Production of interleukin-1β related to mammalian target of rapamycin/Toll-like receptor 4 signaling pathway during Aspergillus fumigatus infection of the mouse cornea

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Abstract

• AIM: To elucidate the effect of rapamycin on regulating the production of interleukin (IL)-1β in Aspergillus fumigatus (A. fumigatus)-induced keratitis and to verify whether the expression of IL-1β in A. fumigatus keratitis is associated with the mammalian target of rapamycin (mTOR)/Toll-like receptor 4 (TLR4) signaling pathway.

• METHODS: Fungal keratitis mouse models of susceptible C57BL/6 mice were established using A. fumigatus. The mice were subsequently treated with rapamycin. The protein levels of p-mTOR, TLR4, and IL-1β in normal and infected corneal tissue were measured by Western blot. The TLR4 and IL-1β mRNA levels were determined by real-time polymerase chain reaction (PCR).

• RESULTS: In C57BL/6 mice, rapamycin treatment decreased the clinical scores and production of the pro-inflammatory cytokine, IL-1β. The expression of TLR4, stimulated by A. fumigatus, was reduced as well when the mTOR signaling pathway was suppressed by rapamycin.

• CONCLUSION: Rapamycin is beneficial for the outcome of fungal keratitis and has an inhibitory effect on IL-1β expression can be associated with the mTOR/TLR4 signaling pathway in A. fumigatus infection in mice.

•KEYWORDS: keratitis; interleukin-1β; mammalian target of rapamycin; Toll-like receptor 4; mice

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INTRODUCTION

Fungal keratitis is a serious ocular disease with a high probability of causing blindness[1-2]. Pathogen-associated molecular patterns (PAMPs), comprising molecular structures unique to microbes, trigger the activation of inflammatory pattern recognition receptors (PRRs), such as Toll-like receptor (TLR), thus, leading to inflammatory phagocytosis and induction of immune responses[3-4]. Toll-like receptor 4 (TLR4), which belongs to the TLR family of PRRs, plays a significant role in activating inflammatory signaling pathways in response to microbial pathogens[5-6], and studies have shown that TLR4 plays an important role in fungal keratitis in mice infected with Aspergillus fumigatus (A. fumigatus)[7-9]. Mammalian target of rapamycin (mTOR) consists of two distinct complexes called mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2), and mTOR signaling impacts downstream cellular processes, including the cell cycle, cell growth, differentiation, survival, and metabolism[10-15]. Rapamycin, a classical mTOR inhibitor[16], was discovered in soil samples from Easter Island in the 1970s and was found to have antifungal activity. Over the past few years, the suppressive effect of rapamycin on T cell activation, as part of adaptive immunity, has been investigated in depth, and clinically rapamycin has been used as an immunosuppressant in allogeneic transplantation. In addition, mTOR has been targeted in the treatment of various types of cancers[17-20]. Now, it is increasingly clear that the mTOR signaling network plays an extremely important role in regulating the functions of innate immune cell populations and may further affect the outcome of infectious diseases[21-26]. Some researchers have reported that the inhibition of mTOR signaling on rapamycin treatment reduced the expression of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-2, IL-6, IL-10, IL-12, and tumor necrosis factor (TNF)-α in vivo or in vitro[18,27-30]. However, the role of mTOR in innate immunity during fungal keratitis has not been reported.

IL-1β is one of the key cytokines involved in antifungal immunity[9,31-32]. TLR4 mediates the production of IL-1β in protective immunity[9,33-34]. Recently, studies have provided evidence that rapamycin suppresses TLR4-triggered IL-6
production in colon tumor cells by inhibiting TLR4 expression and nuclear factor-κB (NF-κB) activation. Rapamycin also reduced lipopolysaccharide (LPS)-induced IL-6 production in head and neck squamous cell carcinoma cells, and this effect was mediated by TLR4[35-37]. Rapamycin may attenuate diabetic nephropathy by suppressing TLR4 signaling and Th17 cell signaling. These findings suggest that the effects mediated by TLR4 are closely correlated with mTOR; however, whether IL-1β production could be regulated through the mTOR/TLR4 signaling pathway in the innate immune response during fungal keratitis has not yet been reported.

In the present study, we investigated whether mTOR participates in the regulation of inflammatory responses via the TLR4 signaling pathway. Our results show that after inhibiting phosphorylated mTOR (p-mTOR), the expression of TLR4 and IL-1β stimulated by A. fumigatus was suppressed during the innate immune response to fungal keratitis. These results suggest a potential role of mTOR in mediating IL-1β signaling, which may occur through the mTOR/TLR4 pathway during A. fumigatus infection in mice. These data may contribute to better understanding of fungal keratitis pathogenesis and provide new avenues for its treatment.

MATERIALS AND METHODS

**Mice and Corneal Infection** Specific pathogen-free (SPF) 8-week-old female C57BL/6 (susceptible) mice were purchased from Changzhou Cavens Laboratory Animal Co., Ltd. (Changzhou, Jiangsu Province, China) and were carefully housed in a 22°C±2°C environment. Their physiological condition was observed every day before and after A. fumigatus infection. The mice were anesthetized with 8% chloral hydrate. The mice were observed using a stereoscopic microscope at a magnification of 40×, and a scratch area with a radius of 2 mm was made on the central corneal epithelium. A 5 μL A. fumigatus inoculum (strain 3.0772, General Microbiological Culture Collection Center, Beijing, China), with a concentration of 1×10⁶ CFU/mL, was applied on the damaged corneal surface. The cornea was covered with a soft contact lens and stored at -20°C. Before subconjunctival and intraperitoneal injection, the rapamycin solution in ethanol was diluted with sterile phosphate buffer saline (PBS) containing 5% Tween-80. To determine the appropriate concentration of rapamycin, it was administered via subconjunctival injection into the left eye at a concentration of 0.1 μg/5 μL and 0.5 μg/5 μL in the preliminary experiments. Results indicated that the concentration of 0.5 μg/5 μL had significantly lower p-mTOR protein levels in the cornea before and after infection (comparative data not shown); thus, all subsequent experiments were performed using this concentration of rapamycin. In addition, in others studies using rapamycin on the eyes[38-39], researchers chose to pretreat mice with rapamycin intraperitoneally at a concentration of 6.0 mg/kg, so an additional 6.0 mg/kg was injected intraperitoneally on the day of infection and day 1 after infection in our experiment, as well. Results showed that this method was effective in inhibiting p-mTOR expression and IL-1β production. The vehicle group was given PBS containing 3% alcohol and 5% Tween-80. Corneal tissue was harvested for the further research.

**Ocular Response to Aspergillus Fumigatus Infection** The severity of keratitis in mice was scored visually, according to photographs taken using a slit-lamp. The clinical scoring criteria were as follows[40]: 0=no or slightly visible ulcer area; 1=mild ulcer and anterior segment was covered partially; 2=moderate ulcer and the pupil was partially or completely covered; 3=cornea was opaque and the complete anterior segment was covered; and 4=corneal perforation. The scores were recorded on day 1 post infection (p.i.) for each infected mouse before harvesting the cornea. Representative corneal tissue was photographed to document the effects of rapamycin vs vehicle treatment.

**Real-time Reverse Transcription Polymerase Chain Reaction** Normal (untreated; n=6/group/time), vehicle (injected with sterile PBS containing 3% alcohol and 5% Tween-80), and rapamycin-treated corneal tissue was harvested for detection of mTOR, TLR4, and IL-1β mRNA levels. cDNA was synthesized by reverse transcription of 2 μg total RNA using the PrimeScript RT Reagent Kit (Takara, Tokyo, Japan) and was diluted to 1:25 with diethylpyrocarbonate (DEPC)-treated water. Quantitative RT-PCR was performed using Eppendorf Mastercycler and SYBR green. The housekeeping gene, β-actin, served as the internal control. The oligonucleotide primers used were as follows: β-actin: F-GATTACTGCTCTGGCTCCTAGC, R-GACTCATGCTACTCCTGCTTG; TLR4: F-CCTGACACGAAGCAGCAAG; and IL-1β: F-CGCAGCAGCACATCACAGGAAGCTTGAA, R-TCTGATCCATGCATTGGTAGGT; mTOR: F-ACCAGCACCACATTATTGAAGAG, R-CTCTGGAGAGTACAGCAGAGG; and IL-1β: F-CCGCAGCGACACATCAAAGAGACG, R-TGTCCCTCATCTGGAAGTGCCACG. Each experiment was repeated at least three times.

**Western Blot** Corneal tissue was lysed in 200 μL radioimmunoprecipitation assay (RIPA; Solarbio, Beijing, China) lysis buffer containing 2 μL phenylmethanesulfonyl
fluoride (PMSF; 100:1; Solarbio) and 2 μL protease inhibitor cocktail (100:1; MCE, Monmouth Junction, NJ, USA) for 2h. The samples were then centrifuged, SDS sample buffer was added, and the samples were boiled after determining the protein concentration. Total proteins were separated on 8%-16% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Merck, Darmstadt, Germany). After blocking with Western blocking buffer (Beyotime, Jiangsu Province, China) at room temperature for 3h, the membranes were incubated overnight at 4°C with a polyclonal antibody against β-actin (1:7000; Bioss, Beijing, China), primary antibody against p-mTOR (1:1000; Ser2448; Cell Signaling Technology, Danvers, MA, USA), monoclonal antibody against mTOR (1:1000; Abcam, Cambridge, UK), primary antibody against TLR4 (1:1000; Proteintech, Wuhan, China), or primary antibody against IL-1β (1:500; Bioss). The membranes were washed in PBS containing 0.05% Tween-20 (Bio-Rad, Hercules, CA, USA) 3 times every 5min. Membranes were incubated with corresponding peroxidase-conjugated secondary antibodies (1:10 000; Abcam) at room temperature for 2h. Finally, the blots were visualized by a chemiluminescence system (ECL; Thermo Fisher Scientific, Waltham, MA, USA), and the protein levels were measured and normalized to the levels of β-actin using ImageJ software.

Statistical Analysis Data were expressed as mean±standard deviation (SD) and were analyzed by GraphPad Prism 5.0 software. Statistical significance of real-time RT-PCR and Western blot data was determined by unpaired two-tailed Student’s t-test. Differences were considered significant at \( P\leq0.05 \).

RESULTS Enhanced Toll-like Receptor 4 and Interleukin-1β Production During Aspergillus fumigatus Infection We examined the mRNA expression of TLR4 and IL-1β in normal and \( A.\ fumigatus\)-infected corneal tissue using real-time RT-PCR. The mRNA levels were significantly increased in \( A.\ fumigatus\)-infected corneal tissue. Compared to those in the normal control, the relative TLR4 \( (P<0.001;\) Figure 1A) and IL-1β \( (P<0.001;\) Figure 1B) mRNA levels were significantly higher in \( C57BL/6\) mice corneal tissues on days 1 and 3 after infection, and both mRNA levels peaked on day 1 after infection.

Disease Responses in \( C57BL/6\) Mouse Corneal Tissues After Rapamycin Treatment To elucidate the role of mTOR in \( A.\ fumigatus\)-induced inflammatory responses, rapamycin was used to inhibit the activity of mTOR in \( C57BL/6\) mice corneal tissues. The effect of rapamycin on the outcome of fungal keratitis was assessed by photographs taken using a slit-lamp (Figure 2A-2C) and clinical score analysis (Figure 2D). The results of the photograph analysis showed that the severity of the disease decreased after rapamycin treatment on day 1 p.i. (Figure 2C) compared with that on treatment with the vehicle (Figure 2B). A significant difference was also observed on the analysis of clinical scores \( (P<0.05;\) Figure 2D). Further, Western blot confirmed the effect of rapamycin on mTOR expression in \( C57BL/6\) mice corneal tissues on day 1 p.i. mRNA and protein levels of mTOR were assessed using real-time RT-PCR and Western blot, respectively (Figure 2A, 2B). There was no significant difference in mTOR expression between the vehicle and rapamycin-treated groups (Figure 2E-2G); however, Western blotting showed that rapamycin treatment downregulated p-mTOR protein levels in infected corneal tissues compared to the vehicle group (Figure 2F, 2H).

Effect of Rapamycin Treatment on Expression of Interleukin-1β The relative mRNA levels of the pro-inflammatory cytokine, IL-1β, were measured in the infected corneal tissue of \( C57BL/6\) mice on day 1 p.i. by RT-PCR. Rapamycin treatment significantly suppressed the expression of IL-1β compared to the vehicle \( (P<0.01;\) Figure 3A). The protein levels of IL-1β stimulated by \( A.\ fumigatus\) were also significantly reduced following rapamycin treatment \( (P<0.001;\) Figure 3B, 3C).
Inhibitory Effect of Rapamycin on TLR4 Upregulated by Aspergillus Fumigatus

To investigate whether the reduced expression of IL-1β after inhibition of mTOR expression is associated with TLR4, relative mRNA levels (Figure 4A) or protein levels (Figure 4B, 4C) of TLR4 were assessed by RT-PCR and Western blot, respectively. After rapamycin treatment, there was a significant decrease in the mRNA levels on day 1 after infection \((P<0.01; \text{Figure } 4A)\) compared with the vehicle group. Protein expression of TLR4 was also significantly decreased on day 1 after infection compared to that in the vehicle group, as observed by Western blot \((P<0.001; \text{Figure } 4B, 4C)\).

**DISCUSSION**

Fungal keratitis is a challenging ophthalmologic condition that requires aggressive treatment to prevent untoward outcomes\(^{[41]}\). Infection with *Aspergillus* species is one of the major causes of fungal keratitis worldwide, resulting in visual impairment and even blindness. Research on the exact pathogenesis of *Aspergillus*-induced corneal infections is thus of great significance.

Considering the high molecular weight of rapamycin and the barrier structure of the eye, we selected subconjunctival injection in mice before infection in order to ensure the drug could effectively reach the cornea. Changes in the expression of mTOR and p-mTOR after rapamycin intervention were detected at the beginning, and they were considered to be indicative of the inhibition of the mTOR signaling pathway; these observations also verified the model of drug intervention in mice. Studies have reported that the mTOR inhibitor, rapamycin, only inhibited expression of p-mTOR, but not of unphosphorylated mTOR\(^{[25,42-43]}\). Our data also showed that the mRNA and protein levels of mTOR were not significantly different between the vehicle and rapamycin-treated groups; however, rapamycin treatment downregulated p-mTOR expression compared to the vehicle group.

A retinal inflammation study indicated that the expression of inflammatory molecules was suppressed by rapamycin in the retina during inflammation\(^{[39]}\). Similarly, a study of LPS-induced acute lung injury suggested that knockdown of mTOR reduces LPS-induced inflammatory responses in human bronchial epithelial (HBE) cells\(^{[25]}\). Another study showed that the decreased expression levels of mTOR reduced the concentrations of IL-1β and IL-6 in a rat model of spinal cord injury\(^{[44]}\). Other studies have shown that rapamycin treatment suppressed the expression of immunostimulatory molecules in *in vitro*-generated human monocyte-derived dendritic cells during...
LPS stimulation and inhibited the expression of IL-12p40, TNF, and IL-6.[10,45] IL-1β is an important pro-inflammatory cytokine in the inflammatory response against fungal infections[9,31-32]. In this process, upon β-glucan stimulation, TLR4 triggers NF-κB and other signaling pathways. TLR4-dependent activation of the signaling network triggers IL-1β induction. In our study, treatment with rapamycin reduced the expression of p-mTOR, *A. fumigatus*-induced TLR4, and IL-1β production after infection. Our in vivo experiments suggested that p-mTOR mediated the induction of IL-1β, likely through the mTOR/TLR4 signaling pathway. These results indicate that mTOR participates in the TLR4-induced IL-1β induction in mouse *A. fumigatus*-induced keratitis, supplementing a regulatory pathway for IL-1β induction triggered by TLR4. However, a study on *Pseudomonas aeruginosa*-induced keratitis showed IL-10 mRNA expression was significantly decreased after rapamycin treatment and that rapamycin treatment leads to the development of a pro-inflammatory environment in BALB/c mice.[38]. This conclusion appears slightly different from our findings. We hypothesized that this discrepancy could be attributed to the different mouse models, different methods of drug interventions, different drug concentrations, and different disease agents (bacteria or fungi).

Therefore, our study showed that the expression of TLR4 and pro-inflammatory cytokine IL-1β, was significantly upregulated during *Aspergillus* infection of the cornea in mice, while rapamycin suppressed this elevation in expression. These data suggest a potential role of mTOR in mediating inflammatory responses in *A. fumigatus*-induced keratitis in mice and prove that rapamycin has an anti-inflammatory effect, which may be mediated via the mTOR/TLR4 pathway in *A. fumigatus*-induced keratitis in mice. Furthermore, our results propose the potential use of rapamycin treatment as a therapy for suppressing the expression of p-mTOR and related inflammatory molecules, thus preventing subsequent visual dysfunction during fungal keratitis. There is clearly more than one mechanism by which rapamycin may regulate innate immunity, and further studies must be conducted to explore these mechanisms.

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REFERENCES
IL-1β related to mTOR/TLR4 signaling pathway


