Acta Pharmacol Sin 2008 Oct; 29 (10): 1202–1208

Full-length article

Aspergillus fumigatus conidia upregulates NOD2 protein expression both *in vitro* and *in vivo*[1]

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Key words

Aspergillus fumigatus; NOD2 protein; pattern recognition receptors

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Received 2008-03-10
Accepted 2008-07-10

doi: 10.1111/j.1745-7254.2008.00860.x

Abstract

Aim: To determine if NOD2 is involved in host recognition of *Aspergillus fumigatus* (*Af*) conidia. **Methods:** An *Af* conidia pulmonary infection murine model was established by intranasal inoculation of *Af* conidia suspensions. Protein levels of NOD2 in lung tissue were determined by immunohistochemistry. A549 and phorbol-12-myristate 13-acetate (PMA)-activated THP-1 cell lines were treated with heat-killed *Af* conidia, then the presence of NOD2 protein in these cell lines was detected by Western blotting. The ability of muramyl dipeptide (MDP) to induce the secretion of TNF-α after incubation with heat-killed *Af* conidia was measured by enzyme-linked immunosorbent assay. **Results:** The expression of NOD2 protein in lung tissue increased after *Af* conidia infection. Heat-killed *Af* conidia significantly upregulated NOD2 protein expression in A549 cells and PMA-activated THP-1 cells. Additionally, *Af* conidia in conjuction with MDP, significantly increased the secretion of TNF-α in A549 cells and PMA-activated THP-1 cells. **Conclusion:** *Af* conidia upregulates NOD2 protein expression *in vitro* and *in vivo*. These findings indicate that NOD2 protein may respond to *Af* conidia.

Introduction

Invasive pulmonary aspergillosis (IPA) is a life-threatening disease that occurs predominantly in immuno compromised patients. *Aspergillus fumigatus* (*Af*) has become the second most common opportunistic fungal infection in immunocompromised patients[1]. Despite recent progress in the development of antimicrobial therapies, the incidence of IPA continues to increase while patient outcomes remain poor[2]. A better understanding of the mechanisms involved in host antimicrobial defense to *Af* infection may contribute to new therapeutic interventions.

Innate immunity is the first component of microbial recognition and serves as a primary host defense. The innate immune system confers rapid recognition of a wide spectrum of pathogens through pattern recognition receptors (PRRs)[3]. Among membrane-bound PRRs, the best known are the Toll-like receptors (TLRs), that sense a wide array of microbial ligands at the cell surface or within endosomes. Cytoplasmic PRRs include the caspase-recruiting domain (CARD) helicases, such as retinoic acid-inducible protein I and melanoma differentiation-associated protein 5, which are involved in antiviral responses, and the nucleotide binding oligomerization domain (NOD)-like receptor (NLR) family that primarily recognize microbial molecules of bacterial origin[4]. NOD2, a caspase recruitment domain (CARD)-15 protein, is a widely studied member of NLR family. The presence of NOD2 is mainly limited to leukocytes, dendritic cells, and epithelial cells[5]. Biochemical and functional analyses have identified muramyl dipeptide (MDP), the minimal motif in all peptidoglycans both from Gram-positive and Gram-negative bacteria, as the essential structure recognized by NOD2[5,6]. Martinon *et al*[7] has proposed that MDP also activated NALP3, another member of the NLR family. However, Sutterwala *et al*[8] found that

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MDP enhanced lipopolysaccharide (LPS)-induced secretion of TNF-α in both NALP3$^{+/+}$ and NALP3$^{-/-}$ macrophages. Additionally, one recently published paper revealed that NOD2 was the dominant NLR involved in MDP recognition[9]. MDP can enhance host defense against infection and can improve the survival rates of animals infected by fungi[10]. It has been demonstrated that MDP can induce cytokine secretion following an initial stimulation to induce NOD2 expression[11]. These results suggest that NOD2 may play an important role in innate immune sensing of microbes.

As *Af* conidia can be internalized by epithelial as well as endothelia cells and can be phagocytized by dendritic cells and macrophages which express NOD2 protein, and as NOD2 protein mediates intracellular detection of bacterial components, we hypothesized that NOD2 contributed to the innate immune recognition of *Af* conidia.

In these experiments, NOD2 expression induced by *Af* conidia stimulation *in vitro* and *in vivo* was investigated. The ability of MDP to induce the secretion of TNF-α in A549 cells and THP-1 cells following treatment with heat-killed *Af* conidia was also studied.

Materials and methods

Animals and cells Specific pathogen-free BALB/c mice, 8–10 weeks old, were purchased from the Shanghai Laboratory Animal Centre (Shanghai, China). Procedures involving animals and their care were conducted in conformity with national and international laws and policies. A549 cells (human lung adenocarcinoma epithelial cell line, ATCC number CCL-185) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 365 mg/L *L*-glutamine, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin. THP-1 cells (human acute monocytic leukemia cell line, ATCC number TIB-202) were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 IU/mL), streptomycin (100 μg/mL), and *L*-glutamine (2 mmol/L).

***Af* strain and culture conditions** The strain of *Af* used in these experiments was obtained from a fatal case of pulmonary aspergillosis at the Infectious Diseases Institute of Huashan Hospital, Fudan University, Shanghai, China. The *Af* was cultured on Sabouraud dextrose agar supplemented with chloramphenicol for 4 d at room temperature. The surface of each plate was then washed with 15 mL of sterile 0.1% Tween-80 in normal saline. The resulting suspension was filtered through sterile gauze to remove clumps and hyphal debris, was washed once, and was then resuspended in 4 mL of 0.1% Tween-80. After extensive washing with saline, the *Af* conidia were counted using a particle counter and diluted to the desired concentrations. Final concentrations were measured again prior to administration. Heat-killed *Af* conidia were obtained by heating the *Af* conidia in a water bath at 100 °C for 1 h, then washed 3 times with sterile phosphate-buffered saline (PBS). The efficiency of this killing treatment was verified by the failure of the heat-killed *Af* conidia to grow when cultured for 7 d in Sabouraud dextrose agar.

Inoculation of mice Mice were inoculated intranasally with either the *Af* conidia suspension (infected group) or sterile normal saline (control group) following the procedure described by Shao *et al*[12]. Each group consisted of 6 to 8 animals. For 2 consecutive days mice were anesthetized with inhaled diethyl ether prior to instillation. The infected group was given a daily suspension of 2×10^7 *Af* conidia in 20 μL of sterile normal saline. Control mice were given 20 μL of sterile normal saline. The suspension was delivered into the nostrils slowly by micropipette with sterile disposable tips. Mice were held in an upright position until the suspension was completely inhaled and normal breathing resumed.

Immunohistochemistry assay Mice were killed by CO_2 asphyxiation at designated time points. The chest cavity was opened aseptically, and the pulmonary vasculature was perfused with PBS *via* the right ventricle. For lung immunohistochemical analysis, whole lungs were fully inflated with 4% paraformaldehyde dissected and placed in fresh paraformaldehyde for 24 h. After fixation, the entire lung tissue was embedded by paraffin. Five μm sections of paraffin-embedded lung tissue were deparaffinized, then rehydrated. After antigen retrieval using microwave heating, the sections were incubated overnight at 4 °C with anti-NOD2 mAb (Cayman Chemical, Ann Arbor, MI, USA) at 4 μg/mL. After 3 washes with PBS, specific staining was detected by incubating sections for 1 h at room temperature with a biotinylated rabbit anti-murine antibody (1:500 dilution) followed by a 1 h incubation with Vectorstain ABC Elite reagent (Vector Laboratories, Burlingame, CA, USA) and then a 1 to 5 min incubation with 3,3′-diaminobenzidine tetrachloride (DAB) peroxidase substrate. Lung tissue sections were then counterstained with haematoxylin before dehydration with ethanol and histosolve. Slides were mounted with permount and visualized by light microscopy.

Electron microscopy THP-1 cells were cultured in 6 cm culture dishes. Fifty ng/mL PMA was added to the

culture to induce macrophage phenotype differentiation. Two hours later, non-adherent cells and PMA were removed with 3 washes of PBS that did not contain calcium and magnesium. Adherent macrophages were incubated in complete RPMI-1640 medium supplemented with 10% heat-inactivated FBS for 24 h, incubated with heat-killed *Af* conidia, and were then examined by transmission electron micrographs at different time points. A549 cells were grown to confluence in 6 cm culture dishes, incubated with heat-killed *Af* conidia, and were then examined by transmission electron micrographs at different time points.

Detection of NOD2 protein by Western blotting A549 and PMA-activated THP-1 cells were grown in 6 well plates. After incubation with heat-killed *Af* conidia for 24 h, the medium was removed, and 300 μL of 1% RIPA lysis buffer (1% Triton X-100, 0.1 mol/L NaCl, 10 mmol/L Hepes pH 5.6, 2 mmol/L EDTA, 4 mmol/L Na_3VO_4, 40 mmol/L NaF，40 mmol/L PMSF, 20 μL/mL β-glycerin) was added. Supernatants of cell lysates containing total cell lysate proteins were obtained by centrifugation at 900×*g* for 10 min. Total cell lysate proteins were then immunoprecipitated with anti-NOD2 monoclonal antibodies. After 3 h incubation at 4 °C, 20 μL magnetic beads were added to each tube. After overnight incubation at 4 °C, the precipitum was obtained by centrifugation at 12 000×*g* for 20 s, 20 μL ice-cold lysis buffer (50 mmol/L Tris–HCl, pH 7.4, containing 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.1% deoxycholate, 5 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L 4-nitrophenyl phosphate, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, and 1 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride) was added to precipitum. After centrifugation at 15 000×*g* for 20 min at 4 °C , the supernatants were mixed with one-fourth volume of 4×SDS sample buffer, boiled for 5 min, and then electrophoresed on 8% SDS-polyacrylamide gel electrophoresis (PAGE) gels. After electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane. Membranes were blocked with 5% skim milk (1 h), rinsed, and incubated with NOD2 monoclonal antiserum in TBS containing 0.05% Tween 20 (TBS-T) and 3% skim milk for 2 h. Excess primary antibody was then removed by washing the membrane 4 times with TBS-T. The membrane was then incubated with 0.1 μg/mL secondary antibody for 1 h. Following 3 washes with TBS-T, bands were visualized by ECL Western blotting detection reagents and exposed to X-ray. The blots were scanned for density assays.

Examination of the concentrations of TNF-α by ELISA A549 and PMA-activated THP-1 cells were grown in 24-well plates in a final volume of 500 μL. After incubation with nothing (control group), MDP, heat-killed *Af* conidia, and a mixture of heat-killed *Af* conidia and MDP for 24 h at 37 °C, in the presence of lipofectamine 2000 0.5 μL/well, the supernatant was collected and stored at –80 °C, until assays of TNF-α concentrations were carried out by a sandwich enzyme-linked immunosorbent assay (ELISA).

Statistical analysis All data were expressed as means ±SEM. Analyses were carried out by SPSS statistical software 10.0. Student's *t*-tests were used to determine the statistical significance of values between experimental groups (significance was defined as $P<0.05$).

Results

The expression of NOD2 protein in lung tissue To delineate the role of the intracellular pattern recognition receptor NOD2 in the process of *Af* conidia infection, an *Af* infected murine model was established. Previous research showed that glucocorticoids had suppressive effects on Toll-like receptor signaling pathways[13]. To avoid the potential effects of such immunosuppressive drugs on NOD2 signaling pathways, a normal murine model was used instead of the classic immunosuppressed murine model. In the present experiment, we took lung tissue from normal mice without treatment to serve as a blank control (Figure 1A). We also took lung tissue from mice that were inoculated intranasally with sterile normal saline as a negative control (Figure 1E-1G). NOD2 protein expression was not detected in lung tissue samples from either of these groups. However, clear staining of lung tissue from the infected group was detected by immunohistochemistry 24 h after inoculation with *Af* conidia (Figure 1C).

Phagocytosis of *Af* conidia by A549 cells and THP-1 cells *Af* conidia internalization is a key step for recognition by cytosolic pattern recognition receptors. NOD2 is a cytosolic sensor protein. To clarify whether *Af* conidia could be recognized by NOD2 or not, *Af* conidia internalization by lung epithelial cells or macrophages was observed. Lung epithelial cell line A549 cells and THP1-derived macrophages were treated with heat-killed *Af* conidia. As shown in Figure 2, by transmission electron microscopy, conidia that had been digested to different extents were observed within membrane-bound phagosomes 24 h after incubation. Also at 24 h after incubation, the internalization of *Af* conidia in THP-1 cells was more obvious than in A549 cells (Figure 2).

The expression of NOD2 protein in A549 and THP-1

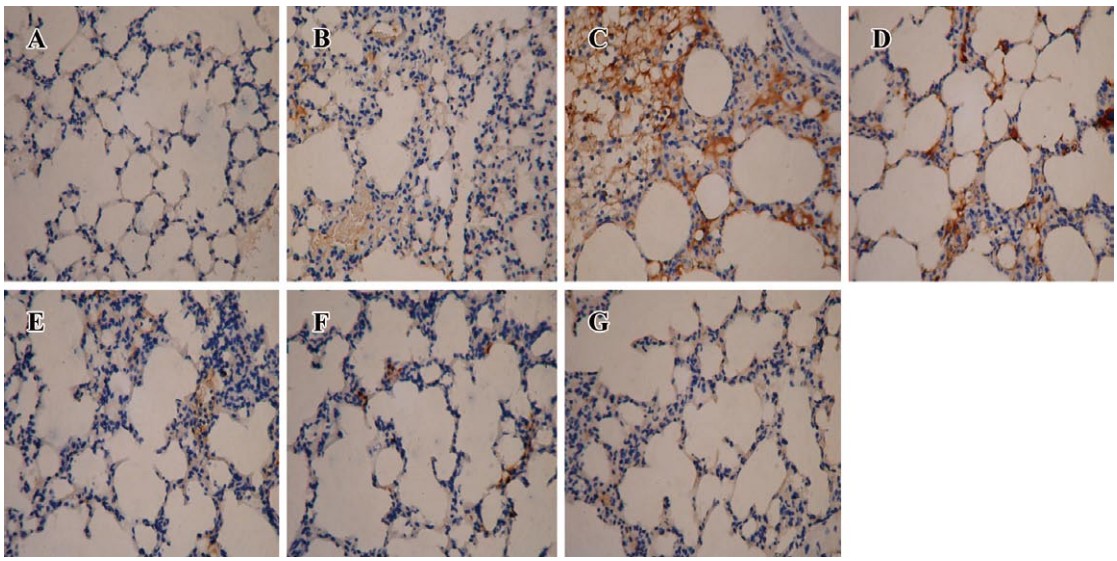

Figure 1. Photomicrographs of immunohistochemistry stained murine lung tissue sections (DAB staining, original magnification×200). Mice were inoculated intranasally with either the *Af* conidia suspension (infected group) or sterile normal saline (NS) (control group). (A) normal murine lung tissue; (B) 12 h post inoculation with *Af* conidia; (C) 24 h post inoculation with *Af* conidia; (D) 48 h post inoculation with *Af* conidia; (E) challenged with NS for 12 h; (F) challenged with NS for 24 h, (G) challenged with NS for 48 h.

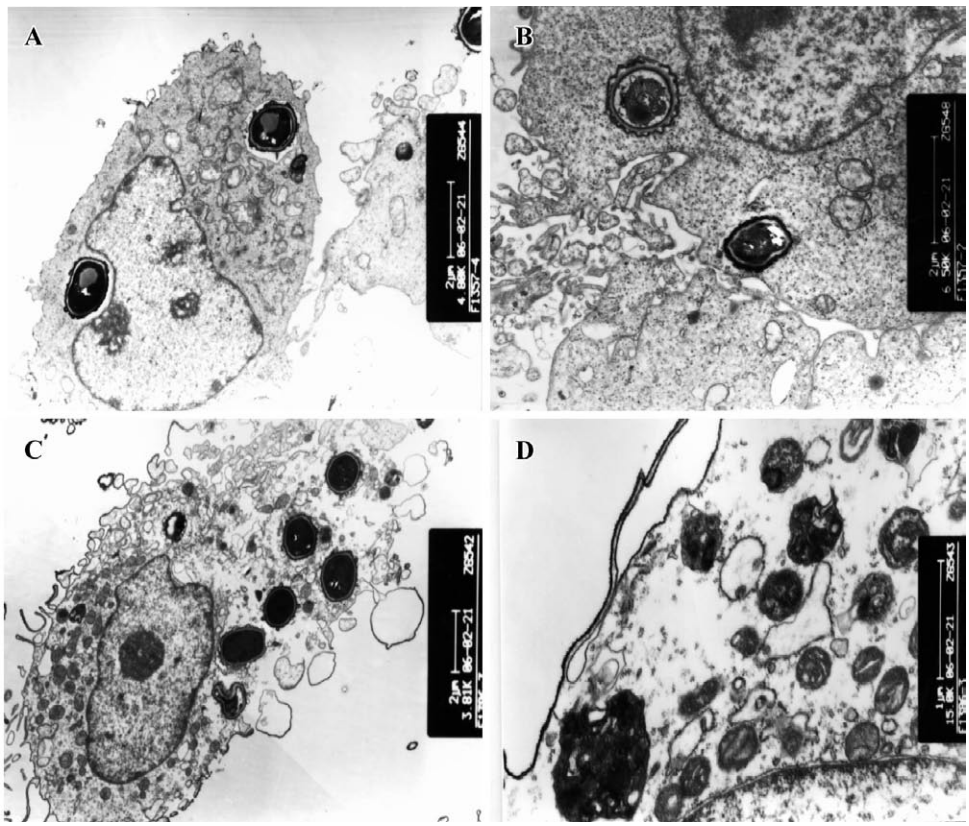

Figure 2. Transmission electron micrographs of A549 cells and THP-1 cells after incubation with heat-killed *Af* conidia for 24 h. THP-1 cells were pretreated with PMA. (A) A549 cells ×4800, (B) A549 cells ×6500, (C) THP-1 cells ×3800, (D) THP-1 cells ×15000.

cells after heat-killed *Af* conidia stimulation The expression of NOD2 protein in A549 and THP-1 cells was measured. There was expression of the NOD2 mRNA in these cells, however, the expression of NOD2 protein was very low and could not be detected directly by Western blotting. Immunoprecipitation and Western blotting were combined in order to measure the expression of NOD2 protein. As shown in Figure 3, exposure to heat-killed *Af* conidia resulted in a remarkable increase in NOD2 protein expression in both A549 and THP-1 cells in a dose-dependent manner.

MDP in conjunction with heat-killed *Af* conidia increases secretion of TNF-α To test the possibility that NOD2 might act as an innate immune response receptor for *Af* conidia or be involved in innate recognition of *Af* conidia, TNF-α secretion following *Af* stimulation in A549 cells and PMA-activated THP-1 cells was investigated. A549 cells and PMA-activated THP-1 cells were stimulated by MDP, heat-killed *Af* conidia and a mixture of heat-killed *Af* conidia and MDP. Because NOD2 is an intracellular protein, lipfectamine was used to deliver higher levels of MDP into cells[14,15]. There was no obvious TNF-α secretion when cells were treated with MDP alone. When treated with *Af* conidia alone, there was a slight increase in TNF-α secretion compared with the control and MDP-only groups (Figure 4). However, heat-killed *Af* conidia combined with MDP significantly increased the secretion of TNF-α in both cell lines ($P<0.05$).

Discussion

The innate immune system recognizes pathogens by sensing pathogen-associated molecular patterns (PAMPs) *via* its PRRs. Toll-like receptors (TLRs) and NOD proteins represent two classes of PRRs in mammals. TLRs are a family of integral membrane proteins, while NOD proteins are cytosolic. Both types of PRRs are involved in detecting potentially harmful microbes through PAMPs recognition and initiating an inflammatory reaction to combat the infection[16]. TLR2 and TLR4 have been demonstrated to be associated with host recognition of *Af*[1,17,18]. Although it is clear that NOD2 and TLR2 have different ligands, there is strong evidence that NOD2 modulates TLR2 signaling[19, 20]. We raised the possibility that these TLRs were likely to serve as first line receptors for *Af*, the NOD2 protein might play a role in a subsequent phase of infection. Since TLRs mediate NF-κB activation and NF-κB binding sites have been identified in the NOD2 promoter[21,22,23], recognition

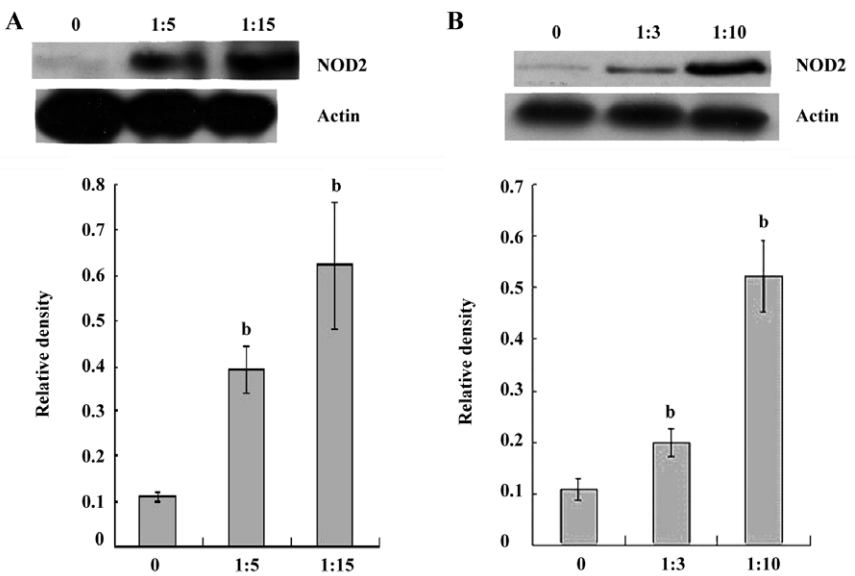

Figure 3. Elevated expression of NOD2 protein in THP-1 and A549 cells after heat-killed *Af* conidia stimulation. THP-1 (A) and A549 (B) cells were either left untreated (0) or exposed to heat-killed *Af* conidia at different cell-conidia ratios (1:5 and 1:15 for THP-1 cells, 1:3 and 1:10 for A549 cells). Densitometric analyses of NOD2 protein bands were shown as relative optical densities normalized to actin protein levels. Date were expressed as mean±SEM. There were significant differences between the groups ($P<0.05$). The experiments were performed three times with independent samples. $^bP<0.05$ *vs* 0.

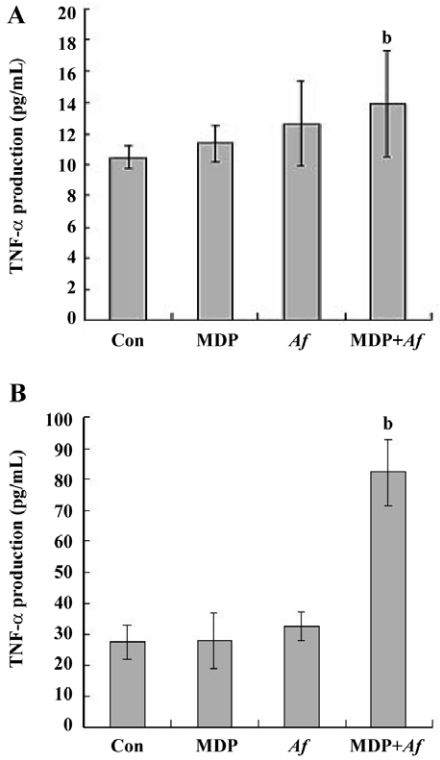

Figure 4. Secretion of TNF-α in A549 and THP-1 cells challenged with MDP and heat-killed *Af* conidia. A549 (A) and PMA-pretreated THP-1 (B) cells were divided into 4 groups: not challenged (control group), or challenged with MDP, heat-killed *Af* conidia, or a mixture of heat-killed *Af* conidia and MDP, respectively, in the presence of lipofectamine ^{TM}2000 0.5 μL/well. After 24 h, supernatants were harvested for analysis of cytokine concentrations by ELISA. $^{b}P<0.05$ *vs* Con.

of *Af* conidia by the TLRs might cause the upregulation of NOD2 and thereby facilitate the immune response of the host against this pathogen. In line with this hypothesis, we found that NOD2 protein levels increased after *Af* conidia infection.

The human A549 alveolar epithelial cell line shows similar features as type II alveolar epithelial cells. This cell line has been previously used as a model of alveolar epithelial cells[24,25]. THP-1, a human monocytic leukemia cell line, differentiates to adherent macrophages through PMA stimulation[26]. Our results have confirmed previous reports[27,28] that *Af* conidia binds to and becomes internalized by A549 lung epithelial cells and THP-1 derived macrophages *in vitro*. Meanwhile, *Af* conidia upregulates NOD2 protein expression in both A549 and THP-1 cell lines. We also tested TNF-α secretion of A549 cells and THP1-derived macrophages. Consistent with a previous study conducted by Marriott *et al*[11], MDP alone fails to elicit significant TNF-α secretion by either of these cell lines, which may be attributable to low levels of NOD2 protein expression under these conditions. Initial stimulation may be required to induce NOD2 expression, thereby rendering both A549 and THP-1 cells sensitive to MDP[11]. When treated with *Af* conidia alone, we found slightly elevated TNF-α secretion compared with the control and MDP-only groups, which may be due to the elevated NOD2 protein expression by *Af* stimulation. *Af* conidia in conjunction with MDP significantly increased the secretion of TNF-α. Taken together, these data suggest that heat-killed *Af* conidia stimulated A549 and THP-1 cells to express functional NOD2 protein that can respond to MDP.

To date, NOD2 has been implicated in the detection of several pathogenic bacteria and induction of innate immune responses to *Streptococcus pneumoniae*[5], *Mycobacterium tuberculosis*[29], *Staphylococcus aureus*[30] and *Listeria monocytogenes*[31]. Only one study, conducted by Chantal *et al*[32] has investigated whether NOD2 is involved in the recognition of fungal infections. Contrary to our results, they concluded that NOD2 was unlikely to play an important role in the recognition of *Candida albicans*. They assessed the prevalence of NOD2 polymorphisms in patients with *Candida* bloodstream infections and in patients with recurrent vulvovaginal candidiasis (RVVC). They also investigated the role of NOD2 in cytokine stimulation by *C albicans* blastoconidia *in vitro*. However, it is difficult to compare the results directly due to variations in cell number, culture media, and experimental procedures. Furthermore, in their study, *C albicans* blastoconidia upregulated NF-κB activity in cells overexpressing NOD2 in a concentration-dependent manner, but the activation of NF-κB in cells overexpressing NOD1 or the control HEK cells was similar to the unstimulated cells (*nb*, the *P* values were not listed in the Chantal *et al* study[32]). This result suggests that when induced by *Candida albicans*, NOD2 contributes to the activation of NF-κB.

In summary, our experiments show that NOD2 protein expression is induced by *Af* conidia stimulation *in vitro* and *in vivo*. Our results suggest that NOD2 protein may respond to *Af* conidia. However, whether NOD2 recognizes *Af* conidia in innate immune response requires further study.

Author contribution

Jie-ming QU, Hui-jun ZHANG, Chang-zhou SHAO, Jing ZHANG, and Li-xian HE designed research; Hui-jun ZHANG performed research; Zheng-hong YUAN contrib-

uted new analytical tools and reagents; Hui-jun ZHANG analyzed data; Hui-jun ZHANG wrote the paper.

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