

The role of Dectin-1/Raf-1 signal cascade in innate immune of human corneal epithelial cells against *Aspergillus fumigatus* infection

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Abstract

• **AIM:** To investigate the expression of the v-raf-1 murine leukemia viral oncogene homolog 1 (Raf-1) and its role in the innate immune response of human corneal epithelial cells (HCECs) infected by *Aspergillus fumigatus*.

• **METHODS:** HCECs were cultured *in vitro*. They were randomly divided into 4 groups, including control group, *Aspergillus fumigatus* group, GW5074 (an inhibitor of Raf-1) group and Laminarin [an inhibitor of Dendritic-cell-associated C-type lectin 1 (Dectin-1)] group. The protein expression level of total Raf-1 and p-Raf-1 was measured by Western blot. The expression of IL-6 and IL-8 mRNA in each group was detected by real-time polymerase chain reaction.

• **RESULTS:** In *Aspergillus fumigatus* group, total Raf-1 protein levels in HCECs remained unchanged at 5, 15, 30 and 45min after infection, while p-Raf-1 expression was significantly enhanced at 30min after infection compared with control group. However, the expression of p-Raf-1 was apparently declined after treated with GW5074 or Laminarin compared with *Aspergillus fumigatus* group. The expression levels of IL-6, IL-8 mRNA were significantly increased after stimulation with fumigatus compared with control group. Pre-treated with GW5074 significantly inhibited *Aspergillus fumigatus*-induced upregulation of IL-8 and IL-6.

• **CONCLUSION:** *Aspergillus fumigatus* stimulation can elevate the expression of p-Raf-1 in HCECs *in vitro*. Dectin-1/Raf-1 signal pathway may play a role on regulating the expression of inflammatory cytokines, including IL-6 and IL-8.

• **KEYWORDS:** Dectin-1/Raf-1 signal pathway; *Aspergillus fumigatus*; innate immune; human corneal epithelial cells

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INTRODUCTION

Fungal keratitis (FK) is a potentially vision-threatening disease with a gradually increased incidence recently^[1-3]. If not treated promptly and properly, FK would result in corneal perforation, permanent vision loss, or even enucleation. *Aspergillus fumigatus* is one of the main pathogen of FK^[4].

Dendritic-cell-associated C-type lectin 1 (Dectin-1) is a member of C-type lectin super family originally found in dendritic cells. It can identify β -glucan, mediate fungal innate immune response and regulate the production of cytokines and chemokines^[5]. Our study had shown that Dectin-1 was also expressed in corneal epithelial cells of human and mouse^[6]. However, the exact mechanism for its defense against fungal infection is unclear.

The v-raf-1 murine leukemia viral oncogene homolog 1 (Raf-1) is an upstream signaling factor as a central link in transmitting cytokine signaling^[7-8]. Many evidences suggested that Raf-1 was poised at a key relay point in a kinase cascade that ultimately regulated cell proliferation, differentiation and development^[9-10]. Recent study^[11] indicated that Dectin-1 induced a second signaling pathway which was independent of the Syk pathway but integrated with it at the level of NF- κ B activation, mediated by the serine-threonine kinase Raf-1, after stimulated by the β -glucan curdlan or *Candida albicans*.

In this study, we investigate the expression of Raf-1 in human corneal epithelial cells (HCECs) infected by *Aspergillus fumigatus*, and explore the role of Raf-1 in regulating inflammatory cytokines production.

MATERIALS AND METHODS

Preparation of *Aspergillus Fumigatus* Hyphae The standard *Aspergillus fumigatus* strain was purchased from the China General Microbiological Culture Collection Center, and grown in Sabouraud medium at 28°C for 5-7d.

Then the fungal conidia were inoculated to liquid medium at 37°C for 3-4d. After centrifuged at 5000 rpm for 10min, the hyphae in the liquid medium were collected, grinded to the size of 20-40 μm fragment, inactivated by treatment with 75% ethanol overnight [12], washed three times in sterile phosphate buffered saline, and adjusted to a concentration of 5×10⁶/mL with DMEM.

Culture of Human Corneal Epithelial Cells HCECs were kindly provided by Ocular Surface Laboratory of Xiamen Ophthalmic Center and cultured in DMEM with 10% FBS, 0.075% epidermal growth factor, 0.075% insulin, and 100 U/mL penicillin G and 100 μg/mL streptomycin sulfate at 37°C in a humidified atmosphere containing 5% CO₂. The medium was replaced every 2d. The HCECs suspensions were seeded onto 6-well and 12-well tissue culture plates at a density of 1 ×10⁵/mL and grew to 70% -80% confluence. Before stimulated by inactive *Aspergillus fumigatus* hyphae, HCECs were incubated in serum-free medium for 12h.

Western Blot Analysis Expression of Raf-1 and its phosphorylated form were measured by Western blot in HCECs infected by *Aspergillus fumigatus*. Housekeeping gene GAPDH level was examined to ensure equal protein loading. Protein samples (30 g) were resolved on a 10% Bis/Tris polyacrylamide gels under denaturing/reducing conditions and transferred to polyvinylidene difluoride membranes in an iBlot electroblotter per manufacturer recommended condition (Invitrogen, Carlsbad, CA, USA). Blotted membranes were then incubated for 1h at room temperature in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% milk for blocking. After washed for 3 times with TBST, the blots were incubated with anti-phospho-c-Raf (Ser338/Try440) antibody (Merck Millipore, Germany) and Raf-1 antibody (BOSTER, China) for 2h. The membranes were then washed for 3 times with TBST, and secondary antibody (conjugated to horseradish peroxidase) in TBST (5% milk) was added and incubated for 1h. After washing with TBST, the membranes were incubated in the enhanced chemiluminescence-plus detection system, followed by exposure on X-ray film (GE Healthcare; Amersham, Piscataway, NJ, USA). All experiments were repeated at least three times.

Real-time Polymerase Chain Reaction The cultured HCECs were harvested and saved at -80°C. Total RNA of the isolated cells was extracted using RNAiso plus reagent (TaKaRa) and rapidly quantified using spectrophotometry. Complementary DNA was generated by reverse transcription of 2 μg of total RNA and then used in the following quantitative polymerase chain reaction (PCR) reactions with SYBR Green using specific primers: 95°C for 30s, followed by 40 cycles of 95°C for 5s, 60°C for 30s, followed by a final stage of 95°C for 15s, 60°C for 30s, and 95°C for 15s. The

oligonucleotide primers used were as follows: GAPDH: CCCCATGTATCCGTTGTG and GTAGCCCAGGATGCCCTTAGT; IL-8: TTTCAGAGACAGCAGAGCACACA A and CACACAGAGCTGCAGAAATCAGG; IL-6: AAGC CAGAGCTGTGCAGATGAGTA and TGTCCTGCAGCCA CTGGTTC. The gene expression levels were quantified by real-time-PCR using the housekeeping gene β-actin as an internal control. Quantification was performed using the 2^{-ΔΔC_t} method. Each experiment was repeated at least three separate times.

Statistical Analysis All data were plotted using GraphPad Prism software. All data were presented as means ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA). Data analysis was carried out with the SPSS version 17.0. Statistical significance was set at the *P* < 0.05 level.

RESULTS

Expression of Raf-1 in HCECs Infected by *Aspergillus fumigatus* To determine the effect of *Aspergillus fumigatus* infection on Raf-1 and p-Raf-1 expression in HCECs, we treated the cells with *Aspergillus fumigatus* (5×10⁷/mL) in 12-well plates. As shown in Figure 1, total Raf-1 protein levels in HCECs remained unchanged at 5, 15, 30 and 45min after *Aspergillus fumigatus* stimulation. However, p-Raf-1 expression was significantly enhanced in HCECs with fungal infection for 30min compared with control group.

To investigate the relationship between Dectin-1 and Raf-1, Raf-1 and p-Raf-1 expression in HCECs with *Aspergillus fumigatus* infection, they were also detected with Western blot by use of Laminarin (an inhibitor of Dectin-1) or GW5074 (an inhibitor of Raf-1). The total Raf-1 was remain unchanged after stimulated with *Aspergillus fumigatus* alone and pretreated with Laminarin or GW5074 before fungal infection. However, the expression levels of p-Raf-1 were apparently declined in GW5074 or Laminarin pretreatment group compared with *Aspergillus fumigatus* stimulation group (Figure 2).

Raf-1 Antagonism in HCECs Infected by *Aspergillus fumigatus* Inhibited Inflammatory Cytokine Production

As shown in Figure 3A and 3B, the expression of IL-8 and IL-6 mRNA was significantly increased in HCECs after exposure to *Aspergillus fumigatus* for 4, 6 and 8h. They reached the peak at 8h after stimulation compared with control group (*P* < 0.01). To examine the function of Raf-1 in inflammatory cytokine production, GW5074 was added to cells that were cultured in 12- or 6-well flat-bottom plates at 10 μmol/L, and then the cells were subjected to *Aspergillus fumigatus* challenge for 8h. Figure 3C and 3D shows that pre-treated with GW5074 significantly inhibited *Aspergillus fumigatus*-induced upregulation of IL-8 and IL-6 (*P* < 0.01).

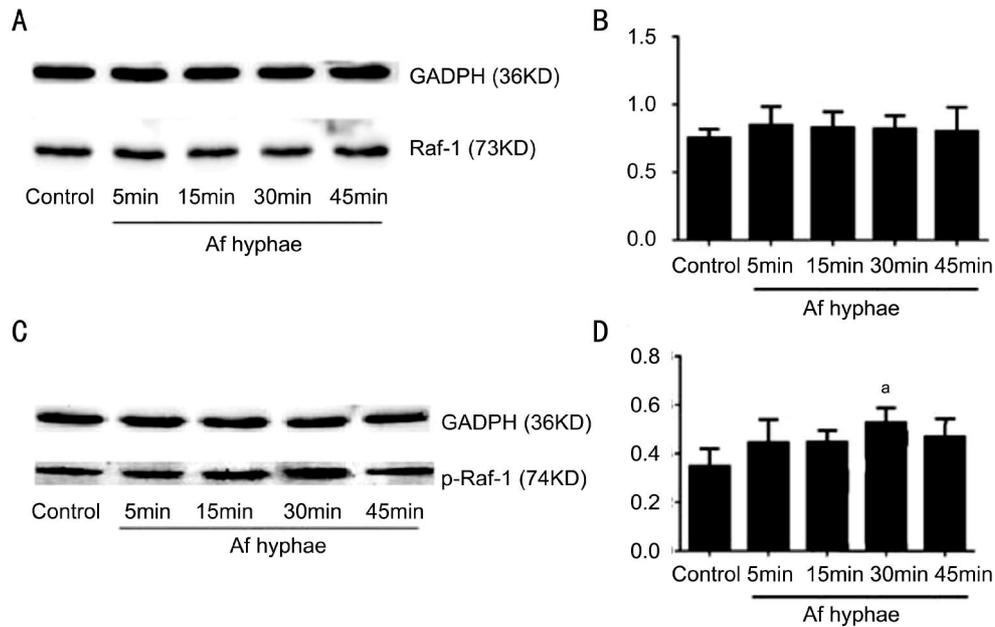


Figure 1 Total Raf-1 and p-Raf-1 expression in cultured HCECs infected with *Aspergillus fumigatus* A, B: Expression of total Raf-1 was determined with Western blot 5, 15, 30 and 45min after incubation with *Aspergillus fumigatus* (5×10^7 /mL). *Aspergillus fumigatus* infection did not increase total Raf-1 expression at these time points; C, D: Western blotting confirmed that *Aspergillus fumigatus* infection increased p-Raf-1 protein expression at 30min after incubation with *Aspergillus fumigatus* (5×10^7 /mL). The data represent the mean \pm standard deviation of three independent experiments. ^a $P < 0.05$ vs control group. Af means *Aspergillus fumigatus*.

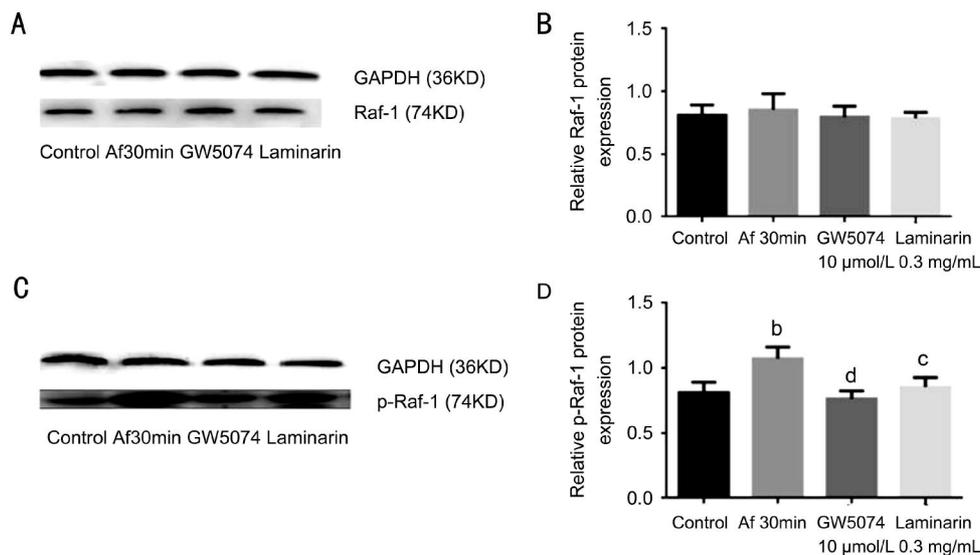


Figure 2 Total Raf-1 and p-Raf-1 expression in HCECs treated with Laminarin or GW5074 HCECs were pretreated with Laminarin (0.3 mg/mL) or GW5074 (10 μ mol/L) for 30min and then incubated with *Aspergillus fumigatus* for 30min for assay of total Raf-1 (A, B) and p-Raf-1 (C, D) expression using Western blot. Laminarin or GW5074 significantly inhibited *Aspergillus fumigatus*-induced upregulation of p-Raf-1 respectively. The data represent the mean \pm standard deviation of three independent experiments. ^b $P < 0.01$ vs control group; ^d $P < 0.01$, ^c $P < 0.05$ vs *Aspergillus fumigatus* group. Af means *Aspergillus fumigatus*.

DISCUSSION

β -glucan receptor Dectin-1 is a member of the C-type lectin family and functions as an innate pattern recognition receptor in antifungal immunity [13-15]. Dectin-1 in corneal epitheliums of rats and humans has been found to play an essential role in regulating infections with *Aspergillus fumigatus* [6], a normally commensal fungus which can cause superficial mucocutaneous infections as well as life-threatening invasive diseases. Previous studies of our research team had shown that Dectin-1 expressed in corneal epitheliums of normal

persons and FK patients. And the expression of Dectin-1 in FK patients in the early innate stage may promote chemokines such as IL-1 β , IL-6, CCL2, CXCL1, CXCL2 to raise neutrophils and macrophages in the body antifungal immunity.

The Raf proteins are a family of serine/threonine-specific protein kinases that form part of a conserved signal transduction module in higher eukaryotes. Raf-1 is a 74 kD cytoplasmic serine/threonine protein kinase implicated in the transduction of signals from cell surface receptors to the

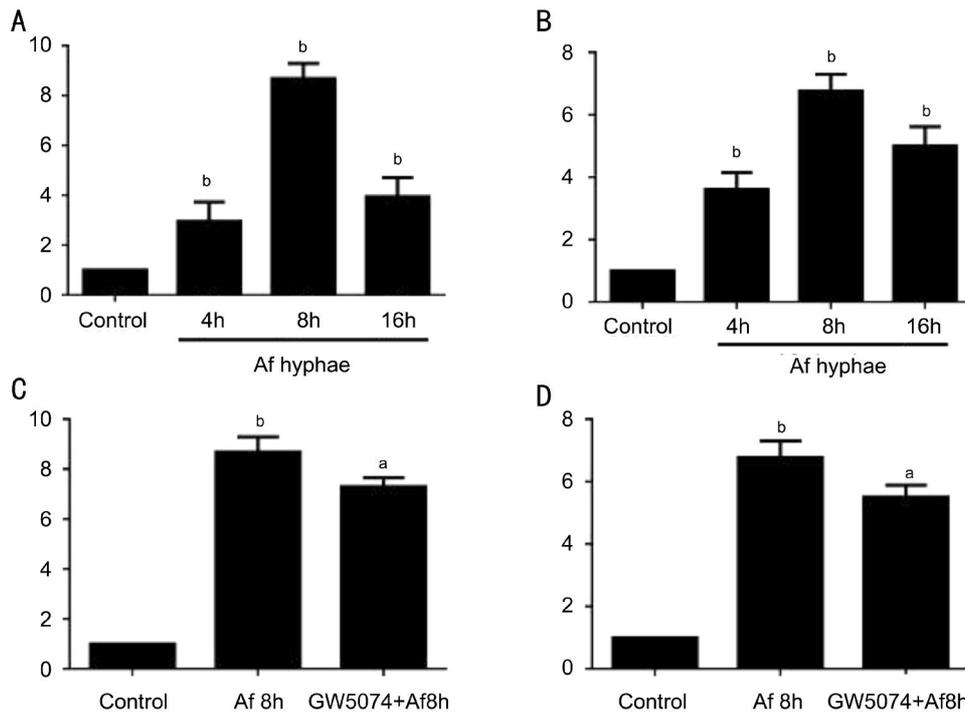


Figure 3 The role of GW5074 on expressions of IL-6 and IL-8 mRNA in HCECs infected by *Aspergillus fumigatus*. A, B: Expressions of IL-6 and IL-8 mRNA in HCECs were determined with real time-PCR 4, 8 and 16h after incubation with *Aspergillus fumigatus* (5×10^7 /mL). *Aspergillus fumigatus* infection increased IL-6 and IL-8 mRNA expression in HCECs peaking at 8h; C, D: Pretreatment with GW5074 (10 μ mol/L) dramatically increased expressions of IL-6 and IL-8 mRNA in HCECs infected by *Aspergillus fumigatus*. The data represent the mean \pm standard deviation of three independent experiments. ^b $P < 0.01$ vs control group; ^a $P < 0.05$ vs *Aspergillus fumigatus* group. Af means *Aspergillus fumigatus*.

nucleus [16-17]. Raf-1 activation requires phosphorylation and two important phosphorylation sites have proved to be critical for the activation of C-Raf. One phosphorylation event occurs on Y341 by Src kinase [18-19]. Another critical phosphorylation event on C-Raf occurs at S338 [20-21]. Treatment of active Raf-1 with protein phosphatases specific for either phosphoserine or phosphotyrosine could result in loss of kinase activity. Raf-1 is predominantly phosphorylated *in vivo*. Activation of the Raf serine/threonine protein kinases is tightly regulated by multiple phosphorylation events [22-23]. Our study was designed to investigate the early expression of Raf-1 and its role in the innate immune response of HCECs infected by *Aspergillus fumigatus*. We found that total Raf-1 protein levels remain unchanged at each time point after *Aspergillus fumigatus* stimulation. But p-Raf-1 expression was significantly enhanced in HCECs with fungal infection for 30min compared with control group. Moreover, the expression levels of p-Raf-1 were apparently declined after treated with GW5074 or Laminarin compared with *Aspergillus fumigatus* stimulation group. The results suggested that *Aspergillus fumigatus* infection induced Raf-1 expression in HCECs and Dectin-1 may play an important role on the expression of Raf-1 in HCECs infected with *Aspergillus fumigatus*.

Gringhuis *et al* [11] proved that Raf-1 kinase activity required phosphorylation of Raf-1 on Ser338, Tyr340 and Tyr341 by

Pak and Src kinases, respectively. Curdlan-induced phosphorylation was blocked by antibody to Dectin-1 but not by the Syk inhibitor piceatannol rapidly and independently of protein synthesis. Our result also suggested that Raf-1 by phosphorylations and Dectin-1/Raf-1 mediated pathway against *fumigatus* challenge in innate responses of corneal epithelial cells.

Ifrim *et al* [24] demonstrated that *Candida albicans* primed the cells for enhanced proinflammatory cytokine production when skin and mucosal surfaces are exposed to a secondary bacterial stimulus through a Dectin-1/Raf-1 mediated pathway. IL-8 is the major mediator of polymorphonuclear neutrophil (PMNs) influx in ocular infection [25], and the major cytokines that recruit PMNs into the infected cornea [26]. IL-6 is an essential protective factor in the corneal inflammation [27]. In our study the expression of IL-8 and IL-6 mRNA was significantly increased in HCECs after exposure to *Aspergillus fumigatus*. Pretreatment with GW5074 can inhibit the up-regulation of IL-8 and IL-6 induced by *Aspergillus fumigatus* in HCECs. The results showed Raf-1 may play a role by up-regulation the expression inflammatory cytokines IL-6 and IL-8 against *Aspergillus fumigatus* challenge.

However, the exact mechanism research of Raf-1 in human corneal epithelial cells for defense against fungal infection is unclear, and it still needs further research.

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