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Evaluation of murine lung epithelial cells (TC-1 JHU-1) line to develop Th2-promoting cytokines IL-25/IL-33/TSLP and genes *Tlr2*/*Tlr4* in response to *Aspergillus fumigatus*

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ABSTRACT

Objective. – The aims of this study were to determine the role of live and heat-killed *Aspergillus fumigatus* conidia in releasing interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP) and to express Toll-like receptor (*Tlr*)2 and *Tlr4* genes.
Materials and methods. – Murine lung epithelial cells were incubated with live and heat-killed *A. fumigatus* conidia at 37 °C for 6, 24 and 48 h. After treatments, ELISA was performed to measure the concentrations of IL-25, IL-33 and TSLP in the supernatants. Quantitative real-time PCR (qPCR) was performed to assess the expression levels of *Tlr*2 and *Tlr4* genes.
Results. – The concentrations of IL-25 and IL-33 significantly increased after exposure to live and heat-killed conidia for various times when compared with untreated control ($P < 0.05$). The secretion of TSLP at different concentrations of heat-killed conidia was significantly higher than both live conidia and untreated control ($P < 0.05$). qRT-PCR results indicated a up-regulation from 1.08 to 3.60-fold for *Tlr2* gene expression and 1.20 to 1.80-fold for *Tlr4* gene expression exposed to heat-killed conidia.
Conclusion. – *A. fumigatus* has a potential ability to stimulate murine lung epithelial cells to produce IL-25/IL-33/TSLP, as well as to express *Tlr2*/*Tlr4* genes, indicating an important role of lung epithelial cells in innate immune responses to *A. fumigatus* interaction.

1. Introduction

Aspergillus fumigatus (*A. fumigatus*) is an opportunistic fungus responsible for allergic phenotypes of aspergillosis, such as *Aspergillus*-induced asthma, allergic bronchopulmonary aspergillosis (ABPA) or allergic *Aspergillus* sinusitis (AAS), in patients [1]. Innate immune cells, such as lung epithelial cells or alveolar macrophages, initiate this inflammatory reaction [2]. Fungi are sensed by innate immune cells through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs) or nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) [3,4]. Activation of these receptors by *Aspergillus* leads to induction of cytokines, recruitment of other immune cells, and can eventually trigger adaptive immune responses such as T helper (Th) responses [3].

A robust induction of the Th1 response during *Aspergillus* infection is associated with the protection and successful clearance, while increased Th2 responses impair fungal clearance [5]. Allergic pulmonary aspergillosis (APA) with the prevalence of 16% is generally regarded as a Th2 disease because increased levels of eosinophils, and Th2 cytokines (IL-4, IL-5 and IL-13) are often observed [2]. *Aspergillus* germinating conidia and hyphae are recognized in vitro by host PRRs on epithelial cells and induce the production of cytokines and chemokines such as interleukin (IL)-6, TNF-α, IL-33, IL-25, thymic stromal lymphopoietin (TSLP), monocyte chemoattractant protein-1 (MCP-1) and IL-8 [6]. In particular, IL-25, IL-33, and TSLP produced and released by lung epithelial cells induce and promote Th2-type airway inflammation and cause remodeling and pathological changes in the airway walls, suggesting pivotal roles in the pathophysiology of allergic aspergillosis. Airway epithelium is not merely a structural barrier,

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but must be considered an active player in the pathogenesis of other allergic disorders [7].

While most *Aspergillus*-related PRRs have been studied in the context of invasive aspergillosis, limited data exist of the *Aspergillus*-associated molecular patterns and their PRRs responsible for triggering the Th2 response in APA [8]. There is accumulating evidence, based on in vitro and in vivo studies, that supports a role for TLRs in *A. fumigatus* sensing. In mouse studies, TLR2 and TLR9 have been described to skew the *Aspergillus*-induced Th response towards a Th2 profile [9]. Findings from various studies demonstrated that *A. fumigatus* recognition by macrophages, neutrophils and dendritic cells depends on the presence of TLR2 and TLR4 [8]. This is of particular interest in the context of the involvement of TLRs in allergic aspergillosis, since lung epithelial cells express all of the different types of TLRs, and activation of these TLRs has been shown to induce the production of various cytokines [10]. In the present study, the first goal was to investigate whether *A. fumigatus* conidia induce release of type 2-promoting cytokines IL-25, IL-33 and TSLP. The second goal was to investigate whether *A. fumigatus* conidia affect epithelial cell responses for expressing *Tlr2* and *Tlr4* genes.

2. Material and methods

2.1. A. fumigatus *strain*

A clinical isolate of *A. fumigatus* (ATCC 204305), purchased from Biomedical Research and Development Material Emerging Infectious Disease Research, was maintained on 2% malt extract agar-MEA (Merck Co., Darmastdt, Germany) slant at 37 °C.

2.2. *Preparation of* A. fumigatus *conidia*

A. fumigatus was sub-cultured onto sabouraud dextrose agar-SDA (Merck Co., Darmastdt, Germany) and incubated at 37 °C for 5 days. *A. fumigatus* conidia were harvested by washing the slant culture with a phosphate-buffered saline (PBS) solution containing 0.5% Tween 20 and gently shaken. Conidia were then washed by centrifugation (5 min at 10,000 × *g*) at room temperature on a Beckmann GS-6 bench centrifuge and suspended in a PBS solution containing 0.1% Tween 20. Conidia concentrations were evaluated by counting with a haemocytometer. The suspension was diluted as needed in order to reach the desired concentration. In this study, the following concentrations were prepared: three concentrations of conidia (3×10^4, 3×10^6 and 3×10^7 cell/mL) inactivated by heat condition (70 °C at 20 min in water bath) and one concentration of live conidia (3×10^4 cell/mL).

2.3. Cell culture and stimulation conditions

Mouse lung epithelial cells (TC-1 JHU-1), obtained from Pasteur Institute of Tehran-Iran, were cultured in roswell park memorial institute (RPMI) 1640 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate supplemented with 0.1 mM nonessential amino acids, 90% fetal bovine serum, penicillin 100 IU/mL and streptomycin 100 IU/mL at 37 °C in 5% CO_2 incubater. The number of viable cells was determined by trypan blue exclusion. A concentration of 3×10^5 cells was plated and grown overnight in 3 mL of medium in 6-well plates prior to cytokine assay [11]. TC-1 JHU-1 cells were stimulated with three concentrations of killed conidia (3×10^4, 3×10^6 and 3 × 107 cell/mL) for various times (6, 24 and 48 h). Lung epithelial cells culture without conidia as negative control and with LPS as positive control were chosen.

2.4. Measurement of cytokines

Levels of IL-25, IL-33 and TSLP in cell culture supernatants were assessed using commercially available sandwich-type ELISA kits (R&D systems, Minneapolis, MN 55413, USA). Assays were performed in accordance with the manufacturer's instructions. Briefly, a 96-well flat bottom plate was coated with capture polyclonal antibody specific to each cytokine. Serially diluted specific standards were added to the respective wells. Following a series of washing, the captured cytokine was detected using the specific conjugated detection antibody. The chromogen/substrate reagent was added into each well and, after color development, the plate was read at 450 nm using a Victor multilabel *plate reader* (Perkin Elmer, Waltham, MA).

2.5. Quantitative real-time PCR (qPCR)

Total RNA was extracted from cell cultures using the RNeasy mini-kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Briefly, samples were spun down, immersed in liquid nitrogen for 5 min, and then ground with plastic mini-pestles (DiaMed, Mississauga, ON, Canada). Tubes were heated for 2 min at 56 °C and passed through the QIAshredder column. RNA was eluted from column to into 50 μL nuclease-free water and was stored at −80 °C until use. RNA concentration and quality were measured using a Nanodrop system (NanoDrop Technology, San Diego, CA, USA). Reverse transcription of total RNA was done using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Real-time PCR was carried out using the SYBR Green method using following primers: *Tlr2*, forward: TGCTGCCATTCTCATTCTTCTG, reverse: AGGTCTTGGTGTTCATTATCTTCC, *Tlr4*, forward: CAACCAAGAACCTGGACCTG, reverse: GAGAGGTGGCTTAGGC, GAPDH, forward: CCACTCCTCCACCTTTGACG, reverse: CCACCACCCTGTTGCTGTAG.

qPCR data were analyzed by the delta delta CT (ddCT) method and normalized to GAPDH [12]. qPCR was performed according to the manufacturer's protocol using an ABI 7900HT system (Applied Biosystems, Foster City, CA, USA). Raw threshold cycle values were normalized using RNU-6B (Cat#4427975, Thermo Fisher Scientific, San Jose, CA, USA) as the internal control .

Table 1
Comparison of gene expression of *Tlr2*, *Tlr4* and GAPDH in mouse lung epithelial cells (TC-1 JHU-1) exposed to *Aspergillus fumigatus* conidia.

Time	Group	Conidia concentration (cell/mL)	2^ΔCT = Expression	
			TLR2	*TLR4*
6 hours	Heat-killed conidia	3×10^4	1.3	1.3
		3×10^6	1.7	1.4
		3×10^7	1.9	1.8
	Live conidia	3×10^4	1.8	0.75
	Control	–	1.2	1
24 hours	Heat-killed conidia	3×10^4	1.5	1.6
		3×10^6	2	2
		3×10^7	2.2	2.1
	Live conidia	3×10^4	2.1	0.27
	Control	–	1.1	1.2
48 hours	Heat-killed conidia	3×10^4	2	1.2
		3×10^6	3.5	1.2
		3×10^7	3.6	1.5
	Live conidia	3×10^4	3.8	0.12
	Control	–	1	1

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