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# Phospholipase Cy2 is critical for Ca<sup>2+</sup> flux and cytokine production in anti-fungal innate immunity of human corneal epithelial cells

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## Abstract

**Background:** Fungal keratitis (FK) is a sight-threatening disease, accounting for a significant portion with its complex presentation, suboptimal efficacy of the existing therapies and uncontrollable excessive innate inflammation. Phospholipase C- $\gamma$ 2 (PLC $\gamma$ 2) is a non-receptor tyrosine kinase that plays an important role at the early period of innate immunity. This study aimed to identify the role of PLC $\gamma$ 2 in Dectin-1-mediated Ca<sup>2+</sup> Flux and its effect on the expression of proinflammatory mediators at the exposure to *Aspergillus fumigatus* (*A. fumigatus*) hyphae antigens in human corneal epithelial cells (HCECs).

**Methods:** The HCECs were preincubated with or without different inhibitors respectively before *A. fumigatus* hyphae stimulation. Intracellular calcium flux in HCECs and levels of PLC $\gamma$ 2 and spleen-tyrosine kinase (Syk) were detected by fluorescence imaging and Western Blotting. The expression of proinflammatory mediators was determined by reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA).

**Results:** We demonstrated that an intracellular Ca<sup>2+</sup> flux in HCECs was triggered by *A. fumigatus* hyphae and could be reduced by pre-treatment with PLC $\gamma$ 2-inhibitor U73122. *A. fumigatus* hyphae induced PLC $\gamma$ 2 phosphorylation was regulated by Dectin-1 via Syk. Furthermore, PLC $\gamma$ 2-deficient HCECs showed a drastic impairment in the Ca<sup>2+</sup> signaling and the secretion of IL-6, CXCL1 and TNF- $\alpha$ .

**Conclusions:** PLC $\gamma$ 2 plays a critical role for Ca<sup>2+</sup> Flux in HCECs stimulated by *A. fumigatus* hyphae. Syk acts upstream of PLC $\gamma$ 2 in the Dectin-1 signaling pathway. The expressions of proinflammatory mediators induced by *A. fumigatus* are regulated by the activation of Dectin-1-mediated PLC $\gamma$ 2 signaling pathway in HCECs.

**Keywords:** PLC $\gamma$ , Dectin-1, Ca<sup>2+</sup>, Innate immunity, Corneal epithelium, *Aspergillus fumigatus*

## Background

FK is a sight threatening disorder associated with multiple risk factors, such as ocular surface disease, extended wear contact lenses, and traumatic ocular surface accidents [1], presenting a therapeutic challenge due to the lack of effective antifungal agents and uncontrollable excessive innate inflammation. Innate immunity is an important defence against microbial infections in cornea.

However, excessive innate inflammatory response could damage normal corneal epithelial cells whilst slowing down the pathological progress of FK [2].

On one hand, cytoplasmic Ca<sup>2+</sup> flux has been demonstrated to play an important role in innate immune response. In airway epithelia, cytoplasmic Ca<sup>2+</sup> regulates *P. aeruginosa*- or *flagellin*- activated innate immune responses [3]. Additionally, cytoplasmic Ca<sup>2+</sup> flux promotes macrophage to recognize carbohydrate structures on pathogenic fungi via C-type lectin receptors (CLRs) [4–7]. In lymphocytes, cytoplasmic Ca<sup>2+</sup> flux is one of the hallmarks of B cell receptor (BCR) signaling [8, 9], in

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which the enzymatic activity of PLC $\gamma$ 2 is essential for the induction of Ca<sup>2+</sup> flux [10]. PLC $\gamma$ 2 is a non-receptor tyrosine kinase, playing an important role at the early period of innate immunity. It is documented that PLC $\gamma$ 2 is the key component in Dectin-2 signaling pathway, mediating anti-fungal innate immune responses in macrophages [11]. However, there is little evidence showing the relationship between PLC $\gamma$ 2 and the induction of Ca<sup>2+</sup> flux in human corneal epithelial cells (HCECs) [12].

On the other hand, pattern-recognition receptors (PRRs) exert the regulatory role in innate immune response [2, 13]. At the early period of fungal infection, the innate immune system recognizes pathogen associated molecular pattern (PAMP) of pathogenic microorganism using PRRs, such as C-type lectin receptors (CLRs), Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [14–17]. Dectin-1 is a CLR that can identify  $\beta$ -glucan of fungal cytoderm, mediating a variety of fungal innate immune responses and triggers signal transduction via its cytoplasmic hemi-ITAM [18]. The phosphorylated ITAM-like motif of Dectin-1 could directly recruit Syk, which subsequently signals downstream to activate mitogen-activated protein kinases (MAPKs) and nuclear factor  $\kappa$ B (NF- $\kappa$ B). Additionally, Syk plays a significant role in Dectin-1 mediated signaling pathway as an antigen-receptor-like manner in macrophages [19, 20]. However, the function of Syk in the Dectin-1 signal pathway in HCECs is still unclear. Recently, we reported that Dectin-1 induced cytoplasmic Ca<sup>2+</sup> flux upon *A. fumigatus* infection in HCECs [21], suggesting a potential relationship between PLC $\gamma$ 2 and Dectin-1, as well as cytoplasmic Ca<sup>2+</sup> flux.

In this study, we demonstrated that the participation of Dectin-1 regulated the expression of PLC $\gamma$ 2 via Syk upon the treatment of *A. fumigatus* in HCECs. Moreover, PLC $\gamma$ 2 is the critical phospholipase in the process of Dectin-1-mediated Ca<sup>2+</sup> flux and the secretion of pro-inflammatory mediators (IL-6, CXCL1 and TNF- $\alpha$ ) in HCECs.

## Methods

### Materials and reagents

RNAiso Plus and RT-PCR kits were from TaKaRa (Dalian, China). RIPA (radioimmunoprecipitation assay) was from Solarbio (Beijing, China). The BCA Protein Assay Kit, polyvinylidene difluoride (PVDF) membranes, confining liquid and enhanced chemiluminescence (ECL) kit were from Beyotime Biotechnology (Shanghai, China). The following reagents were purchased: PLC $\gamma$ 2 inhibitor-U73122 (MilliporeSigma, MO, USA), syk inhibitor-Piceatannol (Selleck, Texas, USA) and Dectin-1 inhibitor-Laminarin (MilliporeSigma, MO, USA). Antibodies used for confocal microscope were from AAT Bioquest (California, USA). Antibodies used for Western

blot were from Cell Signaling (Danvers, MA): anti-PLC $\gamma$ 2, anti-phospho-PLC $\gamma$ 2 (Tyr759), anti-Syk, anti-phospho-Syk.

### The preparation of *A. fumigatus* suspension

*A. fumigatus* standard strain (CPCC 3.0772) was cultured in Sabouroud liquid culture at 37 °C 200 rpm for 2–3 days, and then the harvested mycelia of *Aspergillus fumigatus* was washed twice by sterile phosphate buffered saline (PBS) and sterilized by 70% ethanol at 4 °C for 12 h. The density of the fungal mycelia was read in a blood cell counting board and reached the final concentrations of  $1 \times 10^8$  colony-forming units per 1 ml. The inactivated *A. fumigatus* mycelia was stored at –20 °C [22, 23].

### Cell culture

HCECs were kindly offered by Ocular Surface Laboratory of Xia Men Eye Center and grown in DMEM/F12 with 6.4% Fetal bovine serum (FBS), 7.52 ng/ml Insulin, 7.52  $\mu$ g/ml Epidermal Growth Factor (EGF), 100 u/ml penicillin G and 100  $\mu$ g/ml streptomycin sulfate in a humidified 5% CO<sub>2</sub> incubator at 37 °C. The medium was replaced every 2 days before experiments. HCECs suspensions of  $1 \times 10^5$ /ml were seeded onto 12- or 6-well tissue culture plates and when 90 % of the cells were attached, the medium was replaced.

### HCECs stimulation assay

HCECs untreated were set as controls, others were added with *A. fumigatus* hyphae ( $5 \times 10^6$ /ml). Or HCECs were treated with 0.3 mg/ml laminarin, 5  $\mu$ mol PLC $\gamma$ 2 inhibitor (U73122) or 10  $\mu$ mol syk inhibitor (Piceatannol) for 30 min prior to *A. fumigatus* hyphae antigens stimulation in order to block Dectin-1, PLC $\gamma$ 2 and syk. After 15 min or 8 h' incubation, HCECs were harvested to detect the protein and mRNA expression by western blot and RT-PCR.

### Calcium imaging

For analysis of cytoplasmic calcium, HCECs which seeded on the glass-bottom culture dishes (NEST) were labeled with Fluo-3 AM (5  $\mu$ M; AAT Bioquest) for 60 min. To block PLC $\gamma$ 2, Fluo-3-loaded HCECs were preincubated with U73122 for 30 min at room temperature. After resting for 30 min, cells were stimulated with *A. fumigatus* hyphae ( $5 \times 10^6$ /ml) and cytoplasmic calcium was monitored on a Leica TCS SPE confocal microscope in real time for 6–8 min. The images were acquired using Leica LAS software before and after *A. fumigatus* hyphae were added for each condition. The fluorescence intensity were measured using Image J software [21].

### Western blot

Cells were lysed in RIPA buffer for 1 h, and then were centrifuged. After estimation of protein content, addition of SDS sample buffer, and boiling, total protein was separated on 10% acrylamide SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% BSA liquid, and then were incubated with a monoclonal antibody to human  $\beta$ -actin, and a monoclonal antibody to human Primary antibody at 4 °C overnight. After washed in PBST for three times, the membranes were incubated with corresponding peroxidase-conjugated secondary antibodies at 37 °C for 1 h. Then the blots.

were developed using chemiluminescence (ECL; Thermo Scientific).

### Real-time PCR

RNAiso plus reagent were used to extract total RNA from samples according to the manufacturer's protocol, and the RNA was quantified by spectrophotometry. The first strand cDNA was synthesized by RT from total RNA. The Real-Time PCR was performed in a Mx3005PTM system (Stratagene) with 20ul reaction volume containing 2ul of cDNA. cDNA was amplified by PCR using primers shown below.  $\beta$ -actin was used as the endogenous control. The thermocycler parameters were 95 °C for 30s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30s. A melting curve was used to confirm the specificity of the PCR products following each reaction. The  $\Delta\Delta$ CT method was used for quantization of target gene.

products of stimulated and unstimulated group. Data are expressed as fold of increase in mRNA expression. Each experiment was performed in triplicate. The double-stranded probes used are as follow: The following primers were used (5'-3'): AAGCCAGAGCTGTG CAGATGAGTA(forward) and TGTCCTGCAGCCAC TGGTTC(reverse) for Human-IL-6; AGGGAATTC ACCCAAGAAC(forward) and CACCAGTGAGCTTC CTCCTC(reverse) for Human-CXCL1; TGCTTGTTCTCAGCCTCTT(forward) and CAGAGGGCTGATTA GAGAGAGGT(reverse) for Human-TNF- $\alpha$ ; TGGCACC CAGCACAATGAA(forward) and CTAAGTCATAGTC CGCCTAGAAGCA (reverse) for Human- $\beta$ -actin as housekeeping gene.

### Enzyme-linked immunosorbent assay

According to the manufacturer's protocol Enzyme-linked Immunosorbent Assay, Double-sandwich ELISA for human IL-6, CXCL1 and TNF- $\alpha$  was performed, to detect the concentration of IL-6, CXCL1 and TNF- $\alpha$  protein in conditioned media and culture cell lysates from different treatments. Absorbance was read at 450 nm with a reference wavelength of 570 nm by a VERSAmix microplate reader (Molecular Devices, Sunnyvale, CA) [21].

### Statistical analysis

All data were presented as mean  $\pm$  SD from independent experiments. The data were analyzed using SPSS19.0 statistical package. One-way ANOVA test was used to make comparison among three or more groups, and LSD was used to identify between each two groups.  $P < 0.05$  was considered statistically significant and data are shown as mean  $\pm$  SEM.

## Results

### *A. fumigatus* induces cytokines production in a time- and dose-dependent manner

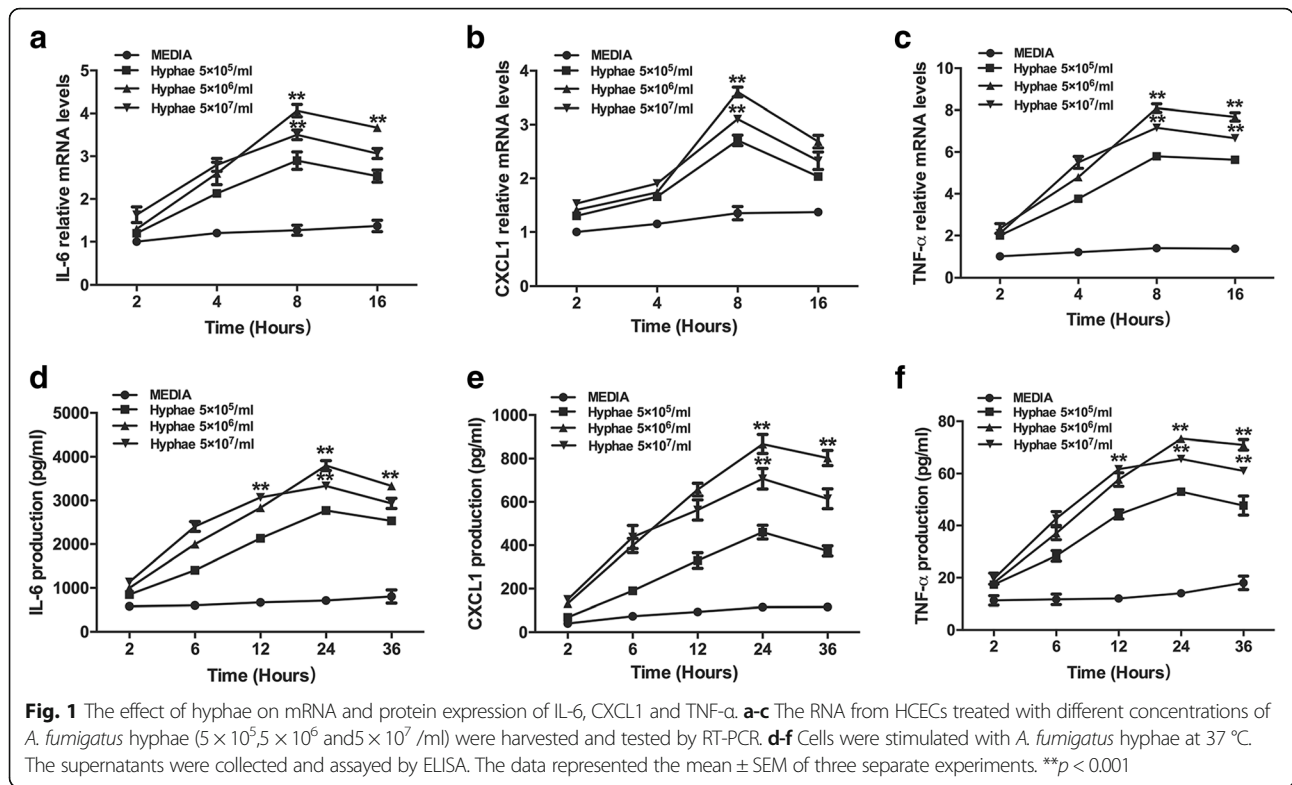
To investigate *A. fumigatus* hyphae-induced IL-6, CXCL1 and TNF- $\alpha$  mRNA expression and protein secretion, hyphae-treated cells and supernatants were analyzed by RT-PCR and ELISA. The assays were performed over a period of 16 h and 36 h, with cells being exposed to  $5 \times 10^5$ ,  $5 \times 10^6$  and  $5 \times 10^7$ /ml of hyphae, respectively. The level of IL-6, CXCL1 and TNF- $\alpha$  mRNA expression was elevated and peaked at 8 h ( $5 \times 10^6$ /ml), then returned to decrease after hyphae stimulation, as shown in Fig. 1a-c. The maximal protein production was recorded at 24 h ( $5 \times 10^6$ /ml) (Fig. 1d-f). The data demonstrated that IL-6, CXCL1 and TNF- $\alpha$  mRNA expression and protein secretion were induced by hyphae in a time- and dose-dependent manner in HCECs.

### PLC $\gamma$ 2 could be activated by *A. fumigatus* and induced by the engagement of Dectin-1 in HCECs

We stimulated HCECs with *A. fumigatus* hyphae, and the data showed that the stimulation led to the activation of PLC $\gamma$ 2 as indicated by their phosphorylation status. The phosphorylation of PLC $\gamma$ 2 was significantly increased after *A. fumigatus* hyphae infection with a time-dependent manner, and peaked at 15 min, whereas no significant difference was seen in total PLC $\gamma$ 2 protein (Fig. 2a). But we wondered whether the engagement of Dectin-1 could increase the activation of PLC $\gamma$ 2 in HCECs. To address this question, we preincubated HCECs with the Dectin-1 inhibitor laminarin before the stimulation of hyphae and data showed that this prior treatment could decrease the expression of p-PLC $\gamma$ 2 (Fig. 2b). Thus, it proved that the engagement of Dectin-1 played an important role in the activation of PLC $\gamma$ 2 with the stimulation of hyphae in HCECs.

### PLC $\gamma$ 2 plays a role in the stimulation of Ca<sup>2+</sup> flux induced by *A. fumigatus*

To confirm whether PLC $\gamma$ 2 could induce Ca<sup>2+</sup> flux in the infected HCECs, we preincubated the cells with U73122, the inhibitor of PLC $\gamma$ 2, prior to *A. fumigatus* hyphae treatment. As shown in Fig. 3, the Ca<sup>2+</sup> flux elicited by treatment of hyphae in HCECs could be

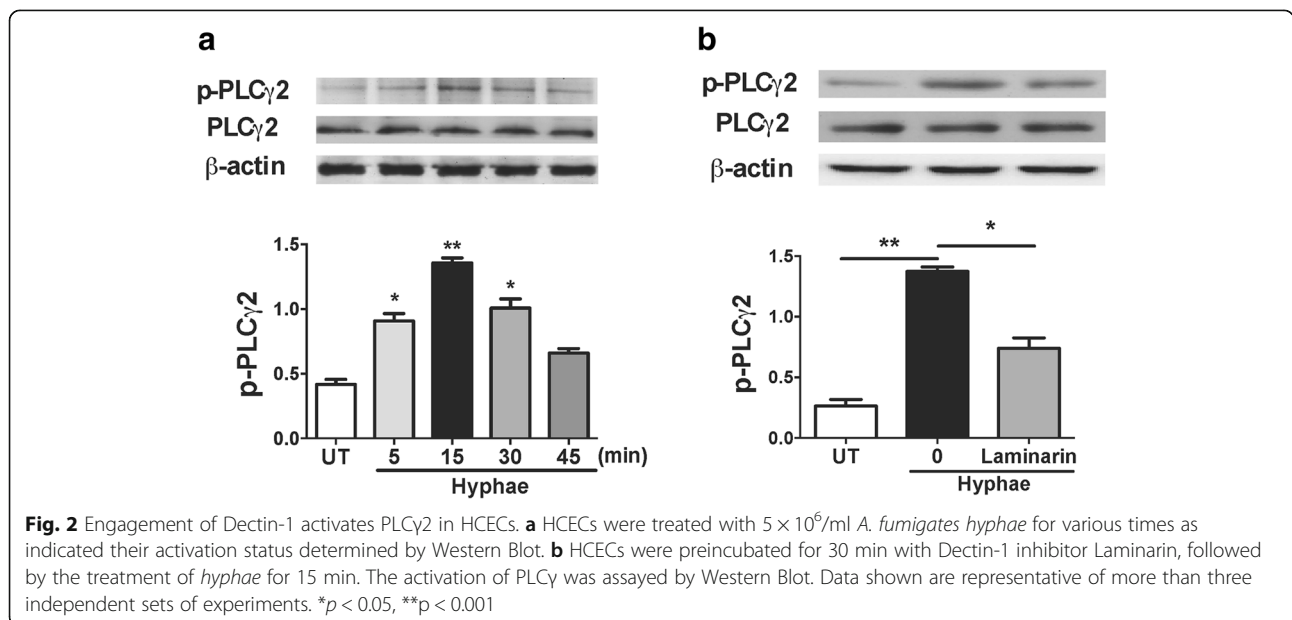


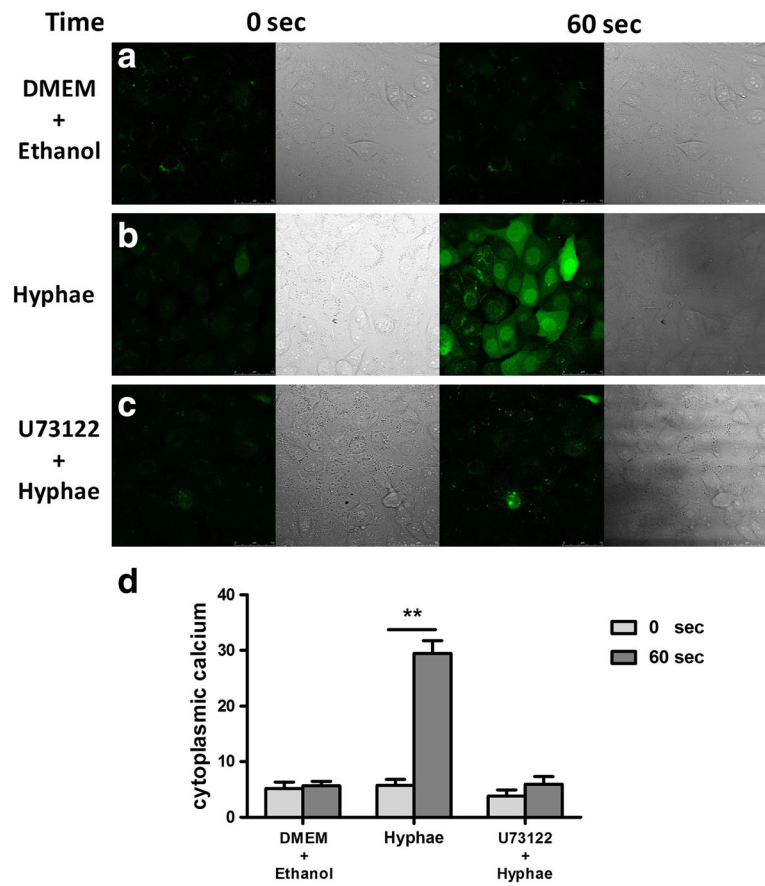
inhibited by U73122, which suggested that PLC $\gamma$ 2 played an important role in triggering Ca<sup>2+</sup> signaling in HCECs.

**Syk is important for Dectin-1-induced PLC $\gamma$ 2 activation**

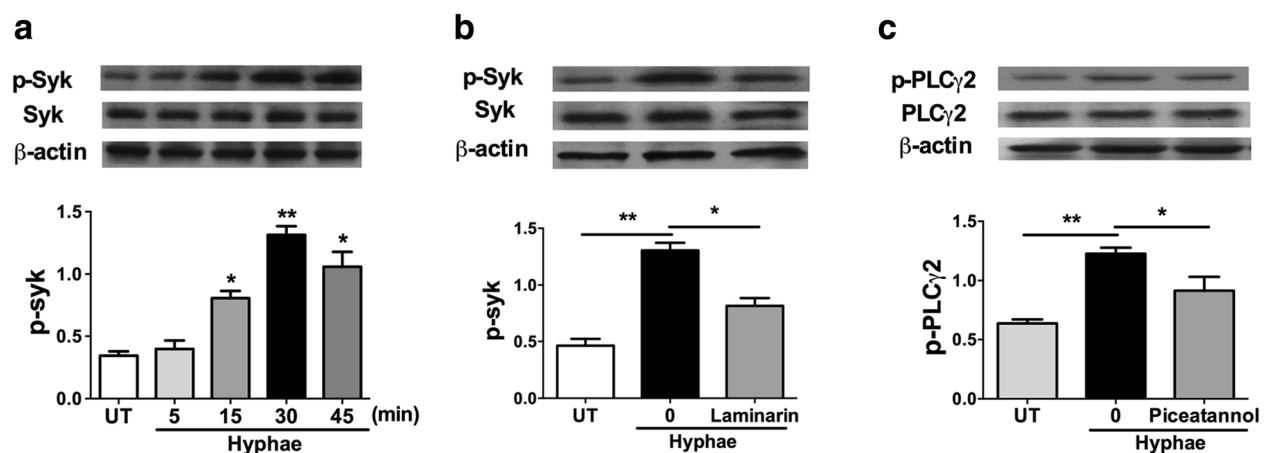
To investigate the stimulatory effects on Syk with the *A. fumigatus* hyphae, the HCECs were incubated with the *A. fumigatus* hyphae ( $5 \times 10^6$ /mL) for 5, 15, 30 and

45 min, tested by western blotting. As shown in Fig. 4a, the findings indicated the phosphorylation of Syk was activated at 30 min by *A. fumigatus* hyphae stimulation in HCECs. We indicated that Dectin-1 was critical in the activation of Syk, because the inhibition of Dectin-1 abrogated hyphae-induced phosphorylation of Syk (Fig. 4b). To assess if the tyrosine kinase is important for





**Fig. 3** PLCγ2 plays an important role for the elicitation of Ca<sup>2+</sup> flux in HCECs. **a-c** HCECs were loaded with Fluo-3 and treated with DMEM in the presence of Ethanol, 5 × 10<sup>6</sup>/ml *A. fumigatus* hyphae or 5 × 10<sup>6</sup>/ml *A. fumigatus* hyphae in the presence of 1 μMol/L inhibitor of PLCγ2-U73122. Confocal images of HCECs showed cytoplasmic calcium expression (green stain). **d** The corresponding results of fluorescence intensity were measured by Image J software. Data are representative of more than three independent sets of experiments. Magnifications 400X. \*\*p < 0.001



**Fig. 4** Syk Plays a crucial role for the activation of Dectin-1-induced PLCγ2 in HCECs. **a** The phosphorylation of Syk was activated after *A. fumigatus* hyphae stimulation in HCECs. The activation status was measured by Western Blot at 5, 15, 30 and 45 min. **b, c** HCECs were preincubated for 30 min with Dectin-1 inhibitor Laminarin or syk inhibitor Piceatannol, followed by treatment with hyphae for 30 or 15 min. The activation of syk or PLCγ was assayed by Western Blot. Results shown are mean ± SD of three independent experiments. \*p < 0.05, \*\*p < 0.001

Dectin-1-induced activation of PLC $\gamma$ 2, we pretreated HCECs with the Syk inhibitor Piceatannol(10  $\mu$ M) for 30 min followed the stimulation of hyphae. Then we examined the phosphorylation status of PLC $\gamma$ 2. As shown in Fig. 4c, hyphae-stimulated phosphorylation of PLC $\gamma$ 2 was markedly reduced when Syk was inhibited by its specific inhibitor Piceatannol in HCECs. Consistent with the finding that Syk play important role in Dectin-1 signaling, our results further suggested that Syk acted upstream of PLC $\gamma$ 2 in the Dectin-1 signaling pathway as their activities are crucial for the activation of PLC $\gamma$ 2.

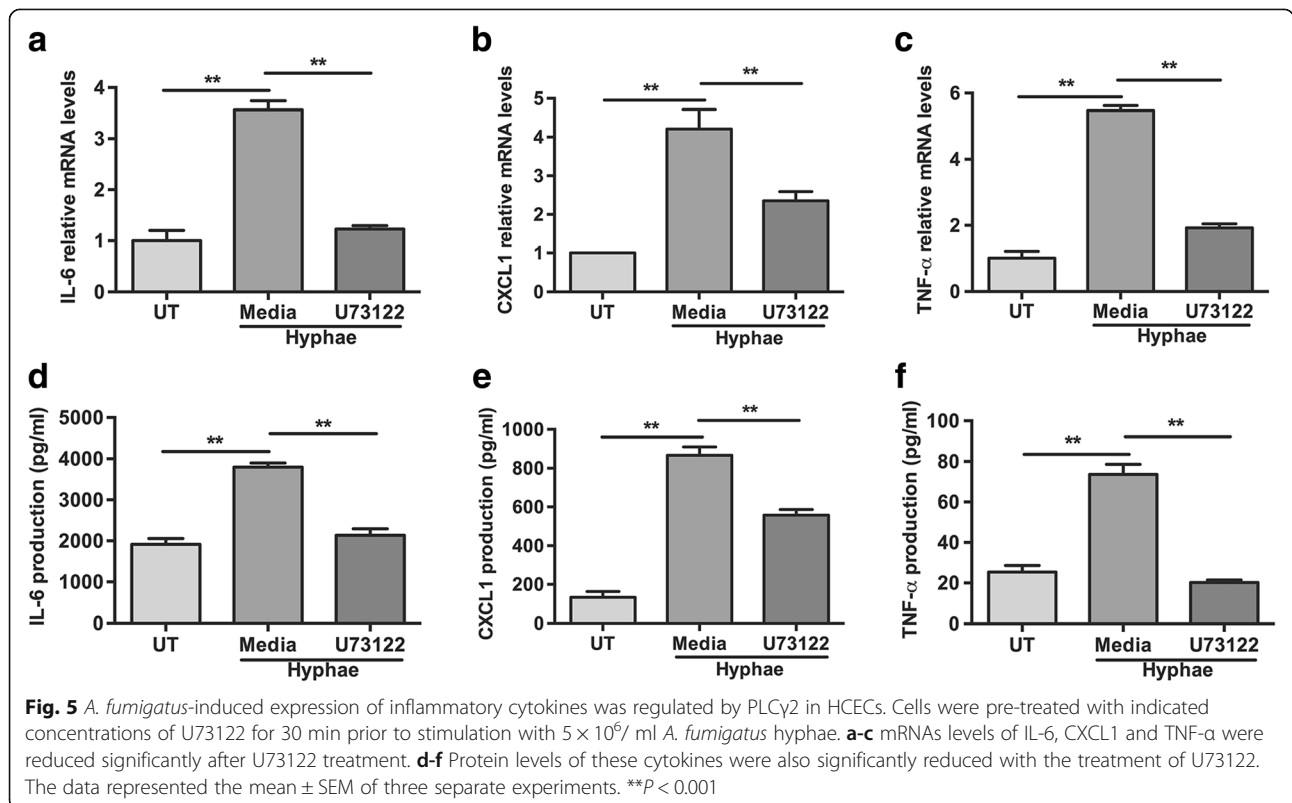
**PLC $\gamma$ 2 is essential for the up-regulation of cytokine production in HCECs**

To determine whether PLC $\gamma$ 2 can regulate cytokine secretion upon *A. fumigatus* hyphae stimulation in HCECs, RT-PCR and ELISA were used to detect the expression of cytokine at 8 and 24 h. As seen in Fig. 5a-c, relative mRNA levels of IL-6, CXCL1 and TNF- $\alpha$  was significantly reduced with the pretreatment of U73122 compared with untreated controls. Protein analysis confirmed the mRNA data, with significant reduction between U73122 treated groups and controls (Fig. 5b-d). It suggested that PLC $\gamma$ 2 signaling critically regulated these cytokines in antifungal immunity.

**Discussion**

FK is a blinding infection of the corneas, accounting for a significant portion in all keratitis. However, the pathogenesis of FK and the underlying molecular mechanisms are still unclear [24]. In the development of FK, innate immune response against *A. fumigatus* plays a crucial role in controlling microbial infection with the participation of inflammatory mediators [19]. In this study, we demonstrated that Dectin-1 mediated PLC $\gamma$ 2 signaling plays a critical role in *A. fumigatus* hyphae induced innate immune response in HCECs, and Syk acts as an upstream mediator in the Dectin-1 PLC $\gamma$ 2 signaling pathway. Above all, we showed that *A. fumigatus* hyphae significantly upregulated the expression of inflammatory factors IL-6, CXCL1 and TNF- $\alpha$  in HCECs. These fungi-induced cytokines resist fungal infection and promote the infiltration of inflammatory cells to remove pathogens.

In addition, our results suggested that PLC $\gamma$ 2 plays an important role in the stimulation of Ca<sup>2+</sup> flux induced by *A. fumigates* in HCECs, which is consistent with that the enzymatic activity of PLC $\gamma$ 2 is required for the induction of Ca<sup>2+</sup> flux in B cells [10]. Cytoplasmic Ca<sup>2+</sup> flux is the hallmark of B cell receptor (BCR) signaling pathway, in which the engagement of PRRs is essential for the elicitation of Ca<sup>2+</sup> flux in lymphocytes [9]. It is in



the agreement with our previous study that the innate PRRs such as Dectin-1 could elicit intracellular  $Ca^{2+}$  flux [21]. It is also consistent with the importance of PLC $\gamma$ 2 for intracellular  $Ca^{2+}$  flux with the engagement of Dectin-1 in DCs [25]. Recently, Lu et al. showed that the maintenance of intracellular  $Ca^{2+}$  homeostasis was involved in the potential mechanisms of hypoxia-induced inflammation and apoptosis in microglia BV2 cells [26]. Besides functioning in BCR signaling, PLC $\gamma$ 2 also plays an critical role in the innate immune system as a key component of the downstream signaling pathway for many receptors in response to fungal infection [11]. A previous study has shown that PLC $\gamma$ 2 functions downstream of Dectin-2 in response to the stimulation by the hyphal form of *Candida albicans* (*C. albicans*), an opportunistic pathogenic fungus [11]. In addition, they found that PLC $\gamma$ 2-deficient mice are defective in clearing *C. albicans* infection in vivo [11]. In this study, our findings showed that the lack of Dectin-1 impaired the phosphorylation of PLC $\gamma$ 2 in response to the infection with *A. fumigatus*, suggesting that Dectin-1 mediated the activation of PLC $\gamma$ 2 in the infected HCECs. Taken together, our data suggested that the intracellular  $Ca^{2+}$  mobilization, as a mechanism of cellular signaling in HCECs, is elicited by Dectin-1-mediated PLC $\gamma$ 2 signaling pathway.

Moreover, Dectin-1 is one of C-type lectin receptors (CLRs), functioning as PRRs to sense fungal infection. However, Dectin-1 induced PLC $\gamma$ 2 signaling pathway remains largely unknown. Here we explored how PLC $\gamma$ 2 was activated by determining which upstream kinase is required for its activation in the Dectin-1 signal transduction pathway. Recent studies demonstrated that Syk played critical roles in Dectin-1 signaling in macrophages [19, 20]. While in HCECs, we indicated that Syk was identified as a critical component downstream of Dectin-1 signaling, because the inhibition of Dectin-1 abrogated hyphae-induced activation of Syk. During BCR signaling, Syk is important for PLC $\gamma$ 2 activation and  $Ca^{2+}$  flux in B cells [27, 28]. Consistent with the results of BCR signaling, we found that the hyphae-stimulated phosphorylation of PLC $\gamma$ 2 was markedly reduced after Syk inhibition by its specific inhibitor Piceatannol in HCECs. Taken together, these findings demonstrated that Syk plays a critical role in Dectin-1-induced PLC $\gamma$ 2 signaling pathways, governing antifungal innate immune responses in HCECs.

Furthermore, it is reported that the lack of PLC $\gamma$ 2 impaired cytokine production in response to infection with *C. albicans* in PLC $\gamma$ 2-deficient macrophages [11]. Its deficiency resulted in the defective activation of NF- $\kappa$ B and MAPK and in a significantly reduced production of reactive oxygen species (ROS) following fungal challenge [11]. In our study, inflammatory

mediators (IL-6, CXCL1 and TNF- $\alpha$ ) stimulated by *A. fumigatus* hyphae were markedly blocked by PLC $\gamma$ 2 inhibitor in HCECs, suggesting that Dectin-1-mediated PLC $\gamma$ 2 signaling pathway is involved in the innate immune response of HCECs against *A. fumigatus* hyphae. PLC $\gamma$ 2 activation is essential to the expression of inflammatory mediators. These findings demonstrated that inflammatory cytokines and chemokines production could be upregulated through activation of Dectin-1-mediated PLC $\gamma$ 2 after *A. fumigatus* hyphae stimulation.

Last, but not least, it is increasingly acknowledged that C-type lectins act critically in the regulation of initiating and sustaining immune response against various pathogens. Therefore, the elucidation of the signaling of these CLRs would make a major impact on our understanding of host defense and microbial spread in the epithelium cells. For example, Dectin-1 is important for host recognition of  $\beta$ -glucan structure that exists in cell wall of *aspergillus*, *yeast*, *candida*, *penicillium* and other fungus [29–32]. Our current study demonstrated that PLC $\gamma$ 2 in Dectin-1 signal transduction could provide new targets for therapeutic intervention to enhance or suppress the host response.

## Conclusions

In conclusion, our findings demonstrate that PLC $\gamma$ 2 plays a critical role for  $Ca^{2+}$  Flux in HCECs stimulated by *A. fumigatus* hyphae. Syk acts upstream of PLC $\gamma$ 2 in the Dectin-1 signaling pathway. The expressions of proinflammatory mediators induced by *A. fumigatus* are regulated by the activation of Dectin-1-mediated PLC $\gamma$ 2 signaling pathway in HCECs. The further study of PLC $\gamma$ 2 pathway will provide new targets for the prevention and therapeutic intervention of fungal infection.

## Abbreviations

*A. fumigatus*: Aspergillus fumigatus; BCR: B cell receptor; *C. albicans*: *Candida albicans*; CLR: C-type lectin receptor; EGF: Epidermal Growth Factor; ELISA: Enzyme-linked immunosorbent assay; FBS: Fetal bovine serum; FK: Fungal keratitis; HCECs: Human corneal epithelial cells; MAPKs: Mitogen-activated protein kinases; NF- $\kappa$ B: Nuclear factor  $\kappa$ B; PAMP: Pathogen associated molecular pattern; PBS: Phosphate buffered saline; PLC $\gamma$ 2: Phospholipase C- $\gamma$ 2; PRRs: Pattern-recognition receptors; RT-PCR: Reverse transcriptase polymerase chain reaction; Syk: Spleen-tyrosine kinase

## Funding

This study was supported by the National Natural Science Foundation of China (No. 81470609; No. 81170825), Youth Project of Natural Science Foundation of Shandong Province (ZR2013HQ007) and Key Project of Natural Science Foundation of Shandong Province (ZR2012FZ001).

## Availability of data and materials

The data have not been placed in any online data storage. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

All of the authors contributed substantially to this study. Conceived and designed the Experiments: GZ JL and XP. Performed the experiments: XP JL CL JQ and YZ. Analyzed the data: JQ YZ and CL. Contributed reagents/

materials/analysis tools: JQ and CL. Wrote the paper: XP GZ and YZ. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

This study followed the tenets of the Declaration of Helsinki.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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Received: 12 December 2017 Accepted: 6 July 2018

Published online: 13 July 2018

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