·Basic Research·

Effects of retinoic acid receptor–γ on the *Aspergillus fumigatus* induced innate immunity response in human corneal epithelial cells

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Abstract

• AIM: To explore the effects of retinoic acid receptor–γ (RARγ) on innate immune responses against *Aspergillus fumigatus* (*A. fumigatus*) in cultured human corneal epithelial cells (HCECs).

• METHODS: The HCECs were stimulated with *A. fumigatus* hyphae for 0, 2, 4, 8, 12 and 16h. RARγ mRNA and protein levels were tested by quantitative real–time polymerase chain reaction (qRT–PCR) and Western blot. Then HCECs were pretreated with or without BMS961 (RARγ agonist, 1 μg/mL). The mRNA and protein expression of Dectin –1 and the downstream cytokines (TNF–α and IL–6) were determined by qRT–PCR, Western blot and enzyme–linked immunosorbent assay (ELISA).

• RESULTS: The expression of RARγ was upregulated after stimulation with *A. fumigatus*. RARγ mRNA began to rise at 4h and peaked at 8h (*P*<0.001). The protein of RARγ reached to the peak at 16h (*P*<0.001). Pretreated with BMS961 before *A. fumigatus* hyphae stimulation, expression of Dectin –1, TNF –α and IL –6 decreased dramatically at mRNA and protein levels.

• CONCLUSION: HCECs can express RARγ and *A. fumigatus* hyphae infection can increase RARγ expression. BMS961 can inhibit the expression of Dectin–1 and pro –inflammatory cytokines, and play an anti –inflammatory role in innate immune responses against *A. fumigatus*.

• KEYWORDS: retinoic acid receptor-γ; innate immunity; *Aspergillus fumigatus*; corneal epithelium

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INTRODUCTION

F ungal keratitis (FK) is one of the most severe corneal diseases with high rate of blindness and visual impairment [1]. *Aspergillus* (*A. fumigatus*) and *Fusarium* (*F. oxysporum*) species are the main pathogenic fungus of FK. *A. fumigatus* is an opportunistic fungal pathogen abundant in external environments, causing fungal infection in immune suppressed individuals and exacerbating the inflammatory response[2-3]. Fungal virulence factors and host defense factors all play paramount roles in the occurrence of FK[4-5].

As the first line of defense against infection, the innate immune system is responsible for responding and recognizing and eliminating invading fungal pathogens [6]. Dectin-1, as an important member of C-type lectin super family, can recognize β-glucans in fungal cell wall. It is a major pattern recognition receptor (PRR) involved in antifungal immune responses [7]. The inflammatory response is necessary for corneal epithelial cells to initiate host defense, kill and clear the invading pathogens [8]. Appropriate inflammation will stimulate efficient host defense responses to kill pathogen, while excessive inflammation will lead to significant corneal damage such as stromal destruction and vision loss [9-10]. Ocular immune privilege is necessary for preventing or modifying innate immune responses occurring in corneal diseases in order to protect host from excessive damage [6]. Retinoic acid receptor-γ (RARγ) is one of the nuclear retinoic acid receptors, though binding to all trans-retinoic acid (atRA) to exert extensive and complex biological function. Several *in vitro* studies have shed light into the role of vitamin A not only as an important factor for normal immune system development, but also as a modulator of both the innate and the adaptive immune responses[11-12].

In this study, we investigated the expression and the regulation of RARγ in innate immune response of human corneal epithelial cells (HCECs) infected by *A. fumigatus* and the possible mechanisms involved in this process. HCECs were pretreated with RARγ agonists (BMS961), the expressions of inflammatory factors (TNF-α and IL-6) were monitored by quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) methods in order to determine the inhibition role of RARγ and to find its relationship with Dectn-1 in FK.

MATERIALS AND METHODS

Preparation of *Aspergillus fumigatus* Hyphae The standard *A. fumigatus* strains (NO3.0772) were bought from China General Microbiological Culture Collection Center (CGMCC) and grown in Sabouroud medium (Babio biotech, Jinan, China) at 28 ℃ for 5-7d. Then the conidia were inoculated to liquid medium at 37℃ for 3-4d. Then hyphae were grinded to the size of 20-40 μm fragment, washed twice by sterile phosphate buffer saline (PBS) and inactivated by 70% ethanol at 4℃ for 12h. Inactive *A. fumigatus* hyphae was washed for 3 times and added in PBS. The hyphae suspension was quantified using a hemacytometer, and saved at -20℃ [13].

Human Corneal Epithelial Cells Culture and Stimulation HCECs (provided by Sun Yat-sen University) were cultured and maintained in HCECs growth medium in a humidified 5% CO_2 incubator at 37 ℃. HCECs growth medium contains 1:1 DMEM/HamF-12 supplemented with 5% fetal bovine serum (FBS), 10 ng/mL human epidermal growth factor (EGF), 5 mg/mL insulin, and 50 mg/mL penicillin and streptomycin [14-15]. For stimulation, HCECs were treated with *A. fumigatus* hyphae (5×10^7 /mL) in different times. And HCECs pretreated with or without BMS961 (1 μg/mL) (Tocris Bioscience, UK) for 0.5h were stimulated by *A. fumigatus* hyphae. Total RNA, protein and supernatant were collected for qRT-PCR, Western blot and ELISA. BMS961 was dissolved in DMSO, preliminary experiments showed no obvious difference between the DMSO group and normal group[16].

RNA Isolation and Quantitative Real-time Polymerase Chain Reaction Assay Total RNA from HCECs were extracted, quantified by its absorption at 260 nm and stored at -80℃ according to the manufacturer's protocol. The total RNA extracted were reverse transcribed and conducted qRT-PCR by using SYBR at the same time. The thermocycler parameters were 95℃ for 30s, and followed by 40 cycles of 95℃ for 5s and 60℃ for 30s. The results of qRT-PCR were analyzed by the comparative threshold cycle method. The cycle threshold (Ct) was determined using the cycle at which the primary (fluorescent) signal crossed a user-defined threshold. Quantification was normalized by the Ct value of β-actin by using the $2^{-\Delta\Delta Ct}$ formula. The double-stranded probes used are as follow: TGGACGAAGATGGATATA (forward) and CCAAGCAC AGGATTCCTA (reverse) for Dectin-1 (human); TGCTTGT TCCTCAGCCTCTT (forward) and CAGAGGGCTGATTA GAGAGAGGT (reverse) for TNF-α (human); AAGCCAGA GCTGTGCAGATGAGTA (forward) and TGTCCTGCAGC CACTGGTTC (reverse) for IL-6 (human); TGGCACCCAG CACAATGAA (forward) and CTAAGTCATAGTCCGCCT AGAAGCA (reverse) for β-actin (human) as housekeeping gene[17].

Western Blot Analysis HCECs were lysed with RIPA containing 1 mmol/L PMSF (100:1) (Solarbio, Beijing, China) for 1h and then were centrifuged. After estimation of protein content, addition of sodium dodecyl sulfonate (SDS) sample buffer, and boiling, total protein was separated on 10% acrylamide sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% bovine serum albumin (BSA) liquid, and then were incubated with primary antibodies of RARγ (1:2000) (Abcam, Cambridge, MA, USA), Dectin-1 (1:1000) (Cell Signaling Technology, Danvers, MA, USA), and β-actin (1:2000) (Beyotime, Shanghai, China) at 4℃ overnight. After washed in phosphate buffer saline with Tween-20 for three times, the membranes were incubated with corresponding peroxidase-conjugated secondary antibodies (1:2000) (Santa Cruz Biotechnology, San Jose, CA, USA) at 37℃ for 1h. Then the blots were developed using chemiluminescence (ECL; Thermo Scientific).

Enzyme-linked Immunosorbent Assay Double-sandwich ELISA for human TNF-α and IL-6 were performed, according to the manufacturer's protocol (Biolegend company, San Diego, CA, USA), to determine the protein concentration of different treatments. Absorbance was read at 450 nm with a reference wavelength of 570 nm by a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis All data were represented as mean ± SEM of three independent experiments performed in triplicate. Statistical comparison was made by one-way analysis of variance (ANOVA) using SPSS 18.0 software with significance set at $P < 0.05$.

RESULTS

Retinoic Acid Receptor -γ Expression Increased in Human Corneal Epithelial Cells Incubated with *Aspergillus fumigatus* To determine the expression of RARγ and identify its potential role in corneal epithelial cells during *A. fumigatus* infection, we treated the cells with *A. fumigatus* hyphae (5×10^7/mL) in 12-well. As shown in Figure 1A, the expression of RARγ mRNA in HCECs with *A. Fumigatus* hyphae significantly increased at 4h ($P < 0.05$), and in a time-depended manner, and reached peak at 8h ($P < 0.001$). Western blot analysis showed that RARγ protein began to rise at 12h ($P < 0.01$; Figure 1B) and peaked at 16h ($P < 0.001$; Figure 1B).

BMS961 Modulates the Dectin-1 Expression Induced by *Aspergillus fumigatus* in Human Corneal Epithelial Cells In order to know whether Dectin-1 expression are modulated in response to the activation of RARγ. HCECs were pretreated with or without BMS961 (1 μg/mL) for 30min, before incubated with hyphae for 8h. The mRNA and protein levels of Dectin-1 were measured by qRT-PCR and Western blot respectively. The mRNA expression of Dectin-1 were increased in HCECs after hyphae stimulation for 8h, but
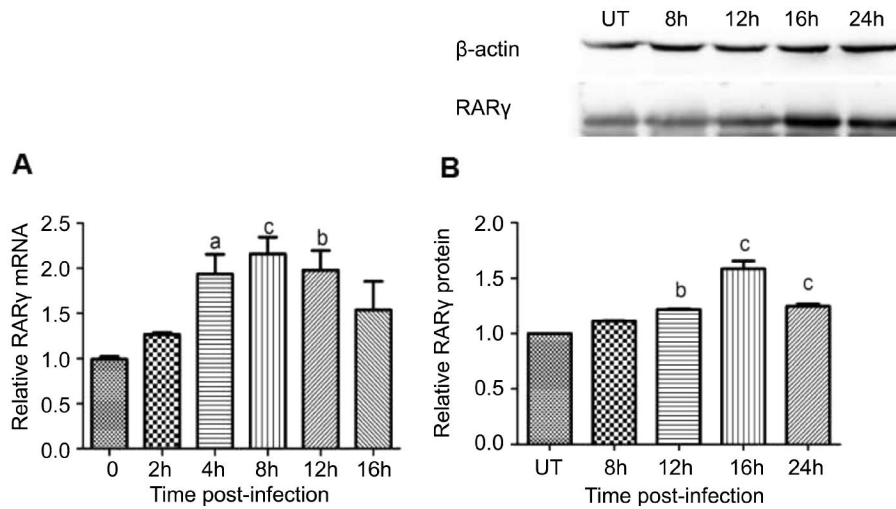
Figure 1 RARγ expression in HCECs infected with *A. fumigatus* A: The mRNA expression of RARγ induced by *A. fumigatus* (5× 10^7/mL) in HCECs were evaluated at 2, 4, 8, 12 and 16h, which compared with untreated normal HCECs; B: RARγ protein expression was elevated in infected HCECs compared with the control at 8, 12, 16 and 24h. [a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$ compared with normal control.
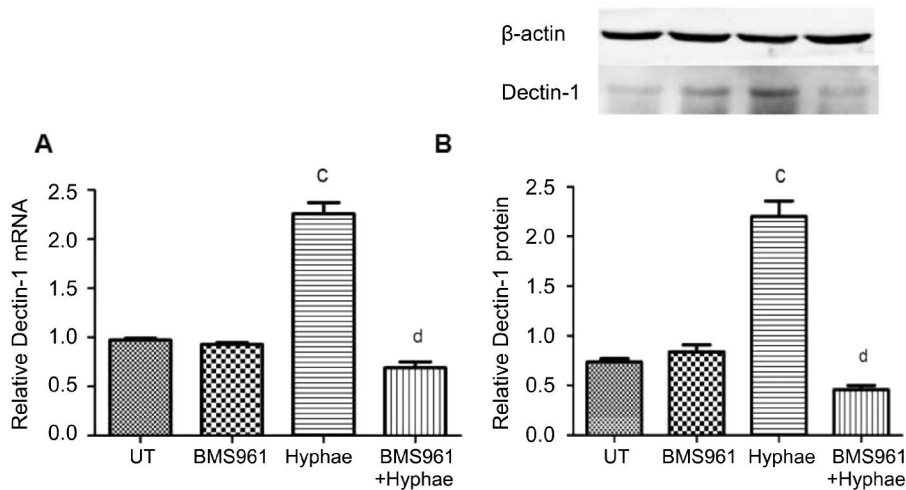

Figure 2 Modulation of the Dectin–1 expression by BMS961 upon *A. fumigatus* infection The HCECs were cultured without or with BMS961 1 μg/mL for 0.5h. Then the cells were stimulated with hyphae (5×10^7/mL) for 8 or 16h. The mRNA and protein levels of Dectin-1 were assayed by qRT-PCR (A) and Western blot (B). [c]$P<0.001$ compared with untreated controls; [d]$P<0.001$ compared with HCECs stimulated by hyphae without BMS961 pretreatment.

the levels were down-regulated by BMS961 pretreatment ($P<0.001$; Figure 2A). Similarly, compared with HCECs exposed to hyphae, the protein levels of Dectin-1 were also inhibited by BMS961 pretreatment ($P<0.001$; Figure 2B).

BMS961 Pretreatment Inhibited Pro –inflammatory Cytokines Production Induced by *Aspergillus fumigatus* To investigate whether RARγ can modulate the innate immune response to *A. fumigatus* in HCECs, HCECs were pretreated with BMS961 (1 μg/mL) for 30min, followed by hyphae for 8h or 16h. The mRNA and protein levels of TNF-α and IL-6 were measured by qRT-PCR and ELISA, respectively. Figure 3 showed that the expressions of TNF-α and IL-6 increased at 8h post-infection with *A. fumigatus* in HCECs compared with the control group. Agitated of RARγ by BMS961 suppressed the production of these cytokines compared with the infection group ($P<0.01$; Figure 3A). Meanwhile, the expressions of TNF-α and IL-6 in the

supernatant were also down-regulated, which were consistent with the change of mRNA levels ($P<0.01$; Figure 3B).

DISCUSSION

FK is a major public health concern in China. Fungal infection of cornea has been become a health threat and clinical problem due to the lack of broad-spectrum, low toxicity and effective anti-fungal drugs, shortages of corneal donor, recurrence and even complications of operation. Along with the development of immunology, anti-fungal immunity, thus growing increasingly important, is started by innate immune receptors to recognize the fungal pathogens[18]. As the first line of defense microbial infection, innate immune response inhibits the reproduction and invasion of fungi in the host through immune cells and immune mechanisms which constitute important components for resisting fungal infection in cornea [19]. Although alveolar macrophages and neutrophils are important for killing
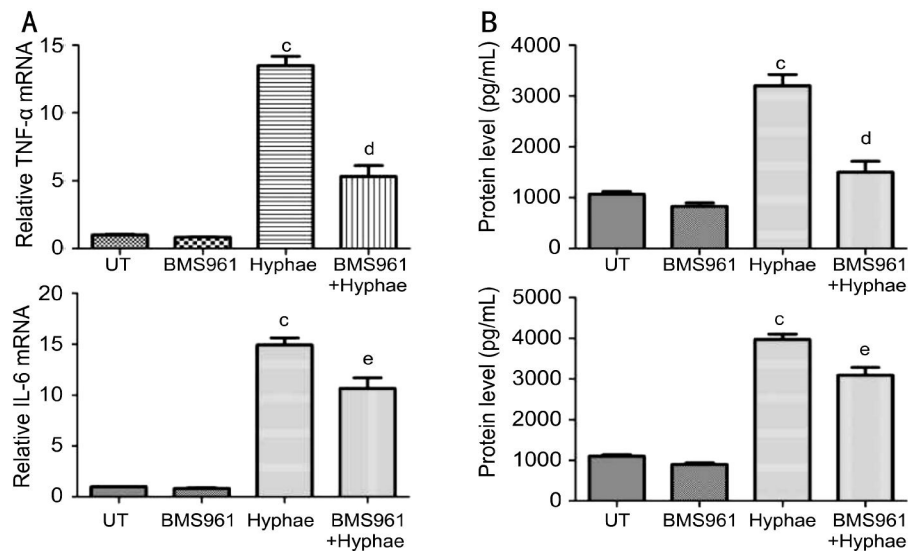
Figure 3 BMS961 inhibited the production of TNF−α and IL−6 induced by *A. fumigatus* in HCECs The HCECs were cultured without or with BMS961 1 μg/mL for 0.5h. After being washed twice with serum-free medium, the cells were stimulated with hyphae (5×10^7/mL) for 8 or 16h. The mRNA and protein levels in supernatant of TNF-α and IL-6 were assayed by qRT-PCR (A) and ELISA (B). $^{c}P<0.001$ compared with untreated controls; $^{e}P<0.01$, $^{d}P<0.001$ compared with HCECs stimulated by hyphae only.

dormant or germinating conidia and hyphae, monocytes, natural killer cells, NKT cells, plasmacytoid dendritic cells, and eosinophils may also critical for providing early protection [20-23]. Although the research of immunomodulatory effects of RARγ becomes more and more interesting, there's no conclusive evidence to prove that RARγ could influence the innate immune response during FK. Previous studied had shown that atRA plays a key role in immune regulation, it can promote T, B cell proliferation, differentiation and maturation, adjust T lymphocyte apoptosis, promote the generation of immune globulin and subtype transformation[24-25]. The inhibition and protection role of atRA in immune mediated inflammatory reaction have been confirmed by autoimmune reactivity disease amimal model[26]. According to this, we wonder if RARγ plays a modulatory role in innate immune response of FK.

In our present study, our data demonstrated that RARγ was stably expressed in HCECs, and its expression significantly increased after fungal infection. By using *A. fumigatus* hyphae to incubate with HCECs, we confirmed that RARγ had higher expression in mRNA and protein levels. In fact, RARγ had been proven to be expressed in the cornea[27]. The elevated expression can explain it plays a critical role in FK. The effect of RARγ on the FK could be according to combine with atRA to exert the biological effect, to regulate the innate immunity and to affect other immune receptors.

To further explore the influence of RARγ for immune response effect of *A. fumigatus* infection in corneal epithelial cells, we used the RARγ agonist BMS961 to observe the changes of Dectin-1 and pro-inflammatory cytokines. Interestingly, in our study, pretreatment with BMS961 led to an almost full abrogation of Dectin-1 mediated expression and secretion of TNF-α and IL-6 in

HCECs. It would be interesting to investigate whether the higher impact of BMS961 observed in our study could be related to the nature of the immunological challenge. This is consistent with reports on other cell types that RARs signaling is critical for enhanced Raldh2 expression. Raldh2 suppresses pro-inflammatory cytokines in DCs *via* induction of Socs3, a well known regulatory of pro-inflammatory responses [28-31]. In our previous study, Dectin-1 plays an important role in defending fungal infection. It can cause a series cellular responses, including respiratory burst, ligand endocytosis and phagocytosis, maturation of dendritic cells, generation of cytokine and chemokine (including TNF-α, IL-6, IL-10, IL-2, GM-CSF, G-CSF). Studies have shown that Dectin-1 was up-regulated in fungal infected corneas, and infected Dectin-1^{-/-} corneas have diminished cellular infiltration and fungal clearance compared with control mice[4]. Under these conditions, the modulatory effect of RARγ resembled the one observed in experiments performed with *A. fumigatus* Our data provide evidence that RARγ had its ability to regulate Dectin-1 expression and activation and had the possible mechanism for the anti-inflammatory effect. Other mechanisms may contribute to the anti-inflammatory effect of RARγ, due to Manicassamy *et al*[32] found that RA signaling attenuates activation of p38 MAPK.

As we all know, corneal inflammatory reaction is like a double-edged sword. Appropriate inflammation will stimulate effective host defense responses and don't bring obvious tissue damage, while excessive inflammation will seriously destroy visual function [10]. From the host's standpoint, a "balanced" response could ensure immune defense, without collateral damage caused by excessive inflammation. Therefore, proper inflammatory response is the key to removing fungi inflammation, once excessive,

inflammation will seriously destroy visual function [10]. In the early stage of the inflammatory response, it is necessary to increase the pro-inflammatory cytokines in order to initiate the inflammatory response. However, especially in the later stages of the inflammatory response, down-regulation of inflammation can reduce corneal tissue damage. When excessive inflammatory reaction occurs, timely appropriate use of immunosuppressive drugs can reduce the damage and the scar formation and can be beneficial to the future healed[33].

In conclusion, RARγ played an indispensible role in FK to fight against corneal keratitis caused by *A. fumigatus*. This study opens up a new approach to explore the role of RARγ in fungal infections and shed light on further molecular mechanisms of its immunomodulatory function. This study also aroused awareness of the clinical importance of anti-fungal and admitted the need for a long-term study on the anti-fungal activity in cornea. Therefore, the ability of RARγ to inhibit Dectin-1 and pro-inflammatory cytokines expression and activation provides a novel therapeutic approach to managing cornea diseases in which Dectin-1-induced fungal inflammation contributes to tissue injury. The effects of RARγ can reduce the damage caused by pro-inflammatory cytokines and promote the ulcer healing.

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