Cyclophosphamide reduces dectin-1 expression in the lungs of naive and Aspergillus fumigatus-infected mice

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Aspergillus fumigatus is an important opportunistic fungal pathogen. Patients treated with chemotherapeutic agent such as cyclophosphamide are susceptible to invasive pulmonary aspergillosis. Dectin-1 is a pattern recognition receptor critically involved in immune responses to A. fumigatus. Therefore, we tested whether cyclophosphamide treatment could cause alterations in dectin-1 expression in the lung, which could contribute to invasive pulmonary Aspergillus infections in patients. We established a murine A. fumigatus infectious model to investigate the kinetics of dectin-1 expression in lung tissues in the presence or absence of cyclophosphamide treatment. During infection, dectin-1 expression was strikingly increased in immunocompetent mice infected with A. fumigatus as compared to those in a non-infected control group. In vitro macrophages stimulated with heat-inactivated A. fumigatus conidia expressed a significantly elevated level of dectin-1. Infected mice treated with cyclophosphamide showed decreased levels of dectin-1 and a higher fungal burden in the lung than the infected mice without cyclophosphamide treatment. These results suggest that dectin-1 is involved in host defense against A. fumigatus infection and that suppression of dectin-1 expression caused by cyclophosphamide may contribute to susceptibility to infections caused by this fungus in the immunocompromised host.

Keywords Aspergillus fumigatus, dectin-1, cyclophosphamide

Introduction

Aspergillus fumigatus is a ubiquitous opportunistic fungal pathogen. Inhalation of its conidia by immunocompetent individuals rarely has any adverse effect, since the conidia are eliminated efficiently by innate immune mechanisms [1]. This pathogen, however, can cause severe and often fatal invasive infections in the patients treated with immunosuppressive agents such as cyclophosphamide. Over the past 20 years, with an increasing population of immunocompromised patients, the incidence of invasive pulmonary aspergillosis (IPA) has been on the rise with mortality more than 60% [23].

The cell wall of A. fumigatus consists mainly of carbohydrates, including mannose-based structures (the manno-proteins), β-glucan and chitin. These carbohydrates serve as pathogen-associated molecular patterns (PAMPs) that can be recognized by a variety of host-expressed pattern-recognition receptors (PRRs). Recognition of invading microorganisms by the innate immune system is the first and essential step in the successful elimination of pathogens [4–6]. Host-cell recognition of -glucan is mediated mainly by dectin-1, a myeloid-expressed type II transmembrane C-type lectin-like receptor [7,8].

Dectin-1 is expressed at high level in the lungs [9] and in many cell types, including macrophages, monocytes and neutrophils [10,11]. In vitro, dectin-1 contributes to the recognition and killing of fungal pathogens [12,13]. However, the expression of dectin-1 during the infection of A. fumigatusin vivo is not completely understood, and the correlation between dectin-1 expression and cyclophosphamide treatment remains unexplored. In this study, we...
established a murine A. fumigatus infectious model to investigate the role of dectin-1 in host defense against A. fumigatus infection and the effects of cyclophosphamide on dectin-1 expression in the lung.

Materials and methods

Preparation of A. fumigatus conidia

A clinical isolate of A. fumigatus (B5233) was grown on slants of potato dextrose agar (PDA) at 37°C for 7 days prior to collection of conidia. Conidia were harvested in physiologic saline with 0.1% Tween 20, and passed through sterile gauze to remove hyphal fragments. The number of conidia was determined by hemocytometer. The final concentration was adjusted to 4 × 10⁷ colony forming units (CFU)/ml and 4 × 10⁸ CFU/ml. For in vitro experiments, conidia were killed by heating at 100°C for 1 h.

Murine model of A. fumigatus infection and cyclophosphamide treatment

Pathogen-free BALB/c mice (male, 6 weeks old, 22–25g) were used in the experiments. Mice were housed in sterilized filter-top cages and had access to sterile food and water containing tetracycline hydrochloride (500 μg/ml, Sigma). All animal studies were approved by the institutional animal care and use committee. Mice were divided into five groups, i.e., normal control mice, mice infected with 1 × 10⁷ A. fumigatus conidia, mice infected with 1 × 10⁷ A. fumigatus conidia, mice treated with cyclophosphamide, and mice treated with cyclophosphamide and infected with 1 × 10⁸ A. fumigatus conidia. For infection, mice were anesthetized with ether and were intranasally administered 30 μl of conidia suspension or physiological saline with 0.1% Tween 20. For cyclophosphamide treatment, the mice were administered i.p. with cyclophosphamide (150 mg/kg of body weight, Sigma) on days -3 and 0 before conidial inoculation.

Samples harvest and preparation

Five to eight mice of each group were sacrificed on day 1, 3, and 5 post-inoculation for bronchoalveolar lavage fluid (BALF) collection according to the method described before [15,16]. Briefly, lung tissues were lavaged 4 times with 0.5 ml of ice-cold Ca²⁺- and Mg²⁺-free PBS through a polyethylene syringe attached to the tracheal cannula. Lavage fluid was centrifuged at 400 g for 8 min at 4°C, and the cell pellet was resuspended in 0.3 ml of RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), and 10% heat-inactivated FCS (Hyclone, USA). The viability of the cells was higher than 95% as judged by trypan blue exclusion. Cells from BALF were cytospun to slides for Wright-Giemsa stain and immunofluorescence.

Quantitative Real-time PCR

Total RNA was isolated from tissues by sequential extraction with TRIzol reagent (Invitrogen, USA) and quantified by spectrophotometric analysis. Prior to amplification, all RNA samples were treated with RNase-free DNase (Promega, USA) to remove genomic DNA contamination. First-strand cDNA was synthesized from 2 μg of RNA using reverse transcription system (Promega, USA) in accordance with the manufacturer’s instructions. The pairs of primers and the TaqMan probes for the target mRNAs were designed based on the mouse mRNA sequences using Primer Express software (Applied Biosystems) (Table 1). The reaction mixture was incubated at 50°C for 2 min, at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. PCR products were analyzed on a 7500 ABI platform (Applied Biosystems). β-actin was used as an internal control. Levels of dectin-1 and β-actin were quantified against standard curves which were established with plasmids containing specific sequences of the gene studied. The relative expression of dectin-1 mRNA was expressed as the ratio of the dectin-1 mRNA to β-actin mRNA.

Western blotting

Tissues were lysed in sample buffer containing 62 mM Tris-HCl, 5% SDS, 0.1 mM sodium orthovanadate, and 50 mM sodium fluoride. Equal amounts of protein (55 μg) were loaded and separated by 10% SDS-PAGE, then transferred to nitrocellulose membranes. The membranes were blocked for 1 h in 5% non-fat milk in TBS-T buffer (150 mMNaCl, 20 mM Tris-HCl, pH 7.4, 0.05% Tween-20), incubated with primary monoclonal antibodies specific for dectin-1(R&D) overnight at 4°C, followed by incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The blots were developed by use of an enhanced chemiluminescence (ECL) detection kit (Applygen Technologies Inc.,China).
Table 1 Primers and probes used for Real-time Quantitative PCR Analysis

<table>
<thead>
<tr>
<th>mRNA (GenBank no.)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin (NM-007393)</td>
<td>5'-AGGCCCAAACGTGAAAAGA</td>
<td>5'-TGTGTTAGCGCCAGAGG</td>
<td>5'-TGAGACCTTCACACCCCA</td>
</tr>
<tr>
<td>Dectin-1 (NM-020008)</td>
<td>5'-GAACCCAAGCCACAGA</td>
<td>5'-CTCTCCATGCTGA</td>
<td>5'-CTCCTCAAGGCCATCCA</td>
</tr>
</tbody>
</table>

Note: β-actin:110bp; dectin-1:116bp

Densitometric analysis of protein bands was performed using NIImage software.

**Macrophage isolation and stimulation with conidia**

Alveolar macrophages were harvested from BALF of normal mice as described previously [15]. Briefly, cells from BALF were washed, resuspended in RPMI 1640 supplemented with 10% FCS and incubated at 37°C, 5% CO₂ for 2 h to allow them to adhere to cover slips. After removing the non-adherent cells, macrophages were challenged with conidia at 37°C, 5% CO₂ for 24 h. The macrophages on the non-adherent cells, macrophages were challenged with 2 h to allow them to adhere to cover slips. After removing the non-adherent cells, macrophages were challenged with conidia at 37°C, 5% CO₂ for 24 h. The macrophages on the cover slips were analyzed by immunofluorescence.

**Immunofluorescence and confocal laser-scanning microscopy**

Macrophages or total cells from BALF were fixed in 4% paraformaldehyde for 30 min, washed three times in PBS and permeabilized with 0.2% Triton X-100 for 5 min as previously described [12]. After three more washes in PBS, cells were incubated with blocking buffer (PBS containing 3% BSA and 10% goat serum) for 1 h and stained with an mAb specific for dectin-1 or an isotype control mAb (R&D, each at 25 μg/ml) at 4°C overnight. After washing, cells were incubated with a 1:2 dilution of goat anti-rat antibody conjugated with FITC at 4°C for 1 h and washed with PBS. Immunofluorescent stained cells were then analyzed with an Olympus FV1000 confocal laser-scanning microscope (Olympus, Japan). Images were acquired using a 60 × DIC oil immersion objective (1.42 NA) at 512 × 512 pixel resolution with averaging (2 × ). Threshold settings were applied uniformly for all paired data sets. Images were analyzed by FV10-ASW software (Version 1.6) and adjusted equally for brightness and contrast in Adobe Photoshop CS (Adobe Systems Incorporated, San Jose, CA).

**Statistics**

Statistics were performed with a one way ANOVA and LSD post hoc test for multiple comparisons. The Mann-Whitney test was used to analyze the differences of fungal burden in the lung (SPSS 10.0 SPSS Inc., Chicago, IL). P < 0.05 was considered significant.

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**Results**

**A. fumigatus infection enhanced dectin-1 expression**

We studied kinetic dectin-1 expression in pulmonary tissues of the immunocompetent mice infected with *A. fumigatus*. On day 1 after inoculation, there was no statistical difference in dectin-1 expression at mRNA levels between immunocompetent mice with a 1 × 10⁶ conidial infection and the normal control group (Fig. 1A). Dectin-1 expression in the immunocompetent mice challenged with 1 × 10⁷ conidia was increased compared to control mice and mice challenged with 1 × 10⁶ conidia (Fig. 1A). On day 3, however, both 1 × 10⁶ conidia- and 1 × 10⁷ conidia-infected mice showed significantly increased dectin-1 expression in the lung compared to the normal control group, with the 1 × 10⁷ conidia-infected group having the highest dectin-1 expression (Fig. 1A). Indirect immunofluorescence revealed that cells from BALF of infected mice exhibited much higher levels of dectin-1 expression than non-infected control mice (Fig. 1B, panels c and a). Similarly, stronger dectin-1 protein bands were detected by western blotting in lung protein extracts from the infected mice (Fig. 1C lanes 2 and 3, Fig. 1D) as compared to non-infected control mice (Fig. 1C lane 1, Fig. 1D). These results demonstrate that during the infection, *A. fumigatus* upregulates dectin-1 expression in vivo and the infection-induced dectin-1 expression levels are correlated with the amount of conidia inoculated.

Since macrophages are important in host immunity to *A. fumigatus* infection we determined whether *A. fumigatus* conidia directly affects dectin-1 expression in these cells. Purified alveolar macrophages were incubated with heat-inactivated *A. fumigatus* conidia for 24 h and then assayed for dectin-1 expression by indirect immunofluorescence. The *A. fumigatus* conidia challenged macrophages (Fig. 2d) exhibited increased dectin-1 expression relative to untreated macrophages (Fig. 2a). These results demonstrate that as with the results from the in vivo studies, *A. fumigatus* conidia upregulates dectin-1 expression by macrophages in vitro.

**Cyclophosphamide depressed dectin-1 expression**

*A. fumigatus* infection upregulates dectin-1 expression in immunocompetent hosts, while the immunosuppressive
Yang et al. suggest that cyclophosphamide inhibits dectin-1 expression in the lung regardless of *A. fumigatus* infection. Cyclophosphamide treatment promoted *A. fumigatus* invasion. Dectin-1 mediates host-pathogen recognition and cyclophosphamide down-regulates dectin-1 expression in our murine *A. fumigatus* infection model. Therefore, we tested whether the cyclophosphamide-caused reduction of dectin-1 expression in mice is associated with an increased susceptibility of the mice to *A. fumigatus* infection. On day 1 after inoculation, the infected mice with or without cyclophosphamide treatment had similar level of the fungal burden (Fig. 4A). On day 3, however, the fungal burden in the cyclophosphamide-treated mice was significantly higher than the infected mice without cyclophosphamide treatment (Fig. 4A). On day 5 after inoculation, the fungal burden levels in both groups of mice were undetectable.

Phagocytosed conidia were readily seen inside macrophages in the lung of immunocompetent mice (Fig. 4B, panel a), while hyphae, indicators for the invasive status of *A. fumigatus* in tissues, were commonly present in cyclophosphamide-treated mice (Fig. 4B, panel b). These results demonstrate that cyclophosphamide treatment is agent cyclophosphamide compromises the host immune system and causes increased susceptibility to invasive pulmonary aspergillosis. To determine whether cyclophosphamide affects dectin-1 expression in the lung, we first compared the dectin-1 expression levels in cyclophosphamide treated and untreated mice. On day 1 and day 3 after inoculation, dectin-1 expression in lung tissues of cyclophosphamide-treated mice was statistically significantly decreased compared to the normal control mice (Fig. 3A). On day 5, the difference between the cyclophosphamide treated and control mice was statistically insignificant (Fig. 3A). These results demonstrate that cyclophosphamide down-regulates dectin-1 expression in the lung.

We then determined whether cyclophosphamide also affects dectin-1 expression in lung tissues of mice during *A. fumigatus* infection. On days 1 and 5, there were no differences in dectin-1 expression between *A. fumigatus* infected mice with or without cyclophosphamide treatment (Fig. 3B). However, on day 3, the dectin-1 expression in *A. fumigatus* infected and cyclophosphamide-treated mice was significantly decreased compared to the mice challenged with the same concentration of conidia but without cyclophosphamide treatment (Fig. 3B). Taken together, our data suggest that cyclophosphamide inhibits dectin-1 expression in the lung regardless of *A. fumigatus* infection.

**Cyclophosphamide treatment promoted *A. fumigatus* invasion**

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Cyclophosphamide reduces dectin-1 expression in the lungs

Dectin-1 in the immune cells such as macrophages. The increased levels of dectin-1 trigger inflammatory responses and subsequent neutrophil recruitment to the airways [13,19]. Dectin-1 contributes to the recognition and efficient phagocytosis of *A. fumigatus* conidia [20–22] and the killing of fungal pathogens in part through the induction of the respiratory burst [23–24] and production of cytokines and chemokines such as TNF and CXCL2 [21,25] by macrophages. In our study, three days after *A. fumigatus* conidia infection, dectin-1 expression in immunocompetent mice inoculated with 1 × 10^6 and 1 × 10^7 conidia was increased significantly compared to the normal control groups. Dectin-1 expression on the normal macrophages was also upregulated in vitro by heat-killed spores. Our

Discussion

Fungal cell walls consist of more than 50% β-glucan [18] and these carbohydrates contribute to the molecular immune recognition of fungal pathogens through an interaction with dectin-1 and the other receptors, such as scavenger receptors, CR3, lactosylceramide etc., on host immune cells [4]. During the infection of *A. fumigatus*, the architecture of the cell wall is changed and more β-glucan is exposed, which would induce more expression of dectin-1 in the immune cells such as macrophages. The increased levels of dectin-1 trigger inflammatory responses and subsequent neutrophil recruitment to the airways [13,19]. Dectin-1 contributes to the recognition and efficient phagocytosis of *A. fumigatus* conidia [20–22] and the killing of fungal pathogens in part through the induction of the respiratory burst [23–24] and production of cytokines and chemokines such as TNF and CXCL2 [21,25] by macrophages. In our study, three days after *A. fumigatus* conidia infection, dectin-1 expression in immunocompetent mice inoculated with 1 × 10^6 and 1 × 10^7 conidia was increased significantly compared to the normal control groups. Dectin-1 expression on the normal macrophages was also upregulated in vitro by heat-killed spores. Our
Aspergillus fumigatus
CP: cyclophosphamide. (B) Wright–Giemsa staining. Three days after
from cyclophosphamide-treated mice (panel b, black arrowhead).
(panel a, black arrowhead), whereas hyphae were seen in the cell aggregates
immunocompetent mice showed conidia phagocytosed within macrophages
infection. BALB/c mice were administered i.p. with physiological saline
control or cyclophosphamide (150 mg/kg of body weight) on days -3 and 0
before being infected with 1 × 10⁶ conidia. (A) Fungal burden. Fungal burden
in the lung were quantified on days 1, 3, and 5 post infection. On day 3, the
infected mice with cyclophosphamide treatment showed significantly higher
levels of fungal burden than the infected mice without cyclophosphamide
infection. Data shown are log₁₀ CFU/g (mean ± SD). N = 5–8. *P < 0.05.
CP: cyclophosphamide. (B) Wright–Giemsa staining. Three days after
Aspergillus fumigatus inoculation, cells aggregated in BALF from
immunocompetent mice showed conidia phagocytosed within macrophages
(panel a, black arrowhead), whereas hyphae were seen in the cell aggregates
from cyclophosphamide-treated mice (panel b, black arrowhead).

Fig. 4 Cyclophosphamide treatment promotes Aspergillus fumigatus
invasion. BALB/c mice were administered i.p. with physiological saline
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in vitro and in vivo findings suggest that dectin-1 is involved in defense mechanisms of the immunocompetent host to
A. fumigatus infection.

Cyclophosphamide, an alkylating agent used clinically as a chemotherapeutic and immunosuppressive drug, induces a dose-dependent decrease of circulating neutrophils and various pulmonary leukocyte populations [26–28]. Although leukopenia is an easily measurable clinical risk factor for the development of invasive aspergillosis in humans, this susceptibility may be also related to other effects of cyclophosphamide on the function of immune effector cells [26]. Previous studies [29,30] have shown that dexamethasone significantly down-regulates dectin-1 expression in peritoneal macrophages and abolishes the TNF-α response to zymosan in vitro, which could be a contributing factor to fungal susceptibility [31]. In our study, the lungs of the cyclophosphamide-treated mice had significantly decreased levels of dectin-1 expression as compared to the normal control mice on days 1 and 3 after conidial inoculation. Dectin-1 expression was also strikingly decreased in the immunocompromised mice in contrast to the immunocompetent mice on day 3 after conidia inoculation. To determine whether cyclophosphamide treatment has effects on the expression of other receptors known for innate immune responses, we assayed TLR2 and TLR4 expression by real-time RT-PCR. There were no statistical differences in TLR2 and TLR4 mRNA expression between cyclophosphamide treated and normal control mice (data not show). These results suggest that cyclophosphamide inhibited the expression of dectin-1 in vivo regardless of A. fumigatus infection. As a consequence, mice treated with cyclophosphamide became more susceptible to A. fumigatus invasion.

In summary, our studies demonstrated that cyclophosphamide down-regulates dectin-1 expression in the lung, likely on macrophages, indicating that reduction in dectin-1 expression caused by cyclophosphamide may contribute to fungal susceptibility in the immunocompromised hosts. Our current findings also suggest that regulation of dectin-1 expression could be a novel therapeutic strategy for the immunocompromised hosts to defend against A. fumigatus infection.

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References

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