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Analysis of infection-associated gene expression and their interaction with differentially expressed miRNA from the prospective of polymicrobial infections in corneal keratitis

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Keratitis is one of the leading causes of eye comorbidities. Bacteria, fungi, viruses, protozoans and helminths are the causative organisms of infectious keratitis. Simultaneous or sequential infection of more than one organism, known as polymicrobial infection, may lead to severe form of the disease. Few studies have highlighted the incidence and epidemiology of polymicrobial infections. However, no attempts were made to understand the changes in gene expression and identify pathways in polymicrobial infections, due to complex experimental design. This study utilizes the computational tools combined with experimental approaches to curate infection-associated genes that were expressed in cornea and secreted into tears. Gene Ontology and KEGG analyses revealed enrichment of pathways relevant to corneal host defense like epithelial polarity, leukocyte activation, antigen presentation, bacterial invasion and adherent junction. Network analysis identified highly interacting genes, including HSPA4, HSPA8, MAPK1, RHOA, STAT1, GSK3B, YWHAZ and TFRC. Integration of highly interacting genes with differentially expressed miRNAs from human fungal keratitis dataset identified regulatory hubs, such as miR-511-5p, miR-618 and miR-124-3p.1, with multiple infection-associated genes as targets. LPS treatment of human corneal epithelial cells modulated expression of several of these genes, further supporting their role in innate immune response. Connectivity map analysis identified perturbagens associated with key targets, like GSK3B and SYK, suggesting potential therapeutic relevance. Together, these findings reveal molecular mechanisms shared across microbial infections and highlight miRNA-gene interaction networks that may influence disease dynamics and outcomes in polymicrobial keratitis. While *in vitro* LPS treatment does not fully replicate the complexity of polymicrobial infection, this study provides a hypothesis-generating framework for future validation in patient derived samples and co-infection models.

Keywords: Connectivity map, Gene ontology, Human corneal epithelial cells, Interactome, Keratitis, miRNA

The eye is structurally and anatomically unique organ and is highly vulnerable to infection. Infection affects different regions of the eye, including eye lid manifesting to hordeolum, blepharitis and chalazion, scleritis and episcleritis in sclera, keratitis in the cornea, conjunctivitis of the conjunctiva and cellulitis in the surrounding tissue of the eye. The infections can be mild or severe. Corneal ulcer or keratitis is considered to be among the severe infections of the eye[1]. Keratitis is characterized by observations such as corneal oedema, infiltration of inflammatory cells and ciliary congestion. Keratitis can be infective and/or non-infective posing considerable threat to the vision[2]. Infectious keratitis is caused by many different microbes or parasites such as the bacteria, fungi, viruses, protozoans and helminths and in severe cases simultaneously by more than one organism.

Bacterial keratitis is one of the most dominant form and accounts for 80% of ulcerative keratitis cases[3] with *Pseudomonas aeruginosa* (*P. aeruginosa*) keratitis being the most common one[4]. Incidence of bacterial keratitis is rare and usually occur as a consequence of pre-disposing ocular trauma or ocular surface damage resulting from corneal surgery,

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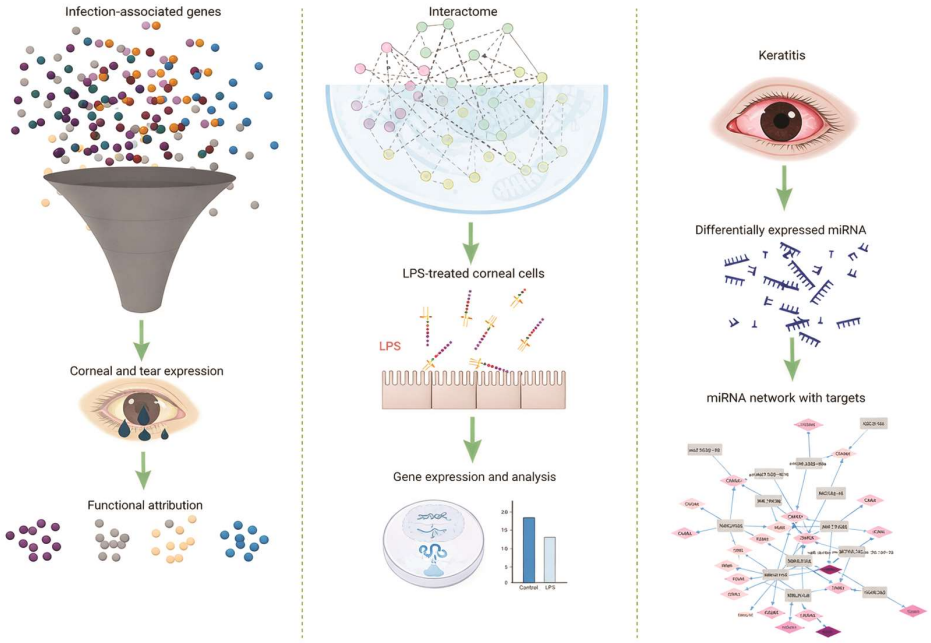

Graphical abstract

systemic diseases, immunosuppression and to certain extent use of contact lenses[5]. Fungal keratitis is mostly vegetative in origin and is caused by aspergillus and fusarium in tropical and subtropical regions and candida in temperate regions[6,7]. The severity of the disease may be attributed to the virulence of the pathogen, host immune response towards pathogen, inflammation, and the suboptimal diagnosis or treatment strategies[8].

Polymicrobial infections consisting of bacteria and fungus are most common which might result due to sub optimal host immune and inflammatory response. Bacterial and fungal co-infections increase the risk of monocular blindness leading to poor prognosis[9]. Studies show twenty per cent fungal keratitis cases were having bacterial co-infection, which can be simultaneous or sequential[10]. Standard treatment options may not be effective to treat these conditions due to different clinical features and diverse risk factors[11]. Polymicrobial interactions among the bacteria results in anti-microbial resistance[12]. Few clinical reports and epidemiological studies on polymicrobial infections were also reported[13,14].

Changes in the gene expression leading to altered levels of coding and non-coding RNA is observed due to changes in the ocular surface integrity. Differentially expressed genes in bacterial, fungal keratitis and genes unique to one of these conditions were reported[15,16]. miRNAs play an important role in the outcome of pathogenic processes including ocular infection[17]. miRNAs are small molecules (20-24 nucleotides long) which regulate target gene expression by interacting with coding and non-coding regions[18]. miRNAs were also differentially expressed and have diverse roles in regulating the gene expression of their target genes. Studies have reported differentially expressed miRNA during bacterial[17] and fungal keratitis[19]. 75 differentially expressed miRNAs were identified in fungal keratitis, among which miR-511-5p, miR-142-3p, miR-155-5p, and miR-451 have significant change in expression[19].

Databases of differentially expressed genes, gene network tools and system biology combined with experimental approaches have enabled and assisted in understanding the molecular mechanisms of pathogenesis which require complex experimental designs. Although polymicrobial keratitis is increasingly recognized for its clinical severity, the underlying molecular interactions that govern the host response to multiple pathogens remain poorly understood. Most studies have examined the pathogens in isolation, which limits the understanding of the pathways engaged when different microbes act together or sequentially. In addition, while corneal and tear-based studies have identified several infection-associated genes, their integration with miRNA regulation was not studied. To bridge this gap, the present work combines systematic curation of infection-associated genes expressed in the cornea and present in tear fluid with miRNAs altered

during human fungal keratitis, followed by network analysis and experimental testing using LPS treated human corneal epithelial cells. Through this integrative approach, this study aims to identify common regulatory nodes and gene-miRNA interactions that may contribute to disease outcomes in polymicrobial keratitis, thereby laying the foundation for more detailed mechanistic studies in co-infection models.

Material and Methods

Chemicals and reagents

MEM alpha medium (#M0644) and LPS (#L9143) were purchased from Sigma-Aldrich, cDNA synthesis kit (#6110A) and TRIzol (#9108) were from Takara. Primers were from Eurofins and SYBER green supermix (#1725121) was from Biorad.

Genes expressed in corneal and tear fluid

Genes associated with infection were retrieved from National Centre for Biotechnology Information (NCBI) from the Uni Gene database using the search words "Infection" and "*Homo sapiens*". The protein coding genes having corneal expression were selected from the complete list of infection-associated genes based on the RNA sequence data of corneal epithelium[20]**.** The proteins which were secreted into the tears were chosen from the reported proteomic analysis of tear fluid[21-29]. The list excludes the proteins with cytoplasmic expression. The genes common in corneal expression and secreted into tear fluid were further curated for pathway enrichment and protein interaction network analysis.

Gene ontology and pathway enrichment analysis

The gene ontology and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed for the genes having corneal expression and were part of tear proteome using Enrichr online tool accessible at URL https://maayanlab.cloud/Enrichr/. Only those pathways and Gene Ontology terms with a P value < 0.05 was considered statistically significant.

Interactome analysis

The human protein-protein physical interaction was downloaded from the HIPPIE database (http://cbdm-01.zdv.uni-mainz.de/~mschaefer/hippie/) and the subnetwork with the genes expressed both in the cornea and secreted into the tear fluid were analysed. The network properties of the subnetwork were analysed using the Cytoscape network analysis tool (https://cytoscape.org). The genes were sorted based on decreasing degree.

Prediction of miRNA targets

Differentially expressed miRNA in the fungal keratitis was obtained from the dataset with GSE64843[19]. TargetScan (version 7.2) was used to predict miRNA-mRNA interactions. Predictions were ranked based on cumulative weighted context++ scores, which estimate repression strength and aggregate P_{ct}, which reflects evolutionary conservation. Since TargetScan does not prescribe fixed cutoff values, all potential targets predicted by TargetScan were selected and the targets were overlapped with genes expressed in infection, cornea and tears. The Jaccard index was calculated to identify miRNAs that are enriched for target genes.

$$\text{Jaccard Index} = \frac{A \cap B}{A \cup B}$$

miRNA-target network

A network of differentially expressed miRNAs in fungal keratitis and their target genes expressed in the cornea and secreted into tears during infection was created using Cytoscape.

Connectivity map analysis

To identify potential therapeutic compounds involved in metabolic pathways associated with corneal infections, the Connectivity Map (CMap) database through the CLUE platform (https://clue.io/) was utilized. This approach allowed us to systematically analyse gene expression signatures derived from our study and match them with bioactive compounds capable of either reinforcing or counteracting these expression patterns. The compounds were ranked based on their connectivity scores, with a threshold of $\leq$ -90 indicating strong reversers and $\geq$ 90 signifying strong mimickers. Further, mechanisms of action (MoA) and molecular targets were investigated using CMap annotations to gain deeper insights into the biological relevance of these compounds. Our focus remained on compounds with well-documented biological activity, particularly those modulating infection-related pathways, offering insights into possible therapeutic interventions.

Cell culture

Human corneal epithelial (HCE) cells were grown in minimum essential medium (MEM) alpha modification. The medium was supplemented with insulin (5 mg/L), epithelial growth factor (0.1 mg/L), heat-inactivated FBS (10% (*v/v*)) and antibiotic and antimycotic solution. Cultures were maintained in a

Table 1 — Primer sequences used to study expression of infection-associated genes

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Tm (°C)
APP	GGTGTTCTTTGCAGAAGATGTG	ATGACGATCACTGTCGCTATG	57
CD44	GCAGGTATGGGTTCATAGAAGG	GGTGTTGGATGTGAGGATGT	60
CDH1	TCGACAAAGGACAGCCTATTT	TTTATCCGACAGGAAACAGCT	60
EEF2	TACGAGCTCTCGGAGAATGA	GGGAGTCAATGAGGTTGATGAG	57
EGF	GGGAATGGTTTATGCCCTAGAT	CGCTGGGAACCATCCATATT	60
GSK3B	TGGAGCCACTGATTATACCTCTA	CAAACTGATCCACACCACTATC	60
HSPA4	GACCTGCCAATCGAGAATCA	CCGCTCCTTCTCCAGTTTATC	60
HSPA8	CCTACTCTTGTGTGGGTGTTT	ACCGTTCAGTGTCCGTAAAG	60
KRAS	GGCTCAGGACTTAGCAAGAA	CGGATCTCTCTCACCAATGTATAA	57
MAPK1	AGAGAACCCTGAGGGAGATAAA	CGATGGTTGGTGCTCGAATA	60
P4HB	CCCAAGAGTGTGTCTGACTATG	GTCGCTGTCGATGAAGATGAA	60
RHOA	CCATCATCCTGGTTGGGAATAA	ATCTCTGCCTTCTTCAGGTTTC	60
STAT1	CACCTACGAACATGACCCTATC	GCTGTCTTTCCACCACAAAC	60
SYK	TCTGGAGCTTTGGAGTGTTG	CATCCGCTCTCCTTTCTCTAAC	60
TFRC	TTTCCACCATCTCGGTCATC	GGGACAGTCTCCTTCCATATTC	60
TLR2	GAAGGTGAGTGGTGCAAGTAT	AATGGGCTCCAGAAGAATGAG	60
YWHAB	CGTGCTATCTCCAGCATTGA	GCAGTTCTGCCTCTATCTTCTC	60
YWHAQ	ACAAAGACAGCACCCTCATC	TTCTGCCGCATCACATTCT	57
YWHAZ	AGCAGAGAGCAAAGTCTTCTATT	GACTGATCGACAATCCCTTTCT	60

humidified atmosphere with 5% CO2 at 37°C. The cells were sub cultured every 72-h using 0.25% of Trypsin-EDTA. Trypan blue dye exclusion method was used to determine cell viability before using the cells for each experiment.

LPS treatment and RNA isolation

Cells were seeded in 100 mm dish overnight, treated with LPS (10 µg/mL) for 24 hrs. Cells were harvested and lysed for RNA isolation by adding TRIzol. To the lysed cell suspension, 200 µL Chloroform was added vortexed and centrifuged at 15,000 rpm for 20 min at 4°C. 500 µL of Isopropanol was added to upper aqueous layer and kept in -20°C for 1 h. After centrifugation at 15,000 rpm for 20 min, the pellet was washed with 1 mL of 75% ethanol, centrifuged at 15,000 rpm for 10 min. Pellet was resuspended in nuclease free water. Total RNA was quantified using Nanodrop.

Real-time PCR analysis

First strand cDNA synthesis kit was used to synthesize the cDNA. Real-time studies were performed in triplicate on Real-Time PCR detection system (BioRad). 10 µL reaction mixture contain 1 µL of template, 10 pmol of each primer (Table 1) and 5 µL of iTaq Universal SYBER green super mix. The real-time results were presented as change in target gene expression of LPS treated relative to untreated. Target gene C_t values were normalized to that of β-actin gene C_t values based on the comparative $2^{\Delta\Delta}$Ct method.

Statistical analysis

Statistical significance between untreated and LPS treated groups was assessed using a two-tailed unpaired Student's t-test performed on ΔCt values in GraphPad, PRISM. Data are presented as mean ± SEM of at least three independent experiments. Significance levels are indicated as follows- ****P< 0.0001, ****P< 0.001, **P< 0.01, *P< 0.05.

Results

Curation of infection associated genes with corneal and tear expression

A total of 2264 human infection-associated genes were retrieved from the NCBI UniGene database initially. Among these infection-associated genes, using the available RNA-seq based expression data from human corneal epithelium or human corneal epithelial cells, 1334 protein coding genes were identified as being expressed in the cornea. From the published tear proteome data 498 genes with secretion into tear fluid were identified. Comparison between two groups revealed 343 genes having both corneal expression and the tear secretome. This forms a core infection-associated gene set for further functional enrichment (Fig. 1 & Suppl. Table S1[20-29]). In the table, these genes were listed broadly based on their cellular

function like metabolism, biosynthesis, cell proliferation and survival, cell death, cell adhesion/motility/structure, immunity, phosphatase/kinase/ other enzyme, receptor/channel/ transporter/ trafficking and intracellular signalling. More precise gene function based on OMIN/ Gene Cards/ NCBI was also listed in this table.

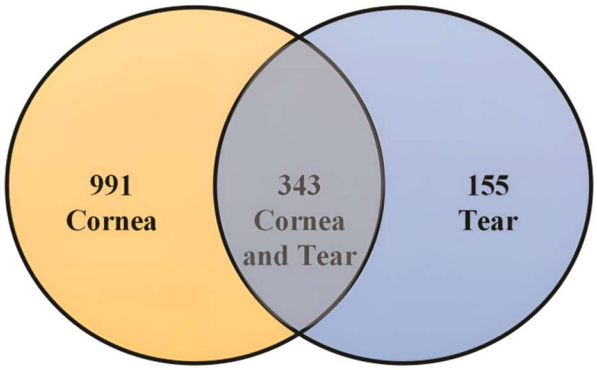

Fig. 1 — Venn diagram showing the number of infection-associated genes expressed in cornea and secreted into tear fluid

Functional annotation of the genes attributed them to essential cellular processes including ubiquitination, DNA repair, nucleic acid metabolism, ribosomal assembly, protein folding, cytoskeleton dynamics and cell-cell interactions, processes known to be disrupted during ocular infections. Notably, many curated genes also mapped to innate immune responses, epithelial barrier maintenance and intracellular signaling, all of which are important in host defense.

Gene Ontology and KEGG pathway enrichment analysis

Gene set enrichment analysis was performed to understand functional and pathway enrichment of the infection-associated genes, expressed both in cornea and secreted into tear fluid. These genes are mainly involved in biological process like positive regulation of leukocyte differentiation, viral entry into host cell, symbiont of entry into host, establishment of epithelial cell apical/basal polarity, positive regulation of protein localization to endosome, antigen processing and presentation of endogenous peptide antigen via MHC Class I (Table 2). These biological processes were

Table 2 — Functional and pathway enrichment analysis of infection-associated genes expressed both in cornea and secreted into tear fluid (GO: Biological Processes (Top 10 Biological processes sorted based on combined score). Adjusted P-value is multiple-hypothesis corrected Fisher exact test p value, Odds Ratio is measure of magnitude of enrichment and combined score multiplies the log of the p-value computed with the Fisher exact test by the z-score computed by odds ratio

Term	Adjusted P-value	Odds Ratio	Combined Score
Positive Regulation of Leukocyte Differentiation (GO:1902107)	4.8E-06	140.72	2418.03
Positive Regulation of Viral Entry into Host Cell (GO:0046598)	4.0E-06	56.44	988.13
Positive Regulation by Symbiont of Entry Into Host (GO:0075294)	4.0E-06	56.44	988.13
Regulation Of Dendritic Cell Differentiation (GO:2001198)	2.5E-05	56.28	829.69
Establishment Of Epithelial Cell Apical/Basal Polarity (GO:0045198)	2.5E-05	56.28	829.69
Regulation Of Protein Localization to Early Endosome (GO:1902965)	1.9E-04	56.12	671.27
Positive Regulation of Protein Localization to Endosome (GO:1905668)	1.9E-04	56.12	671.27
Positive Regulation of Protein Localization to Early Endosome (GO:1902966)	1.9E-04	56.12	671.27
Gland Morphogenesis (GO:0022612)	1.7E-03	55.96	512.34
Antigen Processing and Presentation Of Endogenous Peptide Antigen Via MHC Class I (GO:0019885)	0.00167	55.96	512.34
Top ten KEGG pathways sorted based on combined score			
Other glycan degradation	1E-07	36.01	651.70
Bacterial invasion of epithelial cells	1E-12	16.47	518.66
Pathogenic Escherichia coli infection	2E-16	10.35	424.51
Adherens junction	5E-11	15.48	421.90
Salmonella infection	4E-17	9.25	399.95
Pertussis	1E-10	14.21	372.25
Proteoglycans in cancer	4E-15	9.45	355.53
Phagosome	1E-12	9.95	308.74
Legionellosis	4E-08	13.66	263.97
Tight junction	1E-11	8.79	253.21
Leukocyte trans-endothelial migration	4E-10	10.17	251.79
Shigellosis	4E-13	7.65	251.42

Table 3 — List of highly interacting genes from the infection-associated genes expressed both in cornea and secreted into tear fluid.

Gene	Degree	Gene	Degree
ACTB	147	YWHAZ	106
MAPK1	142	GSK3B	102
HSPA4	140	TFRC	101
EGF	135	TLR2	99
RHOA	127	P4HB	98
ACTG1	125	EEF1A1	97
HSPA8	125	STAT1	97
KRAS	120	YWHAQ	97
CTNNB1	115	EEF2	95
APP	113	CFL1	95
CDH1	109	SYK	92
CD44	107	YWHAB	92

relevant in infection and host pathogen interactions as reported in previous studies. Further, first four KEGG pathways overrepresented in these genes (Table 2), including other glycan degradation, bacterial invasion of epithelial cells, pathogenic *Escherichia coli* infection, adherens junction show that the curated gene functions correlated with gene ontology and KEGG pathway analysis.

Genes with most interacting partners

Different centrality measures such as degree and betweenness centralities were calculated for nodes in the subnetwork comprising of infection-associated genes/proteins (nodes) expressed in the cornea and secreted into tear. Network analysis of the 343 curated genes revealed major hubs with high degree centrality. Genes such as ACTB, MAPK1, RHOA, GSK3B, STAT1, HSPA4, HSPA8, YWHAZ and TFRC emerged as highly interconnected nodes within the infection-associated network (Table 3). These genes represent converging points for cytoskeletal regulation, stress responses, inflammatory signaling and iron homeostasis functional categories relevant to keratitis pathology. The change in expression of these genes with LPS treatment in Human corneal epithelial cells was further studied.

Prediction of miRNA targets

Targets of differentially expressed miRNA in fungal keratitis[19] from infection-associated genes expressed in both in cornea and secreted into tear fluid were listed (Table 4). Among the miRNA differentially regulated, miR-511-5p has the highest number of predicted targets (90 genes). miR-618 and miR-124-3p.1 have 69 and 44 targets, respectively (Tables 4 & 5). Among all the miRNAs, targets of miR-511-5p are mostly enriched in infection. This miRNA is known to be involved in immune response, cell proliferation and tumour suppression. The genes highlighted in the (Table 4) were having highest degree centrality among all the targets of these miRNAs.

Network between differentially expressed miRNA and infection-associated genes

The network constructed between differentially expressed miRNAs in fungal keratitis and their target genes expressed both in the cornea and secreted into tear fluid showed that there are few target genes such as GSKB targeted by multiple microRNAs (miR-124-3p.1, miR-142-5p., miR-144-3p, miR-144-5p, miR-15-5p_16-5p_195-5p_424-5p_497-5p, miR-155-5p, miR-511-5p). However, there are some target genes such as TLR2 which is targeted by only miR-144-5p but show upregulation when validated (Figs. 2 & 3).

Connectivity map analysis

Connectivity Map (CMap) analysis of the key regulatory genes in this study revealed the mechanisms and associated perturbations (Table 6). These findings offer an integrated view of essential genes and their regulators, underscoring their roles in major pathways associated with corneal infections. For instance, GSK3B was associated with known GSK3 inhibitors (SB-216763, AR-A014418), while SYK was linked to Fostamatinib, a SYK inhibitor with known immunomodulatory effects. Structural genes such as ACTB and RHOA were associated with compounds that affect cytoskeletal dynamics. These associations highlight potential therapeutic nodes but remain exploratory.

Expression of highly interacting genes by real-time quantitative polymerase chain reaction

Quantitative PCR analysis of the total RNA isolated from LPS treatment of HCE cells showed significant down regulation of HSPA4, HSPA8, EGF, RHOA, KRAS, CDH1, YWHAZ, GSK3 B, STAT1 and SYK in comparison to untreated control whereas EEF2, APP, CD44, TFRC, TLR2, P4HB and YWHAB were upregulated. Although altered expression was observed in YWHAQ and MAPK1, it was not statistically significant (Fig. 3). These findings indicate that a subset of computationally curated, infection associated genes is dynamically regulated in response to a canonical innate immune stimulus.

Table 4 — Differentially expressed miRNAs in fungal keratitis and their targets from the infection-associated genes expressed both in cornea and secreted into tear fluid.

miRNA	Predicted targets
miR-124-3p.1.	TFRC, RHOA, MAPK1, GSK3B, DIAPH1, TLN1, DUSP3, F11R, PLEC, OSBP, GSN, USP14, SUB1, PLIN3, IQGAP1, CUL5, DNM2, LMAN1, GNAI3, G3BP2, CADM1, MAN2A1, PEA15, HADHA, FUBP1, GNAI1, ITGB1, MAPRE1, FURIN, ITGA3, KPNB1, DCTN4, MYH9, IDE, RPS6KB1, EIF2S1, PPP1R13L, TACC2, RAP1A, RHOG, NAP1L1, PTBP1, PRDX6, VIM, PRPS1, GNAI2, DDX6, VPS4B, CD82
miR-124-3p.2_506-3p.	ACTB, KRAS, PPP1R13L, MAN2A1, PEA15, DDX6, USP14, DCTN4, NAMPT, PTBP1, SUB1, YTHDF1, CDK6, RHOG, F11R, OSBP, CUL5, RPS6KB1, MAPRE1, CADM1, IQGAP1, PPM1A, MYH9, ARF6, PLIN3, PDGFA, TACC2, IDE, DIAPH1
miR-142-3p.1.	CTTN, RAC1, MYH9, STAU1, TGFB2, KIF5B, RAB1A, IL6ST, ATP1B1, HMGB1
miR-142-3p.2.	KRAS, CTNNB1, APP, TFRC, VCL, KIF5B, PDCD6IP, DDX6, ARF4, TRIM23, PLEC, IDH1, HNRNPK, IQGAP1, CLTC, RDX
miR-142-5p.	GSK3B, RHOA, HSPA8, TGFB2, IL6ST, STAU1, PRNP, CADM1, MAN1A1, VCL, SUMO2, SUB1, CXCL14, CDK6, RAP1A, IPO5, ACTR3, PCBP2, ATP1B1
miR-144-3p.	GSK3B, MAPK1, TFRC, APP, VPS4B, ATP1B1, RAB1A, RAB5A, UBE2D3, GNAI3, CUL5, THBS1, GLI2, MET, PDCD4, PRKAA1, STMN1, CADM1, STAU1, RAP1A
miR-144-5p.	APP, STAT1, YWHAQ, GSK3B, TLR2, YWHAB, ACTG1, TGM2, RELN, LMAN1, SOD2, KIF5B, RDX, CD58, ACTR2, MAPK13, UBE2D3, ACTR3, FUBP1, ASS1, HNRNPK, EIF4E, HLA-A, PROS1, ARF6, C3, SLPI, HEXA, PRKAA1, MSN, ITGA3
miR-146-5p.	HNRNPD
miR-15-5p_16-5p_195-5p_424-5p_497-5p.	GSK3B, YWHAQ, APP, KRAS, TFRC, RELN, USP14, RAB35, FUBP1, PSMD7, TUBA1A, GNAI3, ACTR2, TUBA4A, CDK6, PPM1A, SMPD1, CSF1, EIF4E, DIAPH1, PDCD4, FASN, CADM1, PDCD6IP, CX3CL1, FURIN, HNRNPA1, HDGF, PDIA6
miR-155-5p.	GSK3B, YWHAZ, KRAS, EEF2,CD47, TRIM23, RPS6KB1, IL6ST, G3BP2, RDX, PEA15, USP14, DNAJB1, FUBP1
miR-184.	None
miR-204-5p_211-5p.	P4HB, YWHAZ, MAPK1, EZR, IPO5, DNAJB1, DNM2, RAB1A, AIP, LAMP1, HMGB1
miR-21-5p_590-5p.	MAPK1, PDCD4, CADM1, CDK6, CD47, RAD21, PCBP1, VCL, UBE2D3, HNRNPK, MCAM, RDX, PCBP2, TGFB2, IL6ST
miR-223-3p.	IL6ST, RPS6KB1, RPS15A, ATP1B1, KIF4A
miR-451.	YWHAZ, PSMB8
miR-511-5p.	CDH1, ACTG1, SYK, GSK3B, MAPK1, STAT1, EEF1A1, CD44, KRAS, YWHAB, TFRC, CFL1, EGF, TRIM25, PCBP2, CLTC, CADM1, PLA2R1, LANCL2, UBE2D3, MAN2B2, NAP1L1, UBE2N, DNM1L, YTHDF2, CD47, CCL28, TACC2, G3BP2, EIF4E, MAN2A1, FUBP1, SOD2, STAU1, PLG, ACTR3, MET, HADHA, GBP1, B2M, SAMHD1, HNRNPH1, MUC4, PPIL1, TOLLIP, RPL15, IPO5, PSMB9, OSBP, MAN2A2, VCL, MYH9, RAP1A, IL6ST, CD59, GNAI3, LMAN1, CUL5, DCTN4, EIF2S1, PABPC1, TGM2, STAT2, USP14, PROS1, PTPRS, DUSP3, CD9, HMGB1, FTL, CDK6, MAN1A1, XDH, RPS6KB1, SCARB2, ITGA3, RAD21, HDGF, HYOU1, ANXA1, IL16, DNAJC3, RPS3, SUB1, HLA-A, RDX, ITGB1, RAC1, NLRC5, NCL
miR-618.	RHOA, EGF, HSPA4, CTNNB1, HNRNPD, MOGS, CDK6, DDX6, PSMB9, DUSP3, HNRNPA1, EIF4E, TRIM25, FUBP1, TUBA4A, MAPRE1, PTPRF, RPS19, CD59, PSMD7, PPM1A, APEX1, RPS3, PABPC1, VCL, DIAPH1, OSBP, TIMP1, CCL28, HNRNPK, IL6ST, ACO1, CUL5, PPIA, PDCD6IP, BLVRA, THBS1, TLN1, NAP1L1, GOLM1, MSN, EIF4A2, UBE2D3, NAMPT, CSK, FUT3, NQO1, SIRPA, TGFB2, B2M, SOD2, RDX, USP14, ILF3, APOL1, TRIM23, ANXA1, PCBP2, RAB1A, ITGB1, SAMHD1, MAN2A2, RPL4, SUB1

Table 5 — miRNA enrichment with infection gene targets in comparison with overall targets calculated as Jaccard index

miRNA	Infection gene targets	miRNA_targets	JI_infection
miR-124-3p.1	49	1821	0.02
miR-124-3p.2_506-3p	29	1326	0.02
miR-142-3p.1	10	384	0.01
miR-142-3p.2	16	604	0.02
miR-142-5p	19	951	0.01
miR-144-3p	20	1049	0.01
miR-144-5p	31	2077	0.01
miR-146-5p	1	284	0.00
miR-15-5p_16-5p_ 195-5p_424-5p_497-5p	29	1516	0.02
miR-155-5p	14	557	0.02
miR-184	0	30	0.00
miR-204-5p_211-5p	11	792	0.01
miR-21-5p_590-5p	15	385	0.02
miR-223-3p	5	416	0.01
miR-451	2	30	0.01
miR-511-5p	90	5221	0.02
miR-618	64	3330	0.02

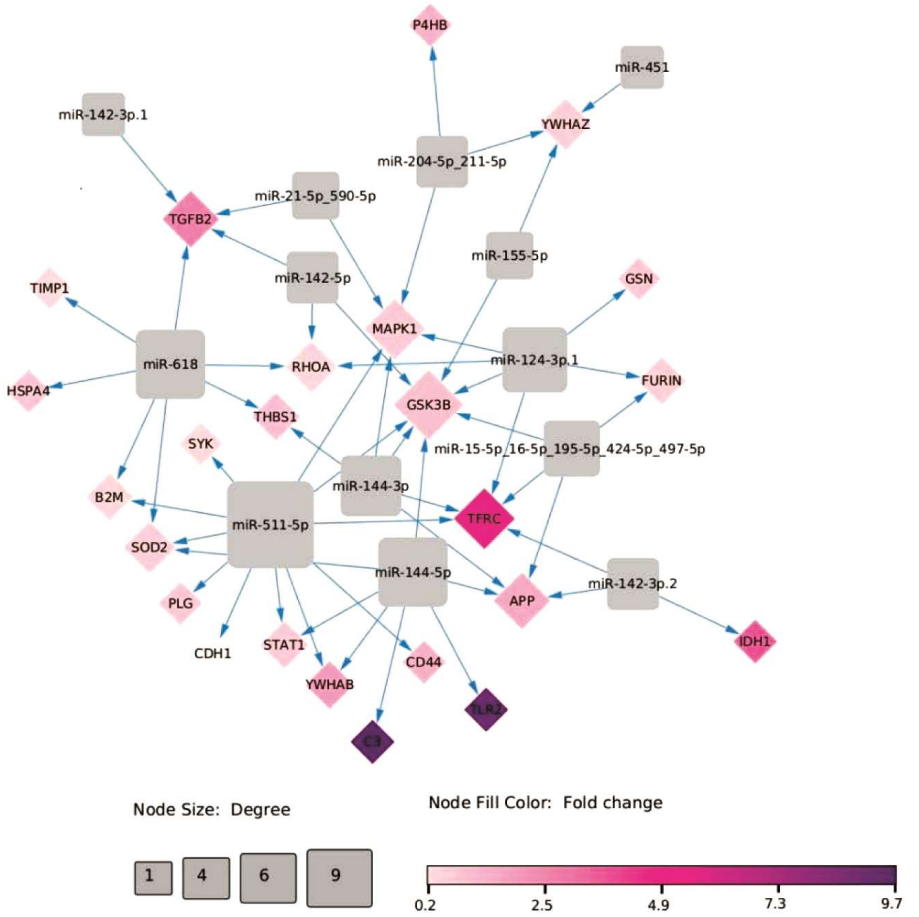

Fig. 2 — Network of differentially expressed miRNA (in square) in keratitis and predicted targets (in rhomboid) which are expressed in infection, cornea and tears. The node size indicates the degree, and the color of the targets represents the fold change upon LPS treatment

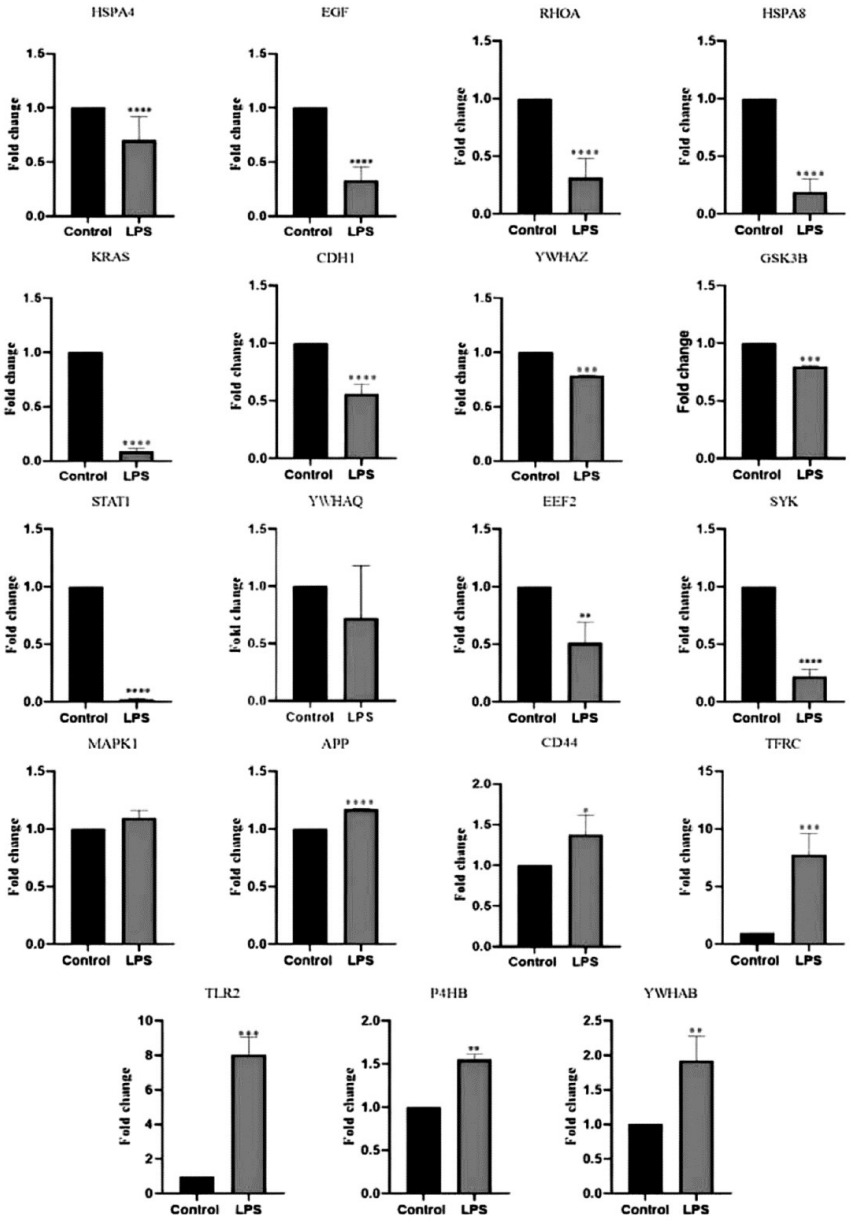

Fig. 3 — Real time PCR analysis of genes, in the LPS treated corneal epithelial cells, with high degree centrality among the infection-associated genes expressed in cornea and secreted into tear fluid. Significance levels are indicated as follows- ****P< 0.0001, ****P< 0.001, **P< 0.01, *P< 0.05

Table 6 — Summarizes the significant genes identified in the study and their associated perturbagens retrieved from Connectivity Map (CMap) analysis

Gene	Perturbagen	Type	Perturbagen ID	Target(s)	Mechanism of Action (MOA)
ACTB	Gene overexpression, gene knockdown	trt_oe, trt_sh.cgs	ccsbBroad304_05763 CGS001-60	ACTB	Actin Microtubule Polymerization inhibitor
MAPK1	LY-294002, Gene knockdown	trt_cp, trt_sh.cgs	BRD-K27305650 CG001-5594	AKT1, CAMK2A, CHEK1, LCK, MAPK1, MAPK11, MAPK12, MAPK14, MAPK8,	calcium/calmodulin dependent protein kinase inhibitor, purinergic receptor antagonist, calmodulin antagonist

(*Contd*.)

Table 6 — Summarizes the significant genes identified in the study and their associated perturbagens retrieved from Connectivity Map (CMap) analysis (*Contd*.)

Gene	Perturbagen	Type	Perturbagen ID	Target(s)	Mechanism of Action (MOA)
HSPA4	Gene knockdown	trt_sh.cgs	CGS001-3308	HSPA4	Not Available
EGF	Gene knockdown	trt_sh.cgs	CGS001-1950	BCL2, BCL2L1, MCL1, BCL2L2, CTGF, EGF	BCL inhibitor, MCL1 inhibitor
RHOA	Narciclasine, gene overexpression, gene knockdown	trt_cp, trt_oe, trt_sh.cgs	BRD-K06792661 ccsbBroad304_00100 CGS001-387	RHOA	Coflilin signaling pathway activator, LIM kinase activator, Rho associated kinase activator
ACTG1	Not Available	Not Available	Not Available	Not Available	Not Available
HSPA8	Gene overexpression, gene knockdown	trt_oe, trt_sh.cgs	ccsbBroad304_06410 CGS001-3312	HSPA8	Not Available
KRAS	Gene knockdown	trt_sh.cgs	CGS001-3845	KRAS	Not Available
CTNNB1	Mesalazine, gene knockdown	trt_cp, trt_sh.cgs	BRD-K28849549 CGS001-1499	PTGS1, PPARG, PTGS2, ALOX5, CHUK, CTNNB1, IKBKB, MPO, NAT1	Cyclooxygenase inhibitor, Lipoxygenase inhibitor, Prostanoid receptor antagonist
APP	RO-90-7501, gene knockdown	trt_cp, trt_sh.cgs	BRD-K58299615 CGS001-351	APP	Beta amyloid inhibitor
CDH1	Gene knockdown	trt_sh.cgs	CGS001-999	CDH1	Not Available
CD44	Gene overexpression, gene knockdown	trt_oe, trt_sh.cgs	ccsbBroad304_05963 CGS001-960	CD44	Not Available
YWHAZ	Gene knockdown	trt_sh.cgs	CGS001-7534	YWHAZ	Not Available
GSK3B	SB-216763, SB-415286, AR-A014418, gene knockdown	trt_cp, trt_sh.cgs	BRD-K59184148 BRD-K76805682 BRD-K67860401 CGS001-2932	GSK3B	Glycogen synthase kinase inhibitor
TFRC	Gene knockdown	trt_sh.cgs	CGS001-7037	TFRC	Not Available
TLR2	Gene overexpression, gene knockdown	trt_oe, trt_sh.cgs	ccsbBroad304_07074 CGS001-7097	Not Available	Not Available
P4HB	Gene overexpression, gene knockdown	trt_oe, trt_sh.cgs	ccsbBroad304_01138 CGS001-5034	Not Available	Not Available
EEF1A1	Not Available	Not Available	Not Available	Not Available	Not Available
STAT1	Gene overexpression, gene knockdown	trt_oe, trt_sh.cgs	ccsbBroad304_01607 CGS001-6772	Not Available	Not Available
YWHAQ	Gene overexpression, gene knockdown	trt_oe, trt_sh.cgs	ccsbBroad304_02579 CGS001-10971	Not Available	Not Available
EEF2	PJ-34	trt_cp	BRD-K11853856	EEF2, PARP1, PARP15, PARP3	PARP inhibitor
CFL1	Gene overexpression, gene knockdown	trt_oe, trt_sh.cgs	ccsbBroad304_05985 CGS001-1072	Not Available	Not Available
SYK	Fostamatinib, ER-27319, gene overexpression, gene knockdown	trt_oe, trt_sh.cgs	BRD-K20285085, BRD-K11927976, ccsbBroad304_07024 CGS001-6850	FLT3, RET, SYK	SYK inhibitor
YWHAB	Not Available	Not Available	Not Available	Not Available	Not Available

Discussion

This study integrates infection associated gene curation, profiling tear and corneal expression, miRNA target analysis and experimental validation of key regulatory genes to reveal molecular networks that may underlie corneal pathology across diverse microbial infections. By curating 343 infection-associated genes expressed both in the corneal epithelium and tear film, a biologically relevant gene set reflective of the ocular surface microenvironment during infection was assembled. These genes spanned across essential cellular processes such as innate immune signaling, cytoskeletal remodelling, epithelial polarity, antigen presentation and protein homeostasis, the functions

frequently disrupted during bacterial and fungal keratitis. Previous studies have emphasized the importance of epithelial barrier stability, leukocyte recruitment and early innate immune activation in controlling corneal infections[30,31]. The enriched pathways, including bacterial invasion of epithelial cells and adherens junction regulation align with prior observations that pathogens such as *P. aeruginosa* rapidly compromise epithelial junctions to facilitate colonization[31].

Fungal keratitis-derived miRNA dataset with curated infection-associated genes was integrated to provide a prospective on cross pathogen regulatory response. miRNAs have emerged as important regulators of corneal inflammation, epithelial repair and immune signaling. The identification of miR-511-5p, miR-124-3p.1 and miR-618 as major regulators with numerous predicted targets suggest that fungal keratitis alters regulatory axes potentially relevant across microbial infections. Protein interaction network analysis revealed, several predicted targets such as GSK3B, STAT1, KRAS, TFRC and YWHAZ as highly connected nodes, showing their importance in shaping host responses during infection. Prior studies have similarly reported the involvement of STAT1 signaling in keratitis[32], the contribution of GSK3β to bacterial corneal inflammation[33], and the roles of 14-3-3 family proteins in cellular stress responses[34]. These biological observations give relevance to the predicted interaction of miRNA and infection-associated genes.

Experimental validation using LPS-treated corneal epithelial cells modulated expression of several high centrality genes, including the downregulation of HSPA4, HSPA8, EGF, CDH1, RHOA, KRAS, and GSK3B, and the upregulation of TFRC, TLR2, YWHAB, and CD44. HSPA4 and HSPA8, heat shock proteins, were previously not shown to be associated with pathophysiology of corneal surface. In the present study, when HCE cells were treated with LPS, significant down regulation of HSPA4 and HSPA8 was observed. Nuclear localization of deacetylated HSPA4 represses the proinflammatory cytokines and attenuates neuroinflammation[35] and that of HSPA8 helps in cell survival during oxidative stress[36]. Our results and those of others highlight the role of HSPA4 and HSPA8 in conditions of infection, inflammation and survival.

Tear secretome contains several growth factors assisting in maintenance of ocular surface integrity. Epidermal growth factor helps in corneal epithelial cell migration and wound healing process[37]. It is expressed by the corneal epithelium and also present in the tear fluid. LPS treatment of HCE cells decreased the expression of EGF in our study. Cornea also expresses Ras homologue gene family A (RhoA) protein. The decrease in expression of RhoA with LPS treatment might delay the corneal wound healing process as it's signaling is associated with cell migration and re-epithelialization. Limited studies showed the involvement of the 14-3-3 family of proteins in corneal infections. Up regulation of YWHAB in tear and lacrimal fluid was previously reported in dry eye disease patients[38], which correlated with increase in expression of YWHAB with LPS treatment of corneal epithelial cells. These genes might play a role in corneal cell proliferation whose expression decreased with LPS treatment, although no such previous studies were reported.

KRas and signal transducer and activator of transcription (STAT) 1 regulates the expression of GSK3 β, which is involved in many cellular processes like proliferation, differentiation, inflammation. In the corneal pathology, GSK3β inhibitor SB216763, significantly reduced the microbial burden and decreased the opacity and keratitis of the cornea caused by *P. aeruginosa*[39]. GSK3β and KRAS are the targets of miRNA, hsa-miR-1910-5p, hsa-miR-143-3p, which are significantly expressed in corneal epithelial stem cells in comparison to corneal epithelial cells[40]. Our results showed significant reduction in expression of KRAS and STAT1 in LPS treated HCE cells and a similar trend was observed with GSK3β expression.

CDH1 (E-cadherin) expression is necessary for maintenance of the epithelial nature of the cells. CDH1 expression decreases transiently to facilitate the cell migration and wound healing during epithelial debridement[41]. In diabetic keratopathy, corneal cell junction abnormalities were observed due to decrease in expression of E cadherin. LPS was shown to alter the barrier function of the corneal epithelial cells by disrupting the tight junctions[42]. In our study, a decrease in the expression of CDH1, was observed which might impact cadherin-based junctions present in the corneal epithelium with LPS treatment.

In *Aspergillus fumigates* keratitis, the expression of TLR2 increases[43] which correlated with our results with LPS treatment. Several other studies also showed elevated expression of several pathogen pattern recognition receptors like TLR2 with increased level of infection[44]. However, decrease in expression of SYK, the

downstream signaling molecule of TLR's was noticed. Although SYK is the target of miR-511-5p, which was differentially over expressed in fungal keratitis, the significant decrease in expression of SYK with LPS treatment, might be of interest to investigate further.

P. aeruginosa was shown to secrete toxins, which modifies eukaryotic elongation factor (EEF) 2, thereby halting protein synthesis in addition to inducing apoptosis[45]. Our results also show decrease in expression of EEF2 with LPS treatment indicating the impact on protein synthesis. Transferrin receptor (TFRC) expression increases with hyperosmolarity conditions in dry eye disease, leading to ferroptosis[46]. This condition was reversed with NAC treatment. Increase in TFRC expression with LPS treatment signifies the role of iron metabolism in corneal inflammation and infection. These consistencies from the previous studies support the relevance of our curated gene list in corneal innate response pathways.

Connectivity Map (CMap) analysis further revealed associations between key genes and known perturbagens, including GSK3 inhibitors (SB-216763, AR-A014418), SYK inhibitors (fostamatinib), cytoskeletal modulators and compounds affecting calcium signaling. These associations align with known roles of these proteins in immune regulation, cytoskeletal integrity and inflammation. Although this study helped in shortlisting candidate genes, the identification of the potential therapeutic targets through CMap and network analysis, these findings are preliminary and require *in vitro* or *in vivo* validation. The knockdown or overexpression of the candidate genes in relevant cell lines, followed by phenotypic assays (e.g., infection assays, viability, response to perturbation), or small molecule screening based in the CMap predicted compounds, may be further experimentally confirmed in future work.

Our experimental validation in which corneal epithelial cells were treated with LPS, which models canonical TLR4-mediated innate immune activation but does not reproduce the full biology of polymicrobial interactions. LPS has been widely used as a bacterial mimic to probe corneal inflammatory pathways and host responses and therefore provides insight into shared innate signaling cascades that are likely engaged during infection[47]. However, LPS treatment alone cannot model direct co-infection, so, our experimental data should be viewed as hypothesis-generating evidence that prioritized infection-associated genes and miRNA interactions in corneal innate responses.

The combined computational and experimental designs used were intended to bridge the infection-relevant candidate genes with an experimentally tractable readout of innate immune activation. Although fungal and bacterial pathogens engage distinct upstream pattern-recognition receptors, they activate conserved and significantly overlapping downstream inflammatory transcriptional programs and innate immune pathways. Consequently, the transcriptional environment elicited by LPS stimulation closely mirrors the key innate response pathways activated during fungal keratitis[48,49]. Prior studies reporting altered miRNA profiles in human fungal keratitis and tear proteome changes in microbial keratitis indicate that the genes studied are relevant[19,48,49]. Nonetheless, it warrants a validation in clinical specimens of polymicrobial infections or more complex co-infection models.

Overall, this study provides insights by mapping of gene and miRNA interactions relevant to corneal infection and highlights conserved host pathways that may operate across bacterial, fungal and potentially polymicrobial infections. While the use of LPS-treated epithelial cells imposes limitations and does not recapitulate the full complexity of polymicrobial keratitis, our findings provide mechanistic hypotheses that can guide future research using human clinical samples and co-infection models. These models will be needed to fully understand regulatory networks and their contribution to disease severity and dynamics in polymicrobial keratitis.

Conclusion

In this study, a set of infection-associated genes expressed in both the corneal epithelium and tear fluid, thereby generating a biologically relevant dataset for understanding ocular surface immunity and homeostasis during infection were curated. Functional enrichment analyses demonstrated that these genes participate in innate immune activation, epithelial polarity, cytoskeletal remodelling and pathogen invasion, pathways central to the corneal pathogenesis during infection. By integrating these genes with miRNAs dysregulated in fungal keratitis, several regulatory miRNA-gene regulations that may operate across diverse microbial infections were identified. Network analysis highlighted highly interacting genes such as GSK3B, STAT1, RHOA, HSPA4, HSPA8 and TFRC as key molecular nodes. Experimental validation using LPS-treated corneal epithelial cells confirmed that several of these high-centrality genes are dynamically

modulated during LPS treatment. The observed expression patterns aligned with established roles of these genes in inflammation, epithelial barrier disruption and impaired wound healing. Connectivity Map associations further suggested mechanistic links to known perturbagens, offering hypothesis-generating leads for therapeutic exploration. Although LPS treatment does not fully model polymicrobial complexity, it effectively probes shared innate pathways engaged during bacterial and fungal infection. Collectively, these findings reveal converging molecular mechanisms involved in corneal infection and highlight the need for validation using patient-derived samples of polymicrobial infections or co-infection models to fully elucidate the biology of polymicrobial keratitis.

Significance and impact of the study

This study provides the first integrated analysis linking infection-associated genes expressed in the cornea and tears with miRNAs known to be dysregulated in fungal keratitis, offering new insights into polymicrobial regulatory networks. By identifying hub genes and pathways responsive to innate immune activation, this work reveals molecular mechanisms that may underlie host responses in bacterial, fungal and polymicrobial keratitis. The curated infection-associated genes serve as a valuable resource for studying ocular infection biology, while the LPS-responsive genes highlight potential regulatory relevance. Overall, the study establishes a hypothesis-generating framework that can guide future investigations using clinical samples and co-infection models.

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Conflict of interest

All authors declare no conflicts of interest.

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