shown that PMN are key inflammatory cells in control of viral load in HSV-1 infected corneas. In fact, these cells enhance the severity of HSK in the mouse model [56]. To test their role in disease, PMN were selectively depleted, and the data provided evidence that inflammatory monocytes, (not PMN), recruited by production of type I interferon by corneal epithelial cells were important in controlling viral load early after infection [86]. Type I interferon production in response to HSV-1 infection is regulated by an innate DNA sensor (IFI-16) [89]. Another study examined early depletion of resident corneal dendritic cells (DCs). Depleting these cells reduced infiltration of natural killer (NK) cells in infected corneas, suggesting their indirect role in controlling HSV-1 infection [90]. It is without question that innate immune cells are essential in controlling viral load in HSV-1 infected corneas. However, the importance of adaptive immunity, especially CD8⁺ T cells, in HSV-1

clearance from infected corneas has been demonstrated in a mouse model of primary ocular HSV-1 infection [89] and also will require further work to understand applicability to human disease. The latter is also true for studies of another virus, the Zika virus, a single-stranded RNA virus of the Flaviviridae family, which has recently been shown to infect and replicate in primary cultures of human corneal epithelial cells [91].

5.2 IL-17 and Foxp3⁺Treg

Studies using mouse models have established that CD4⁺ T cells are a principle effector cell for induction of HSK [87]. In this regard, data has shown that after experimental induction of ocular HSV-1 infection, only infiltrating corneal DCs took up viral antigens and trafficked to draining lymph nodes where they primed virus specific CD4⁺ T cells [92]. It also was shown that these primed cells migrate to the infected corneas where they orchestrate tissue damage. Recently, IL-17 producing CD4⁺ T cells were detected in inflamed corneas during the later stages of disease and in IL-17 knock out mice, significantly reduced ocular lesions were seen [93]. ($\gamma\delta$) T lymphocytes that produce IL-17 also were shown to infiltrate HSV-1 infected corneas early after infection, but whether IL-17 production by these cells during active viral infection is beneficial or detrimental is not clear. Nonetheless, IL-17 expression, which has been reported in HSK patients, is of importance, as these human data suggest IL-17 involvement in HSK pathogenesis [94]. Other studies examined the role of Foxp3 expressing regulatory CD4⁺ T cells in controlling HSK using mice. In vivo treatment with the fungal metabolite drug FTY720 was shown to increase the Foxp3⁺ Treg population, significantly diminishing HSV-1 induced ocular lesions [95]. However, discontinuation of the treatment resulted in relapsed inflammatory lesions. Similarly, in vivo expansion of Foxp3⁺ Tregs during the early stages of corneal HSV-1 infection, using IL-2/anti-IL-2 complex, was shown to reduce the development of HSK lesions but, increased Treg therapy was not effective upon priming and infiltration of pathogenic CD4⁺ T cells [96]. Thus, it appears from these data that increasing the Foxp3⁺ Treg population only would not sufficiently control HSK.

5.3 Hemangiogenesis and lymphangiogenesis

After HSV-1 infection, new blood vessels sprout from the corneal limbus and invade the avascular cornea. Vascular endothelial growth factor-A (VEGF-A), produced in HSV-1 infected corneas, is important for induction of hemangiogenesis in HSK [57]. Infection has been shown to reduce availability of soluble VEGF receptor-1 (sVEGFR-1), a molecule which inactivates VEGF [97]. Reduction in sVEGFR-1 is dependent upon matrix metalloproteinase 9 (MMP-9), produced by infiltrating PMN. Other angiogenic factors are also involved. Studies have shown that mice lacking Robo4, an endothelial receptor that counteracts VEGF downstream signaling, have increased angiogenesis vs control mice after ocular HSV-1 infection [98]. In contrast, microRNA 132 (miR-132) has been shown to enhance VEGF-A mediated angiogenesis [99]. HSV-1 infected corneas have recently been shown to have extensions of lymphatic vessels from the limbal region of the cornea. HSV-1 induced lymphangiogenesis is dependent on VEGF-A produced by virus infected corneal epithelial cells [100]. Besides VEGF-A, VEGF-C (released from infiltrating CD8⁺ T cells), promote lymphatic vessel growth into the central cornea [89]. VEGF-A/ VEGFR2 signaling also induces lymphangiogenesis in HSV-1 infected mouse corneas [51]. Inflammatory

cytokines (TNF and IL-6) potentiate VEGF, promoting virus induced lymphangiogenesis in infected corneas [101]. Novel recent work has focused on metabolic changes in HSK lesions and showed the prevalence of glycolytic metabolism and the development of hypoxia in HSK lesions, information which may provide new targets for treatment development [102].

5.4 Corneal homeostasis

Alteration in homeostasis of resident cells in cornea may also contribute to initiation and or progression of HSK. A recent study used in vivo confocal microscopy, and demonstrated reduced corneal nerve density, branching of nerve trunks and loss of sensation in patients with HSK, in both the infected and uninfected eye [103]. Similarly, in a mouse model of HSK, reduced corneal innervation was detected in the corneal epithelium during the clinical period of disease [104]. Tarsorrhaphy (suturing eyelids), after ocular HSV-1 infection, prevented corneal desiccation and nerve loss, reducing HSK severity. The data suggest that inhibiting nerve retraction in the infected corneas could be important in controlling HSK severity. Corneal nerve terminals are closely associated with superficial corneal epithelial cells and thus, alteration in the corneal epithelium of HSV-1 infected eyes effect corneal innervation. In fact, in the superficial epithelium, increase in cell size, decrease in cell density, and squamous metaplasia and desquamating superficial corneal epithelial cells is reported in diseased eyes of patients with HSV keratitis, suggesting an altered corneal epithelial morphology correlated with changes in corneal innervation [105]. This suggests that further studies directed to the interaction of corneal nerves and immune cells may deliver new therapeutic targets with clinical potential.

6. microRNAs (miRNA)

Several pioneering studies have demonstrated that miRNAs play critical roles in the pathogenesis of infectious keratitis. These findings will be reviewed essentially focusing on how miRNAs modulate infectious keratitis through regulating host-microbe interactions.

6.1 miRNAs in *P. aeruginosa* keratitis

Yang et al. was first to report that miR-155 was induced by *P. aeruginosa* infection in macrophages in vitro and in mouse and human corneas in vivo[106]. Inactivation of miR-155 resulted in a reduced bacterial load, and decreased severity of experimental keratitis, suggesting that miR-155 enhanced bacterial burden and promoted corneal susceptibility to the bacteria. Macrophages of miR-155 knockout mice showed increased phagocytosis and intracellular bacterial killing; while over-expression of miR-155 inhibited them, and expression of induced nitric oxide synthase (iNOS) and the production of nitric oxide (NO)[106]. These effects are mediated by targeting Rheb, a gene known to interact with mammalian target of rapamycin (mTOR) and increase its activity[107]; inhibition of mTOR using rapamycin increased bacterial burden and reduced PMN bactericidal activity (Fig.4A)[108]. Data suggest that miR-155 could be a therapeutic target as knockdown increased phagocytosis and intracellular killing capacity by macrophages, cells that constitute a minority of the infiltrate after infection.

Recently, it has been shown that the conserved, paralogous miRNA cluster, miR-183/96/182 cluster (miR-183/96/182), modulates the corneal response to *P. aeruginosa* infection through its regulation of pathogenesis at multiple levels. The miR-183/96/182 was originally identified as a sensory organ-specific miRNA cluster [109] required for normal development and function of all major sensory domains[110]. Inactivation of the cluster in mice resulted in decreased corneal nerve density in the subbasal plexus and the expression of nociceptor transient receptor potential vanilloid 1 (TRPV1) and multiple pro-inflammatory neuropeptides, including Tac1 [the precursor gene for substance P (sP)], calcitonin gene-related peptide (CGRP) and chemokine (C-X3-C motif) ligand 1 (Cx3cl1)[111]. miR-183/96/182 knockout mice showed a significantly decreased corneal inflammatory response to *P. aeruginosa* infection and reduced severity of disease[111]. Furthermore, the cluster was also expressed on innate immune cells, including macrophages and PMN[111]. Inactivation of miR-183/96/182 resulted in increased phagocytosis and intracellular bacterial killing capacity of both groups of cells (Fig.4A)[111]. miR-183/96/182 targets Nox2, a key enzyme required for generation of superoxide (O_2^-) and other microbicidal products [112]; inactivation of miR-183/96/182 resulted in increased Nox2 and enhanced production of reactive nitrogen (RNS) and ROS in macrophages. In addition, miR-183/96/182 promotes the production of pro-inflammatory cytokines in macrophages by targeting DAP12[112], consistent with the observation of an overall decreased level of pro-inflammatory cytokines in the cornea in response to *P. aeruginosa* infection (Fig.4B)[111]. Data further demonstrated that, in addition to its regulation on circulating macrophages and PMN, miR-183/96/182 has direct regulatory effects on corneal resident macrophages [113].

miR-183/96/182 is not only expressed in innate immune cells, but also it is one of the highest induced miRNAs during helper T (Th)-17 cell differentiation, and promotes Th17 cell pathogenicity by negatively regulating the expression of transcription factor Foxo1, a negative regulator of IL-1R1 [114]. Inactivation of miR-183/96/182 in mice resulted in decreased pathogenicity of Th17 cells and a reduced production of pro-inflammatory cytokines, leading to decreased severity of experimental autoimmune encephalomyelitis [114]. Since IL-17 activity and Th17 have been shown to promote PMN infiltration and the severity of *P. aeruginosa* keratitis[115], miR-183/96/182 may also by regulation of Th17, effect *P. aeruginosa* keratitis.

6.2. miRNAs in herpes simplex virus (HSV)-1 keratitis

At least 18 pre-miRNAs and 27 mature miRNAs have been identified in the HSV-1 genome based on the most updated miRbase (Release 22.1. October 2018). These miRNAs are actively involved in the control of productive infection/latency/reactivation[116]. During latency, viral gene expression is restricted to select non-coding RNAs, including the latency-associated transcript (LAT), which is critical to the HSV-1 latency-reactivation cycle through its anti-apoptotic and immune evasion properties, and viral miRNAs[116,117]. At least ten LAT-associated miRNAs (miRs H1-4, 6-8, H14, 15, 27) have been identified in and near the LAT locus of the HSV-1 genome[118].

6.3 HSV miRNAs modulate viral virulence

LAT-derived miR-H2 is in antisense to infected cell protein (ICP)0 gene, an immediate early gene which promotes viral replication, trans-activates lytic gene expression and facilitates reactivation from latency. It targets and regulates ICP0 expression at protein levels[118]. Disruption of miR-H2 without altering the amino acid sequence of the ICP0 gene resulted in increased production of ICP0 in host cells, and enhanced neurovirulence in mice after ocular infection with HSV-1 and more rapid reactivation[119,120]. Simultaneous inactivation of LAT transcript and miR-H2 resulted in a more robust reaction[120]. These data suggest that miR-H2 suppresses HSV-1 neurovirulence and reactivation and promotes latency through targeting ICP0. miR-H6, a HSV-1 miRNA derived from the promoter region of the LAT gene[118], showed sequence complementarity to ICP4, a transcription factor required for the expression of most HSV-1 genes during productive infection[120]; it also repressed ICP4 protein expression, an essential immediately-early lytic viral gene [118], suggesting a potential role in promoting or maintaining latency. Consistently, in human corneal epithelial cells, miR-H6 inhibited HSV-1 replication[121], but animal modeling is needed to confirm this.

6.4 HSV miRNAs regulate host genes

In addition to its direct regulation of ICP4 and latency, miR-H6 of HSV-1 modulates the expression of IL-6 in human corneal epithelial cells [121]. Overexpression of miR-H6 in these cells significantly reduced HSV-1-induced upregulation of IL-6[121]. These data are derived from an in vitro study and the direct target of miR-H6 mediating this effect is unidentified. However, since IL-6 is known to promote an inflammatory response, reactivation [122] and neovascularization during HSV-1 infection[123], it suggests that miR-H6 has a potential to inhibit the above effects of HSV-1[121]. Further study on miR-H6's regulation on HSV-1 induced IL-6 in the cornea in vivo is warranted.

6.5 HSV-1 can regulate host miRNA expression

During acute HSV-1 infection, miR-183/96/182 is selectively upregulated in rat primary superior cervical ganglion neurons and human primary fibroblasts within 2–3 hours after HSV-1 infection. It is increased up to 30–40 fold by 1 day post-infection, gradually dropping over the next 6 days[124,125]. Lutz et al. further demonstrated that the immediate early protein ICP0, an E3 ubiquitin ligase, is required and sufficient to induce the expression of miR-183/96/182[124]. The induction of miR-183/96/182 by ICP0 is mediated by members of the transcriptional repressor ZEB family, ZEB1 and ZEB2. ZEB1/ZEB2 suppresses miR-183/96/182 transcription; ICP0 promotes the degradation ZEB1 (/ZEB2) proteasome through its E3 ubiquitin ligase activity and therefore releases the inhibition on miR-183/96/182 expression by ZEB1 (/ZEB2)[124]. Once miR-183/96/182 is induced by ICP0 in acute infection, its expression may be maintained at a lower but significant expression level over the latency phase because of a negative feedback regulation of miR-183/96/182 on ZEB1/ZEB2[126–128], when ICP0 expression is downregulated during latency. Although target genes of miR-183/96/182 and its exact functions during HSV-1 infection have not been identified, its upregulation in acute infection and modest expression during latency suggest a role in various phases of disease. In addition, HSV-1 is not the

only herpesvirus to induce the expression of miR-183/96/182. It is among the most strongly upregulated miRNAs during the productive infection cycle of human cytomegalovirus, a β -herpesvirus[129]. The γ -herpesvirus Epstein-Barr virus (EBV) selectively downregulates miR-183/96/182 during type II and III latency[130]. These suggest that miR-183/96/182 may be important, with a conserved role in the pathogenesis of all three major herpesvirus subfamilies.

6.6 Host miRNA can regulate HSV-1 gene expression

A host neuron-specific miRNA, miR-138, targets and represses the expression of ICP0 and other lytic genes of HSV-1. A mutant HSV-1 (M138) with disrupted miR-138 target sites in ICP0 mRNA exhibited increased expression of ICP0 and other lytic proteins in infected neuronal cells in culture[131]. Consistently, in vivo, after corneal inoculation, M138-infected mice showed higher expression of ICP0 and other lytic transcripts in the TG during latency establishment, and exhibited increased mortality and encephalitis symptoms[131]. After full establishment of latency, an increased fraction of TG neurons expressed lytic transcripts in M138-infected mice. These data suggest that host miR-138 represses HSV-1 lytic gene expression and promotes host survival and viral latency[131]. As most of the studies on latency/reactivation are limited to in vitro or ex vivo level, further in vivo studies are needed to test the roles of miRNAs in these processes.

6.7 Host miRNAs regulates immune response to HSV-1

miR-155 is one of the first miRNAs shown to play a role in HSK through its regulation of the host immune system. It is significantly upregulated in the cornea at 2, 7 and 15 days after HSV-1 infection in the mouse[132]. miR-155 knockout mice showed decreased angiogenesis and reduced severity of HSK, and fewer infiltrating CD4⁺ T cells and Th1 and Th17 responses in both the infected cornea and lymphoid organs, including draining lymph nodes and the spleen[132]. miR-155 targets Ship1 and IFN- γ R α , which are important regulators of IFN- γ expression and Th1 differentiation [133,134]. Inactivation of miR-155 resulted in significantly increased expression of both Ship1 and IFN- γ R α in activated CD4⁺ T cells of draining lymph nodes[132], promoting Th1 cell development and a decrease in HSK severity in miR-155 knockout vs wildtype mice[132]. In vivo silencing of miR-155 by conjunctival injection of antigomir-155 nanoparticles at 1 day after infection and at an early clinical stage (5 days after infection) reduced infiltration of CD4⁺ T cells, PMN, pro-inflammatory cytokine production, and decreased the severity of HSK[132].

Corneal neovascularization allows immune/inflammatory cells to gain access to the cornea contributing to the pathogenesis of HSK[135,136]. Controlling neovascularization is critical to treatment of HSK[136]. miR-132 is known to activate the endothelium and facilitate pathological angiogenesis by targeting p120RasGAP- a negative regulator of angiogenic Ras activity[137]. Mulik et al showed that HSV-1 infection upregulates IL-17 production, which induces VEGF expression in the cornea; VEGF further induces the expression of miR-132, which inhibits p120RasGAP expression and contributes to neovascularization in the cornea in response to HSV-1 infection [138]. Knocking down VEGF by VEGF trap (soluble VEGF receptor) or inactivation of IL-17A receptor in mice prevented the

upregulation of miR-132 in HSV-1 infected cornea, suggesting miR-132 mediates VEGF and IL-17A-induced neovascularization[138].

7. Promising new therapeutic targets and future directions

Development of new therapeutic targets is the underlying goal of all studies focused on host-microbe interactions, and as antibiotic resistance remains a growing threat, the urgency for this work is heightened. A few emerging targets include reduction of infection and inflammation by decreasing bacterial load and HMGB1 levels using GLY, which has been shown efficacious in a mouse model of multi-drug resistant *P. aeruginosa* infection, as an example, or use of an anti-miR for miR-155 or knockdown of the miR-183/86/182 cluster. The role of the gut and eye microbiome [139,140] is another area of extreme interest, which provides important clues to bacterial, fungal and viral diseases; and more than likely, not only those involving microbial infections. Additional studies will be needed to test the clinical feasibility of any promising agent that works well in an animal model. How to achieve this goal is the key to future treatment successes.

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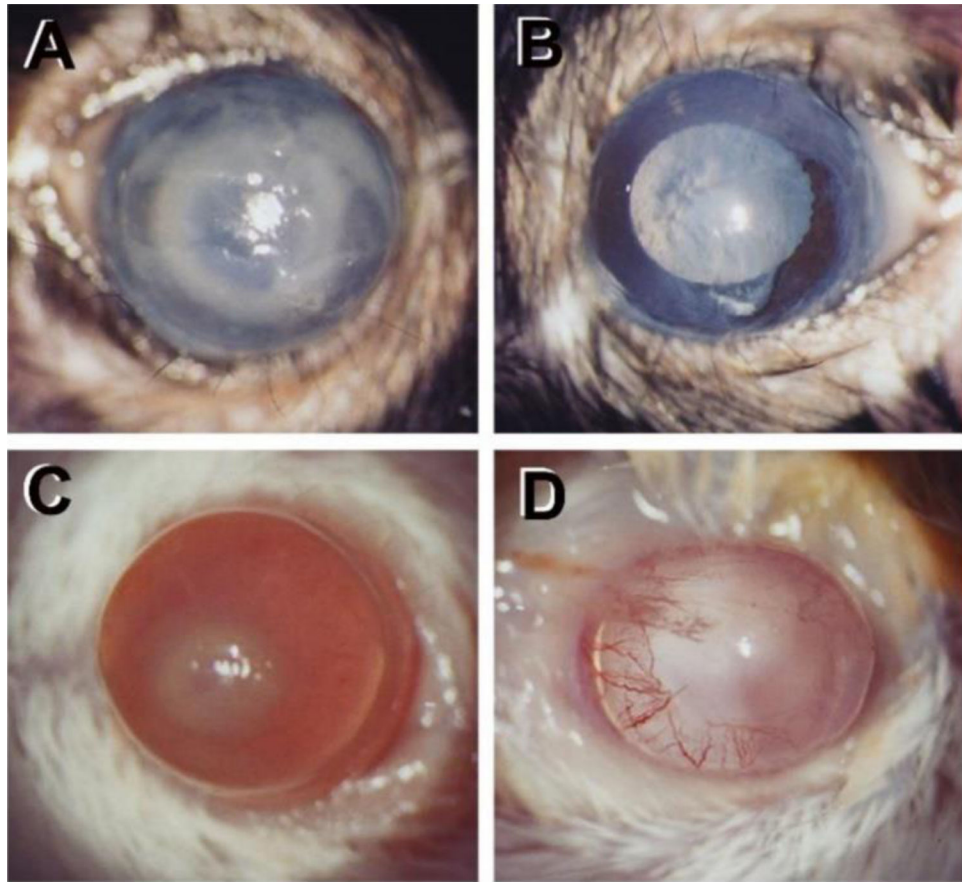


Figure 1.
P. aeruginosa infected mice at 1 day after infection and depletion of PMN with anti-Ly-6G/GR-1 antibody (Ab). (A) Control C57BL/6 mouse cornea shows light opacity over entire cornea. (B) Ab treated C57BL/6 mouse cornea is clear (lack infiltrating PMN). (C) Control BALB/c mouse cornea with opacity over the pupil. (D) Ab treated BALB/c mouse cornea is clear (vessel ingrowth from the periphery).

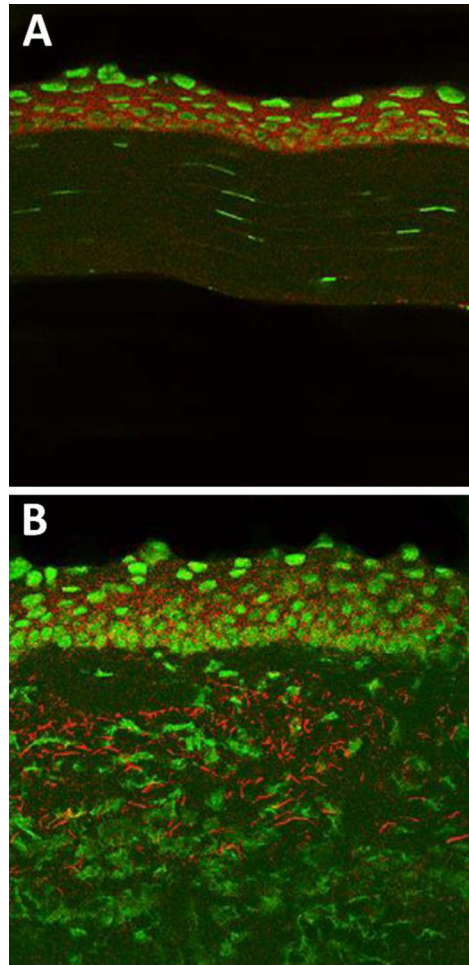


Figure 2. Normal and 5 day post infection C57BL/6 mouse cornea stained with an anti-HMGB1 Ab. (A) HMGB1 epithelial staining (red) in normal, uninfected cornea. (B) HMGB1 staining distributed throughout the epithelium and stroma after 5 days of infection. Nuclear marker=Sytox Green.

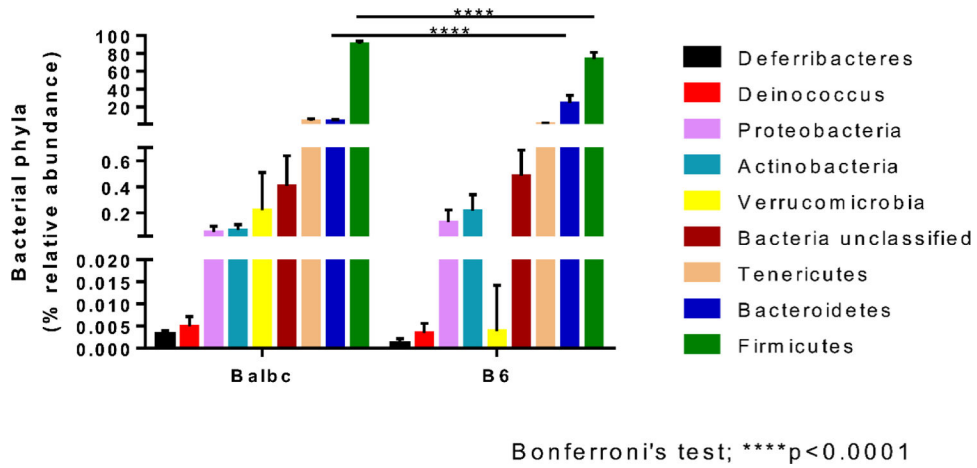


Figure 3. Normal BALB/c and C57BL/6 mice harbor a strain-specific gut bacteria phyla. Results are represented as % relative abundance and were analyzed with two-way ANOVA. Significant differences were present in Firmicutes and Bacteroidetes phyla. For Firmicutes, significantly higher ($p < 0.0001$, Bonferroni's test) levels were found in BALB/c compared to C57BL/6. For Bacteroidetes, BALB/c had significantly reduced ($p < 0.0001$, Bonferroni's test) levels when compared to C57BL/6.

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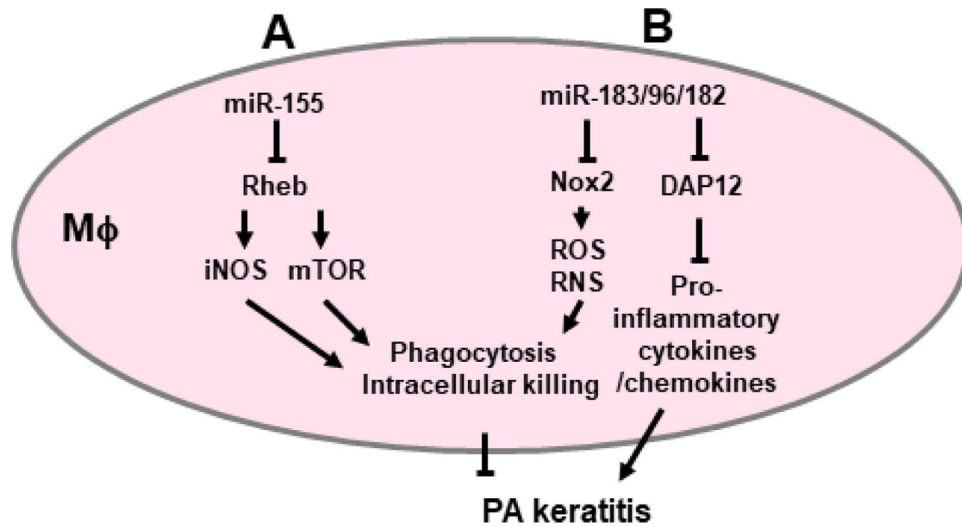


Figure 4. miR-155 and the miR-183/96/182 cluster modulate *P. aeruginosa* keratitis by regulating the function of macrophages (Mφ). A. miR-155 targets the expression of Rheb, which enhances the production of iNOS and mTOR activity to promote phagocytosis and intracellular bacterial killing. B. miR-183/96/182 targets Nox2, important in ROS and RNS production, to promote phagocytosis/intracellular bacterial killing, and DAP12, which limits production of pro-inflammatory cytokines.