• Basic Research •

Indoleamine 2,3-dioxygenase adjusts neutrophils recruitment and chemotaxis in *Aspergillus fumigatus* keratitis

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Abstract

● **AIM:** To explore the effect of indoleamine 2,3-dioxygenase (IDO) on recruitment and chemotaxis function of neutrophils in *Aspergillus fumigatus (A. fumigatus)* keratitis.

● **METHODS:** C57BL/6 mice models of *A. fumigatus* keratitis were established by inoculating hyphae of *A. fumigatus* evenly on the corneas. The clinical scores and inflammatory cytokines expression were measured respectively on the 1st, 3rd, 5th day after infection. The 1-MT (1 mg/mL) was administered by gavage to exert an inhibitory effect on IDO during infection. The mice were divided into control group, 1-MT group, *A. fumigatus* (A.F.) group, and 1-MT+A.F. groups. The corneas were monitored by slit lamp microscopy, and recorded disease scores in 3d after infection. Myeloperoxidase (MPO) assay was done to evaluate the neutrophils infiltration. Immunofluorescence staining was used to detect the recruitment of neutrophils in murine corneas. The mRNA of inflammatory cytokines was measured with reverse transcription-polymerase chain reaction (RT-PCR).

● **RESULTS:** The corneal inflammation and the clinical score reached the peak on the 3rd day after the corneal infection. The mRNA of inflammatory cytokines of the A.F. group reached the highest on the 3rd day after the infection accordingly. Meanwhile, the results of slit light photography indicated that inhibitors of IDO made inflammation more serious contrasted with the A.F. group on the 3rd day. Besides, imunofluorescence staining and MPO indicated that 1-MT enhanced the recruitment, infiltration and chemotaxis of neutrophils obviously in contrast to the A.F. group. RT-PCR indicated that 1-MT increased the expression of CXCL-1, ICAM-1, IL-1β, and IL-8 significantly.

● **CONCLUSION:** IDO participates in the pathogenesis of *A. fumigatus* keratitis and plays an important role in inducing immune protection by inhibiting neutrophils-related inflammatory reaction and suppressing recruitment and chemotaxis of the neutrophils.

● **KEYWORDS:** indoleamine 2,3-dioxygenase; keratitis; neutrophils; *Aspergillus fumigatus*; innate immune

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INTRODUCTION

Fungal keratitis (FK) is an intractable disease which leads to visual impairment even blindness[1]. Agricultural injuries, the overuse of broad-spectrum antibiotics and glucocorticoids, even long-term use of contact lenses can result in an increase in its incidence year by year[2]. *Fusarium, Aspergillus fumigatus* (*A. fumigatus*) and *Candida* are common pathogens of FK[1]. The pathogenic process of FK has initiated once *A. fumigatus* has invaded the cornea[1]. A series of pathological keratopathy appear including corneal vasodilation, a great number of inflammatory cells which are recruited to the site of inflammation, a large number of immune active substances which are released to the corneal tissue after stimulation by *A. fumigatus*[3]. Typically, a series of inflammatory responses happen in the cornea after fungal infection, such as exudation, recruitment, and phagocytosis of cells including neutrophils, macrophages and T lymphocytes that produce proinflammatory, chemotactic and regulatory cytokines, and reactive oxygen species, the release of active nitrogen and protease which have the effect of inhibiting or eliminating pathogens[3-4]. These cells and inflammatory factors are essential to keep the corneal structure, but undue inflammation will destroy the corneal stroma, even cause corneal perforation and vision loss[5].

Indoleamine 2,3-dioxygenase (IDO) is the rate-limiting enzyme which takes part in the kynurenine pathway of tryptophan degradation and induces immune tolerance against *A. fumigatus*[6-8]. IDO has the function of immunomodulator which takes part in the inflammatory response in *A. fumigatus* keratitis, and induces the immune tolerance against *A. fumigatus* by our preliminary study[8]. Furthermore, it is reported that

IDO can promote immune tolerance by suppressing T-cell responses[9]. IDO regulates the balance of immunopathology and protective immunity by acting on neutrophils[10]. IDO can attenuate neutrophils migration in urinary tract caused by bacterial infections[11]. All in all, the results showed that IDO may play an important role by modulating neutrophils in the *A. fumigatus* keratitis. However, the influence of IDO on neutrophils in *A. fumigatus* keratitis has not yet been studied. This study will explore the mechanism of IDO which involved in FK.

MATERIALS AND METHODS

Ethical Approval We purchased Specific pathogen-free (SPF) eight-week-old females C57BL/6 mice from SPF (Beijing) Biotechnology Co., Ltd. The mice were treated for ophthalmic and vision research in accordance with the ARVO Statement.

Murine Models of *A. Fumigatus* Keratitis Murine models of *A. fumigatus* keratitis were built according to the previous methods[12-13]. Mice were placed under a stereoscopic microscope after being anesthetized with 8% trichloroacetaldehyde. We chose the left eyes of mice as the experimental eyes. The 3 mm-diameter central corneal epithelial tissue was removed, inoculated 5-μL hyphae of *A. fumigatus* evenly [1×10^8 colony forming unit (CFU)/mL], in order to ensure the whole corneas were covered. Finally, the aseptic contact lens was paved on the corneas and sutured the eyelids. The corneal epithelium of control group was removed without *A. fumigatus* coverage. The opacity density, opacity area, and surface regularity of corneas were evaluated the severity of keratitis, each of which had a grade of 0 to 4. In summary, the degree of keratitis included normal (0), mild (1-5), moderate (6-9), and severe (10-12)[14]. The mice were divided into four groups randomly: the control group [the mice were scraped corneal epithelial only without infection of *A. fumigates* and treated with phosphate buffer saline (PBS) intragastric administration], the 1-MT group (the mice drank a quantitative filter-sterilized 1-MT suspension every day and scraped corneal epithelial only without infection of *A. fumigates*), the A.F. group (the mice were scraped corneal epithelial with infection of *A. fumigatus* and treated with PBS intragastric administration), and the 1-MT+A.F. group (the mice were treated with 1-MT intragastric administration and scraped corneal epithelial with infection of *A. fumigatus*). In addition, the corneas of the A.F. group on the 1st, 3rd, and 5th day after the infection of murine models were collected. Meanwhile, the corneas of control group were collected on the 5th day for reverse transcription-polymerase chain reaction (RT-PCR) detection. Corneas from those four groups were collected on the 3rd day for RT-PCR and myeloperoxidase (MPO). For immunofluorescence staining, eyeballs on the 3rd day were detached.

Reverse Transcription-Polymerase Chain Reaction The RNAiso Plus Reagent (TaKaRa, China) extracted the RNA in the corneas which could be measured by spectrophotometry. The experimental method of RT-PCR was proceeded in accordance with the kit instructions. Primers for RT-PCR were listed in Table 1.

Table 1 Nucleotide sequences of mouse primers

Gene	Primer sequence (5'-3')
mβ-actin	F: GATTACTGCTCTGGCTCCTAGC
	R: GACTCATCGTACTCCTGCTTGC
mCXCL-1	F: ATCCAGAGCTTGAAGGTGTTGC
	R: GAGCTTCAGGGTCAAGGCAAG
mIL-8	F: CAAGGCTGGTCCATGCTCC
	R: TGCTATCACTTCCTTTCTGTTGC
mICAM-1	F: TTCCGGCATTTATGTGTGTGAAG
	R: GGCACATTTCCACAAGTGCAG
mFoxp3	F: GGCCCTTCTCCAGGACAGA
	R: GCTGATCATGGCTGGGTTGT
mIFN-γ	F: CGGCACAGTCATTGAAAGCCTA
	R: GTTGCTGATGGCCTGATTGTC
mIL-1β	F: CGCAGCAGCACATCAACAAGAGC
	R: TGTCCTCATCCTGGAAGGTCCACG
mIL-17	F: TTTAACTCCCTTGGCGCAAAA
	R: CTTTCCCTCCGCATTGACAC
mIL-23	F: CCAGCGGGACATATGAATCT
	R: AGGCTCCCCTTTGAAGATGT
mTGF-β	F: ACCCTCACACTCAGATCATCTT
	R: GGTTGTCTTTGAGATCCATGC

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

Immunofluorescence Staining The eyeballs of mice were collected and placed in the optimum cutting temperature (Sakura Tissue-Tek, USA), and then were cut into 10 μm-thick sections after being frozen by liquid nitrogen. The tissues were fixed in acetone and sealed goat serum off (1:100). The photos were captured with microscope (400×) after being covered with rat anti-mouse neutrophils marker (1:100; Santa Cruz Biotechnology, USA) and FITC-conjugated goat anti-rat secondary antibody (1:200; Elabscience).

Myeloperoxidase Assay The corneas were collected on the 3rd day after infection in accordance with the MPO kit instructions (Jiancheng Institute, China). Corneas were homogenized in 1 mL of the second agent of the MPO test kit according to the manufacturer's instructions. Every cornea was freeze-thawed and centrifuged, and then supernatant was warmed in a water bath, and the change in absorbency (460 nm) was immediately monitored. The slope of the line was determined for each sample and used to calculate units of MPO per cornea.

Statistical Analysis We determined the significance by *t*-test and the data was expressed as the mean±standard error of mean (SEM). The data was significant when $P<0.05$.

RESULTS

Development of *A. Fumigatus* Keratitis The murine *A. fumigatus* keratitis models were established and took picture of mouse corneas by slit lamp microscope to record the keratitis

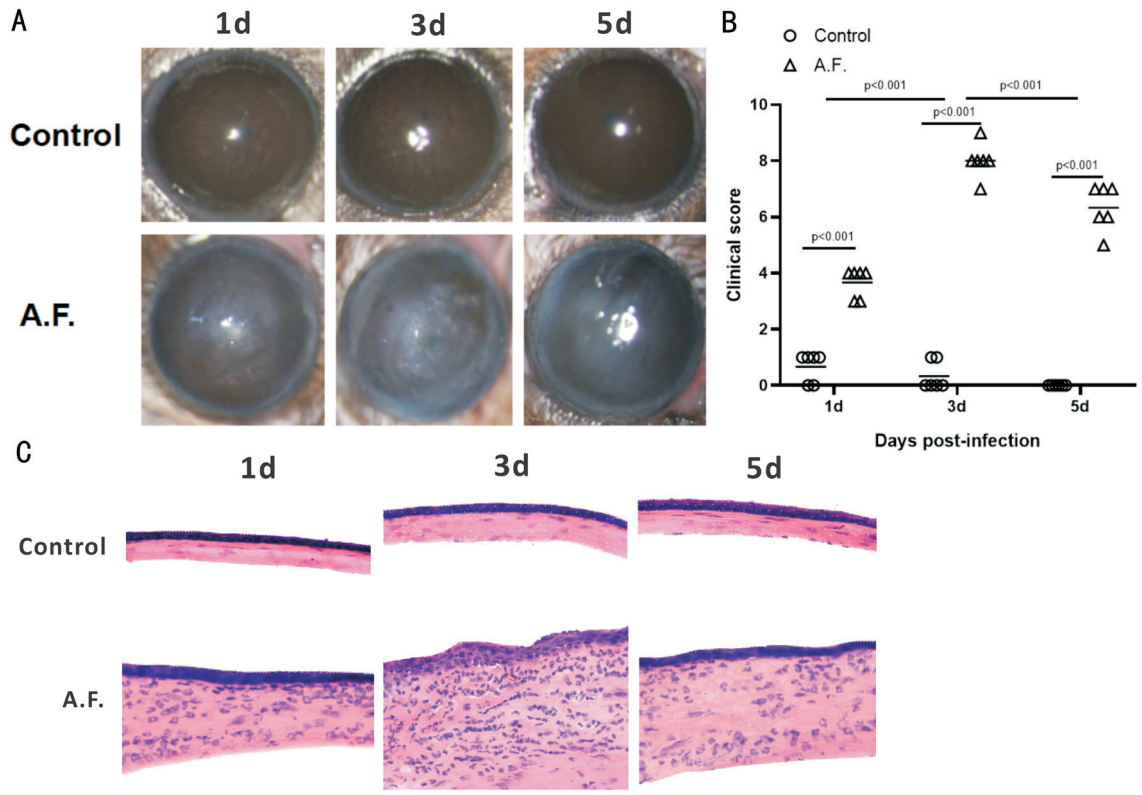

Figure 1 The development of *A. fumigatus* keratitis The photos and clinical score of murine corneas on the 1st, 3rd, 5th day. A: The photos were taken by slit lamp. B: Clinical score of infected corneas and the control corneas (mean±SEM). The peak appeared on the 3rd day after infection (P<0.001). C: The hematoxylin and eosin staining photos of murine corneas on the 1st, 3rd, 5th day (1:400).

development on the 1st, 3rd, 5th day (Figure 1A). Meanwhile, the clinical score of *A. fumigatus* keratitis were recorded (Figure 1B). The corneas of the control group had slight lesion on the 1st, 3rd day after establishing mouse model and recovered on the 5th day. The corneas of the A.F. group appeared different levels of keratopathy. Inflammatory infiltration and corneal edema could be found, and the keratopathy became the most severe on the 3rd day. The keratopathy began to recover, and neovascularization could be found at the corneal limbus on the 5th day without treatment. The clinical scores of the corneas of infection were higher than the corneas without infection significantly (P<0.001). The peak of clinical score of keratitis appeared on the 3rd day after infection (P<0.001).

Inflammatory Cytokines Expression in the Murine Cornea Infected by *A. Fumigatus* To explore whether inflammatory cytokines participate in infection process of *A. fumigatus* keratitis and how to express, the expression of some inflammatory cytokines in corneas by RT-PCR were measured. Results indicated that inflammatory cytokines including Foxp3, IFN-γ, IL-1β, IL-17, IL-23, TGF-β mRNA were measured in the control and infected cornea tissue. The inflammatory cytokines reached the peak in the corneas infected by *A. fumigatus* on the 3rd day and thereafter, began to drop significantly (Figure 2). The results indicated that the level of corneal inflammation was consistent with the expression level of pro-inflammatory cytokines, including IL-1β, IL-17, IL-23, and reached the peak on the 3rd day after infection. In addition, the level of IFN-γ, a cytokine that could activate neutrophils, reached the highest on the 3rd day of infection. Foxp3 reached the peak on the 3rd day when the infection was the most serious. Finally, the level of corneal inflammation in the infected corneas began to recover after 3d without treatment, and the level of corneal infection and various inflammatory cytokines began to decrease afterthat.

Effect of IDO on Mouse *A. Fumigatus* Keratitis To test the effect of IDO on *A. fumigatus* keratitis, the mice were pretreated with 1-MT before establishing *A. fumigatus* keratitis models. The photographs of four groups were taken and the clinical scores were recorded on the 3rd day when infection reached the peak (Figure 3). The keratopathy wasn't found in the corneas of the control group and 1-MT group. The corneas in the A.F. group showed inflammatory infiltration and corneal edema obviously. The corneas of 1-MT+A.F. group had the severer keratitis and higher clinical score, and corneal ulcer was more serious, and inflammation aggravated compared with the A.F. group (P<0.001). There wasn't significant difference in the other two groups.

Effect of IDO on Neutrophils Recruitment in *A. Fumigatus* Keratitis The eyeballs of mice in each group including the control group, the 1-MT group, the A.F. group, and

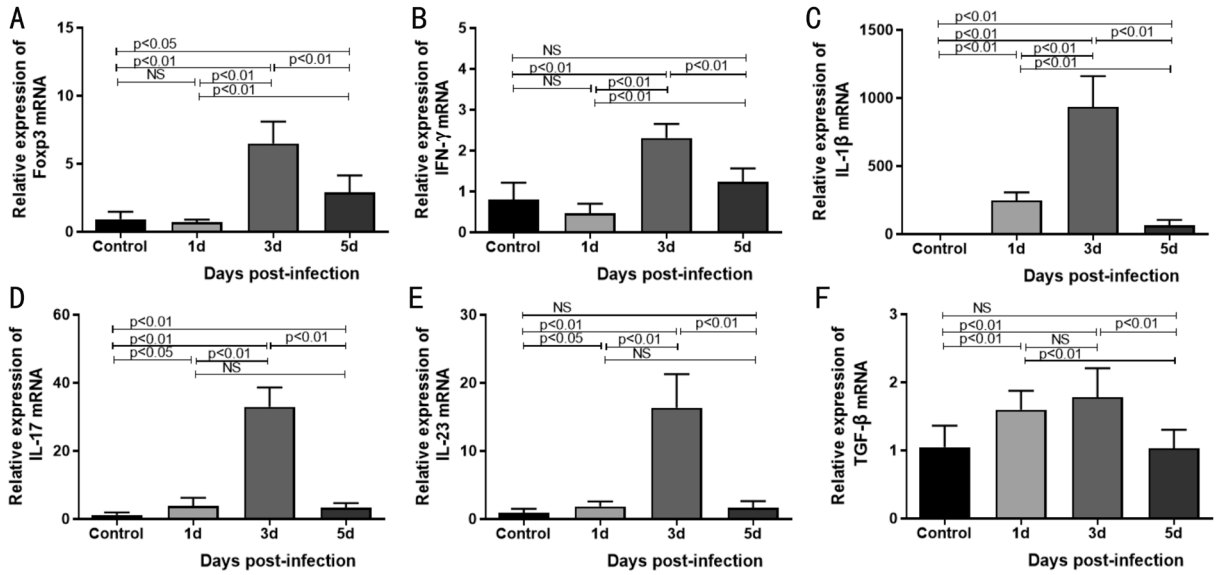

Figure 2 Inflammatory cytokines expression in the murine cornea infected by *A. fumigatus* Inflammatory cytokines expression reached the peak on the 3rd day (P<0.01). Foxp3 mRNA (A), IFN-γ mRNA (B), IL-1β mRNA (C), IL-17 mRNA (D), IL-23 mRNA (E), and TGF-β mRNA (F) in corneas of mice.

the 1-MT+A.F. group were collected on the 3rd day after infection. Immunofluorescence staining could detect the position and number of neutrophils in the corneas. Neutrophils were labeled with green fluorescence in the corneal tissue, and the results confirmed that there was small number of neutrophils in the corneas without infection. There was no significant increase between these two groups. The number of neutrophils increased significantly in the corneas after infection (P<0.001). On the 3rd day after infection, the number of neutrophils which were recruited to the corneas in the 1-MT+A.F. group increased, the degree of ulcer deepened, and the inflammatory response aggravated compared with the A.F. group. The number of recruited neutrophils in the corneas in each group were quantitatively counted under the fluorescence microscope at 400-fold field, and the results suggested that the number of neutrophils in the mouse corneas in the A.F. group was about 35-38, and the number of neutrophils in the 1-MT+A.F. group was about 54-56. The number of neutrophils in corneas of 1-MT+A.F. group was higher than A.F. group, and the difference was significant (P<0.01). Besides, neutrophils were observed in the stromal layer of the cornea and mainly concentrated in the area near the corneal ulcer (Figure 4A). MPO assay was used to quantitatively determine MPO in mature neutrophils and evaluated the recruitment and infiltration of neutrophils in mouse corneas. The recruitment and infiltration of neutrophils significantly increased in the corneas of infection compared with uninfected corneas (P<0.05). MPO of the 1-MT+A.F. group was significantly higher than the A.F. group. It was proved that 1-MT treatment could promote MPO activity of neutrophils in mice with *A. fumigatus* keratitis and the recruitment and infiltration of neutrophils in mouse corneas increased. Meanwhile, there was no significant difference between the control group and the 1-MT group (Figure 5).

IDO Blockade Influenced the Production of Neutrophils-Related Inflammatory Cytokine in Corneas Infected with *A. Fumigatus* In order to explore the effect of IDO on the neutrophils-related inflammatory cytokine production in *A. fumigatus* keratitis, the corneas of mice in each group on the 3rd day after infection were collected. It showed that the expressions of CXCL-1, ICAM-1, and IL-1β mRNA increased in the infected corneas in comparison to the uninfected group (P<0.05; Figure 6A-6C). The 1-MT which blocked out IDO, increased the production of these neutrophils-related inflammatory cytokines observably in comparison to the A.F. group (Figure 6A-6C). And there was no significant increase in the uninfected group. From the result of Figure 6D, we could find that the IL-8 mRNA in mouse corneas is down-regulated after infection in comparison to the uninfected group (P<0.05). Blockage of IDO further increased the production of IL-8 mRNA in comparison to the A.F. group (P<0.05). There wasn't significant increase in the 1-MT group and the control group, too.

DISCUSSION

FK is an intractable disease that will lead to severe visual impairment even blindness. A series of pathological keratopathy appear including corneal ulcer, a great number of inflammatory cells recruited to the site of ulceration with a large number of immune active substances which were released after corneal tissue stimulated by *A. fumigatus*[3-4]. The inflammation has protective function, but excessive inflammation may make disease more serious.

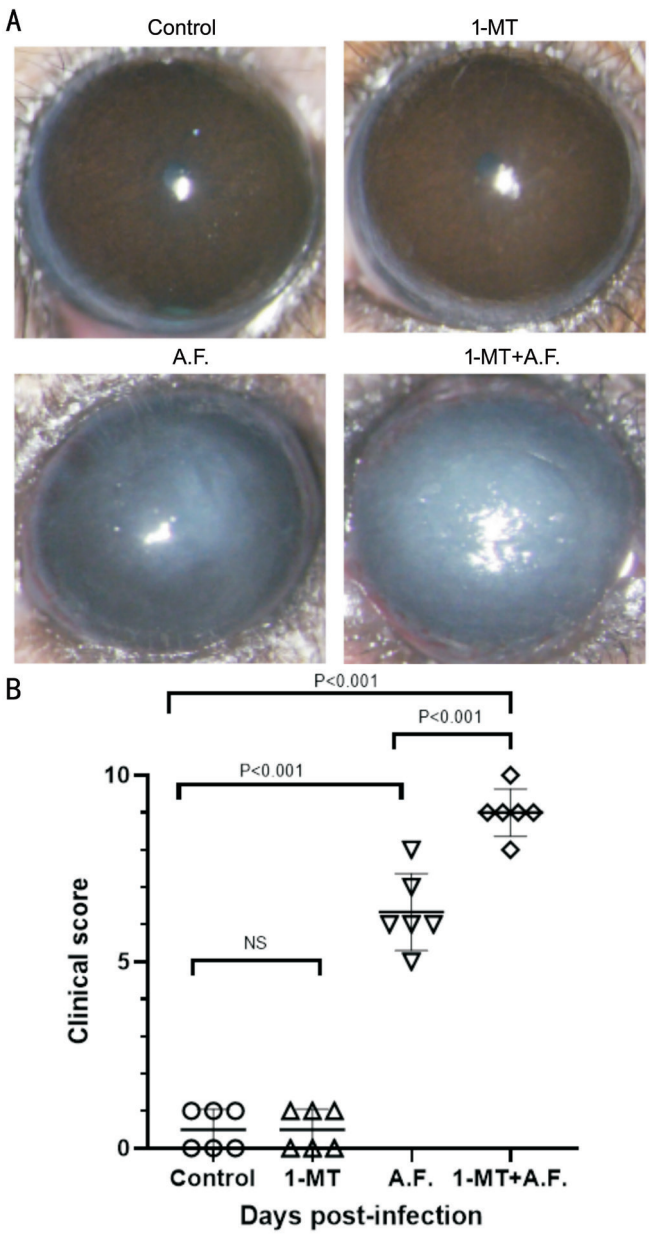

Figure 3 Effects of IDO in mouse *A. fumigatus* keratitis Photographs and the clinical score recorded on the 3rd day when the infection reached the peak. A: The photographs of murine corneas infected by *A. fumigatus* were taken by slit lamp; B: Clinical score of corneas infected by *A. fumigatus* and the control corneas (mean±SEM). The clinical score of 1-MT+A.F. group was higher than the A.F. group (P<0.001).

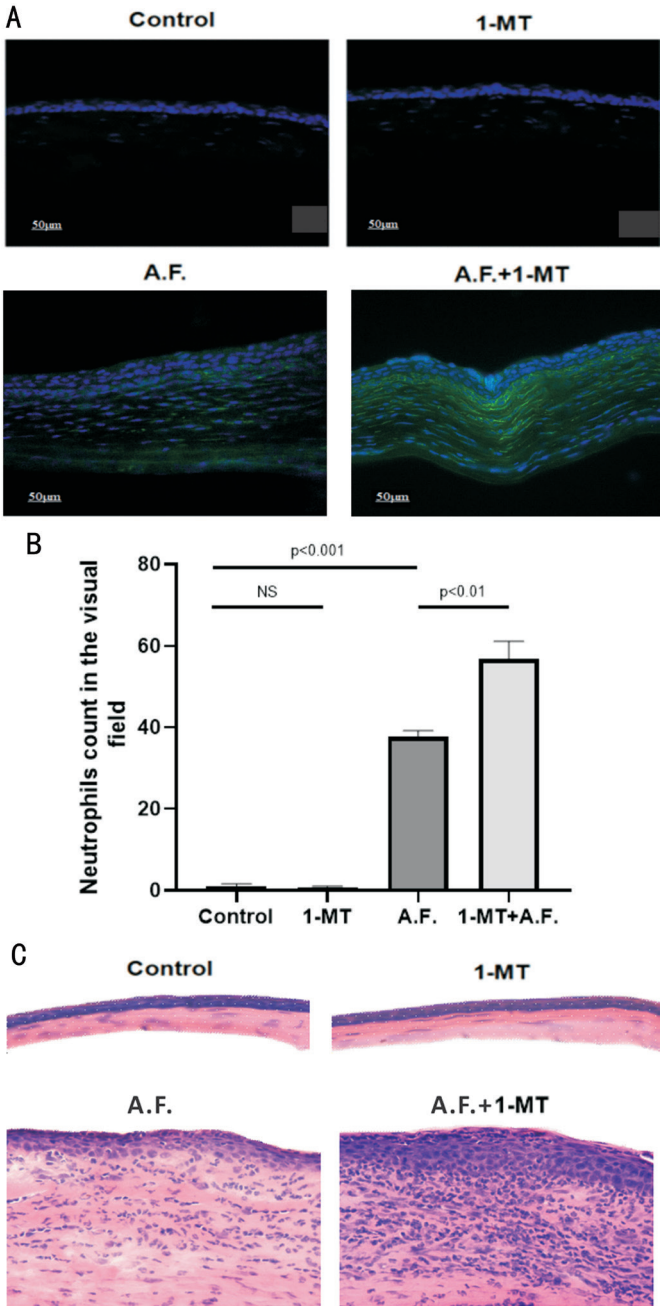

Figure 4 The neutrophils expression in mouse corneas A: Neutrophils (green) were dyed by the NIMP-R14-FITC, showing strong green fluorescence in the stromal layer in the A.F. group and the 1-MT+A.F. group. However, there was few neutrophils in the other two groups. Neutrophils in corneas of the 1-MT+A.F. group was increased obviously in comparison to the A.F. group. B: Neutrophils amount of C57BL/6 mice corneas. C: Hematoxylin and eosin staining of murine corneas (1:400). Scale bar in immunofluorescence images: 50 μm.

Inflammation, edema, and ulcers of corneas appeared after corneal infection by *A. fumigatus* and a range of inflammatory cells and cytokines changed at the same time. To identify the change of corneal inflammation, we set up the murine model of *A. fumigatus* keratitis. It could be informed that there were a large number of inflammatory cells on the corneas infected by *A. fumigatus*, and inflammatory cells secreted proinflammatory cytokines (IL-1β, IL-17, IL-23) on the 3rd day and reached to the highest clincal score of infection. In addition, the level of IFN-γ, a cytokine that can activate neutrophils, was the highest on the 3rd day after infection[15]. Foxp3, as a kind of T-cell-associated transcription factors which could be regulated by TGF-β, reached the peak on the 3rd day when the inflammation was the most severe[16].

IDO plays an important part in the immune regulation of infections, inflammation, autoimmune diseases. Previous study indicated that IDO was expressed in the mouse cornea, played an important part in the pathological process of *A. fumigatus*

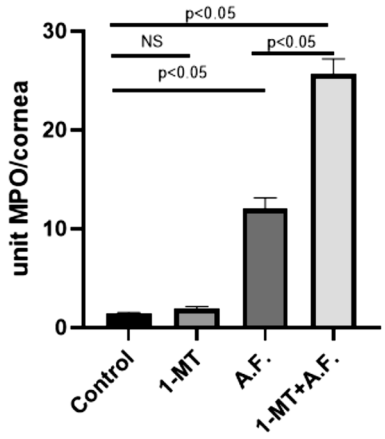

Figure 5 MPO quantitative analysis on the 3rd day MPO of the A.F. group and the 1-MT+A.F. group significantly increased in comparison to the control group and 1-MT group (P<0.05); MPO of infected mouse which was treated with 1-MT was significantly higher than infected group (P<0.05). There wasn't significant difference between control group and 1-MT group.

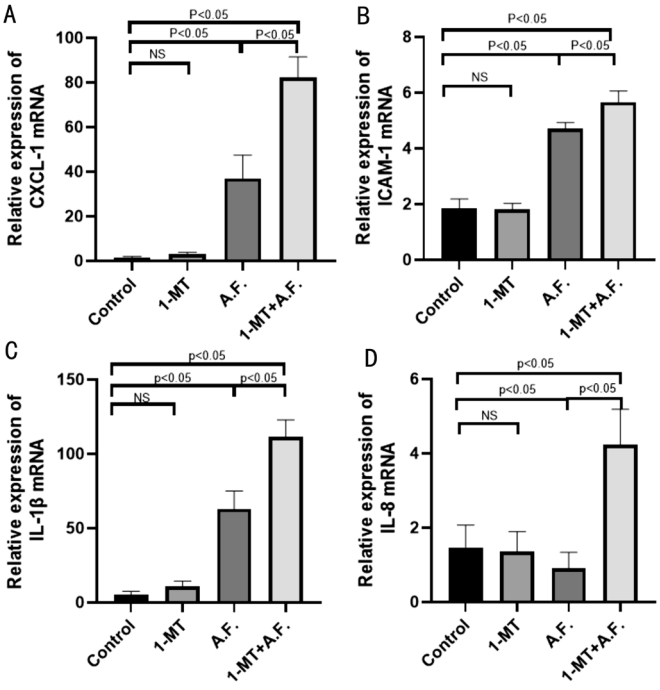

Figure 6 The relative expression of neutrophils-related inflammatory cytokine Contrasted with the two uninfected group, the mRNA expression of CXCL-1 (A), ICAM-1 (B), IL-1β (C) in the infected group increased significantly, and the IL-8 mRNA expression (D) in the A.F. group reduced contrasted with the two uninfected groups (P<0.05). The 1-MT up-regulated the expression of these cytokines in the infected corneas (P<0.05).

keratitis and participated in immunoregulation[17]. It was reported that IDO could be induced to control the process of inflammation in corneal epithelial cells of human which were infected by *A. fumigatus*[18]. Meanwhile, IDO could regulate macrophages function and inflammatory response in *A. fumigatus* keratitis[19].

Neutrophils, as the defense cells acting at the front line in the pathogenic process of FK, can quickly activate immune response and effectively eliminate pathogenic bacteria[20]. Neutrophils could remove to the infected position by chemotactic factors, which were produced by abundant cells, and could inhibit the growth of pathogene[21]. However, excessive neutrophils recriuted to the infected site will result in the release of inflammatory cytokines and proteases that led to the aggravation of corneal tissue damage and the formation of ulcers, which was not conducive to the elimination of fungi[5,21]. And in other studies, Loughman *et al*[11] found that the migration of neutrophils was be restrained by uropathogenicescherichiacoli (UPEC) in bacterial cystitis. In this process, IDO was elicited by UPEC, and mediated local kynurenines production increase, which reacted through the aryl hydrocarbon receptor, a ligand-activated transcription factor to impair neutrophils chemotaxis, and IDO could make effect on local tissues by neutrophils[12,22]. To clarify whether IDO could regulate the process of *A. fumigatus* keratitis by influencing neutrophils, we found IDO induced immune protection by inhibiting neutrophils-related inflammatory factors and suppressing neutrophils recruitment and chemotaxis in immune response against *A. fumigatus*.

Photographs and clinical scores of corneas showed the more serious inflammatory infiltration and corneal edema appeared after the inhibition of IDO, and it confirmed that IDO could alleviate the inflammation. Immunofluorescence staining confirmed that neutrophils recruitment increased in the stroma of cornea after infected by *A. fumigatus*. The recruitment of neutrophils was further upregulated in the corneas of infected mouse by being treated with 1-MT, and the result confirmed IDO could inhibit the recruitment of neutrophils into the corneas. MPO result suggested that IDO could recede neutrophils vitality in mice corneas and reduce the recruitment and infiltration of neutrophils, which were consistent with the results of immunofluorescence. Similarly, Liu *et al*[23] also showed that IDO overexpression restrained pulmonary neutrophils recruitment in pulmonary inflammation partially. In FK, IDO could inhibit the recruitment of neutrophils, and inhibit inflammation.

Chemokines could control the peripheral immune cells migration as chemotactic cytokines[24]. The recruitment and chemotaxis of neutrophils are an important early step in controlling tissue infections or injury after chemokine and cytokine stimulation, and the chemokines CXCL-1, IL-8, IL-1β and adhesion molecules ICAM-1 fulfill this role in this process[25-31]. Our result showed that blockage of IDO further increased these cytokines production in *A. fumigatus* keratitis and IDO inhibition could enhance the recruitment and chemotaxis of neutrophils by promoting the production of

neutrophils chemokines CXCL-1, IL-8, adhesion molecules ICAM-1 and inflammatory cytokines IL-1β. The experiment proved that IDO could reduce the inflammation and inhibit of the neutrophils recruitment and chemotaxis by reducing inflammatory cytokine expression. Hoshi *et al*[31] also found that IDO inhibited CXCL-1 production due to LPS-induced in cultured peritoneal cells

The increased expression of inflammatory cytokines promoted the recruitment and chemotaxis of neutrophils forward the infected tissue in order to get rid of pathogens fastly and efficiently. In *A. fumigatus* keratitis, IDO could promote inflammatory local metabolism and alleviate inflammation by inhibiting neutrophils recruitment and chemotaxis by reduction the release of inflammatory cytokines. Excessive inflammation is bad for tissue homeostasis, and further aggravates the inflammatory response. Therefore, the role of neutrophils regulated by IDO in inflammatory response is particularly important[32].

As an important chemokine of neutrophils, IL-8 expression is down-regulated during late inflammation to maintain the feedback regulation mechanism of inflammatory response of inflammatory cytokines release, promotes the healing of corneal tissue, and maintains the stability of inflammatory response and corneal tissue immune response. Dobosz *et al*[33] found that monocyte chemoattractant protein-1-induced protein-1 (MCPIP-1) could regulate the transcription of IL-8 and play an important role in maintaining immune homeostasis and preventing excessive inflammation in the epithelium under infection. When infection occurs, MCPIP-1 could control the inflammatory process, promote tissue repair and maintain tissue homeostasis by inhibiting the production of IL-8. It may be of great significance for IL-8 to maintain the feedback regulation of inflammatory response.

It's reported that IDO could generate broadly bioactive L-kynurenine metabolites which was the metabolites in the IDO pathway and led to impair neutrophils chemotaxis[11,32]. In another study, pathogen stimulated NF-κB and IDO, the produced L-kynurenine which could inhibit NF-κB, resulting in the decrease of CXCL-1 production[21]. IDO has an important role that IDO-mediated tryptophan degradation can reduce pathogens growth, meanwhile, IDO activation can reduce the availability of this essential amino acid under local tissue microenvironments, and induces breakdown of tryptophan and suppresses immune cell proliferation. Thus, IDO modulates immune cell function and thus regulates inflammatory process[34]. All in all, in our study, we observed that IDO played a critical part in immune protection in the infection of *A. fumigatus*.

In conclusion, our research demonstrated that IDO inhibition resulted in *A. fumigatus*-infected exacerbation. IDO plays a critical part in the whole infection process of *A. fumigatus* keratitis by inhibiting neutrophils recruitment and chemotaxis. Thus, it is a necessary mechanism for IDO to influence an immunoregulation by altering neutrophils function. Meanwhile, it's necessary to explore the specific mechanism of IDO regulates neutrophils, and the role of IDO in the prognosis of *A. fumigatus* keratitis needs to be studied more thoroughly.

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Conflicts of Interest: Guo SX, None; **Jiang N,** None; **Zhang L,** None; **Jiang W,** None; **Ma JJ,** None.

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