• Basic Research •

NADPH oxidase 2 plays a protective role in experimental *Aspergillus fumigatus* keratitis in mice through killing fungi and limiting the degree of inflammation

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Abstract

● **AIM:** To explore whether nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) is expressed in fungal keratitis in mice and investigate its role in this disease.

● **METHODS:** NOX2 expression was detected in C57BL/6 mice. After testing the inhibitory effect of diphenyleneiodonium chloride (DPI) on NOX2, its impact on clinical performance, myeloperoxidase levels, the number of colonies forming units, the level of H3, the generation of reactive oxygen species (ROS) and the release of cytokines [NF-κB, interleukin-17A (IL-17A), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), Nrf2, IL-10, and TGF-β] were compared. A one-way ANOVA and an unpaired, two-tailed Student's *t*-test was used to determine the statistical significance.

● **RESULTS:** NOX2 expression was significantly increased after *Aspergillus fumigatus* injection in corneas and that this increase could be reduced by treatment with DPI. DPI treatment produced more severe inflammation and resulted in higher clinical scores, more neutrophils infiltration, a weakened ability to clear fungi, the release of fewer ROS and the formation of neutrophil extracellular traps. Treatment with DPI increased the expression of the proinflammatory cytokines NF-κB, IL-17A, IL-6, and TNF-α and decreased the expression of the anti-inflammatory cytokines Nrf2, IL-10 and TGF-β compared to their expression levels without DPI treatment.

● **CONCLUSION:** NOX2 plays an important role against *Aspergillus fumigatus* in the mouse cornea through killing fungi and limiting the degree of inflammation.

● **KEYWORDS:** NOX2; cornea; *Aspergillus fumigatus*; mice
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INTRODUCTION

As a common blinding disease that mostly occurs in developing countries, fungal keratitis (FK) is difficult to diagnose and treat. Neutrophils play a critical role in the defense against invasive fungal infections[1], and fungal infection can cause many effects in neutrophils, including reactive oxygen species (ROS) production by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) system, the formation of neutrophil extracellular traps (NETs), and the release of bactericidal and fungicidal peptides[2]. NOX is the major origin of ROS in phagocytes. NOXs can transport electrons between the intracellular and extracellular environment, leading to the reduction of oxygen to superoxide[3]. NOXs play diverse roles in different organisms through redox signaling[4]. In the genetic disorder chronic granulomatous disease (CGD), in which NOX2 is defective, patients are hypersensitive to life-threatening bacterial and fungal infections due to their inability to kill invasive pathogens. In CGD patients, *Aspergillus fumigatus* (*A. fumigatus*) is the main etiological agent[5]. *A. fumigatus* is very susceptible to ROS, and phagocytes kill *A. fumigatus* by NOX-dependent mechanisms[6].

The role of NOX2 in *A. fumigatus* keratitis remains not well understood. The study showed that hyphae of *A. fumigatus* could activate neutrophil NOX through CD18 and NOX activation was essential for killing hyphae[7]. The effect of *NOX2* gene deficiency on immune function has been confirmed by many experiments, but the effect of exogenous interference with NOX2 in normal mice on fungal infection is still unknown. Thus, the current study explored the expression of NOX2, its role in immunity against *A. fumigatus* infection in the cornea of C57BL/6 mice and the signaling pathway used in this process. Our data showed that NOX2 expression was upregulated after *A. fumigatus* infection in mouse corneas.

Treatment with an inhibitor of NOX2, diphenyleneiodonium chloride (DPI), reduced NOX2 expression. Blocking NOX2 decreased clinical performance and resulted in the infiltration of more neutrophils, a decrease in the ability to clear fungi, the release of fewer ROS and the formation of NETs.

MATERIALS AND METHODS

Ethical Approval Eight-week-old female C57BL/6 mice were purchased from Jinan Pengyue Laboratory Animal Co., Ltd. (Jinan, China) and treated according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of the Affiliated Hospital of Qingdao University.

Mice and Fungal Culture The central corneal epithelium (3 mm diameter) was removed from the right eye, and a 5 μL aliquot of *A. fumigatus* was dropped onto the ocular surface. Then, the cornea was covered with a soft contact lens, and the eyelid was sutured. The control groups were treated by removing the central corneal epithelium without introducing fungal infection and covering the cornea with a soft contact lens. At 24h post infection (p.i.), the suture was removed. Before each cornea was harvested, its clinical score was recorded according to the standard for evaluation.

A. fumigatus (Number 3.0772, China General Microbiological Culture Collection Center, Beijing, China) was cultured at 29℃ for 5d. After shaking at 37℃ and 120 rpm for 3d in liquid medium, the hyphae were collected and cut into pieces 20-40 μm in length [1×10^8 colony forming units (CFU)/mL].

The inhibition of NOX2 was carried out by subconjunctivally injecting the experimental eyes with 5 μL DPI (1 μmol/L, Sigma Aldrich, Saint Louis, USA) 24h before infection with fungi. After infection, an additional 0.03 μg/100 μL of the DPI solution was immediately injected intraperitoneally (*i.p.*); control corneas were injected with dimethyl sulfoxide (DMSO) at the same concentration in phosphate buffered saline (PBS). After the sutures were removed, 5 μL DPI was subconjunctivally injected every day.

Isolation of Neutrophils C57BL/6 mice were injected *i.p.* with 1 mL 9% casein (Sigma, China) and again after 24h. Three-hours after the second injection, we began to collect neutrophils in the abdominal cavity by injection of 10 mL Dulbecco's modified Eagle's medium (DMEM; Gibco, San Diego, CA, USA). After centrifugation at 300× g for 10min, the neutrophils were collected and purified using 64% and 80% Percoll solutions. Then, the cells were cultured in DMEM with 10% fetal bovine serum (FBS; Gibco) for 2h and used for subsequent experiments. To stimulate *A. fumigatus*, the fungal hyphae were added at a final concentration of 5×10^6 CFU/mL. One micromolar of DPI was added 30min before the fungus, and 0.3 mg/mL laminarin (Solarbio, Beijing, China), 5 μmol/L PRT062608 (MedChemExpress, New Jersey, USA) and 0.5 μmol/L rottlerin (Tocris, Bristol, UK) were added 1h before the fungus to carry out the inhibition test. DMSO at the same concentrations was added to control samples.

Real-Time Reverse Transcription-Polymerase Chain Reaction Each cornea or cell was placed in a well in a 12-well plate and was considered to be a single sample (n=6/group/time). After the collection of each sample, the total RNA was isolated with RNAiso plus reagent (Takara, Dalian, China), and then the RNA was transformed to cDNA using the PrimeScript RT reagent kit with gDNA Eraser according to the manufacturer's instructions (Takara, Dalian, China). After dilution, a total reaction volume of 20 μL was used with SYBR Premix Ex Taq (Takara, Dalian, China). The cycling parameters were as follows: 95°C for 30s, followed by 40 cycles of 95°C for 5s, 60°C for 30s, and a final stage of 95°C for 15s, 60°C for 30s, and 95°C for 15s. Relative transcription levels were calculated by using the relative standard curve method, which compares the amount of the target normalized to β-actin. The primer sequences used in this study are listed in Table 1.

Western Blot Analysis Six corneas in each well in a 6-well plate were sampled for Western blot analysis. The samples were ground in radio immunoprecipitation assay (RIPA; Solarbio, Beijing, China) lysis buffer with 1 mmol/L phenylmethylsulfonyl fluoride (PMSF; Solarbio, Beijing, China) for 2h and then centrifuged, the supernatant was collected. The total protein was separated by 12% acrylamide SDS-polyacrylamide gelelectrophoresis (PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes (Solarbio, Beijing, China) and then blocked. The membranes were incubated with polyclonal antibodies against β-actin (1:6000; Bioss, Beijing, China), GADPH (1:7000; Elabscience, Wuhan, China), NOX2 (1 μg/mL; Abcam, Cambridge, UK) at 4°C overnight. Then, the membranes were incubated with the corresponding secondary antibodies (1:8000, Abcam, Cambridge, UK) at room temperature for 1h. Finally, the blots were developed by using chemiluminescence (ECL; Beyotime, Shanghai, China).

Enzyme-linked Immunosorbent Assay Each cornea (n=6/group/time) was homogenized in 500 μL PBS with 0.1% Tween 20 and a protease inhibitor cocktail (CWbiotech, Beijing, China). The neutrophil culture supernatants were collected 4h p.i., and 100 μL of each sample was assayed in duplicate to test for TNF-α and H3 according to the manufacturer's instructions (Elabcience, Wuhan, China).

Immunofluorescent Staining Eyeballs were embedded in optimal cutting temperature (OCT) compound (Sakura Tissue-Tek®, USA), frozen in liquid nitrogen and sliced into 10 μm sections. After fixation for 5min with acetone and blocking, the sections were incubated with NOX2 (20 μg/mL) and NIMP-R14 (1:100; Santa Cruz Biotechnology, CA, USA)

Table 1 Primer sequences

Gene	GenBank No.	Primer sequence (5'-3')
β-Actin	NM_007393.3	F-GAT TAC TGC TCT GGC TCC TAG C
		R-GAC TCA TCG TAC TCC TGC TTG C
NOX-2	NM_007807.4	F-TGA TCC TGC TGC CAG TGT GTC
		R-GTG AGG TTC CTG TCC AGT TGT CTT C
Nrf-2	NM_010902.4	F-CCC CAT TCA CAA AAG ACA AAC A
		R-GGC GGC GAC TTT ATT CTT ACC
IL-1β	NM_008361.3	F-CGC AGC AGC ACA TCA ACA AGA GC
		R-TGT CCT CAT CCT GGA AGG TCC ACG
TNF-α	NM_013693.2	F-ACC CTC ACA CTC AGA TCA TCT T
		R-GGT TGT CTT TGA GAT CCA TGC
NF-κB	NM_008337.3	F-GCT TTG CAA ACC TGG GAA TA
		R-TCC GCC TTC TGC TTG TAG AT
IL-6	NM_031168.1	F-CAC AAG TCC GGA GAG GAG AC
		R-CAG AAT TGC CAT TGC ACA AC
IL-10	NM_010548.2	F-TGC TAA CCG ACT CCT TAA TGC ACG AC
		R-CCT TGA TTT CTG GGC CAT GCT TCT C
IL-17*a*	NM_010552.3	F-TTT AAC TCC CTT GGC GCA AAA
		R-CTT TCC CTC CGC ATT GAC AC
TGF-β	NM_011577.1	F-AAC AAT TCC TGG CGT TAC CTT
		R-CTG CCG TAC AAC TCC AGT GA

at 4°C overnight. Then, samples were incubated with FITC-conjugated goat anti-rat secondary antibody (CWbiotech, Wuhan, China; 1:500) for 1h at room temperature. Digital images were captured with a Zeiss Axiovert microscope.

Myeloperoxidase Assay At 3d p.i., the corneas (n=6/group/time) were harvested and homogenized using an myeloperoxidase (MPO) test kit (Njjcbio, Nanjing, China) according to the manufacturer's instructions and then monitored by their absorbance at 460 nm. The results were reported as U/wet weight of cornea. One unit of MPO activity is equivalent to ~2×10^5 neutrophils.

Colony Forming Units Corneas (n=5/group/time) were collected 3d p.i. and ground evenly in 1 mL 0.9% stroke-physiological saline solution (NS). One hundred microliters of homogenate were painted onto the surface of Sabouraud dextrose agar and incubated at 29°C for 48h. The number of fungal colonies were counted manually. The log10 CFU/cornea values were used for statistical analysis.

Measurement of Reactive Oxygen Species The detection of ROS was carried out both *in vivo* in mouse corneas and *in vitro* in peritoneal neutrophils (n=6/group/time). Corneal ROS were measured with 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA; Njjcbio, Nanjing, China) by flow cytometry. After corneal cell suspensions were prepared with Liberase (Sigma Aldrich, Saint Louis, USA), they were incubated with 10 mmol/L DCFH-DA 1h at 37°C. The cells were collected and washed with PBS, and the DCFH-DA fluorescence was measured on a FITC channel (CytoFLEX, Beckman Coulter). Data were recorded with the use of CytExpert software as the 'P1 percentage' fluorescence variation. *In vitro*, ROS generation was detected in cultured 2h p.i. by incubation with 10 mmol/L DCFH-DA for 45min at 37°C.

Statistical Analysis All experiments were repeated once to ensure reproducibility, and data from each representative experiment are shown as the mean±standard error of the mean (SEM). A one-way ANOVA and an unpaired, two-tailed Student's t-test was used to determine the statistical significance. Data were considered significant at P<0.05.

RESULTS

NOX2 Expression in the Corneas of C57BL/6 Mice We detected the mRNA and protein expression of NOX2 by real-time reverse transcription-polymerase chain reaction, Western blot and immunofluorescent staining. The results shown in Figure 1A indicated that relative NOX2 mRNA levels were significantly higher in the *A. fumigatus*-infected corneas than those in the normal and control groups (Figure 1A; P<0.05, <0.01, <0.01 at 1, 3, and 5d p.i., respectively). As shown in Figure 1B, NOX2 protein levels were low in normal corneas, but after *A. fumigatus* infection, NOX2 expression was increased at 1, 3, and 5d p.i. As shown in Figure 1C, there was little positive staining for NOX2 in the normal corneas, while 3d p.i. there was stronger staining for NOX2 in the corneal epithelial cells, and some positively stained cells could have been neutrophils and macrophages in the stroma of the cornea.

Changes in Clinical Performance After the Inhibition of NOX2 Mice were treated with different concentrations of DPI to inhibit the expression of NOX2. As shown in Figure 2A, corneal NOX2 mRNA levels were significantly reduced following treatment with 1 μmol/L DPI (P<0.01). Western blotting also verified the inhibitory effect of DPI on NOX2 protein expression in corneas (Figure 2B). After verification of the inhibitory effect of DPI on NOX2, clinical performance was observed to detect the role of NOX2. As shown in Figure 2C, with DPI treatment, clinical performances were worse after *A. fumigatus* infection and marked by a larger ulcer area, more severe edema, and more neovascularization than those observed without infection. The analysis of the clinical scores is presented in Figure 2D (P<0.01, <0.05, <0.01 at 1, 3, and 5d p.i., respectively).

Effect of NOX2 on Neutrophils *In vivo* treatment with DPI increased the infiltration and intensity of neutrophil labeling with NIMP-R14 after *A. fumigatus* infection (Figure 3A). The expression of myeloperoxidase (MPO) in the corneas increased significantly 3d p.i., and with DPI treatment, its expression was enhanced (Figure 3B; P<0.01, <0.05, respectively). DPI treatment increased the number of CFUs counted (Figure 3C; P<0.05). Twenty-four hours after the corneas were infection with fungi, the concentration of H3 was increased, while after treatment with DPI, the concentration of H3 was decreased

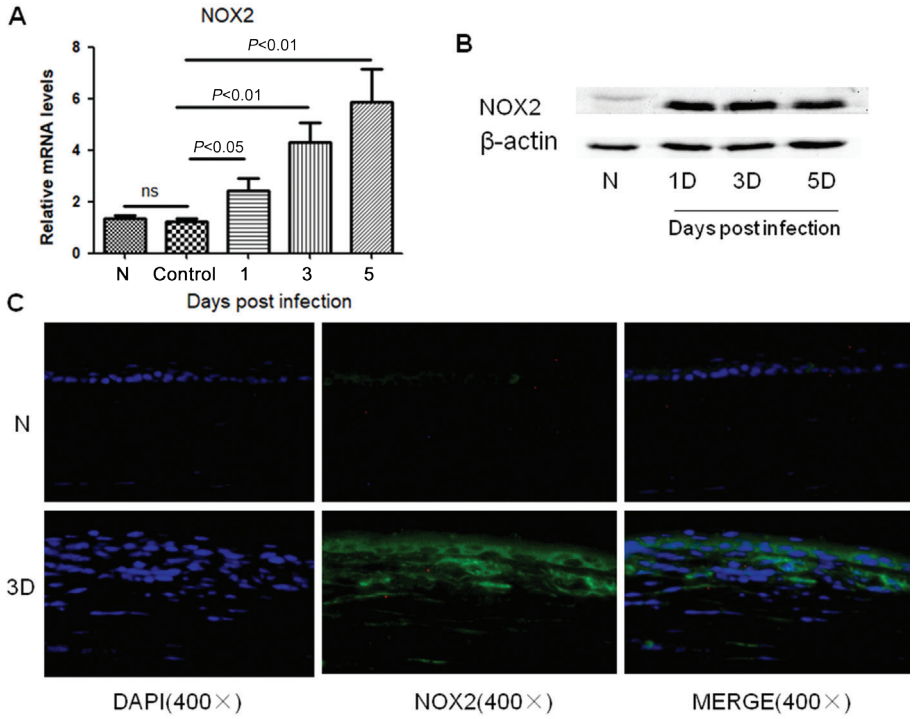

Figure 1 Expression of NOX2 in the normal and *A. fumigatus*-infected corneas of C57BL/6 mice A: The relative mRNA levels of NOX2 were significantly higher in the *A. fumigatus*-infected corneas than in the normal and control groups (P<0.05, <0.01, <0.01 at 1, 3, and 5d p.i., respectively); B: The NOX2 protein expression in the normal corneas was low, but 1, 3 and 5d p.i., it was obviously increased; C: Immunofluorescent staining showing normal and *A. fumigatus*-infected corneas 3d p.i. (400×).

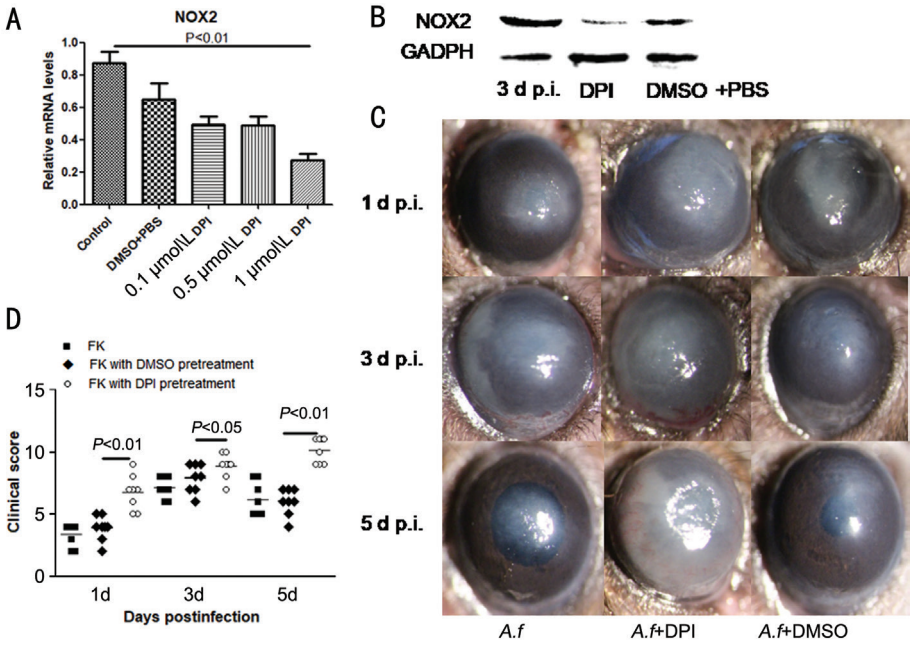

Figure 2 Changes in clinical performance after NOX2 inhibition A: NOX2 mRNA levels in corneas were significantly reduced by treatment with 1 μmol/L DPI (P<0.01); B: NOX2 protein levels in corneas were inhibited by DPI 3d p.i.; C: With DPI treatment, the corneas had a larger ulcer area, more severe edema and more neovascularization after *A. fumigatus* infection than those observed without DPI treatment; D: Analysis of clinical scores (P<0.01, <0.05, <0.01 at 1, 3, and 5d p.i., respectively).

(Figure 3D; P<0.01, <0.01, respectively). In neutrophils 4h p.i., the concentration of H3 in the culture medium increased significantly compared to that observed for normal cells, while following treatment with DPI, the H3 concentration decreased (Figure 3E; P<0.01, <0.05, respectively).

Effect of NOX2 on ROS Generation The corneas were collected 24h p.i. to detect ROS expression *in vivo* by flow cytometry. Compared with the *A. fumigatus*-infected group (43.98% ROS-positive) and the DMSO control group (47.04% ROS-positive), there were fewer ROS-positive cells (17.34%)

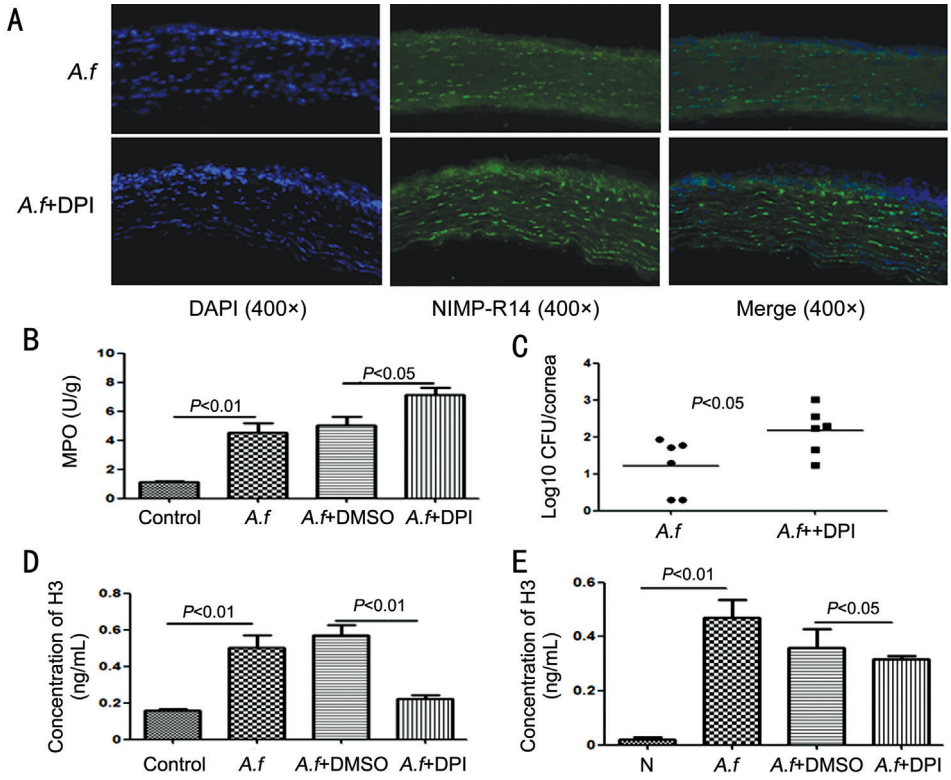

Figure 3 The effect of NOX2 on neutrophils A: Treatment with DPI increased the number of neutrophils labeled with NIMP-R14 after *A. fumigatus* infection compared to those observed without DPI treatment; B: The expression of MPO in the corneas increased significantly (P<0.01) 1d p.i., and with DPI treatment, its expression was enhanced compared with that observed in the control group (P<0.05); C: Treatment with DPI increased the number of CFUs (P<0.05) compared to those measured in samples without DPI treatment; D: Twenty-four hours after *A. fumigatus* infection of the corneas, the concentration of H3 increased (P<0.01), while after treated with DPI, the H3 concentration decreased (P<0.01); E: In neutrophils, the concentration of H3 in the culture medium increased significantly 4h p.i., while treatment with DPI decreased the concentration of H3 compared to that observed without DPI treatment (P<0.01, <0.05, respectively).

in the DPI treatment group (Figure 4A-4C), which showed that the inhibition of NOX2 by DPI suppressed ROS expression in mouse FK. Then, immunofluorescent staining of ROS in neutrophils was performed (Figure 4D).

Effect of NOX2 on Cytokines After *A. Fumigatus* Infection

Three days p.i., the corneas were collected to detect mRNA levels of the following cytokines: Nrf2, NF-κB, IL-17A, IL-6, IL-10 and TGF-β (Figure 5). Compared with their levels in the normal corneas, the relative mRNA levels of Nrf2, NF-κB, IL-17A, IL-6, IL-10, and TGF-β increased following fungal infection (P<0.05, <0.05, <0.05, <0.01, <0.05, <0.05, respectively). Treatment with DPI increased the mRNA levels of NF-κB, IL-17A, and IL-6 (P<0.05, <0.05, <0.05, respectively) and decreased the mRNA levels of Nrf2, IL-10 and TGF-β (P<0.05, <0.05, <0.01, respectively) compared to those observed in control corneas.

DISCUSSION

A small numbers of neutrophils exist in normal corneas, and upon fungal infection, the neutrophils rapidly enter into the site of inflammation from limbal blood vessels. Recent studies from our team regarding how neutrophils work were mainly focused on the role of various pathogen recognition receptors (PRRs) such as PAR-2 and LOX-1[8-9]. Less is known about the method by which neutrophils kill fungi. NOX is the main origin of ROS in phagocytes. The production of NOX-derived ROS, also called an oxidative burst, can mediate many biological functions[10-11]. ROS, as inflammatory mediators, have been studied in the cornea, and their levels in the corneal epithelium increase in dry eyes and are the etiology of alterations in the corneal epithelium[12].

As a critical member of the NOX family, NOX2 transports electrons across biological membranes and converts oxygen into superoxide to mediate antimicrobial reactions. CGD patients who have a genetic disorder that results in defects in NOX2 (or its associated subunits), showed impaired ROS production by phagocytes. They are highly susceptible to severe and invasive bacteria and fungi and endure excessive inflammatory reactions. The degree of NOX2 impairment is related to the severity of this disease. As the most common etiological agent of invasive aspergillosis, which is the leading cause of death for CGD patients, *A. fumigatus* is very sensitive to ROS and NOX2 defects[13]. After NOX2 knockout, the ability

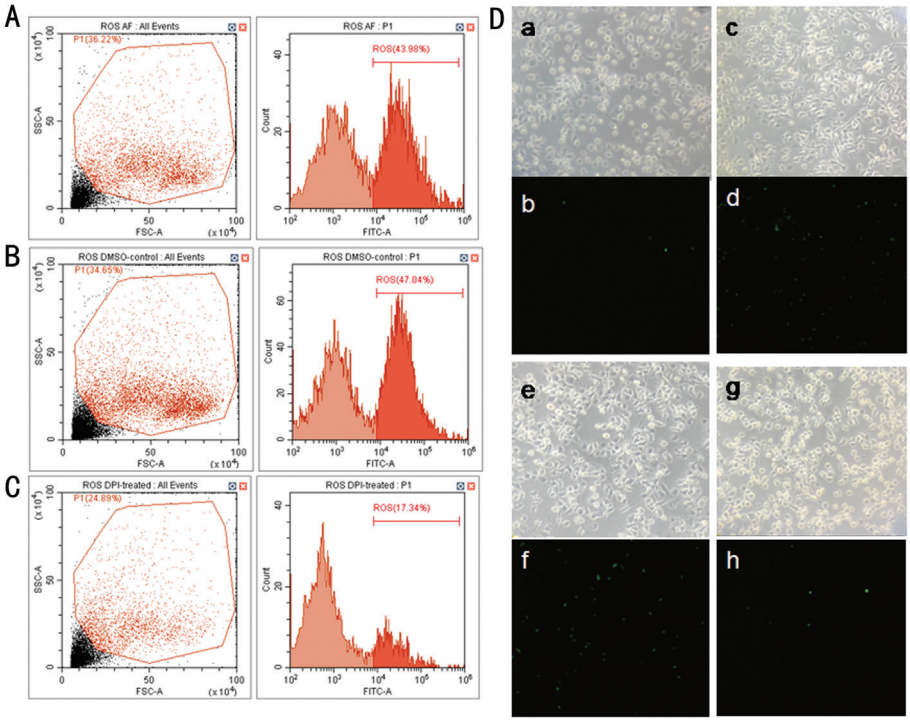

Figure 4 Effect of NOX2 on ROS generation Compared with the ROS-positive cells in the *A. fumigatus*-infected group (A; 43.98% ROS-positive) and the DMSO-treated control group (B; 47.04% ROS-positive), there was a lower percentage of ROS-positive cells (17.34%) in the DPI-treated group (C). ROS immunofluorescent staining in neutrophils. D: Normal cells, showing little staining (a, b); An increase in FITC staining at 2h after infection (c, d); At 2h after infection with DMSO treatment (e, f), showing staining similar to that in d; At 2h after infection with DPI treatment (g, h), showing less staining than that in d and f.

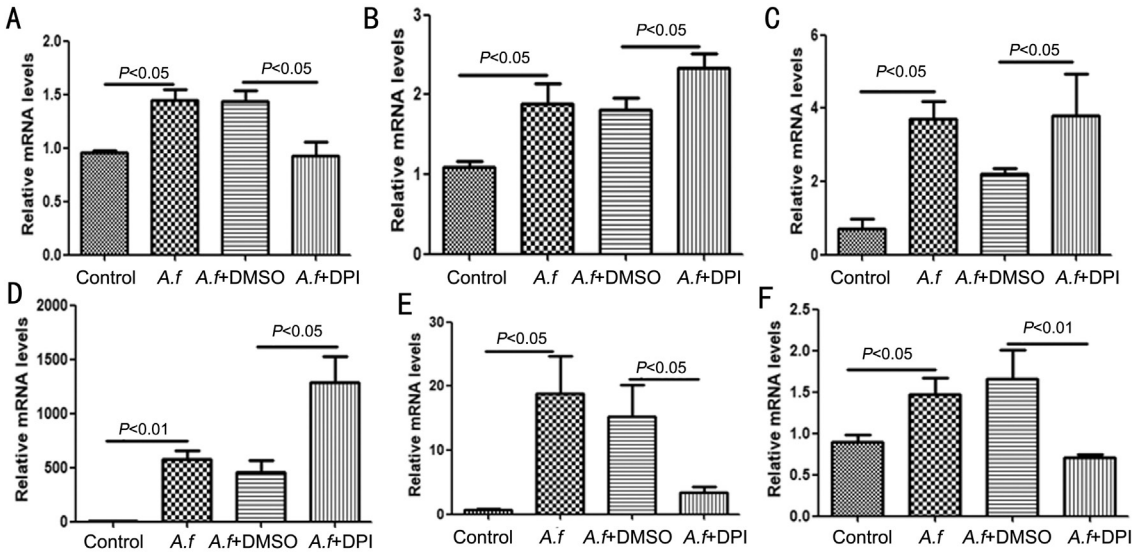

Figure 5 Effect of NOX2 on cytokines after *A. fumigatus* infection The relative mRNA levels of Nrf2, NF-κB, IL-17A, IL-6, IL-10 and TGF-β increased (P<0.05, <0.05, <0.05, <0.01, <0.05, <0.05, respectively) after fungal infection. Treatment with DPI increased the expression of NF-κB, IL-17A, and IL-6 (P<0.05, <0.05, <0.05, respectively) and decreased the expression of Nrf2, IL-10, and TGF-β (P<0.05, <0.05, <0.01, respectively).

of *A. fumigatus* hyphae to kill is obviously decreased. Studies have shown that NOX is expressed in corneal epithelial and stromal cells in both rabbits and humans[14-15]. In experimental alkali burn-induced corneal injury mouse models, NOX2 participated in the inflammatory and neovascular responses, and the inhibition of NOX2 by DPI effectively attenuated alkali burn-induced ROS production by regulating oxidative stress, inflammatory responses and choroidal neovascularization (CNV)[16-17]. To date, there has been no report on the role of NOX2 in keratitis, especially its role in FK. Therefore, we chose *A. fumigatus,* which was closely connecting to ROS and NOX2, to determine how NOX2 affects the process of FK in mice.

Similar to many other infectious diseases, NOX2 was significantly activated by fungal infection in corneas. DPI, an inhibitor of NOXs, reduced the activity of NOX2 and interdicted the production of ROS[18]. We suppressed NOX2 by DPI and found excessive inflammation with less ROS production both *in vivo* and *in vitro*. The clinical performances of NOX2-defective mice were more severe compared with those of control mice, and the mice did not show signs of improvement over time. More neutrophil infiltration was observed at the infection site in both the staining and MPO assays. The termination of neutrophil inflammation is necessary to inhibit excessive tissue damage. Although the direct effects of NOX2 activation such as ROS generation and protease activation appear to increase tissue damage, NOX2 activation can also limit the degree of acute inflammation by accelerating apoptosis and the clearance of neutrophils[19]. Impaired apoptosis and the increased recruitment of neutrophils together may lead to excessive inflammatory reactions in patients with CGD. NOX2-deficient neutrophils have impaired suppressive function (anti-inflammation)[20]. Similar to the results of our tests, NOX2-deficient mice showed strong pulmonary neutrophil inflammation and proinflammatory cytokine response stimulated by zymosan, and the inflammatory response became self-limited after transfection with NOX2[21]. The stimulation of *A. fumigatus* hyphae also resulted in strong and long-term pulmonary inflammation. Another new study showed that neutrophil infiltration in the intestinal mucosa led to severe epithelial injury and impacted wound healing, and MPO was an important regulator of this process[22], which inspired us to investigate the role of neutrophil infiltration in the repair of corneal epithelial defects in FK in our next experiments.

Neutrophils kill invasive fungi through ROS production, the formation of NETs and the release of fungicidal peptides. We found that their ability to clear fungi in corneas was weakened as more CFUs were observed after NOX2 inhibition and that the release of H3 was reduced both *in vivo* and *in vitro* compared to that measured without NOX2 inhibition. H3 normally resides in the nucleus. During the formation of NETs, H3 is released due to the rupture of the cellular and nuclear membranes. Therefore, the H3 concentration largely reflects the formation of NETs. The formation of NETs is now recognized as an important antifungal effector mechanism[23], and NOX is necessary for NET formation in the innate immune response against *Aspergillus* infection[24].

By challenging NOX2-deficient mice with zymosan, lung inflammation was limited by the attenuation of proinflammatory NF-κB and the activation of Nrf2, a key redox-sensitive anti-inflammatory regulatory transcription factor[25]. NOX2 was necessary for Nrf2 activity, and Nrf2 was reduced while NF-κB activity was enhanced after NOX2 was blocked [26]. NADPH oxidase ROS impact multiple pathways triggered in response to fungal cell walls and other inflammatory stimuli, including attenuating activation of NF-κB[27]. Our data were consistent with these tests and showed that Nrf2 decreased while NF-κB increased with DPI treatment. In addition, following DPI treatment, the expression of the proinflammatory cytokines IL-6 and TNF-α increased, while the expression of the anti-inflammatory cytokines IL-10 and TGF-β decreased, which was evidence that the inhibition of NOX2 resulted in an excessive inflammatory response. However, there NOX2 inhibition had a completely opposite effect on NF-κB. The use of a high dose of another NOX2 inhibitor, apocynin, to reduce ROS production suppressed the NF-κB pathway and subsequent proinflammatory cytokine transcription, attenuated lung injury and improved survival in a rat hemorrhagic shock model[28]. And in a mouse experimental autoimmune encephalomyelitis (EAE) model, Nox2 deficiency attenuates EAE-induced neural damage and reduces disease severity, pathogenic immune cells infiltration[29]. The discrepancy between these results may be caused by the different disease models used and may be explained by the distinction between infectious and noninfectious inflammation.

NOX2 also participates in adaptive immunity by regulating the differentiation of T cell subtypes. After NOX2 knockout, tryptophan catabolism was impaired, which led to Th17 upregulation, defective Treg activity and the dominant production of IL-17 in acute inflammatory lung injury[30] .We detected IL-17A levels and found that DPI treatment increased IL-17A expression in corneas and neutrophils. NOX2 may also limit inflammation by inhibiting the Th17 response. Next, we plan to test the differentiation of Th17/Treg cells by flow cytometry.

In summary, the data presented herein indicated that increased NOX2 levels in mouse corneas infected by *A. fumigatus* were inhibited by DPI treatment. The inhibition of NOX2 resulted in an excessive inflammatory response with increased neutrophil infiltration and decreased fungal clearance than that observed in control cells. Fewer ROS were produced, fewer NETs were formed. With DPI treatment, the expression of proinflammatory cytokines increased while the expression of anti-inflammatory cytokines decreased. These data suggest that NOX2 plays an important role in FK. Further studies are needed to investigate the role of NOX2 and its pathway in the pathogenesis of FK.

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Authors' contributions: Zhao GQ conceived of the research and participated in its design and coordination. Xu Q carried out the whole experiments and drafted the manuscript. Wang Q and Li C participated in the design of the research. Hu LT

and Lin J performed the statistical analysis. Jiang N helped preparing *A. fumigatus* antigen. Peng XD helped taking mice experiment. All authors read and approved the final manuscript.

Conflicts of Interest: Xu Q, None; **Wang Q,** None; **Hu LT,** None; **Lin J,** None; **Jiang N,** None; **Peng XD,** None; **Li C,** None; **Zhao GQ,** None.

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