

Expression and role of aryl hydrocarbon receptor in *Aspergillus fumigatus* keratitis

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Abstract

• **AIM:** To observe the expression and role of aryl hydrocarbon receptor (AhR) in the immune response of mouse cornea infected with *Aspergillus fumigatus* (*A. fumigatus*).

• **METHODS:** Murine models of *A. fumigatus* keratitis were established by scraping the central epithelium of mouse cornea, daubing *A. fumigatus* on the cornea and covering with a contact lens. The mice were randomly divided into the control group and the *A. fumigatus*-infected (A.F.) group for 1, 3 and 5d respectively, which corneas were daily monitored by a slit lamp microscope and the clinical scores were also recorded timely after infection. In this study, immunofluorescence staining was used to detect the expression and localization of AhR in mouse corneas, and the mRNA and protein of AhR were detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot. In addition, mouse peritoneal macrophages were stimulated by *A. fumigatus* with or without the pretreatment of AhR antagonist CH223191 and AhR agonist FICZ, and the tumor necrosis factor alpha (TNF- α), inducible nitric oxide synthase (iNOS), interleukin-10 (IL-10) and Arg-1 mRNA were detected by RT-PCR.

• **RESULTS:** According to the results of the slit light photography, it was clearly indicated that the corneal inflammation were the most severe and the clinical score became the highest as well on the 3rd day after the infection of *A. fumigatus*. Contrasted with the control group, the expression of AhR in the corneal epithelial cells infected with *A. fumigatus* was significantly increased detected by immunofluorescence staining. AhR mainly expressed in the nucleus and cytoplasm of corneal epithelial cells. Consistent

with the transcriptional level of AhR mRNA, the expression level of AhR protein reached the peak on the 3rd day after infection which was detected by Western blot. Furthermore, RT-PCR showed that CH223191 up-regulated the expression of TNF- α and iNOS and down-regulated the expression of IL-10 and Arg-1 in peritoneal macrophages; inversely, FICZ reduced the expression of TNF- α and iNOS while elevated the expression of IL-10 and Arg-1.

• **CONCLUSION:** AhR is involved in the pathogenesis of *A. fumigatus* keratitis and induced immune protection in anti-*A. fumigatus* immune response by inhibiting M1 and increasing M2 phenotype macrophage-related inflammatory factors.

• **KEYWORDS:** aryl hydrocarbon receptor; keratitis; *Aspergillus fumigatus*; innate immune response

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INTRODUCTION

Infectious keratitis remains the foremost cause of corneal blindness, especially in developing countries. In agricultural countries, fungal keratitis was reported to account for more than 60% of the total infectious keratitis, and it was of great significance to investigate the immune mechanism of fungal keratitis (FK)^[1]. It is essential to emphasize the fact that FK is more intractable than bacterial keratitis due to its meager response to treatment and its resistance to antifungal drugs^[2].

Aspergillus spp. was widely present in the biological community and considered as a major pathogen of keratitis^[2]. Typically, the innate immunity was the first line of defense for host to resist fungal infection. After infection, inflammatory cells (such as lymphocytes, neutrophils and macrophages *etc.*) were recruited to eliminate pathogens by producing chemokines and cytokines, which were vital to maintain the normal structure of the cornea^[3]. However, excessive immune response could give rise to damnification.

Aryl hydrocarbon receptor (AhR) was a ligand-activated transcription factor^[4]. Recent studies have shown that

structurally different chemicals can bind and/or activate AhR-dependent gene expression. These results indicate that AhR has mixed ligand binding sites and may produce varied effects when binding to different ligands^[5]. When AhR combined with ligands, the conformational changes occurred and AhR was transferred from cytoplasm to nucleus^[6-7]. Originally, AhR was deemed as an important factor affecting the generation and development of regulatory T cells (Tregs) and Th17 cells, ultimately interleukin-10 (IL-10) produced by Tregs may impel a homeostasis response in chronic fungal infections which was regulated by the immune system of the host^[8]. However, recent researches on animal models confirmed that the activation of AhR could restrain inflammatory response in diverse inflammatory diseases. It was reported that the probiotic *Lactobacillus reuteri* could sensitize innate lymphoid cells (ILC3) to produce IL-22 through the AhR pathway, which function as anti-fungal and protective role in mucosal inflammation^[9]. Some other investigations had indicated that AhR-deficient mice also represented multiple abnormal allergies^[10]. Above-mentioned studies all confirmed that AhR contributed to mediate protective immunological tolerance in fungal infections.

This study investigated the orientation and tendency of AhR expression in mice with *A. fumigatus* keratitis, and the effects of AhR on the expression of macrophage-related cytokines *in vitro* were also estimated. Further investigation on this mechanization may contribute to grope for new strategies for the treatment of FK.

MATERIALS AND METHODS

Ethical Approval Specific pathogen-free (SPF) C57BL/6 mice (8-week-old females) were acquired from Jinan Peng yue Laboratory Animal Co. LTD (Jinan, China). All mice were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animal Models of Keratitis The mouse models were established by referring to the previous experimental methods^[11-12]. Mice were anesthetized with 8% trichloroacetaldehyde and placed under a stereoscopic microscope (40× magnification). The left eyes of each mouse were selected as the experimental eyes and the central corneal epithelial area (2-4 mm diameter range) was removed softly. Daubed 5-μL aliquot of *A. fumigatus* [1×10^8 colony forming unit (CFU)/mL, strain 3.0772, China General Microbiological Culture Collection Center], covered with aseptic contact lens and then sutured the eyelids gently. Corneas in control group were treated without *A. fumigatus* inoculation. For reverse transcription-polymerase chain reaction (RT-PCR) and Western blot detection, infected corneas (A.F. group) were collected on the 1st, 3rd and 5th day after the establishment of mouse models, while uninfected corneas (control group) were collected on the 5th day. For

Table 1 Nucleotide sequences of mouse primers for RT-PCR

Gene	GenBank No.	Primer sequence (5'-3')
mβ-actin	NM_007393.5	F: GAT TAC TGC TCT GGC TCC TAG C R: GAC TCA TCG TAC TCC TGC TTG C
mAhR	NM_001314027.1	F: ATT GCC AAG TGC CAA ATG GA R: GGT TAA GTG TTC AGG TGC TGT AAG A
mTNF-α	NM_013693.2	F: ACC CTC ACA CTC AGA TCA TCT T R: GGT TGT CTT TGA GAT CCA TGC
miNOS	NM_010927.3	F: TGT CTG CAG CAC TTG GAT CAG R: AAA CTT CGG AAG GGA GCA ATG
mArg-1	NM_007482.3	F: TGG GTG ACT CCC TGC ATA TCT R: TTC CAT CAC CTT GCC AAT CC
mIL-10	NM_010548.2	F: TGC TAA CCG ACT CCT TAA TGC AGG AC R: CCT TGA TTT CTG GGC CAT GCT TCT C

m: Mouse; F: Forward; R: Reverse.

immunofluorescence staining, eyeballs were detached on the 3rd day after infection.

Acquisition of Macrophages Thioglycollate powder (3 g) was dissolved in 100 mL ddH₂O, followed by stirring in a water bath pot until it completely liquefied. Thioglycollate medium was sterilized by high temperature steam sterilization. C57BL/6 mice were pretreated by intraperitoneal injection with cooling-solution (1 mL). On the 7th day after pretreatment, the skin of the mouse abdominal wall was cut while preserving the peritoneum intact. The RPMI-1640 medium (5 mL) was injected into the mouse abdominal cavity by syringes. After gently massaging the abdomen for 2min, the culture medium was drawn back to obtain the primary macrophages^[13]. In order to obtain more macrophages, repetitive operation of peritoneal lavage was performed.

Cell Culture and Pretreatment of Stimulants Macrophages were cultured according to the previous methods^[14-15]. Macrophages were pretreated with or without AhR antagonist (CH223191, 10 μmol/L, Sigma, St. Louis, MO, USA) and AhR agonist (FICZ, 300 nmol/L, Sigma, St. Louis, MO, USA) for 2h, then stimulated by inactivated *A. fumigatus* hypha (5×10^6 CFU/mL) for a certain amount of time (0, 4, 8, 12h).

Reverse Transcription-Polymerase Chain Reaction The total RNA, which were extracted from the corneas and cells by the RNAiso plus (TaKaRa, Dalian, China), were stored at -80°C and determined by spectrophotometry. The cDNA was synthesized by reverse transcription reagents (TaKaRa, Dalian, China) with 2 μg of total RNA. RT-PCR was performed by the RT-PCR Master Mix with SYBR and specific primers (TAKARA, Dalian, China). The cycle parameters of the reaction were as below: 95°C for 30s, then 40 cycles of 95°C for 5s and followed by 60°C for 30s, with the last steps at 95°C for 15s, 60°C for 30s, and 95°C for 15s. The cycle threshold (Ct SYBR) of β-actin was used as internal control. Primers were showed in Table 1.

Immunofluorescence Staining The eyeballs of C57BL/6 mice were acquired and placed in the optimum cutting temperature (OCT) compound solution (Sakura Tissue-Tek, Torrance, CA, USA), followed by being frozen with liquid nitrogen. The eyeball specimens were cut into 10 mm sections and dried at 37°C for 6h. The tissues were fixed with acetone for 5min. Sealed antigen off with sealing solution for 30min at room temperature (PBS contains 10% donkey serum). Then the slides were placed at 4°C overnight with anti-AhR antibody (1:100, Abcam, Cambridge, UK), then incubated with FITC-bound donkey anti-rabbit secondary antibody (1:500, Bioss, Beijing, China). Finally, the digital images were captured with a Zeiss Axiovert microscope at 400× and 1000× magnification.

Western Blot The concentration of the corneal proteins, which were extracted with RIPA lysis buffer (Solarbio, Beijing, China) plus 1 mmol/L PMSF (Solarbio, Beijing, China), was determined by using a BCA protein analysis reagent (Solarbio, Beijing, China) after centrifuging at 4°C and 12 000 rpm for 5min, followed by denaturing with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer at 109.8°C for 10min. In Tris/SDS/glycine buffer, the protein (60 µg/well) was separated by 10% SDS-PAGE and electroblotted onto the PVDF membranes (Millipore, Billerica, MA). After blocking with a blocking buffer (Solarbio, Beijing, China) at room temperature for 1.5h, membranes were incubated with beta tubulin polyclonal antibody (1:2000, Elabscience, Wuhan, China) and AhR antibody (1:500) overnight at 4°C, then incubated with an anti-rabbit secondary antibody (1:2000, Abcam, Cambridge, UK) for 1h. The immobilized western chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) was used for blot detection.

Statistical Analysis In order to compare the differences between two groups, the significance was determined by *t*-test (GraphPad Prism). The data was represented as the mean± standard error of mean (SEM), and *P*<0.05 was considered significant.

RESULTS

Corneal Disease Procession after *A. Fumigatus* Infection In order to record the disease procession of *A. fumigatus* keratitis, we established the mouse model of *A. fumigatus* keratitis by inoculating *A. fumigatus* on corneas, photographed the mouse corneas with slit lamp microscope (Figure 1A) and recorded the clinical score (Figure 1B). Control group had mild keratopathy at early stage after the establishment of mouse model and gradually returned to normal transparency after the 3rd day. The clinical scores of A.F. group were significantly higher than those of control group at 1, 3 and 5d (*P*<0.001). On the first day after *A. fumigatus* inoculation, irregular inflammatory infiltration and obvious corneal edema were

observed in mouse cornea, accompanied by ulcers of various degree. The highest clinical score of keratitis appeared on the 3rd day after infection. At this time, the area of corneal ulcer enlarged and the degree of inflammation deepened. Since then, the keratopathy began to relieve or recover without the external intervention. On the 5th day, obvious neovascularization appeared at the corneoscleral limbus.

Expression of AhR in Mouse Corneas Inoculated with *A. Fumigatus* In order to confirm whether AhR was involved in the pathological process of *A. fumigatus* keratitis, we firstly detected the expression and localization of AhR in corneas of C57BL/6 mice by immunofluorescence, which labeled AhR with strong green fluorescence (Figure 1C). Results showed that there were very few AhR-positive cells located in the cytoplasm of corneal epithelial cells in the corneas of control group. Compared with the control group, the infiltration of inflammatory cells and the expression of AhR in mouse cornea infected by *A. fumigatus* were significantly increased. AhR mainly expressed in the nucleus and cytoplasm of corneal epithelial cells, which also partially expressed in inflammatory cells that gathered into corneal stroma. In order to make further efforts on observing the expression of AhR in *A. fumigatus* keratitis, mRNA levels of AhR in mouse corneas, lymphatic glands and spleens were detected by RT-PCR, and protein levels of AhR in mouse corneas were detected by Western blot. Results confirmed that a spot of AhR mRNA expression could be detected in control group. AhR mRNA in mouse corneas tended to be up-regulated on the 1st day (*P*<0.001) after infection, and reached the peak on the 3rd day (*P*<0.01). There was significant difference among all of the groups (*P*<0.05; Figure 2A). The tendency of AhR mRNA expression in mouse lymphatic glands (Figure 2B) and spleens (Figure 2C) was basically the same as that in corneas, which had reached the peak on the 3rd day after infection.

Western blot was used to further detect the expression of AhR protein (Figure 3). AhR protein began to raise on the 1st day after infection, reached the peak on the 3rd day, and decreased slightly on the 5th day. The expression of AhR protein was significantly different between the 1st day and the 3rd day, but no dissimilarity between the 3rd day and the 5th day.

Effects of AhR on the Expression of Macrophage-Related Inflammatory Factors In order to evaluate the effects of AhR on topical inflammatory microenvironment, we cultured C57BL/6 mouse peritoneal macrophages *in vitro* and detected the mRNA expression of inflammatory factors by RT-PCR. First of all, we detected the expression of AhR mRNA expression in mouse peritoneal macrophages which were stimulated by inactivated *A. fumigatus* hyphae. Results showed that the expression of AhR mRNA reached the peak after 4h stimulation by inactivated *A. fumigatus* hyphae, and then

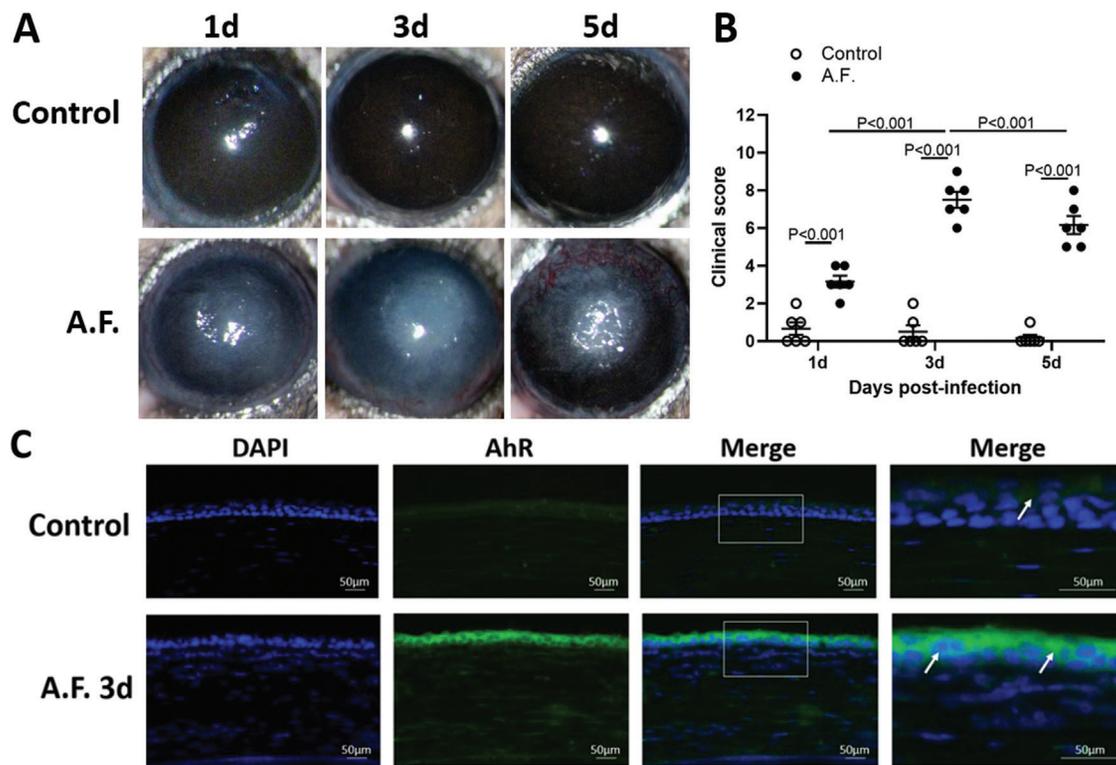


Figure 1 The expression and localization of AhR in corneas of C57BL/6 mice A: Corneal photographs taken under a slit lamp. B: Disease scores evaluated with the scoring system in all corneas (mean±SEM). The highest clinical score of keratitis appeared on the 3rd day after infection. C: Serial sections of control and *A. fumigatus*-infected (A.F.) corneas at 3d were stained using AhR antibody and FITC-conjugated second antibody (for AhR, green). As indicated by the control group, few green fluorescence was observed in corneal epithelium. However, in the A.F. group, strong green fluorescence in corneal epithelium was detected, and there was also a small amount of fluorescence in the corneal stromal cells. As shown in enlarged images at 1000× magnification, AhR staining mainly localized in the nucleus and cytoplasm of corneal epithelial cells in the A.F. group while very few AhR staining localized in cytoplasm of corneal epithelial cells in the control group. Scale bar in immunofluorescence images: 50 μm.

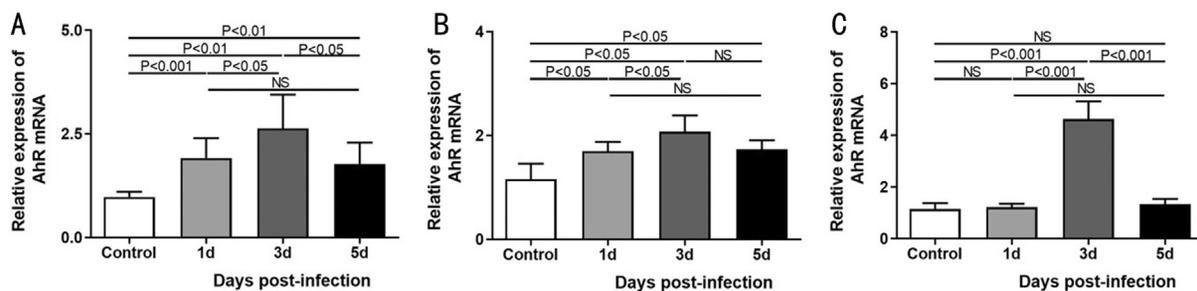


Figure 2 The expression of AhR mRNA in mice with *A. fumigatus* keratitis A: AhR mRNA in mouse corneas tended to be up-regulated on the first day ($P<0.001$) after *A. fumigatus* infection, and reached the peak on the 3rd day ($P<0.01$); B: The tendency of AhR mRNA expression in mouse lymphatic glands had reached the peak on the 3rd day after *A. fumigatus* infection ($P<0.05$); C: The tendency of AhR mRNA expression in mouse spleens had reached the peak on the 3rd day after *A. fumigatus* infection ($P<0.001$).

decreased gradually (Figure 4A). Therefore, we pretreated with or without AhR agonist FICZ (300 nmol/L) and antagonist CH223191 (10 μmol/L) for 2h and inactivated *A. fumigatus* hyphae for 4h. The expression of TNF-α, iNOS, IL-10 and Arg-1 in mouse peritoneal macrophages was detected by RT-PCR. Results showed that compared with A.F. group, FICZ upregulated the expression of IL-10 and Arg-1 and down-regulated that of TNF-α and iNOS. On the contrary, CH223191

down-regulated the expression of IL-10 and Arg-1 and up-regulated that of TNF-α and iNOS.

DISCUSSION

FK is a fungal infectious disease characterized by global distribution. The primary pathogeny of this disease correspondingly changed based on climate status, geographical location and socio-economic conditions. In the tropical, subtropical and temperate regions, the genus of *Aspergillus*,

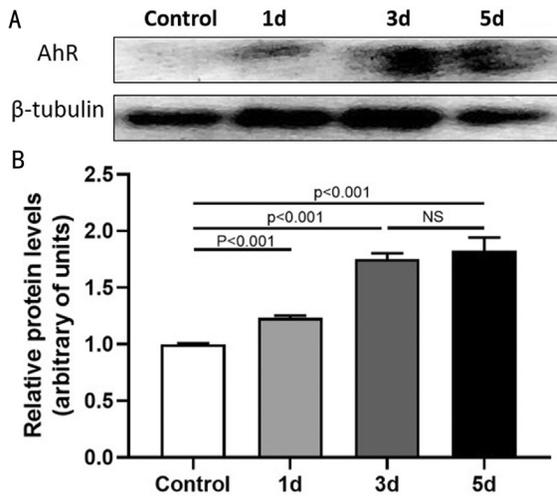


Figure 3 The tendency of AhR protein expression in mouse corneas with *A. fumigatus* keratitis A: The protein immunoblotting of AhR and β -tubulin in the mouse corneas of control, 1, 3, 5d groups were shown. B: Western blot analysis showed AhR protein was elevated at 1, 3, 5d after *A. fumigatus* infection compared with control group ($P < 0.001$). At the 3rd day and 5th day, AhR protein was higher than that of the 1st day ($P < 0.001$).

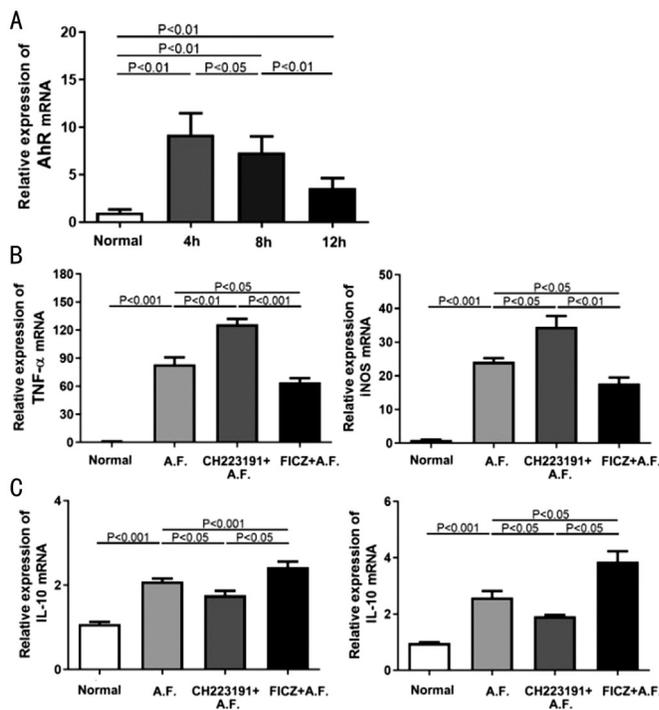


Figure 4 Effects of AhR on the expression of macrophage-related inflammatory factors Mouse peritoneal primary macrophages was stimulated by inactivated *A. fumigatus* hypha. A: Contrasted with the control group, the mRNA expression of AhR in mouse peritoneal macrophages increased significantly ($P < 0.01$), and reached the peak at 4h; B: After the mouse peritoneal macrophages stimulated by inactivated *A. fumigatus* hypha for 4h, the results of RT-PCR showed that, compared with A.F. group, the expression of TNF- α and iNOS were promoted by CH223191 ($P < 0.05$), while inhibited by FICZ ($P < 0.05$); C: Compared with A.F. group, the expression of Arg-1 and IL-10 were promoted by FICZ ($P < 0.05$), while inhibited by CH223191 ($P < 0.05$).

Fusarium and *Candida* are common pathogens of FK^[16]. The potential molecular pathological mechanisms related to fungal infection include host factors and toxic factors, which were involved in the pathogenetic process of keratitis and led to varying degrees of ocular tissue injury. Therefore, on the purpose of minimizing the damage and renovating damaged tissue, the treatment of fungal keratitis should lay much stress not only on eliminating the causative agent, but also on neutralizing destructive factors. A comprehensive cognition of the pathogenesis of fungal keratitis will be propitious to the rational implementation of therapeutic measures^[17]. The intrinsic tolerance of the infection caused by *A. fumigatus* manifested that the emergence of the regulatory mechanism provided the host with sufficient defense ability, and that the presence of such a mechanism prevented fungal infection from causing irreparable damage to the host. Consequently, regulation was an important part of the immune mechanism that could resist the *A. fumigatus* infection and host allergy^[18]. Tryptophan was recognized as an essential amino-acid for T cells executes its function. During the process of its catabolism, the metabolite L-kynurenine has been proved to be the physiological ligand of AhR, which can stimulate the expression of AhR dependent genes at a physiologically obtainable concentration^[19-20]. Tryptophan persists a central position at the host/fungal interface. AhR held a critical function in the fungal community in connecting tryptophan catabolism and the host's tryptophan degradation. Esser^[21] authenticated that all mice with AhR-deficient behaved various abnormalities which included life shorten, weight loss and a decline of liver function due to hepatic vascular dysplasia. Besides, AhR-deficient mice can also represent inflammatory lesions and fibrosis, as well as abnormal hematopoietic dysplasia^[10]. The anterior results indicated that AhR may be involved in the immune regulation of the host inflammatory response. However, the relationship between AhR and fungal keratitis has not been clearly clarified.

To investigate the location and expression of AhR expression in mice with *A. fumigatus* keratitis, we established the murine models of *A. fumigatus* keratitis by scraping the central epithelium of mouse cornea, daubing *A. fumigatus* on the cornea and covering with a contact lens. Consistent with previous data, the clinical score of mouse *A. fumigatus* keratitis reached its highest level on the 3rd day after the infection of corneas^[22]. The expressive location of AhR in the mouse corneas was detected by immunofluorescence. After infection by *A. fumigatus*, the expression of AhR mainly localized in the nucleus and cytoplasm of corneal epithelial cells in the A.F. group while very few AhR staining localized in cytoplasm of corneal epithelial cells in the control group. Additionally, the mRNA expression of the AhR was detected to exist in mouse

corneas by RT-PCR. After inoculated with *A. fumigatus*, the expression of AhR in mouse corneas was up-regulated and reached the peak on the 3rd day, the mRNA expression of AhR in mouse lymphatic glands and spleens raised congruously. Furthermore, Western blot indicated that the protein expression level reached the apex on the 3rd day after the infection. The above researches showed that AhR was involved in the immune mechanism of *A. fumigatus* keratitis.

Previous investigations verified that expression of AhR-dependent genes was positively correlated with IL-22 production under the presence of AhR agonist FICZ. Meanwhile, the production of IL-22 secreted by innate lymphocytes (ILCs) was more directly dependent on the activation of AhR^[23]. IL-22 played a major role in tissue repair of epithelial barrier and host defense^[24], and may affect homeostasis between intestinal immunity and microbial communities^[9,25]. These suggest that AhR may be involved in regulating the immune tolerance against fungi. In order to further evaluate the effects of AhR on the immune response of *A. fumigatus* and the possible pathomechanism of FK, mouse peritoneal macrophages were cultured *in vitro* and then stimulated with inactivated *A. fumigatus* hyphae for a certain period of time, after which the expression of its various inflammatory factors was detected by RT-PCR. TNF- α and iNOS were proinflammatory cytokine secreted by M1 macrophages, which could effectively evoke pro-inflammatory stimulation^[26] and eliminated microorganism. IL-10 and Arg-1 are M2 macrophage-related inflammatory factors, which may participate in antimicrobial immunomodulatory and act as an important part in inflammation inhibition and organizational repair^[27-28]. The results confirmed that the excitement of AhR promoted the expression of IL-10, Arg-1 and inhibited the expression of TNF- α , iNOS. Contrarily, the inhibition of AhR inhibited the expression of IL-10, Arg-1 and promoted the expression of TNF- α , iNOS. It was well illustrated that AhR could restrain the expression of M1 macrophage-related inflammatory factors and promote the expression of M2 macrophage-related inflammatory factors, which mediated protective immune tolerance in anti-*A. fumigatus* immune response and served as a significant part in host defense and organizational repair. Besides, experiments have shown that the AhR/IL-22/Stat3 signaling pathway regulate the biological effects of intestinal microbial flora and regulate the production and secretion of antimicrobial molecules^[29]. Similarly, we will preliminarily confirm that which AhR-related signaling pathway may be involved in the modulation of cornea anti-*A. fumigatus* molecules.

In conclusion, in the anti-inflammatory response of *A. fumigatus*, AhR-mediated immune protection engendered by inhibiting the secretion of M1 macrophage-related

inflammatory factors and promoting the secretion of M2 macrophage-related inflammatory factors. Next, the stimulation mechanisms and other roles of AhR in *A. fumigatus* keratitis are important issues which warrants further investigation so as to support its function in the prognosis of fungal keratitis.

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