Original Article

Overexpression of Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Nonintegrin in Dendritic Cells Protecting against Aspergillosis

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Abstract

Background: Dendritic cells (DCs) play an important role in host defense against pathogen infection. DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (SIGN) is a group II C-type lectin receptor and specifically expressed on the surface of DCs. This study aimed to determine whether DC-SIGN affects intracellular signaling activation, Th1/Th2 imbalance and aspergillus immune evasion in aspergillus infection, and explore the application of DC-SIGN-modified DCs in immunotherapy.
Methods: DCs were first obtained from the mononuclear cells of peripheral blood. The interferon (IFN)-γ and dexamethasone (Dex) were used to stimulate DCs. The expression of DC-SIGN, Th1 and Th2 cytokines, and the capacity of DCs in stimulating T cells proliferation and phagocytosis, and nuclear factor (NF)-κB activation were analyzed. In addition, adenovirus expression vector Ad-DC-SIGN was generated to transfect DCs. Mannan was used to block DC-SIGN signaling for confirming the involvement of DC-SIGN function in *Aspergillus fumigatus* (Af)-induced DCs maturation. The unpaired, two-tailed Student's *t*-test was used in the comparisons between two groups.
Results: Exogenous IFN-γ could activate Af-induced DCs and promote the Th0 cells toward Th1 profile (interleukin [IL]-12 in IFN-γ/Af group: 50.96 ± 4.38 pg/ml; control/Af group: 29.70 ± 2.00 pg/ml, $t = 10.815$, $P < 0.001$). On the other hand, Dex inhibited the secretion of Th2 cytokines (IL-10 in Dex/Af group: 5.27 ± 0.85 pg/ml; control/Af group: 15.14 ± 1.40 pg/ml, $t = 14.761$, $P < 0.001$)), and successfully caused immunosuppression. After transfection with Ad-DC-SIGN, DCs have improved phagocytosis (phagocytosis rates in Ad-DC-SIGN group: 74.0% ± 3.4%; control group: 64.7% ± 6.8%, $t = 3.104$, $P = 0.013$). There was more Th1 cytokine secreted in the Af-induced DC-SIGN modified DCs (IL-12 in Ad-DC-SIGN/Af group: 471.98 ± 166.31 pg/ml; control/Af group: 33.35 ± 5.98 pg/ml, $t = 6.456$, $P = 0.001$), correlated to the enhanced NF-κB activation.
Conclusion: Overexpressing DC-SIGN in DCs had a protective function on aspergillosis.

Key words: *Asepergillus fumigatus*; Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Nonintegrin; Dendritic Cells; Immunity

Introduction

Pulmonary aspergillosis is an acute or chronic lung disease due to aspergillus infection and daily inhalation of aspergillus conidia. Clinically, it is classified into chronic pulmonary aspergillosis, allergic bronchopulmonary aspergillosis , and invasive pulmonary aspergillosis (IPA). IPA is the most common subtype.[1] The high IPA morbidity is observed in the immunocompromised patients under conditions such as the history of organ transplantation, the concurrence of malignant tumor and the history of receiving therapy with corticosteroids or immunosuppressant.[2,3] The early

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diagnosis of IPA is very difficult because of its nonspecific symptoms and rapid symptom progression. The mortality is high up to 80–95%.[4]

Aspergillus infection induces natural immunity and acquired immunity. Innate immune cells such as alveolar macrophages (AMs) and neutrophilic granulocytes can internalize and kill the invading aspergillus conidia and inhibit conidia germinating into hyphae through oxidation and nonoxidation mechanisms.[5] T cells play an important role in acquired immunity after aspergillus infection. Among immune competent patients, Th1 immune responses are predominantly induced. Th1 cytokines such as interleukin (IL)-12, interferon (IFN)-γ, tumor necrosis factor (TNF)-α can promote the differentiation of Th0 cells into Th1 cells, and eliminate aspergillus.[6] However, in immune-compromised patients, AMs and neutrophilic granulocytes cannot kill hyphae and conidia effectively. After patients were infected, Th2 immune responses were predominantly induced and lead to imbalanced Th1 and Th2 responses.[7] The increased Th2 cytokines such as IL-10 and IL-4 promoted the differentiation of Th0 cells into Th2 cells, and inhibited the immune response and facilitated aspergillus infection.[6]

Dendritic cells (DCs) are the most powerful professional antigen-presenting cells, and responsible for antigen recognition and procession.[8] They could initiate primary immune response and play an important role in host defense against pathogen infection and tumor progression. The pattern recognition receptors on DCs recognize pathogen-associated molecular patterns. Toll-like receptors (TLRs) can activate DCs by recognition of lipids, lipopolysaccharide, nucleic acid, etc.; whereas C-type lectin receptors (CLRs) can recognize the mannose-specific ligand on pathogen surface or glycoprotein of autoantigens.[7,9] TLRs and CLRs synergistically activate DCs to produce various pro-inflammatory, anti-inflammatory cytokines and reactive oxygen species.[10]

DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (SIGN), also called CD209, is a group II CLR,[11] specifically expressed on the cell surface of DCs.[12,13] It has been reported that DC-SIGN participated in host defense against pathogen infection.[7] In recent years, some studies have proved that DC-SIGN participated in the elimination of multiple pathogens such as human immunodeficiency virus, Ebola virus, mycobacterium tuberculosis, and fungi.[7,9] In immunocompetent patients with pulmonary aspergillosis, DC-SIGN could recognize the mannose domain on the cell wall of aspergillus conidia,[7] then recruit TLRs[14-17] and lead to promote DC endocytosis and activation of nuclear factor (NF)-κB.[16,18] Currently, it is accepted that DC-SIGN recognizes aspergillus conidia and induces inflammation through TLR signaling pathway,[14,15] while Mitogen-activated protein kinases and NF-κB are activated in DCs and cause a panel of inflammatory cytokines release.[18-20] However, in immunocompromised patients, DCs failed to engulf conidia via phagocytosis. After DCs recognize aspergillus conidia, they migrate through blood circulation into other tissues and cause aspergillosis. DCs secret more Th2 cytokines such as IL-4 and IL-10 instead of Th1 cytokines, which results in the imbalance of Th1 and Th2 immune response and antigen immune evasion. The underlying mechanism has not been elucidated yet. Does DC-SIGN participate in intracellular signaling activation or Th1/Th2 balance? What's the underlying molecular mechanism? Whether does DC-SIGN participate in aspergillus immune evasion? All these questions have not been elucidated. To answer these questions, this study performed the following experiments to elucidate the possible mechanisms via modulation of DC-SIGN expression in DCs after aspergillus infection.

Methods

Ethical approval

The study was approved by the Ethical Committee of Fundan University.

Generation of monocyte-derived dendritic cells and co-culture with stimuli

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood obtained from Zhongshan Hospital, Fudan University using Ficoll-Paque (Biowest, France) density gradient centrifugation and Lymphocyte Separation Medium (Biowest, France). The low-density PBMCs were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium at 37°C for 3 h to allow monocytes adherent, and then non-adherent cells were removed. For DCs, 2×10^6 cells/ml monocytes were cultured in RPMI 1640 Medium with 10% fetal bovine serum (FBS) for 7 days in the presence of 50 ng/ml human recombinant granulocyte-macrophage-colony-stimulating factor (Prospec, Israel), and 20 ng/ml human rIL-4 (Peprotech, America). The DC cultures were received an additional dose of GM-CSF and IL-4 every other day.[21]

On day 6, the cultured monocyte-derived DCs (MDDCs) were stimulated with 10^{-4} mol/ml dexamethasone (Dex; Shandong Xinhua Pharmaceutical Co., Ltd., China) for 24 h as Dex group. The 50 ng/ml IFN-γ (Peprotech, America) was added for 12 h as IFN-γ group. The 2 μg/ml Mannan was added for 24 h as Mannan group. The cultured cells were then stimulated with inactivated *Aspergillus fumigatus* (Af) (DC:Af = 1:10) for 24 h. Cells were labeled separately as control, IFN-γ, Dex, control/Af, IFN-γ/Af, Dex/Af, and Mannan/Af groups.

Preparation of inactivated *Aspergillus fumigatus*

Conidia of Af (ATCC 13073, an organism originally isolated from a patient with invasive aspergillosis) were harvested after 4 days of culture on Sabouraud dextrose agar (Difco, Detroit, USA), filtered through sterile gauze, killed by heating in a water bath at 100°C for 1 h, washed with saline solution, and stored at 4°C.

Cytokine assay

The level of IL-12 and IL-10 was determined using IL-12 and IL-10 ELISA kits (Immunotools, Friesoythe, Germany).

Phagocytosis assay

The cultured DCs was incubated with Af for 24 h (cell: Af = 1:10). Conidia that were not engulfed were counted using blood cell counting plate. The phagocytic rate was calculated as formula: (Total conidia–unengulfed conida)/Total conidia × 100%.

Western blot analysis for detection of dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin

The DCs were washed with phosphate-buffered saline and were lysed with radioimmunoprecipitation assay (RIPA) buffer. The protein concentration was measured, and 50 μg of total protein was used for electrophoresis. The Western blot analysis was performed under wet conditions at 240 mA for 2 h. Polyvinylidene difluoride (PVDF) membrane (Milipore, USA) was blocked with Tris Buffered Saline with Tween® 20 (TBST) buffer containing 5% skimmed milk powder with shaking for 2 h and incubated with primary antibodies (DC-SIGN antibody; R&D, USA). Then, it was incubated with the second antibody for 1 h. The PVDF membrane was washed with TBST and developed with the eSports Champion League substrate. Software Quantity One was used to calculate the ratio of the gray level of the objective strap to the internal reference strap.

Construction of pDC316-mCMV-EGFP-dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin

DC-SIGN recombinant plasmid VRC4409-DC-SIGN was provided by Barney S. Graham (Viral Pathogenesis Laboratory, NIH, USA). DC-SIGN gene was amplified by polymerase chain reaction (PCR) with the primers: forward, 5'-ATAAGAATGCGGCCGCATGAGTGACTCCAAG-3', and reverse, 5'-CCCAAGCTTCTACGCAGGAGGGGG-3', and following reaction condition: 95°C, 5 min–95°C, 30 s–58°C, 40 s–72°C, 30 s for 30 cycles, and then 72°C, 7 min. Restriction enzyme NotI and HindIII (Takara, Japan) were used to digest the amplified DC-SIGN product and pDC316-mCMV-EGFP plasmid. Both products were harvested by gel extraction kit (QIAGEN, Germany) and linked by T4 DNA ligase (TaKaRa, Japan). DH5α competent cells (Tiangen Company, China) were transfected by the product. The recombinant plasmid was extracted from the *Escherichia coli* by plasmid extraction kit (QIAGEN, Germany). The DC-SIGN gene in recombinant pDC-mCMV-EGFP-DC-SIGN was identified by restriction enzyme digestion and electrophoresis.

Generation of recombinant adenovirus

The 293 cells (Microbix Biosystems, Canada) were transfected with pDC-mCMV-EGFP-DC-SIGN and helper plasmid PPE3 by lipofectamine 2000 (Invitrogen) for 9–14 days to obtain adenovirus vector encoding DC-SIGN. After repeated filtering, the adenovirus vectors were purified. DC-SIGN gene in recombinant adenovirus DNA was extracted by the QIAGEN DNA Blood Mini kit (QIAGEN, USA) and confirmed by PCR with the primers DC-SIGN-F and DC-SIGN-R. The amplified virus titer was analyzed by TCID50. In the meanwhile, AdNull adenovirus without target gene inserted was generated in the same way.

T cells proliferation assay

The capacity of DCs to stimulate T cells proliferation was detected by allogeneic mixed lymphocyte reaction (MLR). T cells from PBMCs were purified through the nylon wool column: PBMCs were incubated at 37°C for 3 h and then nonadherent cells were collected. Before use, the column was equilibrated by washing with 20 ml RPMI-1640 and 10% FBS. Cells subjected to nylon wool purification were resuspended in 2 ml of RPMI-1640-10% FBS, and loaded onto the column. The column was sealed with 2 ml RPMI-1640 and incubated at 37°C for 60 min. Cells were eluted and plated at the concentration of 5×10^5/ml.[22] On the other hand, IFN-γ and Dex stimulated DCs were incubated with Mitomycin C on day 7 at 37°C for 45 min and resuspended by RPMI-1640 at the concentration of 5×10^4/ml. The 0.1 ml purified T cells and 0.1 ml DCs were put in culture in 96-well plate for 72 h and then, 20 μl CCK8 was added into each well. The MLR assays were carried out by calculating the stimulation index with optical density value.

Ad-dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin transfected dendritic cells

MDDCs on day 4 were harvested and transfected with Ad-DC-SIGN or control vector AdNull (multiplicity of infection = 200) for 48 h in RPMI-1640 medium, supplied with 10% FBS, GM-CSF and IL-4. The treated cells were divided into Ad-DC-SIGN and AdNull groups. On day 7, the cells were incubated with the inactivated Af (DC:Af = 1:10) for 24 h, divided into Ad-DC-SIGN/Af group and AdNull/Af group.

Nuclear factor-κB activation assay

Nuclear extracts from the treated DCs were obtained by human protein extraction kit (Biovision, USA). RIPA cell lysis buffer was used for extraction of cellular protein. The concentration of total protein and nuclear protein was measured by bicinchoninc acid protein assay. The expression of total NF-κBp65 and activated pho-NF-κBp65 which were transported to the nuclei were analyzed by the Western blot analysis.

Statistical analysis

Statistical analysis was performed using SPSS version 24.0 (IBM, Chicago, IL, USA). The data were presented as mean ± standard deviation (SD). The unpaired, two-tailed Student's *t*-test was used in the comparisons between two groups. A $P < 0.05$ was considered statistically significant.

Results

Expression of dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin of interferon-γ/dexamethasone stimulated dendritic cells

The DC-SIGN expression level in IFN-γ group decreased, compared to control group (0.894 ± 0.026 vs. 1.058 ± 0.041, $t = 5.333$, $P < 0.001$). Compared to control/Af group

(1.112 ± 0.053), the DC-SIGN expression in IFN-γ/Af (1.256 ± 0.119, t = 2.710, P = 0.030) and Dex/Af groups (1.207 ± 0.065, t = 2.752, P = 0.020) were much higher [Figure 1a].

Interferon-γ/dexamethasone affected the dendritic cells capacity in stimulating T cells proliferation and phagocytosis

We performed DCs capacity in stimulating T cells proliferation. There was no difference after IFN-γ or Dex treatment on DCs (control group: 2.03 ± 0.32; Dex group: 2.25 ± 0.02; IFN-γ group: 2.12: ± 0.36; $t_{(\text{control vs. Dex})}$ = 1.682, $P_{(\text{control vs Dex})}$ = 0.123; and $t_{(\text{control vs. IFN-}\gamma)}$ = 0.458, $P_{(\text{control vs. IFN-}\gamma)}$ = 0.657). After infected by Af, the DCs capacity in stimulating T-cell proliferation was significantly increased in control/Af group (2.96 ± 0.32), compared to control group (2.03 ± 0.32, t = 5.034, P = 0.001). In addition, there were significant differences in stimulating T-cell proliferation between IFN-γ/Af and IFN-γ groups (IFN-γ/Af: 2.66 ± 0.06, t = 3.624, P = 0.014), Dex/Af and Dex groups (Dex/Af: 3.55 ± 0.43, t = 7.401, P = 0.001). After Dex treatment, DCs with Af showed increasing capacity in stimulating T-cell proliferation compared to control/Af group (t = 2.696, P = 0.020). However, there was no significant difference among IFN-γ/Af and control/Af groups (t = 2.257, P = 0.070). The results suggested that stimulation with Dex could promote DCs capability in stimulating T-cell proliferation [Figure 1b].

Dex treatment enhanced phagocytic activity of DCs following aspergillus conidia infection, compared to the control group (phagocytosis rates: 91.0% ± 3.4% in Dex group; 67.1% ± 10.1% in the control group, t = 5.493, P = 0.001); whereas IFN-γ did not enhance phagocytic activity of DCs following aspergillus conidia infection (phagocytosis rate in IFN-γ group: 66.7% ± 5.9%, t = 0.084, P = 0.935). It suggested that the inflammatory environment did not change DCs' capacity of phagocytosis and stimulating T cells proliferation. However, DCs from patients administered with immunosuppressant have enhanced phagocytosis.

Interferon-γ and dexamethasone affected Th1 and Th2 cytokine expression

Interferon-γ and dexamethasone increased interleukin-12 expression of dendritic cells

Compared to control group (20.98 ± 1.08 pg/ml), IL-12 expression level of DCs was significantly increased in IFN-γ (29.70 ± 2.68 pg/ml, t = 7.392, P < 0.001) and Dex groups (41.61 ± 2.88 pg/ml, t = 16.429, P < 0.001). Compared to control/Af group (29.70 ± 2.00 pg/ml), the IL-12 levels in IFN-γ/Af (50.96 ± 4.38 pg/ml, t = 10.815, P < 0.001) and Dex/Af groups (41.18 ± 1.84 pg/ml, t = 10.347, P < 0.001) were increased. The IL-12 level in control/Af group was also increased, compared to control group (t = 9.397, P < 0.001; Figure 2a).

Interferon-γ and dexamethasone decreased interleukin-10 expression of the *Aspergillus fumigatus* stimulated dendritic cells

There was no significant difference in IL-10 expression between IFN-γ (20.80 ± 2.12 pg/ml) and control group (22.07 ± 4.32 pg/ml, t = 0.646, P = 0.533). Dex suppressed IL-10 expression compared to control group (dex group: 5.78 ± 1.40 pg/ml, t = 8.787, P < 0.001). Compared to the control/Af group (15.14 ± 1.40 pg/ml), IL-10 levels in IFN-γ/Af (4.70 ± 1.45 pg/ml, t = 12.688, P < 0.001) and Dex/Af (5.27 ± 0.85 pg/ml, t = 14.761, P < 0.001) groups were significantly decreased. Stimulation with Af (control/Af group) could significantly decrease IL-10 expression compared to control group (t = 3.738, P = 0.010; Figure 2b).

Dendritic cells over-expressed dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin after adenoviral vector-mediated gene delivery of dendritic cells-specific intercellular adhesion molecule-3-grabbing nonintegrin

Ad-DC-SIGN was purified after propagation in 293 cells. The Ad-DC-SIGN DNA was isolated, and the *DC-SIGN*

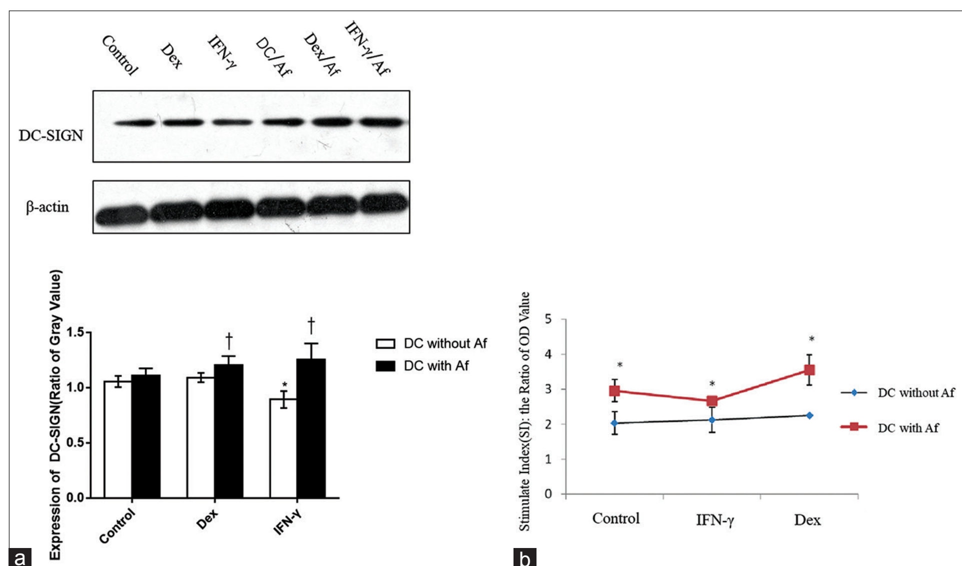

Figure 1: The expression of DC-SIGN by Western blotting analysis (n = 6 in each group; a). The ability of DC stimulating T cells proliferation (b). *P < 0.05 versus the same group cells without Af; †P < 0.05 versus control without Af (b). DC: Dendritic cell; Af: *Aspergillus fumigates*.

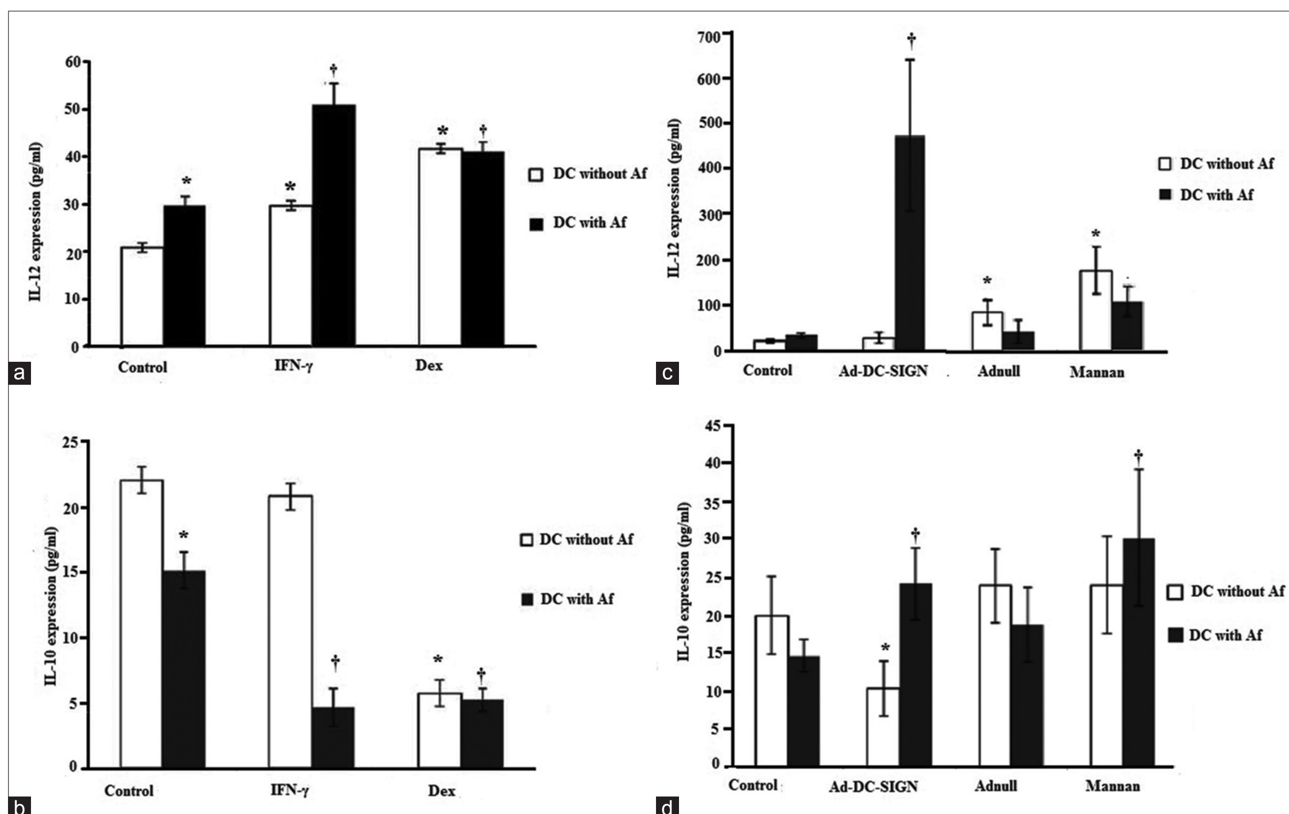

Figure 2: IFN-γ group and Dex affected Th1 and Th2 cytokines secretion from DCs: IL-12 (a) and IL-10 (b) secretion from DCs. Th1 and Th2 cytokines secretion from Ad-DC-SIGN transfected DCs: IL-12 (c) and IL-10 (d) secretion from DCs. *$P < 0.05$ versus control group; †$P < 0.05$ versus control/Af group. DC: Dendritic cell; Af: *Aspergillus fumigates*; Dex: Dexamethasone; INF: Interferon; IL: Interleukin.

gene in the vector was identified by PCR analysis. A 1200 bp band was obtained that was specific for DC-SIGN insert gene [Figure 3a], but the band was not observed in the empty vector AdNull. To detect vector transduction efficiency, the transfected DCs and 293 cells were observed under fluorescence microscope [Figure 3b]. DC-SIGN of 293 cells and DCs were extracted for measurement of protein concentration by the Western blot analysis [Figure 3c]. The results showed that the DC-SIGN modified 293 cells successfully expressed DC-SIGN protein, but DC-SIGN protein was not detected in naïve 293 cells or cells transfected with AdNull. Similarly, Ad-DC-SIGN transfected DCs expressed a higher level of DC-SIGN than the naïve cells or cells transfected with AdNull. The generated Ad-DC-SIGN can successfully express DC-SIGN in the target cells.

Higher expression of dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin in dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin modified dendritic cells and Mannan-stimulated dendritic cells

DCs were transfected with Ad-DC-SIGN and AdNull. Meanwhile, the Mannan was used to stimulate DCs. These three groups were Ad-DC-SIGN, AdNull, and Mannan. The expression level of DC-SIGN in DCs was analyzed before and after aspergillus infection. Ad-DC-SIGN and Mannan groups expressed higher levels of DC-SIGN than control group (Ad-DC-SIGN group: 1.306 ± 0.049; Mannan group: 1.520 ± 0.098; control group: 1.028 ± 0.023; $t_{(\text{Ad-DC-SIGN vs. control})} = 12.515$, $P_{(\text{Ad-DC-SIGN vs. Control})} < 0.001$; $t_{(\text{Mannan vs. control})} = 11.948$, $P_{(\text{Mannan vs. control})} < 0.001$). After aspergillus infection, there was no significant difference in the DC-SIGN level between Ad-DC-SIGN/Af and control/Af group (1.840 ± 0.054 vs. 1.833 ± 0.033 $t = 0.253$, $P = 0.806$), whereas Mannan/Af group (2.027 ± 0.025) showed higher DC-SIGN level than control/Af group ($t = 11.585$, $P < 0.001$; Figure 4).

Capacity of dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin modified dendritic cells in phagocytosis and stimulating T cells proliferation

Phagocytic function of DC-SIGN modified DCs was enhanced, compared with the control group (phagocytosis rates: 74.0% ± 3.4% in DC-SIGN modified DCs; 64.7% ± 6.8% in control group, $t = 3.104$, $P = 0.013$). However, the effects were reversed when DCs were stimulated by Mannan (phagocytosis rate: 44.1% ± 0.1% in mannan stimulated DCs, $t = 7.420$, $P = 0.001$).

The DCs function in promoting T cells proliferation did not significantly change among Ad-DC-SIGN/Af, AdNull/Af and control/Af groups. It indicated that DCs capacity in promoting T cells proliferation was not apparently affected by DC-SIGN gene over-expression.

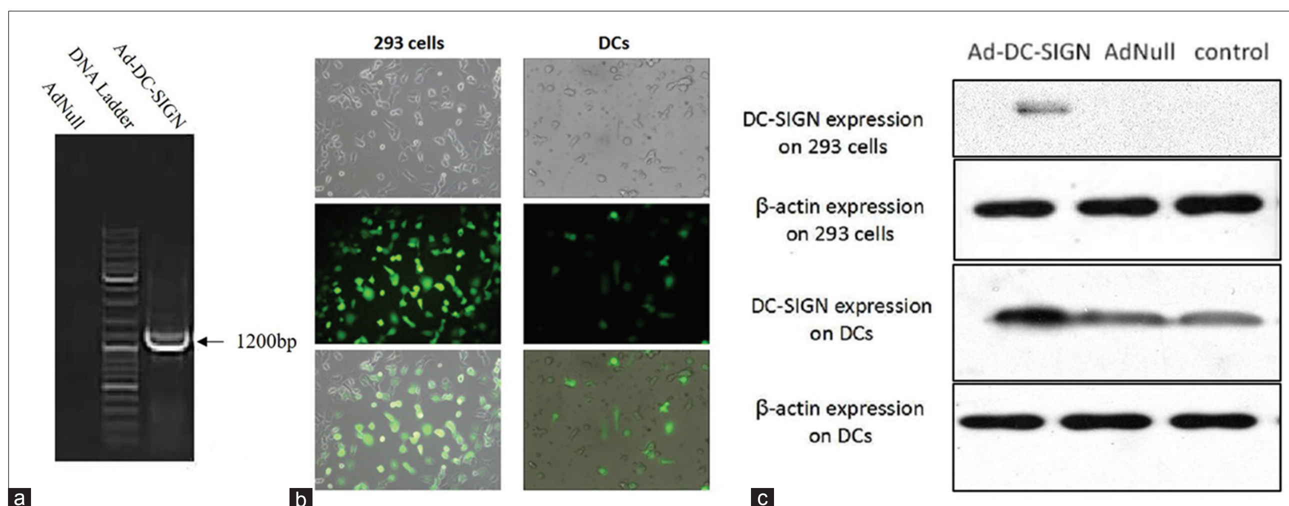

Figure 3: The identification of recombinant adenovirus vector (a); The 293 cells and DC turned green after transfection with Ad-DC-SIGN under the fluorescence microscope (original magnification, × 200; b). Western blotting analysis of DC-SIGN protein fragment expressed in 293 cells and DCs (c). DC: Dendritic cell.

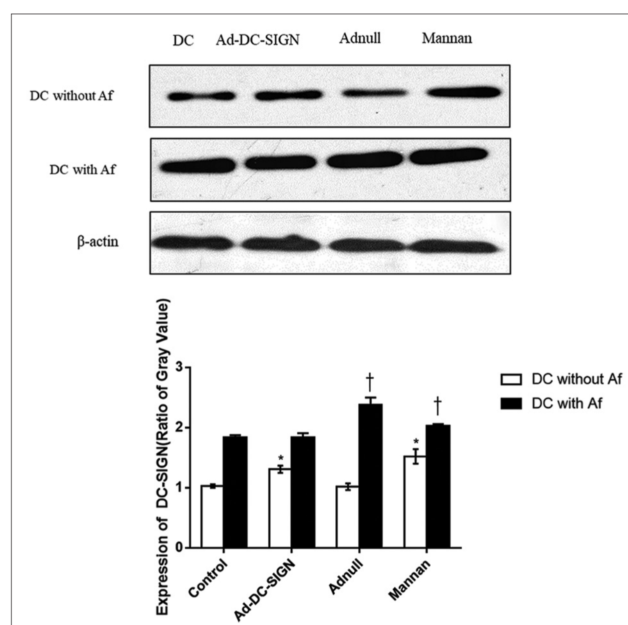

Figure 4: The analysis of DC-SIGN expressed in DC-SIGN modified DCs and mannan-stimulated DCs: Western blotting film and the ratio of DC-SIGN/β-actin. *$P < 0.05$ versus control group; †$P < 0.05$ versus control/Af group. DC: Dendritic cell.

Over-expression of dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin in *Asepergillus fumigatus*-infected dendritic cells increased Th1 cytokine interleukin-12

There was no significant difference in IL-12 expression between Ad-DC-SIGN and control group (27.78 ± 11.96 pg/ml vs. 21.32 ± 4.90 pg/ml, $t = 1.224$, $P = 0.263$). Mannan group secreted more IL-12 than control group (Mannan group: 176.48 ± 51.07 pg/ml, $t = 7.408$, $P = 0.001$). After infection with Af, Ad-DC-SIGN/Af group secreted 14-fold more amount of IL-12 than control/Af group (471.98 ± 166.31 pg/ml vs. 33.35 ± 5.98 pg/ml; $t = 6.456$, $P = 0.001$). In addition, the Mannan/Af group also secreted a slightly more IL-12 than control/Af group (Mannan/Af: 107.9 ± 32.47 pg/ml, $t = 5.531$, $P = 0.002$; Figure 2c).

Overexpression of dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin in *Asepergillus fumigatus*-infected dendritic cells altered Th2 cytokine interleukin-10 expression

Ad-DC-SIGN group secreted less IL-10 than control group (10.34 ± 3.65 pg/ml vs. 19.90 ± 5.12 pg/ml, $t = 3.724$, $P = 0.004$), indicating that Th2 cytokine expression was suppressed. After infection with Af, Ad-DC-SIGN/Af group secreted more IL-10 than control/Af group (Ad-DC-SIGN/Af group: 24.13 ± 4.69 pg/ml; control/Af group: 14.61 ± 2.14 pg/ml; $t = 4.523$, $P = 0.003$). In addition, Mannan/Af group secreted more IL-10 than control/Af group (Mannan/Af group: 30.17 ± 8.94 pg/ml; $t = 4.146$, $P = 0.007$), suggesting that Mannan and overexpression of DC-SIGN could promote secretion of IL-10 from Af-infected DCs [Figure 2d].

Nuclear factor-κB was activated in Ad-dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin transfected dendritic cells after infection with aspergillus

The expression of pho-NF-κB p65 was analyzed usng the Western blot analysis. The results showed that overexpression of DC-SIGN in Ad-DC-SIGN transfected DCs could stimulate NF-κB to transport into nuclei and activate in the Ad-DC-SIGN transfected DCs with and without infection with Af. In addition, Mannan-stimulated DCs could not activate NF-κB [Figure 5]. The results indicated that DC-SIGN was closely related to NF-κB signaling pathway.

Discussion

DCs play an important role in immunity against aspergillosis and exert anti-aspergillus infection through multiple important signaling pathways.[8] Recent studies have shown

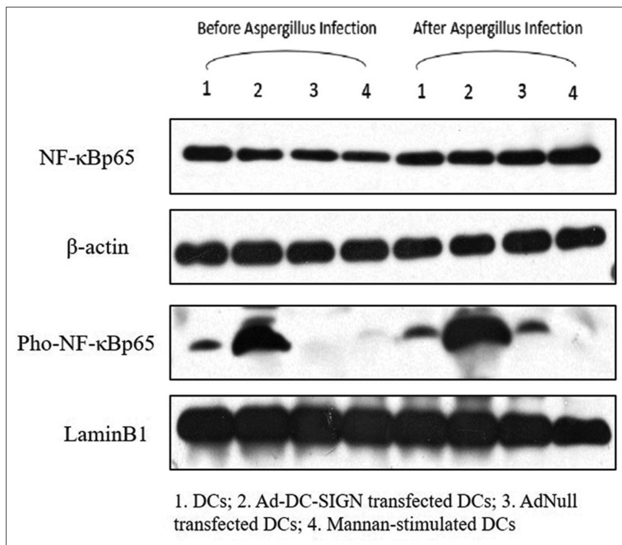

Figure 5: The activation of NF-κB in the DC-SIGN modified DC. DC: Dendritic cell; NF-κB: Nuclear factor-κB.

that DC-SIGN was closely related to intracellular signaling in DCs after aspergillus infection.[7] In this study, the role of DC-SIGN in the processing of DC antigen presentation and T cells activation was investigated. We hypothesized that over-expression of DC-SIGN might has protective function on aspergillosis.

In this study, a large amount of immature DCs after stimulation with GM-CSF and IL-4 were successfully obtained. The immature DCs had strong antigen recognition ability. After antigen uptake, DCs became mature and showed an efficient ability in antigen processing and activating T cells.[23]

IFN-γ and Dex were used to stimulate immature DCs. DCs of IFN-γ/Af group secreted more IL-12 and less IL-10, inducing immune response toward Th1 profile, which was consistent with the previous study.[24] Dex group showed greater phagocytosis ability. However, there was little change in the expression level of IL-12 and IL-10 before and after stimulation with Af, suggesting that glucocorticoid could inhibit DCs activation. The results were in line with the previous report on Dectin-1, showing that glucocorticoid could interfere with DCs activation and function.[25] DC-SIGN might participate in immune tolerance induced by Dex. However, it was paradoxical that DCs in Dex group secreted less amount of IL-10, which should be further investigated and discussed. In addition, this study also concluded that inflammatory (IFN-γ) or immunosuppressed (Dex) factor could not affect the capacity of DCs in stimulating T cells proliferation. The underlying mechanisms might be complicated and further comprehensive study should be performed.

It was well documented that the activated DCs secreted more Th1 cytokines and polarized the immune response toward a protective Th1 profile against fungal infection.[6] DC vaccine could improve the efficiency of antigen presentation, so it has been widely used in animal models and human subjects. In our following experiment, we induced over-expression of DC-SIGN on DCs through adenoviral vector-mediated gene delivery of the DC-SIGN gene. Our results *in vitro* revealed the beneficial effects of DC-SIGN overexpression on DCs activation, as evidenced by the elevated expression level of Th1 cytokine IL-12 and phagocytosis of aspergillus.

This study used adenovirus as a vector for DC-SIGN gene delivery into DCs. DC-SIGN modified DCs could overexpression DC-SIGN, which was confirmed by quantitative reverse transcriptase-PCR, Western blot analysis, and fluorescent staining. Mannan was used to interfere with the binding of Af with DC-SIGN. The results showed that Mannan group had less phagocytosis of Af, but the Mannan/Af group expressed less amount of IL-12 than Ad-DC-SIGN/Af group. The results indicated that DC-SIGN recognized aspergillus conidia and participated in the immune response. The results of this study *in vitro* showed that the DC-SIGN modified DCs could highly express DC-SIGN and secret greater amount of IL-12 (471.98 ± 166.31 pg/ml) after infection with Af. Furthermore, the DC-SIGN modified DCs secreted more IL-10 than control/Af group; IL-10 expression remained at a relative low level (24.13 ± 4.69 pg/ml). The DC-SIGN over-expressing DCs could promote immune response toward Th1 profile and exert potent immune responses against aspergillosis. Mannan-stimulated DCs had similar effects as Ad-DC-SIGN transfected DCs. The elevated cytokine expression might be mediated by increased crosstalk between TLR and CLR.[26,27]

In addition, this study did not observe significant effects of DC-SIGN modified DCs on T cells proliferation, suggesting that DCs stimulating T-cell proliferation and phagocytosis might be mediated through different molecular mechanisms. As previously reported, DC-SIGN had various functions, positively or negatively regulating immune responses against aspergillus infection.[28] After DC-SIGN modified DCs were infected with Aspergillus, NF-κB was activated and led to the elevation of pro-inflammatory cytokine production. Thus, we confirmed that high expression of DC-SIGN could promote immune responses against aspergillus infection through the NF-κB signaling pathway.

The findings of this study confirmed that DC-SIGN signaling pathway was one of the mechanisms involved in immune response against aspergillosis. DC-SIGN might participate in immune tolerance of aspergillus. However, the crude preps of Af were used for cell stimulation in this study, so the antigens or epitopes of Af might activate other signaling pathways. In addition, the cultured DCs might be contaminated with other cell types, such monocytes, etc. The factors possibly affected the results of this study. Due to the limitations of our experimental system, it was difficult to determine the antigen-specific T-cell priming and memory T-cell development. Further study should be performed to support the findings of this study regarding the effects of DC-SIGN in DCs.

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Conflicts of interest

There are no conflicts of interest.

References

1. Kanj A, Abdallah N, Soubani AO. The spectrum of pulmonary aspergillosis. Respir Med 2018;141:121-31. doi: 10.1016/j.rmed.2018.06.029.
2. Davda S, Kowa XY, Aziz Z, Ellis S, Cheasty E, Cappocci S, *et al.* The development of pulmonary aspergillosis and its histologic, clinical, and radiologic manifestations. Clin Radiol 2018. pii: S0009-9260(18)30266-6. doi: 10.1016/j.crad.2018.06.017.
3. Bassetti M, Pecori D, Della Siega P, Corcione S, De Rosa FG. Current and future therapies for invasive aspergillosis. Pulm Pharmacol Ther 2015;32:155-65. doi: 10.1016/j.pupt.2014.06.002.
4. Sam QH, Yew WS, Seneviratne CJ, Chang MW, Chai LY. Immunomodulation as therapy for fungal infection: Are we closer? Front Microbiol 2018;9:1612. doi: 10.3389/fmicb.2018.01612.
5. Espinosa V, Rivera A. First line of defense: Innate cell-mediated control of pulmonary aspergillosis. Front Microbiol 2016;7:272. doi: 10.3389/fmicb.2016.00272.
6. Dewi IM, van de Veerdonk FL, Gresnigt MS. The multifaceted role of T-helper responses in host defense against *Aspergillus fumigatus*. J Fungi (Basel) 2017;3. pii: E55. doi: 10.3390/jof3040055.
7. Serrano-Gómez D, Domínguez-Soto A, Ancochea J, Jimenez-Heffernan JA, Leal JA, Corbí AL, *et al.* Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin mediates binding and internalization of *Aspergillus fumigatus* conidia by dendritic cells and macrophages. J Immunol 2004;173:5635-43. doi: 10.4049/jimmunol.173.9.5635.
8. Velasquez LN, Stüve P, Gentilini MV, Swallow M, Bartel J, Lycke NY, *et al.* Targeting *Mycobacterium tuberculosis* antigens to dendritic cells *via* the DC-specific-ICAM3-grabbing-nonintegrin receptor induces strong T-helper 1 immune responses. Front Immunol 2018;9:471. doi: 10.3389/fimmu.2018.00471.
9. Goyal S, Castrillón-Betancur JC, Klaile E, Slevogt H. The interaction of human pathogenic fungi with C-type lectin receptors. Front Immunol 2018;9:1261. doi: 10.3389/fimmu.2018.01261.
10. Lahiri A, Lahiri A, Das P, Vani J, Shaila MS, Chakravortty D, *et al.* TLR 9 activation in dendritic cells enhances salmonella killing and antigen presentation via involvement of the reactive oxygen species. PLoS One 2010;5:e13772. doi: 10.1371/journal.pone.0013772.
11. Schetters ST, Kruijssen LJ, Crommentuijn MH, Kalay H, Ochando J, den Haan JM, *et al.* Mouse DC-SIGN/CD209a as target for antigen delivery and adaptive immunity. Front Immunol 2018;9:990. doi: 10.3389/fimmu.2018.00990.
12. Geijtenbeek TB, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, *et al.* Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 2000;100:575-85. doi: 10.1016/S0092-8674(00)80693-5.
13. van Kooyk Y, Unger WW, Fehres CM, Kalay H, García-Vallejo JJ. Glycan-based DC-SIGN targeting vaccines to enhance antigen cross-presentation. Mol Immunol 2013;55:143-5. doi: 10.1016/j.molimm.2012.10.031.
14. Schaefer M, Reiling N, Fessler C, Stephani J, Taniuchi I, Hatam F, *et al.* Decreased pathology and prolonged survival of human DC-SIGN transgenic mice during mycobacterial infection. J Immunol 2008;180:6836-45. doi: 10.4049/jimmunol.180.10.6836.
15. Bellocchio S, Montagnoli C, Bozza S, Gaziano R, Rossi G, Mambula SS, *et al.* The contribution of the toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens *in vivo*. J Immunol 2004;172:3059-69. doi: 10.1534/g3.116.035873.
16. Feng D, Wang Y, Liu Y, Wu L, Li X, Chen Y, *et al.* DC-SIGN reacts with TLR-4 and regulates inflammatory cytokine expression via NF-κB activation in renal tubular epithelial cells during acute renal injury. Clin Exp Immunol 2018;191:107-15. doi: 10.1111/cei.13048.
17. Ramirez-Ortiz ZG, Specht CA, Wang JP, Lee CK, Bartholomeu DC, Gazzinelli RT, *et al.* Toll-like receptor 9-dependent immune activation by unmethylated CpG motifs in *Aspergillus fumigatus* DNA. Infect Immun 2008;76:2123-9. doi: 10.1128/IAI.00047-08.
18. Gringhuis SI, den Dunnen J, Litjens M, van Het Hof B, van Kooyk Y, Geijtenbeek TB, *et al.* C-type lectin DC-SIGN modulates toll-like receptor signaling via raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. Immunity 2007;26:605-16. doi: 10.1016/j.immuni.2007.03.012.
19. Zhu W, Yan H, Li S, Nie W, Fan F, Zhu J, *et al.* PPAR-γ agonist pioglitazone regulates dendritic cells immunogenicity mediated by DC-SIGN via the MAPK and NF-κB pathways. Int Immunopharmacol 2016;41:24-34. doi: 10.1016/j.intimp.2016.09.028.
20. Bellocchio S, Moretti S, Perruccio K, Fallarino F, Bozza S, Montagnoli C, *et al.* TLRs govern neutrophil activity in aspergillosis. J Immunol 2004;173:7406-15. doi: 10.4049/jimmunol.173.12.7406.
21. Datta B, Njau F, Thalmann J, Haller H, Wagner AD. Differential infection outcome of *Chlamydia trachomatis* in human blood monocytes and monocyte-derived dendritic cells. BMC Microbiol 2014;14:209. doi: 10.1186/s12866-014-0209-3.
22. Wohler JE, Barnum SR. Nylon wool purification alters the activation of T cells. Mol Immunol 2009;46:1007-10. doi: 10.1016/j.molimm.2008.09.017.
23. Ramirez-Ortiz ZG, Means TK. The role of dendritic cells in the innate recognition of pathogenic fungi (*A. fumigatus*, *C. neoformans* and *C. albicans*). Virulence 2012;3:635-46. doi: 10.4161/viru.22295.
24. Vieira PL, de Jong EC, Wierenga EA, Kapsenberg ML, Kaliński P. Development of th1-inducing capacity in myeloid dendritic cells requires environmental instruction. J Immunol 2000;164:4507-12. doi: 10.4049/jimmunol.164.9.4507.
25. Kotthoff P, Heine A, Held SA, Brossart P. Dexamethasone induced inhibition of dectin-1 activation of antigen presenting cells is mediated via STAT-3 and NF-κB signaling pathways. Sci Rep 2017;7:4522. doi: 10.1038/s41598-017-04558-z.
26. Horrevorts SK, Duinkerken S, Bloem K, Secades P, Kalay H, Musters RJ, *et al.* Toll-like receptor 4 triggering promotes cytosolic routing of DC-SIGN-targeted antigens for presentation on MHC class I. Front Immunol 2018;9:1231. doi: 10.3389/fimmu.2018.01231.
27. Konieczna P, Schiavi E, Ziegler M, Groeger D, Healy S, Grant R, *et al.* Human dendritic cell DC-SIGN and TLR-2 mediate complementary immune regulatory activities in response to *Lactobacillus rhamnosus* JB-1. PLoS One 2015;10:e0120261. doi: 10.1371/journal.pone.0120261.
28. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, *et al.* Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767-811. doi: 10.1146/annurev.immunol.18.1.767.

树突状细胞表面DC-SIGN过表达对于曲霉感染起保护性作用

摘要

背景：树突状细胞（dendritic cells, DCs）在机体抗病原体感染中具有重要作用。DC-SIGN是主要表达于DCs表面的C型凝集素样受体中的一员。本研究旨在探讨DC-SIGN是否在曲霉感染中影响DCs细胞内信号传导、辅助T细胞Th1/Th2的平衡及曲霉菌免疫逃逸, 同时探索DC-SIGN基因修饰的树突状细胞用于免疫治疗的可能性。

方法：首先从外周血中提取了DCs。用IFN-γ和地塞米松分别刺激DCs后检测DC-SIGN的表达、Th1和Th2细胞因子分泌水平、DCs吞噬功能及刺激T细胞增殖能力、以及NF-κB的激活水平。此外，构建DC-SIGN腺病毒载体用于DCs转染。用甘露聚糖阻断DC-SIGN信号传导以明确DC-SIGN在烟曲霉刺激DCs成熟中的作用。未配对的双尾t检验用于组间比较。

结果：外源性IFN-γ刺激可以激活DCs并促使辅助T细胞向Th1细胞分化（IL-12水平在IFN-γ/Af 组为: 50.96±4.38pg/ml; 在control/Af组中为: 29.70±2.00 pg/ml, t = 10.815, P < 0.001）。此外，地塞米松抑制Th2细胞因子分泌（IL-10水平在Dex/Af组为：5.27±0.85pg/ml; 在control/Af 组为: 15.14±1.40pg/ml, t = 14.761, P < 0.001），并引起免疫抑制。在DC-SIGN基因转染的DCs中，Th1细胞因子的分泌（IL-12 水平在Ad-DC-SIGN/Af组为: 471.98±166.31 pg/ml; 在control/Af组为: 33.35±5.98 pg/ml, t = 6.456, P = 0.001）及NF-κB的激活均有上调，DCs的吞噬功能也有所增加（吞噬率在Ad-DC-SIGN 组为: 74.0±3.4%; control组为: 64.7±6.8%, t = 3.104, P = 0.013）。

结论：树突状细胞表面DC-SIGN过表达对于曲霉感染起保护性作用。