Fungal keratitis remains a leading cause of blindness, especially in developing countries, where the rates of corneal ulceration far exceed rates in industrialized countries [1]. Fungi can penetrate deep into the stroma and through an intact Descemet membrane, thus gaining access to the anterior chamber and becoming very difficult to eradicate [2]. Clinically, even with accurate diagnosis and appropriate treatment, 20% of patients with fungal keratitis may experience corneal perforation [3]. Inflammatory cells, such as neutrophils, monocytes, macrophages, and lymphocytes, are required for protection against fungal pathogens in fungal keratitis [2]. The innate response mediated by inflammatory cell activation leads to the recognition of membrane-bound pattern recognition receptors (PRRs), which enable the direct (or nonopsonic) recognition of fungi [2]. The activation of PRRs, including C-type lectin receptors (e.g., dectin-1), Toll-like receptors, and NOD-like receptors [4], can efficiently induce the expression of interferons (IFNs), which are downstream of PRRs. IFNs have marked effects on innate and adaptive immune cells during infections [5-7] because of their immunomodulatory and anti-proliferative activities.

Fungal keratitis interacts with voriconazole to reduce the severity of fungal keratitis by suppressing IFN-related inflammatory responses and concomitant FK506 and voriconazole treatment suppresses fungal keratitis

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Purpose: To investigate the expression and roles of type I and II interferons (IFNs) in fungal keratitis, as well as the therapeutic effects of tacrolimus (FK506) and voriconazole on this condition.

Methods: The mRNA and protein expression levels of type I (IFN-α/β) and II (IFN-γ) IFNs, as well as of related downstream inflammatory cytokines (interleukin (IL)-1α, IL-6, IL-12, and IL-17), were detected in macrophages, neutrophils, lymphocytes, and corneal epithelial cells (A6(1) cells) stimulated with zymosan (10 mg/ml) for 8 or 24 h. A fungal keratitis mouse model was generated through intrastromal injection of *Aspergillus fumigatus*, and the mice were then divided into four groups: group I, the PBS group; group II, the voriconazole group; group III, the FK506 group; and group IV, the voriconazole plus 0.05% FK506 group. Corneal damage was evaluated with clinical scoring and histological examination. In addition, the mRNA and protein expression levels of type I (IFN-α/β) and type II (IFN-γ) IFNs, as well as related inflammatory cytokines, were determined at different time points using quantitative real-time PCR (qRT-PCR) and western blotting.

Results: After zymosan stimulation of mouse neutrophils, lymphocytes, macrophages, and A6(1) cells, the IFN mRNA and protein expression levels were markedly increased until 24 h, peaking at 8 h (p<0.001). The mRNA and protein expression levels of inflammatory cytokines (IL-1α, IL-6, IL-12, and IL-17) were also upregulated after zymosan stimulation. Moreover, type I (IFN-α/β) and type II (IFN-γ) IFN expression levels were increased and positively correlated with the progression of fungal keratitis in vivo. FK506 administered with voriconazole reduced the pathological infiltration of inflammatory cells into the cornea and downregulated the expression levels of IFNs and related inflammatory cytokines.

Conclusions: In conclusion, this study demonstrated that type I and II IFN levels were markedly increased in fungal keratitis and that FK506 combined with voriconazole decreased the severity of fungal keratitis by suppressing type I and II IFNs and their related inflammatory responses.

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IFNs, type II IFNs are encoded only by the gene for IFN-γ [11]. Type III IFNs comprise IFN-κ1, IFN-κ2, and IFN-κ3, and recent studies have demonstrated that type III IFNs do not play as effective a role in fungal infections as type I and type II IFNs [12,13]. In infectious diseases, type I and II IFNs released by infected cells produce unique signals directed toward neighboring cells, instructing the cells to tighten their defense mechanisms and eradicate the pathogen. These signals lead to such activities as the induction of certain enzymes, the inhibition of cell proliferation, and the enhancement of immune activities, including increased phagocytosis by macrophages and augmented specific cytolysis in T lymphocytes [5]. It has been widely reported that IFNs are important for host defenses against viruses, as IFNs can restrict viral infection and activate inflammatory responses [14-16]. Moreover, IFNs have diverse direct and/or indirect effects on innate and adaptive immune cells during bacterial and parasitic infections [17-19]. However, there have been few reports on IFNs in fungal keratitis, and thus far, there are conflicting results about whether IFNs have protective or detrimental effects during fungal infections [5].

In fungal keratitis, the pathogenic virulence and host immune response contribute to the pathogenesis of corneal disease [20]; thus, antifungal therapy and immunosuppressive agents play considerable roles in treating fungal infections. Voriconazole, a triazol antifungal agent, can be effective against yeast and filamentous fungal infections [21,22]. In the presence of voriconazole, the levels of IFN-γ were remarkably increased in peripheral blood mononuclear cells, defending against Candida albicans and C. krusei in a study by Fidan [23]. Tacrolimus (FK506) is an immunosuppressive agent and macrolide molecule that suppresses the activation of T cells, T helper cell–mediated B cell proliferation, and the secretion of cytokines [24]. Moreover, FK506 has been demonstrated to downregulate IFN-γ and related inflammatory cytokines in keratinocytes [25]. Our previous study demonstrated that FK506 can inhibit the inflammation induced by fungi and alleviate the severity of corneal damage at an early stage of fungal keratitis [26]. However, whether the combination of voriconazole and FK506 effectively regulates IFNs in fungal keratitis remains unknown. The present study was designed to investigate the expression levels and roles of type I and II IFNs in fungal keratitis, as well as the therapeutic effects of FK506 and voriconazole treatment on this condition.

**METHODS**

*Isolation and culture of bone marrow–derived neutrophils, macrophages, and intraepithelial lymphocytes: Bone marrow–derived neutrophils (denoted here as neutrophils) and intraepithelial lymphocytes (denoted here as lymphocytes) were isolated from 8-week-old healthy female C57BL/6 mice, as described by Khajah and Andonegui [27] and Montufar-Solis and Klein [28]. To isolate neutrophils, marrow cells were flushed from the femur and tibia with ice-cold Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Carlsbad, CA) and then rinsed and treated with erythrocyte lysis buffer to remove the red blood cells. Resuspended cells were overlaid on discontinuous Percoll density gradients (52%, 64%, and 72%) and centrifuged at 400 × g for 30 min at room temperature, after which neutrophils were isolated from the bottom layer (64–72%). For macrophages, after the resuspended cells were acquired, wash medium was used to collect the cells in a sterile 50-ml conical centrifuge tube on ice, which was then centrifuged at 500 × g for 10 min at room temperature. The cell pellet was resuspended in macrophage complete medium which consists of RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS; cat. 12657-029; Gibco, Carlsbad CA), 30% L929-cell conditioned medium (LCCM), a source of macrophage colony stimulating factor [29], 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Cells were seeded in Optilux Petri dishes (BD Biosciences, San Jose CA) and incubated at 37 °C in a 5% CO₂ atmosphere. After 4 days, an extra 10 ml of medium was added to each plate, and the cells were incubated for an additional 3 days. The supernatants was discarded, and the attached cells were washed with 10 ml of sterile PBS (1X; 12 mM NaCl, 20 mM KCl, 10 mM NaPO₄, 5 mM KPO₄, pH 7.4) after differentiation [30].

For lymphocyte isolation, the small intestine tissues were removed, and Peyer’s patches were excised. The tissues were then flushed of fecal material, opened longitudinally, and cut into 3- to 4-mm pieces in RPMI 1640 supplemented with fetal calf serum (10% v/v; Nutricell, Campinas, SP, Brazil) and gentamicin at 20 µg/ml (GE Healthcare Biosciences, Pittsburgh, PA). The tissue fragments were rinsed several times in Ca²⁺/Mg²⁺-free PBS, transferred to Ca²⁺/Mg²⁺-free PBS containing 5 mM EDTA (Sigma-Aldrich, St. Louis, MO) and 2 mM dithiothreitol, and shaken in a warm bath at 37 °C for 30 min. The cell suspensions were subsequently filtered through 20-ml syringe barrels containing wetted nylon wool, centrifuged, suspended in 3 ml of 40% isotonic Percoll (Sigma-Aldrich), layered on top of discontinuous Percoll density gradients (52%, 64%, and 72%) and centrifuged at 400 × g for 30 min at room temperature. The cell pellet was resuspended in macrophage complete medium which consists of RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS; cat. 12657-029; Gibco, Carlsbad CA), 30% L929-cell conditioned medium (LCCM), a source of macrophage colony stimulating factor [29], 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Cells were seeded in Optilux Petri dishes (BD Biosciences, San Jose CA) and incubated at 37 °C in a 5% CO₂ atmosphere. After 4 days, an extra 10 ml of medium was added to each plate, and the cells were incubated for an additional 3 days. The supernatants was discarded, and the attached cells were washed with 10 ml of sterile PBS (1X; 12 mM NaCl, 20 mM KCl, 10 mM NaPO₄, 5 mM KPO₄, pH 7.4) after differentiation [30].

**Cell culture and stimulation:** The neutrophils and lymphocytes were cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, and 5% 2 mM L-glutamine.
on a 12-well culture plate, which the macrophages culture media added one more component, 5% LCCM. Trypan Blue (Invitrogen, Carlsbad, CA) exclusion indicated that the cell viability was approximately 85%. A microscopy evaluation demonstrated that more than 85% of the isolated cells were neutrophils, macrophages, or lymphocytes. The A6(1) mouse corneal epithelial cell line was a gift from Dr. Peggy Zelenka (National Eye Institute/NIH) and was cultured following her protocol [31]. In A6 (1) mouse corneal epithelium cell line, twelve short tandem repeat (STR) loci were amplified using the commercially available EX20 Kit from Beijing Microread Genetics Co., Ltd. (Beijing, China, Appendix 1). The cell line sample was processed using the 3730xl DNA Analyzer (Applied Biosystems®). Data were analyzed using GeneMapper® ID-X v1.2 software (Applied Biosystems, Beijing, China). Appropriate positive and negative controls were run and confirmed for the sample submitted. The STR analyses are presented in Appendix 1.

Macrophages, neutrophils, lymphocytes, and A6(1) cells were divided into three groups at a seeding density of 1 × 10^6 cells/well in 12-well plates: Group I was the control group (which received PBS), group II received 10 mg/ml zymosan (Invitrogen) for 8 h, and group III received 10 mg/ml zymosan for 24 h. The concentration and administration duration of zymosan were based on a previous study [26].

Aspergillus fumigatus spore preparation: The strain of Aspergillus fumigatus used in this investigation was AS 3.0772, and it was purchased from the China General Microbiological Culture Collection Center, Beijing, China. The yeast strain was grown on Sabouraud dextrose agar (Difco, Detroit, MI) at 30 °C for 4 days. Spores were harvested and washed in sterile PBS and then diluted to a concentration of 1 × 10^7 colony-forming units (CFUs)/ml (resulting in an A. fumigatus spore preparation, or AFSP).

Mice and murine model of Aspergillus fumigatus fungal keratitis: Six- to eight-week-old inbred female C57BL/6 mice were purchased from the Guangdong Provincial Centre for Animal Research (Guangzhou, China). The mice were housed in a standard animal facility with a controlled temperature (22–24 °C) and photoperiod (12 h:12 h light-dark cycle) and were given free access to food and water. The animal experiments complied with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research (Guangzhou, China; approval ID: 20120308) and The Institutional Review Board of the Zhongshan Ophthalmic Center. The mice were anaesthetized intraperitoneally with xylazine (1.9 mg/ml) and ketamine (37.5 mg/ml), and every effort was made to minimize suffering. Each experiment was repeated three times.

The right eye of each mouse was punctured using a 30-gauge needle to form a tunnel in the corneal stroma. Next, a 33-gauge Hamilton syringe was inserted through the tunnel, and 2 µl of a 1 × 10^6 conidia solution was injected into the corneal stroma [32]. The eyelids were then rubbed together for several seconds to distribute the inoculum evenly over the corneal surface. The A. fumigatus–infected mouse corneas exhibited obvious corneal edema, with stromal infiltration 1 day post-infection. The mice were examined using a slit-lamp microscope (Carl Zeiss Meditec, Dublin, CA), and those that did not meet this standard were excluded.

Mice in which fungal keratitis was successfully induced were randomly divided into four groups: group I, the PBS group (n=6/group/time); group II, the voriconazole group (n=6/group/time); group III, the FK506 group (n=6/group/time); and group IV, the voriconazole plus 0.05% FK506 group (n=6/group/time). Group I received PBS topically four times per day for 7 consecutive days, and group II received 10 µg/ml voriconazole topically four times per day for 7 consecutive days. The voriconazole powder (99.9% purity) was obtained directly from Pfizer Central Research (VFEND; Pfizer, New York, NY) and was suspended in 0.3% Noble agar to a concentration of 1 mg/ml. Group III received 0.05% FK506 solution topically four times per day for 7 consecutive days. Finally, group IV was subconjunctivally injected with 5 µl of a 0.05% FK506 solution 24 h before infection, after which the mice with infected eyes received solutions containing 0.05% FK506 and 10 µg/ml voriconazole topically four times per day for 7 consecutive days; the applications of the two eye drops were separated by 5 min. The 0.05% FK506 solution was supplied by Zhongshan Ophthalmic Center, Guangzhou, China, and was prepared as described previously [33]. FK506 (Fujisawa Pharmaceutical co Ltd, Osaka, Japan) was obtained in pure powder form and reconstituted with ethanol to obtain a concentration of 5%. The solutions of Tween-80 (0.01 ml), polyethylene hydrogenated castor oil (0.03 g), and CMC (0.3 g), which were previously prepared in sterile water, respectively, were added to 1 ml of the FK506 solution. Then glycol (0.015 ml), sodium citrate (0.05 g), and merthiolate (0.002 g) were added. Adequate sterile water was replenished to reach the volume of 100 ml, which made the final concentration of FK506 to 0.05%. The suspensions were mixed vigorously by a magnetic stirrer. The formulation was a mixture of normal saline, hydrogenated castor oil (20% w/v), Tween-80 (5.5% v/v), hydroxymethyl cellulose (0.3% w/v), glycerol (8% v/v), and thiomersalate (0.002% w/v).

To determine the clinical scores on day 1, day 3, day 5, and day 7 post-infection, all infected corneas were imaged to visualize disease progression. The two observers were
blinded to the test groups, and the observers scored the same image in turn and performed mutual checks. The area and depth of corneal opacity and surface regularity were evaluated with a scoring system, in which each item has a score of up to 4 to grade the severity of corneal damage. Ocular disease was graded using clinical scores ranging from 0 to 12 according to the scoring system developed by Wu and Wilhelmus [32]. A total score of 5 or lower indicated mild keratitis, a score of 6 to 9 (including 9) represented moderate keratitis, and a score of 9 to 12 indicated severe keratitis. The animals were euthanized at the indicated end points of the experiments with cervical dislocation under isoflurane anesthesia.

Real-time PCR: With a cataract knife, the cornea was perforated at the limbus, making an opening large enough to insert the tip of a pair of corneal scissors. Under an operating microscope, each whole cornea of each mouse was then carefully cut off through the corneal limbus with the corneal scissors. The total RNA in each cornea was extracted using an RNeasy Micro Kit (Qiagen Sciences, Germantown, MD) according to the manufacturer’s protocol. Additionally, the total RNA in primary neutrophils, lymphocytes, and macrophages and A6(1) cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. The RNA was quantified using a NanoDrop 2000C spectrophotometer (Thermo Scientific, West Palm Beach, FL) and reverse transcribed into cDNA using a kit (Fermentas, St. Leon-Rot, Germany). The primer sequences used for IFNs (IFN-α, IFN-β, and IFN-γ), inflammatory cytokines (IL-1α, IL-6, IL-12, and IL-17), and β-actin are listed in Table 1. Quantitative real-time PCR was performed using a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA) and the SYBR Green Master Mix (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. The data were analyzed according to the comparative Ct (ΔΔCT) method and were normalized to the expression of β-actin in each sample. In the real-time PCR, the thermal cycle was programmed for 30 s at 98 °C for initial denaturation, followed by 35 cycles of 10 s at 98 °C for denaturation, 10 s at 59°C for annealing, 10 s at 72 °C for extension, and 1 min at 72 °C for the final extension. The melting curves and gel electrophoresis of the end products were obtained to confirm the specificities of the PCR reactions.

Western blotting: For corneal expression of IFNs, whole corneas (n=6/group/time) were collected and pooled from normal uninfected and infected C57BL/6 eyes at day 1, day 3, and day 5 post-infection. For detection of zymosan-infected macrophages, neutrophils, lymphocytes, and A6(1) cells, the cultured cells were washed three times with ice-cold PBS and then treated with lysis buffer. The specific steps of protein extraction and analysis of the protein levels were performed according to Chen et al.’s (2013) study [34]. Protein extraction was specifically performed using the Pierce BCA Protein Assay Kit (Thermo Scientific, West Palm Beach, FL). In total, 40 µg protein was loaded and separated by 12% (for IFN-β) or 10% (for IL-1α) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was accomplished, followed by transfer onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen). The membrane was then blocked for 2 h with 5% (for IFN-β) or 3% (for IL-1α) bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween-20 and incubated with a monoclonal rat anti-mouse IFN-β antibody (Abcam, Cambridge, UK) or a polyclonal goat anti-mouse IL-1α antibody (R&D Systems, Minneapolis, MN) overnight at 4 °C. After the membrane was washed, it was incubated with an anti-rat or peroxidase-conjugated secondary antibody (Abcam, Cambridge, UK) for 2 h. Finally, the blots were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences, Frederick, MD) according to the manufacturer’s protocol. For normalization, within the same membrane, β-actin was used as a loading control.

Histological examination: Eyes from different groups at each time point were fixed in 4% formaldehyde in 0.075 M phosphate buffer for 24 h, dehydrated in increasing concentrations of ethanol (70–99%), and embedded in paraffin (Merck, Darmstadt, Germany) at 60 °C. Sections 5 mm in

| Table 1. Nucleotide sequences of the specific primers used for PCR amplification. | Gene | Primer sequence (5′-3′) |
|---|---|---|
| IFN-α | F: AGTGGCTGACGAAACCAAG | R: TGAGCAATGTGCAATCCTT |
| IFN-β | F: TCGAAAGCTTTACAGAACT | R: TGCAGTATGTCCTACAG |
| IFN-γ | F: TCGGCTGACCTAGAGAC | R: ATGCAATGCTGACGTCAT |
| IL-1α | F: ATCAAGATGCGCAATGTTCC | R: TGTGCAAGTCATGAATGG |
| IL-6 | F: CTCAAGGTGTATTTGAGCT | R: TTTCCGAGAGGAGTACAAAC |
| IL-12 | F: CCATCGCTTCTCTCATATCC | R: CTTCGCTAACACATGAG |
| IL-17 | F: CCAAGCGCCTCAACAGAAG | R: TGGAGGGCAGACAATTCGA |
| β-actin | F: GCCTCCTCTGAGGCAG | R: CATCTGCTGGAAGTGGACA |
thickness were then cut and rinsed with deionized water at 45 °C; single sections were mounted on SuperFrost Plus glass slides (Menzel-Glaser, Braunschweig, Germany). The slides were subsequently dried at 60 °C for 1 h. Hematoxylin and eosin (H&E) staining was then performed to determine the degrees of corneal edema, inflammatory cell infiltration, and corneal irregularity.

**Statistical analyses:** The clinical scores are reported as the mean ± standard error of the mean (SEM), and the one-way ANOVA test was used to determine the statistical significance of the differences among the control, voriconazole, and voriconazole plus FK506 groups, as well as the differences in the RNA expression levels of inflammatory cytokines in the in vivo and in vitro studies. Differences were considered statistically significant at a p value of less than 0.05.

**RESULTS**

**Zymosan increased type I and II IFN expression levels in neutrophils, lymphocytes, macrophages, and A6(1) cells:** To test the potential of zymosan, a component of the fungal cell wall, in stimulating the expression of IFN transcripts, we stimulated neutrophils (Figure 1A), lymphocytes (Figure 1B), macrophages (Figure 1C), and A6(1) cells (Figure 1D) with zymosan. The mRNA expression levels of IFN-α, IFN-β, and IFN-γ were highest at 8 h after stimulation (all p<0.05) and then decreased rapidly at 24 h compared with the levels at 8 h (all p<0.05), regardless of cell type. The protein levels of one of the IFNs, IFN-β, were also tested in neutrophils, lymphocytes, macrophages, and A6(1) cells after stimulation with zymosan. The western blot data showed that IFN-β expression was statistically significantly increased at 8 h and then gradually decreased up to 24 h after infection in these four cell types (p<0.05; Figure 1E,F). We concluded that zymosan stimulation increased the expression of type I and II IFNs, a fungal keratitis mouse model:

Type I and II IFN expression levels were elevated in the fungal keratitis mouse model: To determine the expression levels of type I and II IFNs, a fungal keratitis mouse model was established, in which corneal swelling, corneal ulcers, and corneal infiltration gradually advanced from day 1 to day 5 post-infection and then decreased on day 7 (Figure 3A), after which the fungal keratitis gradually healed. The clinical score increased in a time-dependent manner and peaked on day 5, with a score of 9, compared with scores of 4 and 6 on day 1 and day 3, respectively; the clinical score then increased up to 24 h after infection in these four cell types (p<0.05; Figure 1E,F). We concluded that zymosan stimulation increased type I and II IFN RNA and protein expression levels in neutrophils, lymphocytes, and macrophages.

**Zymosan enhanced inflammatory responses in neutrophils, lymphocytes, macrophages, and A6(1) cells:** To ascertain the in vitro function of zymosan in neutrophils, lymphocytes, macrophages, and A6(1) cells, the levels of selected inflammatory cytokines downstream of type I and II IFNs were evaluated with real-time PCR and western blotting. In neutrophils, the mRNA expression level of the inflammatory cytokine IL-1α increased dramatically, by approximately 200-fold, at 8 h after zymosan stimulation (p<0.001) and then decreased slightly at 24 h (p<0.05) compared with the level in the unstimulated control (Figure 2A). The mRNA levels of IL-6, IL-12, and IL-17 were also increased at 8 h by approximately 10-, 35-, and 55-fold (all p<0.001), respectively, and then were reduced to 5-, 18- and 25-fold, respectively, their prestimulation levels compared with the levels in the unstimulated control at 24 h (all p<0.01; Figure 2A). In lymphocytes, zymosan enhanced the mRNA expression level of IL-1α by approximately 2,000-fold at 8 h (p<0.001) and by 1,000-fold at 24 h (p<0.001; Figure 2B). The mRNA expression levels of IL-6, IL-12, and IL-17 were also increased at a similar manner as in the neutrophils at 8 h and 24 h (Figure 2B). In macrophages, the mRNA expression levels of all of the inflammatory cytokines (IL-1α, IL-6, IL-12, and IL-17; Figure 2C) showed changes similar to those observed in the neutrophils and lymphocytes. Finally, in A6(1) cells, the mRNA expression levels of all inflammatory cytokines peaked at 8 h and then gradually and statistically significantly decreased up to 24 h (Figure 2D). Additionally, the pro- and mature protein levels of IL-1α were checked in the neutrophils, lymphocytes, macrophages, and A6(1) cells, and the proform and the mature form were elevated increasingly to 8 h and then decreased up to 24 h after infection with zymosan statistically (p<0.05), in which the expression level of the proform was higher than that of the mature form (Figure 2E,F).

**FK506 and voriconazole lessened inflammatory responses in fungal keratitis:** Given that the mRNA expression levels of type I and type II IFNs and downstream inflammatory cytokines were statistically significantly upregulated in vitro and in vivo during fungal infection, we suppressed fungal activity alone or in conjunction with immune response inhibition to further examine the impact of fungal infection on the expression of type I and II IFNs. In particular, FK506 and voriconazole were used to suppress immune responses and fungal activity, respectively.

The administration of voriconazole and FK506 was helpful for inhibition of immune responses, and slit-lamp images showed the slightest corneal opacity and surface...
Figure 1. The expression levels of type I and II IFNs in neutrophils, lymphocytes, and macrophages after zymosan stimulation. The mRNA expression levels of type I interferons (IFNs; normalized to β-actin), including IFN-α and IFN-β, and the type II IFN IFN-γ peaked at 8 h after stimulation in neutrophils (A), lymphocytes (B), macrophages (C), and A6(1) cells (D). The protein expression level and the band intensity of IFN-β (E and F) was also determined in neutrophils, lymphocytes, and macrophages. The data are presented as the mean ± standard error of the mean (SEM) and represent three individual experiments with five samples. *, p<0.05; **, p<0.01; ***, p<0.001. IDV, integrated density value.
Figure 2. The expression levels of IFN-related inflammatory cytokines in neutrophils, lymphocytes, and macrophages after zymosan stimulation. Zymosan increased the mRNA expression levels of interferon (IFN)-related inflammatory cytokines (interleukin (IL)-1α, IL-6, IL-12, and IL-17; normalized to β-actin) as well as the pro- and mature protein levels of IL-1α (E and F), which peaked at 8 h in neutrophils (A), lymphocytes (B), macrophages (C), and A6(1) cells (D) after zymosan stimulation. The data are presented as the mean ± standard error of the mean (SEM) and represent three individual experiments with five samples. *, p<0.05; **, p<0.01; ***, p<0.001.
regularity on day 1 to day 7 among the four groups, whereas the corneal inflammation seemed to be more severe on day 5 in the voriconazole or FK506 group. The voriconazole group and the FK506 group exhibited similar inflammatory severity, and the control group had the most serious inflammation (Figure 4A). The clinical scores (Figure 4B) were lowest in the voriconazole plus FK506 group (p<0.05), followed by the voriconazole group and the FK506 group (both p<0.05), with no statistically significant difference between the two groups (p<0.05), and the control group had the highest score (p<0.05). Furthermore, H&E staining confirmed that the corneas in the voriconazole plus FK506 group showed the mildest pathological changes, with the least inflammatory infiltration in the stroma and anterior chamber on day 5 post-infection. The control group exhibited the most serious corneal pathological changes, with the greatest inflammatory infiltration in the stroma and anterior chamber on day 5 post-infection. The control group demonstrated slightly reduced corneal infiltration and relatively slight inflammation compared with the control group (Figure 4C).

FK506 and voriconazole inhibited the expression levels of type I and II IFNs in fungal keratitis: Notably, as shown in Figure 5, the levels of type I (IFN-α/β) and II (IFN-γ) IFNs gradually increased following the initial infection, peaked on day 5, and then declined on day 7. FK506 combined with voriconazole suppressed the mRNA expression levels of type I and II IFNs obviously, as did voriconazole alone and FK506 alone. The voriconazole plus FK506 group exhibited statistically significantly lower mRNA expression levels of type I and II IFNs (all p<0.05; Figure 5A–C), followed by the voriconazole group and the FK506 group, which did not statistically significantly differ for any IFN (all p>0.05). The control group had the highest mRNA expression levels of type I and II IFNs (Figure 5A–C). Furthermore, the protein levels of IFN-β were examined, and the results indicated that the control group had the highest protein expression levels of IFN-β and that the voriconazole plus FK506 group had the lowest levels (Figure 5D), while there was no obvious differences between the voriconazole group and the FK506 group shown in Figure 5E (p>0.05).

FK506 and voriconazole regulated the inflammatory responses downstream of type I and II IFNs in fungal keratitis: To further explore the inflammatory responses regulated by FK506 and voriconazole, the inflammatory cytokine mRNA levels were measured in the control, voriconazole, and voriconazole plus FK506 groups. Compared with the control and voriconazole groups, the voriconazole plus FK506 group exhibited statistically significantly decreased expression levels of inflammatory cytokines, including IL-1α (Figure
6A, p<0.05), IL-6 (Figure 6B, p<0.05), IL-12 (Figure 6C, p<0.05), and IL-17 (Figure 6D, p<0.05), at day 1, day 3, day 5, and day 7 post-infection. However, the voriconazole group and the FK506 group had no statistically significant differences in the expression levels of IL-1α, IL-6, IL-12, and IL-17.

The proform and the mature form of protein expression levels of IL-1α were also explored, revealing a similar tendency as the mRNA expression levels, and the protein levels of the proform of IL-1α were higher than those of the mature form. The voriconazole plus FK506 group exhibited the lowest protein levels of IL-1α, followed by the voriconazole group and the FK506 group, and the control group had the highest protein levels of IL-1α statistically (p<0.05; Figure 6E,F).

**DISCUSSION**

Fungal keratitis remains a clinically challenging disease, and the recognition of fungi via PRRs is a highly complex and dynamic process [35,36]. Specifically, once fungi invade the mucosa, the host response is mediated by innate immune cells, such as neutrophils, macrophages, lymphocytes, and monocytes, and by soluble mediators, such as complement molecules or different peptides. IFNs, as important immunomodulatory factors downstream of PRRs, have considerable effects on innate and adaptive immune cells during infection [5-7] because of the IFNs’ immunomodulatory and antiproliferative activities. Although many aspects of the pathogenesis of fungal keratitis have been explored, the details of the signaling pathways involved remain unclear; thus, further exploration of the underlying mechanism of fungal keratitis is necessary.

IFNs are a family of proteins that are released by various cells in response to a variety of infections [37]. IFN release can be induced in virtually all cell types upon PRR recognition, especially in leukocytes, fibroblasts, and natural killer cells [38]. Nearly all phases of the innate and adaptive immune responses are affected profoundly by IFNs. In malaria infection, the absence of type I IFN signaling restrains parasite growth [39], and the production of IFN-γ increases during the parasite blood stage [40]. In inflammatory responses to viral infections, IFN-α/β and IFN-γ levels are increased, and these cytokines play harmful roles by triggering more inflammation and tissue damage, exacerbating the disease [41,42]. IFN-α/β may also be involved in protection against bacterial infections such as *Chlamydia pneumoniae* infections through a cooperative interaction with IFN-γ that induces antimicrobial effectors, thus suppressing bacterial survival [43]. However, the roles of type I and II IFNs in fungal keratitis remain largely unclear.
In this study, we first demonstrated that type I and II IFN levels were markedly elevated in fungal keratitis. Moreover, the mRNA and protein levels of type I and II IFNs revealed similar increasing trends as those observed for ocular inflammation and clinical scores, suggesting that IFN-mediated signaling is involved in various processes. During fungal infection, in the main inflammatory infiltrating cells, as well as the corneal epithelial cells (A6(1), that is, neutrophils, followed by macrophages and lymphocytes [44], type I and II IFN levels were increased after zymosan stimulation. Notably, these results were in agreement with those of another study, which demonstrated that IFN-mediated signaling attracted neutrophils and inflammatory monocytes to the disease site in C. albicans infection [45]. The present study demonstrated that type I and II IFNs actively participate in modulating inflammatory cells to defend against fungi and that upregulated type I and II IFN levels are important signs in fungal keratitis.

Moreover, the inflammatory cytokines (IL-1α, IL-6, IL-12, and IL-17) downstream of type I and II IFNs showed a tendency similar to that of type I and II IFN expression after zymosan stimulation. The protein levels of the proform IL-1α were higher than those of mature IL-1α in this study, which the former has been reported to be a proinflammatory activator of transcription [46]. Moreover, in Mycobacterium tuberculosis infections, IL-1α was recently shown to inhibit IFN-α/β induction in mouse and human macrophages [47]; thus, we speculated that IL-1α, especially the proform of IL-1α, may play a synergistic role rather than a suppressive role in IFN-α/β induction in fungal infection. IFN-α/β-mediated signaling has been shown to be necessary for IL-12 production in dendritic cells [48]. The results of the present study demonstrate that type I and II IFNs may also induce the production of IL-6 and IL-12 by other inflammatory cells, such as neutrophils, lymphocytes, and macrophages. Moreover, IFN-α/β is detrimental to the host, restricting...
Figure 6. The expression levels of IFN-related inflammatory cytokines in the control, voriconazole, and voriconazole plus FK506 groups in fungal keratitis. The mRNA expression levels of interferon (IFN)-related inflammatory cytokines (interleukin (IL)-1α, IL-6, IL-12, and IL-17; A to D; normalized to β-actin) and the protein levels and band intensity of the pro- and mature form of IL-1α (E and F) in the voriconazole and voriconazole plus FK506 groups decreased gradually after fungal infection. The data are the mean ± standard error of the mean (SEM) and represent three individual experiments with six animals/group/time. *, p<0.05; **, p<0.01; ***, p<0.001.
the development of a protective IL-17-producing γδ T cell response [49].

FK506 and voriconazole, which have strong inhibitory effects on the inflammation induced by fungal antigens [26,50], were demonstrated in the present study to have collaborative roles in inhibiting fungal keratitis. Voriconazole can work with FK506 to reduce pathological inflammatory cell infiltration in the cornea and reduce the levels of IFNs and downstream cytokines. Our previous study showed that FK506 relies on several molecular mechanisms to exert anti-inflammatory effects [26], thus suppressing the host immune response during fungal infection. Therefore, we inferred that inhibition of IFNs may be necessary for voriconazole to inhibit fungal growth and for FK506 to exert its anti-inflammatory effects against fungal keratitis simultaneously. In the fungal keratitis animal model, the levels of type I and II IFNs were reduced after voriconazole was used to inhibit fungal activity, and these levels then decreased further when immune responses were also controlled by FK506. These results show that the expression levels of IFNs not only are influenced by the degree of fungal activity but also may be determined by the degree of the immune response in fungal keratitis. Additionally, type I and II IFNs exhibited similar trends in fungal keratitis, which may help to confirm Lasfar and Cook’s results [51], showing that the control of the IFN-γ response (type II IFN) by type I IFNs may depend on endogenous type I IFN ligands and that IFN-γ may be involved in the control of IFN-β (type I IFN) responses. In other words, type I and type II IFNs may regulate biologic activities through common IFN receptor complexes. The present in vitro study showed that type I and II IFNs may regulate biologic activities through a distinct pathway; thus, we speculated that IFNs may have a common receptor complex and act via a distinct pathway after activation in fungal keratitis.

In conclusion, this study demonstrated that type I and II IFN expression was increased considerably in fungal keratitis. Additionally, voriconazole plus FK506 inhibited fungal activity more strongly than voriconazole alone in fungal keratitis, and the effect of FK506 may be mediated by the suppression of type I and II IFN levels, as well as the levels of downstream inflammatory cytokines. Moreover, we hypothesized that IFNs may have a common receptor complex and act via a distinct pathway after activation, which merits further study. These findings provide a better understanding of the roles and mechanisms of IFNs, and these results lay a foundation for future clinical therapies for fungal keratitis.

APPENDIX 1. STR ANALYSIS.

To access the data, click or select the words “Appendix 1”.

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We would like to acknowledge the contributions of all group members: Jin Yuan conceived and designed the experiments; Jin Yuan and Jing Zhong wrote the paper; Jing Zhong and Lulu Peng performed the experiments; Bowen Wang and Henan Zhang analyzed the data; and Saiqun Li, Ruhui Yang, Yuqing Deng, and Haixiang Huang contributed reagents/materials/analysis. This investigation was supported by grants from the National Natural Science Foundation of China to JY (81670826).

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