Transcriptome Analysis of the Gene Expression Profiles Associated with Fungal Keratitis in Mice Based on RNA-Seq

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PURPOSE. Fungal keratitis (FK) is an eye disease that can lead to blindness and has a high incidence worldwide. At present, there is no effective treatment for this disease. There are innate immune response mechanisms that protect against fungal infections. One example is C-type lectin receptors (CLRs), which can identify fungal invaders and trigger signal transduction pathways and cellular responses to eliminate pathogens. However, previous studies have focused mostly on single-receptor factors, and a systematic analysis of the genetic factors underlying the pathogenesis of FK has not been conducted. This study aimed to investigate the molecular mechanisms of FK in terms of genomics and to further elucidate its pathogenesis.

METHODS. We performed a transcriptome analysis of a mouse model of FK using RNA sequencing to obtain the relevant gene expression profiles and to identify differentially expressed genes, signaling pathways, and regulatory networks of the key genetic factors in the pathogenesis of murine FK.

RESULTS. Several genes that are significantly associated with FK and serve as markers of FK, such as the inflammatory cytokine genes IL1B, IL6, IL10, IL23, and TNF, were identified. The mRNA and protein expression patterns of IL-1β, IL-6, and TNF-α in the corneas of mice with FK were validated by quantitative RT-PCR and Luminex multiplex assay technology. The Wnt, cGMP–PKG, and Hippo signaling pathways were significantly enriched during fungal infection of mouse corneas.

CONCLUSIONS. Our study may help to elucidate the mechanisms of FK pathogenesis and to identify additional candidate drug targets for the treatment of FK.

Keywords: fungal keratitis, RNA sequencing, signaling pathways, gene, pathogenesis

Fungal keratitis (FK) is a severe corneal infection that can lead to blurred vision, blindness, and redness and pain in the eyes that do not improve when contact lenses are removed.1,2 Compared with the incidence in temperate regions, the incidence of FK is higher in tropical and subtropical regions. FK is more common in hot and humid climates and in tropical areas with large populations engaged in agriculture, such as India. The spectrum of FK in humid climates and in tropical areas removed.1,2 Compared with the incidence in temperate regions, the incidence of FK is higher in tropical and subtropical regions. FK is more common in hot and humid climates and in tropical areas with large populations engaged in agriculture, such as India. The spectrum of FK in}

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Here, we performed a transcriptome analysis of a mouse model of disease based on high-throughput sequencing technology to systematically study the molecular mechanisms that regulate FK. To some extent, this analysis may provide basic support for the diagnosis of patients and the treatment of FK.

**Materials and Methods**

**Fungal Strains and Establishment of the Mouse Model**

The strain of *Aspergillus fumigatus* (*A. fumigatus*) was CCTCC AF 93048, which was obtained from the China Center for Type Culture Collection (Wuhan, China). The strain was cultured for 3 to 4 days on potato dextrose agar, and suspensions of fresh conidia were prepared by scraping the conidia from the surface of the medium. The samples were quantified using a hemocytometer and adjusted to a final concentration of 5 × 10⁴ conidia/μl in PBS. C57BL/6 mice (Charles River Laboratories, Beijing, China), 6 to 8 weeks old, were adaptively fed for 1 week and randomly divided into two groups. The corneal epithelium in the experimental group (named the Af group) was scraped, and an *A. fumigatus* spore suspension was injected into the corneal stroma. The mice that served as negative controls were mock inoculated with sterile PBS (named the PBS group). Only the right eyes of each mouse in the Af group and PBS group were treated for the experiments. The corneal samples from the Af group at 2 days and 5 days post-injection were labeled Af-2 and Af-5, respectively. The mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experiments were approved by the Animal Ethics Committee of Anhui Medical University.

**Clinical Scoring and Corneal Fungal CFU Analysis in Mice**

We randomly selected five mice from each group and scored them. Then, the corneas were excised, and the fungal colony-forming units (CFUs) per cornea were determined. The standard of clinical scoring and CFU analysis were based on a study by Wu et al. A score of 0 to 4 was assigned to each of the following three criteria: area of opacity, density of opacity, and surface regularity. The final corneal pathology score of each mouse was the sum of the three scores described above. Thus, the minimum value was 0, and the maximum value was 12. To assess fungal viability, each cornea was homogenized in 100 μl PBS. Subsequently, serial dilutions were performed and plated onto potato dextrose agar plates. *A. fumigatus* was cultured at 37°C for 3 days.

**Histopathology Examinations**

The infected corneas treated with either *A. fumigatus* or the control PBS were enucleated from the euthanized mice, fixed in 4% paraformaldehyde, and then embedded in paraffin. Sections (4 μm) were cut and stained with hematoxylin and eosin (H&E) for general histological analysis. Gomori methenamine silver (GMS) staining was performed to identify the fungal hyphae and spores.

**Immunohistochemistry**

Corneal paraffin sections with thicknesses of 4 μm were used. Immunohistochemistry was performed by using the 3,3′-diaminobenzidine staining method. The endogenous peroxidase was inactivated by incubating the sections with 3% H₂O₂ for 20 minutes. The rabbit anti-mouse IL-1β, IL-6, IL-10, IL-23, and TNF-α antibodies (1:200; Bioss, Beijing, China) were applied overnight at 4°C. The sections were washed three times with PBS for 5 minutes each and then incubated with peroxidase-conjugated goat anti-mouse IgG (1:1000; Zsbio, Beijing, China) for 20 minutes at room temperature. PBS buffer was used as a negative control. The cells with brownish-yellow particles in the cytoplasm and cell membrane were considered positive.

**Cytokine Analysis**

The mouse corneas were excised 2 days and 5 days after injection with *A. fumigatus* or PBS and homogenized in 200 μl of PBS–0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). We removed the cellular particles by centrifugation and used the supernatants for cytokine analysis. The concentrations of cytokines, including IL-1β, IL-6, IL-10, IL-23, and TNF-α, were measured using Luminex multiplex assay technology (Thermo Fisher Scientific, Waltham, MA, USA). The plate was read on a Luminex 200TM instrument. The data preprocessing and analysis were conducted using Luminex xPONENT software.

**mRNA Extraction and Sequencing**

RNA samples were extracted from the resected corneas of the PBS-2, Af-2, and Af-5 groups using TRIzol (Thermo Fisher Scientific) and pooled together within each group. cDNA from each sample was synthesized from 1 μg of total RNA using reverse transcriptase (Takara Biomedical Technology Co., Ltd., Beijing, China) according to the manufacturer’s instructions. The preparation of the cDNA library and RNA sequencing were performed by Nanjing Personal Gene Technology Co., Ltd. (Nanjing, Jiangsu, China). The cDNA originating from the RNA fragments was paired and sequenced using the HiSeq X10 high-throughput sequencing platform (Illumina, San Diego, CA, USA), and an average of 6 G reads per sample were obtained. The total RNA was extracted from the cornea samples of the mice in the PBS-2, Af-2, and Af-5 groups and the quality of the RNA was examined with a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The RNA integrity number values of all the sequencing samples were more than 7.0, and they were 9.2, 8.6, and 8.8 for the PBS-2, Af-2, and AF-5 groups, respectively (Table 1). The sequencing data for the three groups of samples are shown in Table 1. The results show that the quality levels of the three groups of samples were above 95% and 90%, respectively. Our sequencing data are available in the functional genomics database of ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-7907.

**Differentially Expressed Gene Analysis**

We analyzed the RNA-Seq data based on the regular protocol. The data source of the selected reference genome was the Ensembl database (http://www.ensembl.org/). The mouse reference genome of *Mus musculus* GRCm38.dna.fa
Table 1. Quality of Sequencing Samples and Data

| Sample | RNA Integrity Number | Reads (n)   | Q20 (%) | Q30 (%) | Clean Reads |
|--------|----------------------|-------------|---------|---------|-------------|
| PBS-2  | 9.2                  | 70,082,806  | 97.91   | 94.75   | 69,859,772  | 99.68       |
| AF-2   | 8.6                  | 76,228,368  | 97.74   | 94.37   | 75,974,668  | 99.66       |
| AF-5   | 8.8                  | 75,979,188  | 97.80   | 94.52   | 75,721,772  | 99.66       |

Figure 1. Phenotypic results of the fungal keratitis mouse model. (A) The corneal lesions of the mice in the experimental group (Af group) and the control group (PBS group) were observed under a microscope on the second or fifth day. (B) The corneal pathology scores were assessed in each group (*P < 0.05). (C) The fungal viability was assessed in the Af group. The CFUs in the Af-2 group were significantly higher than in the Af-5 group (**P < 0.01). (D) H&E and GMS staining (400×) was performed in each group. The black arrow indicates conidia, and the blue arrow indicates hyphae.

Pathway Enrichment Analysis

We counted the number of differentially expressed genes at different levels of the KEGG pathway (https://www.genome.jp/kegg/pathway.html) and then determined the metabolic pathways and signaling pathways in which the differentially expressed genes were primarily involved. Based on the whole genome, hypergeometric distribution was used to calculate the pathway in which the DEGs were significantly enriched.
Quantitative PCR

Primers for the target genes were designed using Primer 3.0 input software. The quantitative PCR (qPCR) experiment was performed with the TransStart Tip Green qPCR Super-Mix (Takara). The expression level of each transcript was normalized to that of glyceraldehyde-3-phosphate dehydrogenase and analyzed using the $2^{-\Delta \Delta CT}$ method. All of the experiments were performed in triplicate.

RESULTS AND DISCUSSION

Construction and Phenotypic Observation of the Fungal Keratitis Mouse Model

Under the microscope, we observed the corneal lesions of the mice in the experimental group (Af group) and the control group (PBS group), which are shown in Figure 1A. In the control group, the corneas regained the normal appearance on the second day and remained unchanged on the fifth day. In the experimental group, corneal edema aggravated on the second day, showing white porcelain changes, local ulcers, or perforation. On the fifth day in the Af group, the severity of corneal infection was mitigated, and if the area of the ulcer was smaller then there was a trend of scar healing. Compared to those of the PBS groups, the clinical scores of the Af-2 and Af-5 groups were significantly higher, which indicated that the symptoms of fungal keratitis in the Af groups were more severe (Fig. 1B). Based on the assessment of the fungal viability in the Af group, the CFUs in the Af-2 group were found to be significantly higher than in the Af-5 group ($P = 0.029$) (Fig. 1C). Compared with that of the other groups, the corneal stroma in the Af-2 group was heavily infiltrated, according to H&E staining, and fungal hyphae and spores were observed, according to GMS staining (Fig. 1D). These phenotypic results indicate that we successfully constructed a mouse model of fungal keratitis.

Assessment of Sequencing Data Quality

In addition, we detected the expected number of fragments per kilobase of transcript sequence per million reads mapped (FPKM) density and saturation distribution at the transcriptome level in the three groups of sequencing samples. The statistical results of the expression characteristics of all the genes in each sample are shown in Figures 2A and 2B. The results of the principal component analysis and sample correlation tests show that there is clear heterogeneity among the three groups of sequencing samples, as shown in Figures 2C and 2D. The above information shows that
**FIGURE 3.** Results of the DEGs associated with fungal keratitis in mice. (A) Volcano plot of the significant DEGs between the PBS-2 group and Af-2 group. (B) Volcano plot of the significant DEGs between the Af-5 group and Af-2 group. The x-axis represents the log2 fold change and the y-axis represents –log10 (P value) of each significant DEG. (C) A heatmap of each significant DEG. (D) Comparison of the DEGs at two different time points. The Venn diagram shows the overlapping up- or downregulated DEGs at two different time points.

the high-throughput sequencing data of the three groups of samples are of high quality and meet the requirements for subsequent transcriptomics analysis.

**Significant DEGs Associated with Fungal Keratitis in Mice**

Based on the alignment with the mouse reference genome, a total of 16,548 transcripts were identified. By comparing the gene expression profiles of the PBS-2 group and Af-2 group (Af-2 group/PBS-2 group), and based on the threshold criterion of log2 fold change > 1 and P < 0.05 significance, we screened 646 differentially expressed genes, including 455 upregulated genes and 191 downregulated genes (Fig. 3A). Excluding the factor of zero value, expression of the SPDEF (SAM pointed domain-containing Ets transcription factor) gene indicated that it was the most significantly upregulated gene, and this gene was increased 154-fold in the Af-2 group compared to the PBS-2 group. SPDEF, which is a member of the ETS transcription factor family, has been reported to play an important role in conjunctival goblet cell differentiation and pulmonary Th2 inflammation. Among the 191 significantly downregulated genes, expression of the GALNT15 (polypeptide N-acetylgalactosaminyltransferase 15) gene in the Af-2 group was the lowest; the expression of this gene was approximately 57 times lower in the Af-2 group than in the PBS-2 group. However, to the best of our knowledge, there have been no reports on the function of GALNT15.

By comparing the gene expression profiles of the Af-5 group and Af-2 group (Af-5 group/Af-2 group), the total number of differentially expressed genes was 739, including 650 upregulated genes and 89 downregulated genes (Fig. 3B). Among the 650 significantly upregulated genes, expression of the MARCO (macrophage receptor with collagenous structure) gene in the Af-5 group increased the greatest extent; the expression of this gene was approximately 124 times higher in the Af-5 group than in the Af-2 group. In a study of Cryptococcus neoformans-infected mice, it was demonstrated that deficiency of the scavenger receptor MARCO could lead to impaired fungal control during the afferent phase of cryptococcal infection. It was also reported that MARCO might significantly mediate adenovirus infection and the subsequent innate responses in macrophages.

In addition, we further compared the differentially expressed gene sets identified above. A heatmap of the expression patterns of these differentially expressed gene sets is shown in Figure 3C. The comparison of the significantly DEGs at both infection time points showed that there were three commonly downregulated genes, including GLDC (glycine decarboxylase), ALDH1A1 (aldehyde...
Af-5/Af-2 upregulated; Af-2/PBS-2
and this production can be regulated by the Dectin-1/Syk
pathway play an important role in the occurrence and
WNT5A
signaling site family, member 5A), were downregulated in the
tivity to oxidative stress in keratoconus (KT) cornea fibrob-
and signal transduction are closely related to the inflam-
matory response, suggesting that IL-1α is an important proinflammatory factor.24 IL-1β
infection were identified as playing a significant role in regulat-
ing various inflammatory processes.30,31 Aldehyde dehydro-
genase (ALDH1A1) was found to be expressed in immature

Table 2. Common DEGs During Fungal Infection in the FK Mouse Model

| Regulation | Genes |
|------------|-------|
| Af-5/Af-2 downregulated; Af-2/PBS-2 downregulated (n = 3) | GLDC, ALDH1A1, PPP1R3C |
| Af-5/Af-2 downregulated; Af-2/PBS-2 upregulated (n = 33) | CCL3, MSH6, GOS2, SLPI, CCL4, TNEAP3, SRGN, SLC26A4, RDH12, EDN1, ACO2D, RETNLG, SAMS21, DNAP, ITFIM3, HCD, IL1B, CELF3, CXCL3, CXCL1, NBP5, LIF, LARS2, CSF3, CCL22, VTCN1, CD14, HBB-B5, IFI205, S100A8, CXC2L2, HBA-A1 |
| Af-5/Af-2 upregulated; Af-2/PBS-2 downregulated (n = 93) | SERPINF1, SLCL13A2, COL6A1, COL1A1, PEG3, APOE, FGCRT, AQP1, SCARF2, ZFP651, SCUBE1, MATN4, PLOD1, DPEAP1, LAMA2, DCN, KERA, COL1A2, MRC2, CACNA1G, FBN1, OGN, GALNT5, WNT5A, APOD, P3H3, SERPING1, SMOC2, ADAMTS10, PDGFRB, ZFHX4, ITIH5, TEAP2B, COL5A2, DPT, RG55, DNM1, COL5A1, ADAM33, POSTN, OLFML3, COL11A1, DKK2, COL2A1, ITIH5, SPARC1L, COL1A2, PCOLCE, AZM, MGP, CFPX2, ITMA2, SRPX2, BGN, FGER1, NDK1, CDH11, MMP2, BMER, ITGA11, L0X1L1, PTTH1R, ITGB1L1, PODX2, TXN, LUM, ADAMTS2, CPZ, DCHS1, ISLR, LMX1B, IGBPBP2, KCNS1, SLC26A7, COL1A1, PIEZO2, FMOD, PRELP, KIRREL, MEFAP4, POU3F3, PENK, COL27A1, OLFML2A, COL6A3, CEMP, CYP2F2, JAM2, NTRK2, TNS1, COL8A2, CPED1, SRPX |
| Af-5/Af-2 upregulated; Af-2/PBS-2 upregulated (n = 49) | ITGB2, CFP, CYP2A5, CYYB, MMP9, CC19, MOXD1, GABRP, IGBPBP3, NCKAP1L, ADAMTS1, CLEC4N, MYO1F, TMEM173, CASP12, COL3A1, IL1R2, FGRFB, TNC, LAPTMS5, SORC2S, PF4, ITGA4, ST3GAL4, CAR12, PLOD2, NT5E, ITF, SCARF3, ACTA2, SERPINE1, GCS, SECTIN1B, SH3KBP1, SERPINA3G, SPRR2D, PIG2, MMP12, KLK3, H2-EB1, KCNJ13, PI15, LYZ2, CSN1S1, H2-AB1, SPRR2A3, 4833423E24RIK, CLEC7A, R3GNT7 |

dehydrogenase family 1, subfamily A1), and PPP1R3C3 (protein phosphatase 1, regulatory subunit 3C). There were 49 commonly upregulated genes in both groups, such as CASP12 (caspase 12) and IL1R2 (interleukin 1 receptor, type II). Interestingly, 33 genes, including the IL1B gene, were upregulated in the comparison of Af-2/PBS-2 but downregulated in the comparison of Af-5/Af-2. A total of 93 genes, including the WNT5A gene (wingless-type MMTV integration site family, member 5A), were downregulated in the comparison of Af-2/PBS-2 but upregulated in the comparison of Af-5/Af-2. The details of the results described above are shown in Figure 3D and Table 2. Most of these commonly identified genes have been reported in previous studies of fungal ophthalmopathy or immunology. IL-1α and IL-1β are two types of IL-1, which is a cytokine that is produced by monocytes, endothelial cells, fibroblasts, and other types of cells in response to infection that bind to the same receptor of the immunoglobulin superfamily. Previous studies have shown that IL-1β derived from neutrophils participates in corneal antifungal responses and immune defense as an important proinflammatory factor.24 IL-1β production can be induced by fungal infection in the mouse cornea, and this production can be regulated by the Dectin-1/Syk pathway.24 Casp11 and Casp1 participate in the modification of IL-1β from its precursor to mature form.26,27 Wnt5a is a secretory glycoprotein and a member of the highly conserved Wnt protein family. WNT5A expression regulation and signal transduction are closely related to the inflammatory response, suggesting that WNT5A and its signaling pathway play an important role in the occurrence and development of inflammatory diseases. According to in vivo and in vitro experiments, WNT5A was identified as a critical component of the antifungal immune response that can produce Dectin-1- and LOX-1-induced inflammatory signatures in response to *A. fumigatus* infection.28 Caspase-12 may play an important role in the process of hypersensitivity to oxidative stress in keratoconus (KT) cornea fibroblasts.29 Through analysis of gene expression profile data, GLDC was identified as playing a significant role in regulating various inflammatory processes.30,31 Aldehyde dehydro-
| Signaling Pathway | FDR     | Downregulated Genes, n | Upregulated Genes, n | Downregulated Genes | Upregulated Genes |
|------------------|---------|------------------------|----------------------|---------------------|------------------|
| TNF              | 2.32E-11| 0                      | 24                   | —                   | EDN1, CXCL2, CXCL3, NOD2, ICAM1, TNFAIP3, BIRC3, TRAF1, BCL3, CEBPB, MMP9, FAS, IL18R1, LIF, TNF, MAPK11, IL6, PTGS2, CSF1, TNFRSF1B, IL1B, CXCL1, S0CS3, NFκBIA |
| NF-κB            | 2.66E-05| 1                      | 14                   | CARD14              | DYN, RELB, CCL4, ICAM1, TNF, TNFAIP3, BIRC3, PTGS2, TRAF1, IL1B, CD14, BCL2A1A, BLNK, NFκBIA |
| P3K-Akt          | 3.86E-05| 14                     | 17                   | OS0, PIK3R5, PIK3AP1, NR4A1, SPP1, CSF3, ITGB6, THBS1, PDGFB, CSF3R, TNC, IL4RA, CSF1, IL3RA, IL6, JAK2, IL7R |
| JAK–STAT         | 7.70E-05| 2                      | 17                   | CACNA1G, NTRK2, EFNA3, FGF10, FGFRI, PDGFRB | IL3RA1, OSM, IFNGR1, CSF2RB2, CSF3, LIF, PDGFB, IL10RA, CSF3R, IL4RA, IL6, IL3RA, JAK2, S0CS3, IL7R, CSF2RB, IL20RA |
| MAPK             | 2.03E-03| 6                      | 17                   | GACNA1G, NTRK2, EFNA3, FGF10, FGFRI, PDGFRB | RELB, DUSP1, NR4A1, DUSP5, CD14, GADD45A, IL1A, GADD45G, FAS, GADD45B, RAC2, PDGFB, RASGRF1, TNF, MAPK11, CSF1, IL1B |
| Rap1             | 1.46E-02| 6                      | 10                   | RAPGEF3, GNAO1, EFNA3, FGF10, FGFRI, PDGFRB | ITGB2, THBS1, RAC2, ITGAM, PDGFB, ICP2, MAPK11, CSF1, APBB1IP, FPRI |
| HIF-1            | 3.54E-02| 0                      | 9                    | —                   | EDN1, TMP1, HM0X1, IFNGR1, SERPINE1, IL6, CYBB, HK3, NOS2 |

TABLE 3. Enrichment Results of Signal Transduction-Related Pathways in the Af-2/PBS-2 Comparison
TABLE 4. Enrichment Results of Signal Transduction-Related Pathways in the Af-5/Af-2 Comparison

| Signaling Pathway | FDR    | Downregulated Genes, n | Upregulated Genes, n | Downregulated Genes | Upregulated Genes |
|-------------------|--------|------------------------|----------------------|---------------------|-------------------|
| PI3K-Akt          | 4.05E-09 | 1                      | 42                   |                     | CSF3, TNN, ITGA1, TXN, KDR, FLT4, ITGA11, NTRK2, ITGA7, COL16A6, GNB4, RELN, TEK, COL4A6, PDGFR, CSF1R, PDGFRB, CREB3L1, NTC, COL1A2, KIF1, ITGB3, LAMA2, THBS2, COL1A1, COL4A2, FGFR1, F2R, LAMA4, COL4A1, ITGA4, FN1, FGFR3, COL16A2, ITGA9, COL6A1, COL4A4, COL4A3, PDGFC, FLT1, COL6A3, ITGA5, VWF |
| MAPK              | 3.58E-03 | 4                      | 21                   |                     | CD14, IL1B, DUSP7, RASGFR1, CACNA1G, KDR, FLT4, FGFR1, ARRB1, NTRK2, CACNA1C, TGFBR2, MEF2C, TEK, PDGFR, FGFR3, IL1R1, CSF1R, PDGFRB, TGBF2, FLNC, FLT1, PDGFC, IGF1, TGBF3, GM5431, VCAM1, MMP3, CREB3L1, MMP9 |
| TNF               | 1.15E-02 | 7                      | 5                    |                     | TNFAP3, EDN1, IL1B, CXCL1, LIF, CXCL2, CXCL3, GM5431, VCAM1, MMP3, CREB3L1, MMP9 |
| Rap1              | 2.90E-02 | 0                      | 17                   |                     | —                  |
| Wnt               | 3.62E-02 | 1                      | 12                   |                     | FOSL1, FZD2, WISP1, SERPINF1, NEATC4, DKK2, NDK1, WNT11, SOX17, AXIN2, NDK2, SFRP2, WNT5A |
| cGMP–PKG          | 4.10E-02 | 0                      | 14                   |                     | —                  |
| Hippo             | 4.27E-02 | 0                      | 13                   |                     | ITGB2, FZD2, CTGF, TEAD2, SERPINE1, TGBF2, TRP73, WNT11, D1G4, TGBF3, AXIN2, WNT5A, TGBF2 |
Compared to the control group, the experimental group revealed seven different signaling pathways that were significantly involved in the pathogenesis of murine keratitis on the second day of fungal infection (seen in Table 3 and Fig. 4A). According to the significance level, the pathways included the TNF signaling pathway (false discovery rate [FDR] = 2.32E-11), NF-κB signaling pathway (FDR = 2.66E-05), phosphatidylinositol 3-kinases (PI3K)–Akt signaling pathway (FDR = 3.86E-05), Janus kinase (JAK)–signal transducers and activators of transcription (STAT) signaling pathway (FDR = 7.70E-05), MAPK signaling pathway (FDR = 2.03E-03), Rap1 signaling pathway (FDR = 1.46E-02), and hypoxia-inducible factor 1 (HIF-1) signaling pathway (FDR = 3.54E-02). The details of the dysregulated genes in each significantly associated signaling pathway in the comparison of Af-2/PBS-2 are shown in Table 3.

It has been reported that crosstalk of the TGF-β and NF-κB signaling pathways may enhance corneal epithelial senescence through the RNA stress response.35 TNF-α can inhibit the expression of CX43 and GJIC in human corneal fibroblasts; this inhibition is mediated by the c-Jun N-terminal kinase (JNK) signaling pathway and may play an important role in corneal inflammation.36 Activating or inhibiting the signal transduction pathways related to the proliferative potential and phenotype maintenance of corneal endothelial cells has been considered an important research direction for the development of new therapies for corneal endothelial dysfunction.37 In a previous study conducted in corneal peripheral vascular endothelial cells and a mouse model, the occurrence and development of fungal keratitis could be inhibited by IL-17, which suppressed CX43 expression through the Akt signaling pathway.38 Therefore, corneal endothelial cell dysfunction might play a role in the pathogenesis of fungal keratitis and might be regulated by the identified targets through associated signaling pathways. It has been confirmed in rabbit models that the combined activation of PI3K/Akt and Smad2 leads to the expansion of phenotypic and functional corneal endothelial cells in vitro and expanded cells contribute to the recovery of corneal endothelium.39 These findings may provide a new method for treating corneal endothelial diseases. The MAPK signaling pathway family is widely distributed in eukaryotic cells and has been associated with a variety of extracellular signals or stimuli, especially inflammatory responses. The physiological response mediated by the MAPK signaling pathway contributes to the progression and healing of ocular trauma.40 The effect of JAK/STAT inhibitors on the activity of IL-17-producing neutrophils impaired reactive oxygen species production and fungicidal activity but also inhibited elastase and gelatinase activity, which may lead to tissue damage.41 Rap1 (telomere repeat binding factor 2 interacting protein) has been found to be a regulator of NF-κB, whose deficiency contributes to corneal recovery after injury and may lead to the design of specific NF-κB inhibitors in the treatment of ocular injury.42 Hypoxia or decreased oxygen tension is considered to be the core of the pathogenesis of neovascular and degenerative diseases of the eye. Previous studies have reported a new mechanism by which hypoxia induces CD36 expression by activating the HIF-1 and PI3K pathways.43 HIF-1 directly upregulates expression of the inhibitory Per/Arnt/Sim (PAS) domain protein (IPAS) gene through a mechanism other than RNA splicing, providing additional gene regulation via negative feedback for the adaptive response under hypoxic/ischemic conditions.44 In hypoxia-exposed corneal cells, the high expression of cystic fibrosis transmembrane conductance regulator and basal activity of NF-κB may increase the susceptibility to keratitis.45

Compared with the second day, the fifth day of fungal infection revealed seven different signaling pathways that were significantly enriched in the pathogenesis of keratitis in mice (shown in Table 4 and Fig. 4B). These pathways were the PI3K–Akt signaling pathway (FDR = 4.05E-09),
MAPK signaling pathway (FDR = 3.58E-03), TNF signaling pathway (FDR = 1.15E-02), Rap1 signaling pathway (FDR = 2.90E-02), Wnt signaling pathway (FDR = 3.62E-02), GMP-cGMP-dependent protein kinase (PKG) signaling pathway (FDR = 4.10E-02), and Hippo signaling pathway (FDR = 4.27E-02). The first four signaling pathways involved in this stage were common to the previous stage, whereas the latter three signaling pathways were unique to this stage. The details of the dysregulated genes in each significantly associated signaling pathway in the comparison of Af-5/Af-2 are shown in Table 4. Corneal epithelial stratification in mice is the result of the coordinated development of mesenchymal epithelial interactions. It has been shown that the interaction between the Wnt/β-catenin/bone morphogenetic protein 4 (BMP4) axis in the stroma and BMP4/P63 signaling in the epithelium may play a key role in corneal epithelial stratification. To better understand the factors that lead to KT corneas, the researchers used RNA sequencing to conduct a comprehensive transcriptome analysis of human KT corneas. The results showed that the collagen synthesis and maturation pathways were widely disrupted, and the core elements of the TGF-β, Hippo and Wnt signaling pathways were significantly downregulated in the corneal tissue. KCa3.1 (IK1/SK4/KCNN4) is widely expressed in the congenital and adaptive immune systems. KCa3.1/IK1 channels regulated by PKG can form a new immune regulatory feedback system in microglia. In summary, the disease severity of mice was alleviated on the fifth day of fungal infection due to the immune function of the mice, which could be improved by the reduction in proinflammatory gene expression on day 5.

### Validation of the Expression Patterns of Inflammatory Cytokines

Furthermore, we selected some inflammatory cytokines, including IL-1β, IL-6, IL-10, IL-23 and TNF, and used qPCR to validate their gene expression patterns in mouse corneas during fungal infection. The qPCR primers for all of the selected target genes are shown in Table 5. The qPCR results of the different samples are shown in Figure 5. The expression patterns of these inflammatory cytokine genes in the

| Genes  | Forward Primers | Reverse Primers |
|--------|----------------|-----------------|
| IL1B   | tccagctcgaatctccgac | aggtgctcaggtctcc |
| IL6    | ccctgacccaaccaacaagt | ctacatttgccgaagjccc |
| IL10   | aagctgagaccaaccaacca | aagagaatcgatagacgccc |
| IL23   | tgagaagctgtagatgcgg | agctggctgaaacgctgag |
| TNF-alpha | ccctagccctctctccctce | agatgcagctgactgcgtgg |
| WNT5A  | tgcgggacacaatgacacta | aagatcagtggtggtgtgtg |
| TNSB   | gaccacccacctctcctca | ccacccacacccacccctgtc |
| COL6A1 | gctcaaatgtagtgcgtgtg | gtctggtgtggtggtgatga |
| COL6A2 | ggtctcttctcttctctct | agttgtggagtgtggtgtgtg |
| COL6A3 | gcatactgacagcctctctg | actacgagccacctctgac |
| COL1A2 | cccgtgggcaacatggatag | accttggacccctctgac |

**Figure 5.** Gene expression patterns of inflammatory cytokines revealed by qPCR. The expression patterns of five inflammatory cytokine genes, including IL1B (A), IL6 (B), IL10 (C), IL23 (D), and TNF-α (E), were validated by qPCR. The experiment was performed in triplicate for each group and was repeated three times. The x-axis represents three groups: PBS-2, Af-2, and Af-5. The y-axis represents the relative expression of each gene in the different groups. The gene expression value in PBS-2 was set to 1.
corneal tissue of mice changed significantly during fungal infection. No inflammation was observed in the control group based on the low cytokine gene expression and the lack of obvious corneal opacity. On the second day of fungal infection, the expression of these cytokines reached the highest peak (some, such as the IL1B gene, even increased thousands of times). On the fifth day of infection, the expression of these genes showed a downward trend. Each of these inflammatory cytokine genes displayed the same pattern of expression, which included a peak at day 2 of infection and a decrease by day 5 of infection.

The changes in the corneas of the mice mouse at different time points after fungal or PBS injection were observed by immunohistochemistry. As shown in Figure 6, inflammatory cytokines, such as IL-1β, IL-6, IL-10, and TNF-α but not IL-23, were abundant in the Af groups. These cytokines were mainly expressed in the cytoplasm of the corneal epithelial and stromal cells. The expression of five inflammatory factors was detected in the Af and PBS groups at different time points by using the Luminex multiplex assay technology (Fig. 7). The concentration of IL-23 in the cornea was lower than the minimum limit of detection of the chip, so the value could not be obtained. On the second day of fungal infection, the expression of IL-1β, IL-6, IL-10, and TNF-α increased. Compared with that in the Af-5 and PBS-2 groups, the expression of IL-1β, IL-6, and TNF-α, but not IL-10, in the Af-2 group was significantly different (P < 0.05). In summary, the cytokine expression patterns of IL-1β, IL-6, and TNF-α were consistent with the results obtained by qPCR. These three inflammatory cytokines can be further used as candidate targets for the treatment of fungal keratitis.

CONCLUSION

Based on our transcriptome analysis of fungal keratitis in mice by RNA-Seq, we identified a variety of significant signal transduction pathways and their targets. In particular, some inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and pathways, such as the Wnt, cGMP–PKG, and Hippo signaling pathways, were significantly associated with the pathogenesis of fungal infection in the murine cornea. Targeting these genes and pathways may be useful for the treatment of fungal keratitis.

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FIGURE 7. Cytokine expression in fungal keratitis-infected mice tested by multiplex assay. The expression of cytokines, including IL-1β (A), IL-6 (B), IL-10 (C), and TNF-α (D), in the AF-2 and AF-5 groups (n = 5 mice in each group) and in the PBS-2 and PBS-5 groups (n = 5 mice in each group) was measured by Luminex multiplex assay technology. The significance level was determined by one-way ANOVA tests (**P < 0.01, *P < 0.05).
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