International Immunology, Vol. 18, No. 12, pp. 1637–1646
doi:10.1093/intimm/dxl097
© The Japanese Society for Immunology. 2006. All rights reserved.
For permissions, please e-mail: journals.permissions@oxfordjournals.org

Differential induction of T_h1-prone immunity by human dendritic cells activated with *Sporothrix schenckii* of cutaneous and visceral origins to determine their different virulence

Takeshi Uenotsuchi[1], Satoshi Takeuchi[1], Tetsuo Matsuda[1], Kazunori Urabe[1], Tetsuya Koga[1], Hiroshi Uchi[1], Takeshi Nakahara[1], Shuji Fukagawa[1], Masako Kawasaki[2], Hideko Kajiwara[3], Shin-ichi Yoshida[3], Yoichi Moroi[1] and **Masutaka Furue[1]**

[1]Department of Dermatology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashiku, Fukuoka 812-8582, Japan
[2]Department of Dermatology, Kanazawa Medical University, Ishikawa, Japan
[3]Department of Bacteriology, Faculty of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashiku, Fukuoka 812-8582, Japan

Keywords: dendritic cells, *Sporothrix schenckii*, T_h1

Abstract

Sporotrichosis is caused by a thermo-dependent dimorphic fungus, *Sporothrix schenckii*. The major clinical manifestations occur in the skin; however, cases of visceral manifestations have also been increasingly reported with some being observed in immune compromised patients. Different virulence of individual *S. schenckii* strain as well as immune status of the host could contribute to form such different clinical manifestations. Thus, the purpose of the study was to investigate whether different virulence of individual *S. schenckii* could be a factor for such clinical difference. We investigated the interactions between human monocyte-derived dendritic cells (MoDCs) and *S. schenckii*, assessed by (i) morphological features, (ii) surface marker expressions, cytokine productions, (iii) signaling pathways and (iv) allostimulatory activity of the activated MoDCs. Immature MoDCs, obtained from peripheral blood monocytes supplemented with granulocyte macrophage colony-stimulating factor and IL-4, were stimulated with *S. schenckii* strains of both yeasts and conidia forms of different origins (cutaneous isolates: KMU4649, IFM5906 and IFM46010; visceral isolates: KMU4648, IFM41598 and ATCC26331) to be used for various assays. Through the analysis, we found that the cutaneous *S. shenckii* of cutaneous origins were more potent to activate MoDCs to induce strong T_h1 response, as evidenced by abundant IFN-γ production, while the *S. shenckii* of visceral origins induced only minimal dendritic cell activation and T_h1 induction. The p38 mitogen-activated protein kinase and c-Jun N-terminal kinase signaling pathways appeared to be associated with the differential activation of the MoDCs by *S. schenckii* of cutaneous and the visceral origins. Overall, we concluded that the differential activation of MoDCs by *S. schenckii* of cutaneous and visceral origins to induce T_h1 response, other than immune status or the host, may be a factor for their different clinical manifestations.

Introduction

Sporotrichosis is caused by a thermo-dependent dimorphic fungus, *Sporothrix schenckii*. The hyphal form that is present in the normal environment consists of both conidia and hyphae, while the yeast form develops at 37°C (1). The conidia and/or hyphae enter the body through either traumatic implantation or inhalation (2, 3). However, *S. schenckii* is observed only as the yeast form in biopsies or excised specimens (4, 5). Such conversion from hyphal to yeast form seems to occur in both the implantation and inhalation sites.

Clinical manifestations of sporotrichosis are variable. The major clinical manifestations occur in the skin and present

Correspondence to: T. Uenotsuchi; E-mail: uenotti@dermatol.med.kyushu-u.ac.jp
Received 13 July 2005, *accepted* 5 September 2006
Transmitting editor: S. Koyasu
Advance Access publication 11 October 2006

primarily as either fixed cutaneous or lymphocutaneous forms (4, 5). However, cases of disseminated cutaneous and/or visceral forms in immunosuppressed patients have been reportedly increased (2, 6).

Both pathogenic factors of the individual *S. schenckii* strains and the immunologic status of the host could determine the clinical manifestations of sporotrichosis. However, the factors involved in the pathogenesis of *S. schenckii* and the mechanisms to determine their virulence remain unclear.

Cell-mediated immunity to *S. schenckii* antigen is a key immunologic defense mechanism that controls the infection (7). Both $CD4^+$ T cells and macrophages are required for the development of granuloma formation, which is a critical and essential component of normal host defense against the pathogens (8, 9). IFN-γ mRNA was detected in the granulomatous skin lesions of sporotrichosis (10), and immunohistochemical analysis demonstrated the existence of that IFN-γ-producing $CD4^+$ T cells in the periphery of such lesion (9). These findings indicate that the granuloma formation in sporotrichosis may be associated with T_h1 response in the skin lesions as evidenced by the local detection of IFN-γ. Such T_h1 response would eventually activate macrophages to kill intracellular *S. schenckii*.

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs), playing key roles in cell-mediated immunity and CD83, a specific marker for DC maturation, is recently found to be expressed at a high level on immunocompetent, activated mature DCs (11). Such small, but presumably significant $CD83^+$ DC sub-population was also observed in the granulation tissue of sporotrichosis (12). These findings indicate that activated DCs may play important roles in the T_h1 immune response against *S. schenckii*. However, the precise interaction between DCs and *S. schenckii* has not been fully elucidated.

Thus, the purpose of the current study was to find any different responses by human monocyte-derived dendritic cells (MoDCs) when exposed to *S. schenckii* isolated from cutaneous and visceral lesions by which the different manifestations may occur.

Methods

Fungi

Sporothrix schenckii of cutaneous (C) origin KMU4649, IFM5906 and IFM46010 and visceral (V) origin KMU4648, IFM41598 and ATCC26331 were used in the experiments. Among the cutaneous isolates, KMU4649 was isolated from a patient under oral corticosteroid therapy. IFM5906 was isolated from the patient with normal immunity. The background information of the host immune status for IFM46010 was not available. Among the visceral isolates, KMU4648 was isolated from the bone marrow of a patient under continuous oral corticosteroid therapy, and IFM41598 and ATCC26331 were isolated from sputum and lung, respectively, but the background information of the hosts' immune status for these isolates was not available. IFM5906, IFM46010 and IFM41598 were kindly provided by K. Nishimura from the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan. These fungi were cultivated in 200 ml of Sabouraud's broth (Difco Laboratories, Detroit, MI, USA) with reciprocal shaking (90 cycle min^{-1}) for 7 days at room temperature to obtain hyphal forms, which consist of conidia and hyphae. The fungi were also cultivated in Petri dishes of brain heart infusion agar (Difco Laboratories) with 1% glucose at 37°C for 5–28 days to obtain yeast forms. Both fungi cultures were suspended in PBS and heat inactivated by incubation for 2 h in a 60°C water bath. These fungi cultures were filtered through a paper filter (Toyo Roshi, Tokyo, Japan) for removal of hyphae or pseudohyphae to obtain pure conidia or yeasts and washed three times with PBS. The conidia and the yeast were counted with a Neubauer chamber and stored at −80°C until use.

Generation of MoDCs

Buffy coats were obtained from healthy volunteer donors according to the institutional guidelines. PBMCs were separated by Ficoll-Paque (Amersham Biosciences, Sweden) density gradient centrifugation. PBMCs (40×10^6 ml^{-1}) were incubated for 1 h at 37°C in a plastic Petri dish with 10 ml of a culture media (RPMI-1640 supplemented with 5% heat-inactivated FCS, 2 mmol l^{-1} L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, all these productions were obtained from GIBCO-BRL, Grand Island, NY, USA), and after vigorous washing, adherent cells to the plastic bottom of the Petri dishes were recovered using manual scraping. More than 85% of such recovered adherent cells were considered to be monocytes as evidenced by being positive for CD14, a representative marker for monocytes. The recovered monocytes cells were then cultured in a DC/T cell culture media; RPMI-1640 with 10% heat-inactivated FCS, 10 mmol l^{-1} HEPES, 1% non-essential amino acids, 1 mmol l^{-1} sodium pyruvate, 2 mmol l^{-1} L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (all from GIBCO-BRL), 50 mmol l^{-1} 2-mercaptoethanol (2-ME) (Sigma, Buchs, Switzerland), 10 ng ml^{-1} recombinant human (rh) granulocyte macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ, USA) and 50 ng ml^{-1} rh IL-4 (PeproTech) for 5 days. The cultured monocytes were fed with fresh medium containing 100 mmol l^{-1} 2-ME, 20 ng ml^{-1} rh GM-CSF and 100 ng ml^{-1} rh IL-4 on days 2 and 4. CD14+ monocytes cultured with IL-4 and GM-CSF acquire the phenotype and function of APCs, and are widely approved as so-called 'myeloid DC' phenotype (13–15). Immature MoDCs (2.5×10^5 ml^{-1}) generated in such manner were then incubated for 48 h with 20 ng ml^{-1} LPS (*Escherichia coli*, strain 055:B5; Difco Laboratories), *S. schenckii* yeast (2.5×10^6) or *S. schenckii* conidia (2.5×10^6) in 1 ml of DC/T cell media.

Antibodies

FITC-labeled CD80 mouse antibody (mAb) (mouse IgM, clone BB1) was purchased from PharMingen (San Diego, CA, USA). PE-labeled CD86 mAb (mouse IgG2b, clone HA5.2B7), PE-labeled CD83 mAb (mouse IgG2b, clone HB15A), FITC-labeled CD1a mAb (mouse IgG1, clone NA1/34) and isotype controls (FITC- and PE-labeled mouse IgG1) were from Immunotech (Marseille, France). Biotin-conjugated HLA-DR mAb (mouse IgG2a, clone CR3/43) was from

Becton-Dickinson (Mountain View, CA, USA). PE-Texas RED (ECD)-labeled streptavidin was from Beckman Coulter (Tokyo, Japan). PE-labeled OX40 ligand mAb (mouse IgG1, clone ANC10G1) was from Ancell (Bayport, MN, USA). Rabbit polyclonal antibodies to phosphorylated inhibitory nuclear factor κB (IκB), p42/44 extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), p46/54 c-Jun N-terminal kinase (JNK) were purchased from Cell Signaling (Beverly, MA, USA). Delta1 (mouse IgG2b, clone 251127), Jagged1 (mouse IgG2b, clone 18831) and Jagged2 (goat IgG immunized with purified recombinant human Jagged2) were from R&D Systems, Mineapolis, MN, USA. Delta4 (polyclonal goat IgG) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), HRP-labeled goat anti-rabbit IgG was from Bio-Rad (Hercules, CA, USA), and goat anti-mouse IgG of donkey and anti-goat IgG were from R&D Systems.

Flow cytometric analysis

After incubation with LPS or heat-inactivated fungi for 48 h, MoDCs were washed, re-suspended at a concentration of 0.5×10^5 to 1×10^5 cells in 50 μl of cold PBS containing 0.1% sodium azide, 10 mg ml^{-1} BSA and 200 μg ml^{-1} mouse IgG (Sigma) and were incubated for 15 min on ice for blocking. Subsequent staining with either fluorescent-labeled mAb or appropriate isotype controls was performed for 30 min on ice. Such stained cells were washed, re-suspended in 300 μl of cold PBS containing 0.1% sodium azide, 10 mg ml^{-1} BSA and 10 μg ml^{-1} 7-amino actinomycin D (Sigma) and analyzed for three-color immunofluorescence by flow cytometry (Coulter, Tokyo, Japan). Cellular debris was eliminated from the analysis using a gate on forward and side scatter. A viability gate was set based on 7-amino actinomycin D staining, which discriminates living cells from necrotic, and/or apoptotic cells. A minimum of 10^4 cells were analyzed for each sample. Results were processed using Flow Jo software (Treestar, San Carlos, CA, USA).

Detection of various cytokines and growth factors

IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IFN-γ, transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) in each culture supernatant were measured using cytokine-specific ELISA kits (BioSource and Endogen, Woburn, MA, USA) according to the manufacturer's protocols.

Allogenic mixed lymphocyte reaction and assessment of T_h1/2 polarization

Allogenic T cells from peripheral blood of healthy volunteers were obtained through Ficoll-Paque gradient centrifugation, removal of adherent cells and passage of the non-adherent cells over a nylon wool column (Wako, Osaka, Japan). The purity of recovered $CD3^+$ T cells in such manner was always >90% (data not shown). $CD3^+$ T cells were distributed at a concentration of 1×10^5 cells per well into round-bottom 96-well microplates and cultured for 5 days within graded numbers of irradiated (at a dose of 3000 rad with ^{137}Cs source) mature DCs that had been incubated with LPS, *S. schenckii* of different forms (yeasts or conidia) of different origins (cutaneous and visceral) in 200 μl of DC/T culture medium containing 10% FCS. T cell proliferation was assessed 8–14 h after incorporation with $[^3H]$thymidine (1 μCi per well; New England Nuclear, Boston, MA, USA) through standard procedures. The results are expressed as the mean ± SEM of triplicate cultures. IFN-γ and IL-4 (Biosource and Endogen) in mixed lymphocyte reaction (MLR) supernatants were assessed as previously described.

Western blot analysis for intracellular signaling pathways and Notch ligands

Immature DCs were incubated with either *S. schenckii* conidia or yeasts of different origins (cutaneous or visceral) either 1 or 2 h for intracellular signaling pathways and 24 h for Notch ligands. DCs treated in such manners were washed twice with cold PBS and incubated with 60 μl of a cell lysis buffer,50 mM Tris–HCl, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 40 mM glycerophosphate, 50 mM NaF, 10 mM sodium pyrophosphate, 200 μM Na_3VO_4, 0.3 mM leupeptin, 1 mM pepstatin A, 1 mM phenylmethylsulfonyl fluoride and 100 nM okadaic acid, pH 7.4, until they got homogenized. The homogenates were centrifuged at 390 × *g* (Ex-125, TOMY, Tokyo, Japan) for 15 min at 4°C. Cell lysates were electrophoresed on 4–12% gradient Bis–Tris gels and transferred to nitrocellulose membranes for western blot analysis. After blocking with 5% fat-free dried milk for 1 h at room temperature, membranes were incubated for 2 h with antibodies to phosphorylated IκB, ERK1/2, p38 MAPK, JNK, Delta1, Delta4, Jagged1 and Jagged2. The membranes were washed and incubated with HRP-labeled goat anti-rabbit IgG, goat anti-mouse IgG or donkey anti-goat IgG for 90 min. Immunoreactive bands were visualized by ECL detection reagent (Amersham, Piscataway, NJ, USA). After stripping, the membranes were reprobed with total IκB, p38 MAPK, ERK1/2, JNK antibodies (Cell Signaling) as controls for their phosphorylated counterparts or with β-actin antibodies (mouse IgG, clone AC-15, Sigma–Aldrich, St Louis, MO, USA) as controls for amount of sample loaded per lane for the examination of Delta1, Delta4, Jagged1 and Jagged2.

Statistical analysis

Data were analyzed using Fisher's protected least significant difference or Dunn's procedure as a multiple comparison procedure and a *P*-value <0.05 was considered to be statistically significant.

Results

Sporothrix schenckii of cutaneous origin induces more pronounced morphological changes of MoDCs than that of visceral origin

We compared the morphological features of MoDCs stimulated with *S. schenckii* of the cutaneous or visceral origins at 1:10 ratio of MoDCs: fungi (Fig. 1). MoDCs stimulated with *S. schenckii* of cutaneous isolates in both forms (yeast and conidia) as well as those with LPS presented longer and wider dendrites than those with visceral isolates. Internalization of *S. schenckii* of cutaneous and visceral origins by co-cultured

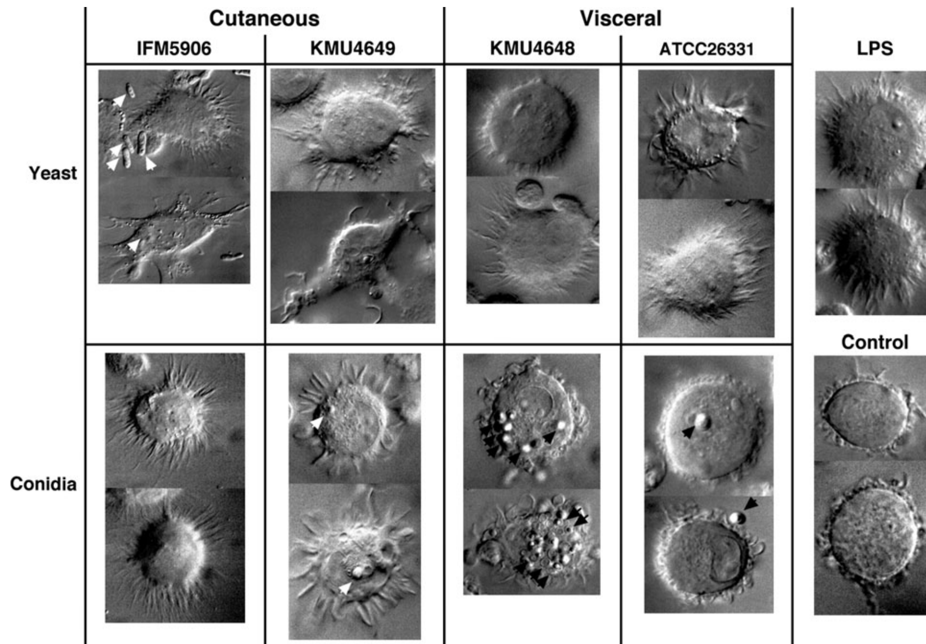

Fig. 1. Phase microscopic appearance of DCs co-cultured with the cutaneous (IFM5906 and KMU4649) and visceral (KMU4648 and ATCC26331) *Sporothrix schenckii*. White and black arrows point to ingested yeasts and conidia (original magnification, ×1000). Results were representative of five separate experiments that yielded similar results.

MoDCs appeared comparable, regardless of the presenting forms (conidia or yeasts).

Sporothrix schenckii of cutaneous origin is a more potent inducer for T_h1-prone DC activation than that of visceral origin

To determine the effect of *S. schenckii* of cutaneous or visceral origin on DC maturation, surface marker expression of MoDC was analyzed using flow cytometry after various stimulations mentioned above (Fig. 2). We found that the cutaneous isolates induced significantly higher expressions of HLA-DR and co-stimulatory molecules, CD80 (B7.1) and CD86 (B7.2) and CD83 and OX40 ligand than visceral isolates.

We also examined the production of IL-2, IL-6, IL-10, IL-12p40, IL-12p70, TGF-β and TNF-α, from the culture supernatants (Fig. 3), and found that cutaneous isolates were significantly more potent inducer for IL-12p40 and TNF-α production than visceral isolates. The cutaneous isolates also tended to induce IL-6 and IL-12p70, although these differences were not statistically significant. The production of immune regulatory cytokine, such as IL-10 and TGF-β, were only minimally detected after exposure to either isolates regardless of their existing forms.

Like what was seen in CD83 expression, the conidia are more potent for TNF-α production than the yeasts when *S. schenckii* of cutaneous origin was used, while no such difference was observed. Taken together, these data suggest that *S. schenckii* of cutaneous origin are more potent to activate MoDCs than that of visceral origin, as evidenced by up-regulations of various activation markers and enhanced cytokine productions with somewhat differential capability between conidial and yeasts forms for the induction of selected forms.

Differential allo-T cell stimulatory capability by DCs activated with S. schenckii of different origins of different existing forms

To further find differences on immune reaction caused by *S. schenckii* of cutaneous and visceral origins, we examined their effect on T cell stimulation, as determined by allogenic MLR and the cytokine profile in the culture media.

In the conidial form, MoDCs incubated (at MoDCs versus fungi ratio = 1:10) with the cutaneous origin was more potent to proliferate allo-T cells (Fig. 4A, left), and stimulated T cells to produce significantly higher IFN-γ (Fig. 4A, middle) than that of visceral origin. IL-4 production was minimal and unaltered by *S. schenckii* of either origin (Fig. 4A, right).

Meanwhile in the yeasts forms, the allo-T cell stimulatory capacity of MoDCs incubated with the *S. schenckii* of cutaneous origin was almost as equal as that of visceral origin (Fig. 4B, left). The MoDCs incubated with cutaneous origin stimulated T cells to produce significantly higher IFN-γ than that of visceral origin (Fig. 4B, middle), and even more interestingly, MoDCs with visceral origin stimulated T cells to produce significantly higher IL-4 than that of cutaneous origin (DC/T cells ratio = 1/10) (Fig. 4B, right).

Sporothrix schenckii of cutaneous origin induces stronger phosphorylation of JNK, p38 MAPK and ERK1/2 of the co-cultured DCs than that of visceral origin

We examined whether the phosphorylation of the DC signaling molecules IκB, JNK, p38 MAPK and ERK1/2 are differentially regulated by the *S. schenckii* of cutaneous and visceral origins using western blot analysis (Fig. 5) and found that both the yeasts and conidial forms of cutaneous origin induced stronger phosphorylation of JNK, p38 MAPK and ERK1/2 than did the visceral origin. There were no differences

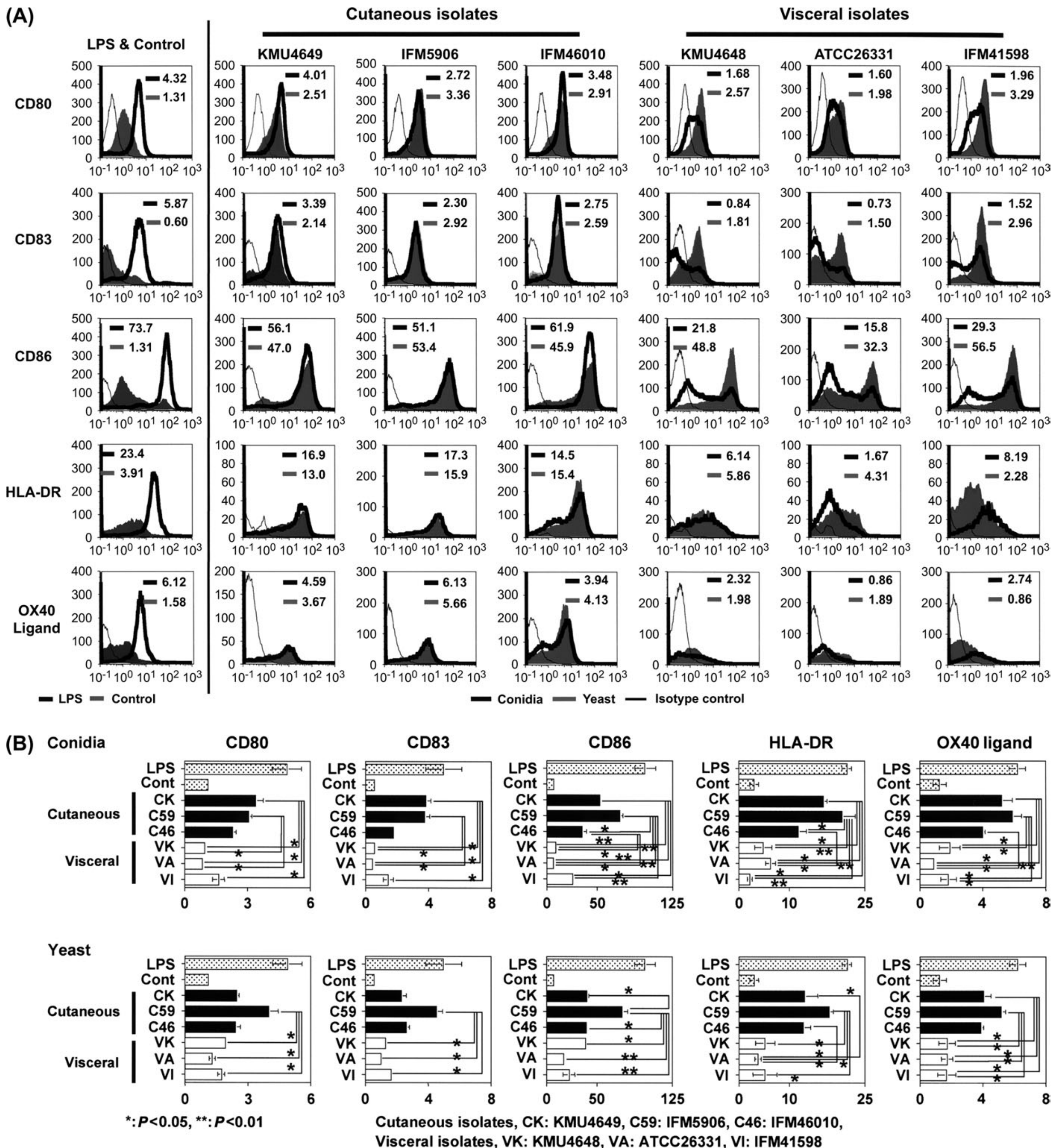

Fig. 2. (A) Flow cytometric analysis of DCs incubated with the cutaneous and visceral isolates of *Sporothrix schenckii*. The bold black line indicates conidia, gray line indicates yeast and dashed line is the isotype-matched control. Numbers in the distinct graph show mean fluorescent intensity (bold black line, conidia; gray line, yeast). Results were representative of five separate experiments that yielded similar results. (B) Mean flow intensity of CD80, CD83, CD86, HLA-DR and OX40 ligand. Data are analyzed using Dunn's procedure as a multiple comparison procedure. The results are the means of three independent experiments. Values are presented as means ± SEM. Brackets indicate statistical comparisons; $^{*}P < 0.05$ and $^{**}P < 0.001$.

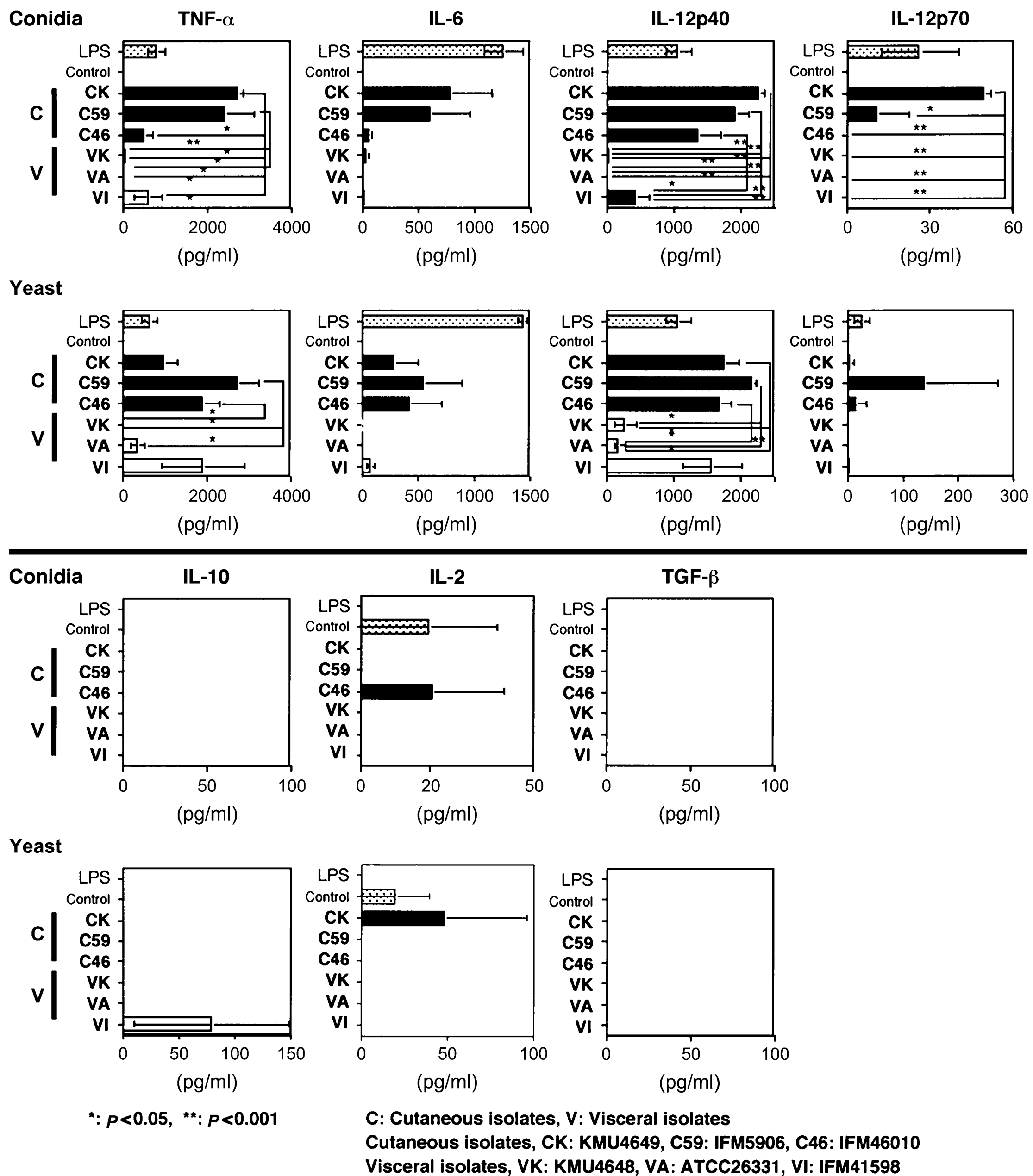

Fig. 3. Production of cytokines from DCs stimulated with cutaneous and visceral isolates of *Sporothrix schenckii*. Using ELISA kits, IL-2, IL-6, IL-10, IL-12p40, IL-12p70, TGF-β and TNF-α of the culture supernatants were measured. Data are analyzed using Dunn's procedure as a multiple comparison procedure. The results are the means of three independent experiments. Values are presented as means ± SEM. Brackets indicate statistical comparisons; *$P < 0.05$ and **$P < 0.001$.

in phosphorylation of IκB between the *S. schenckii* of cutaneous and the visceral origins.

No differences were observed in the expression of Notch ligands, Delta1, Delta4, Jagged1 and Jagged2 between cutaneous and visceral origins

Recently, Notch signaling pathways have been implicated in directing naive T_h differentiation toward the T_h1, T_h2 or regulatory T cell lineage. APCs, DC in particular, express a number of Notch ligands such as Delta1, Delta4, Jagged1 and Jagged2 and to interact with Notch receptors on T cells to determine their $T_h1/2$ commitment (16, 17). Therefore, we examined whether the expressions of such Notch ligands on activated MoDCs are differential expressions of these molecules on the activated MoDCs are different after stimuli with *S. schenckii* of cutaneous and visceral origins (MoDCs versus fungi ratio = 1:10). In our hands, there were no apparent differential expressions of these molecules on the activated MoDCs by *S. schenckii* of cutaneous and visceral origins (Fig. 6).

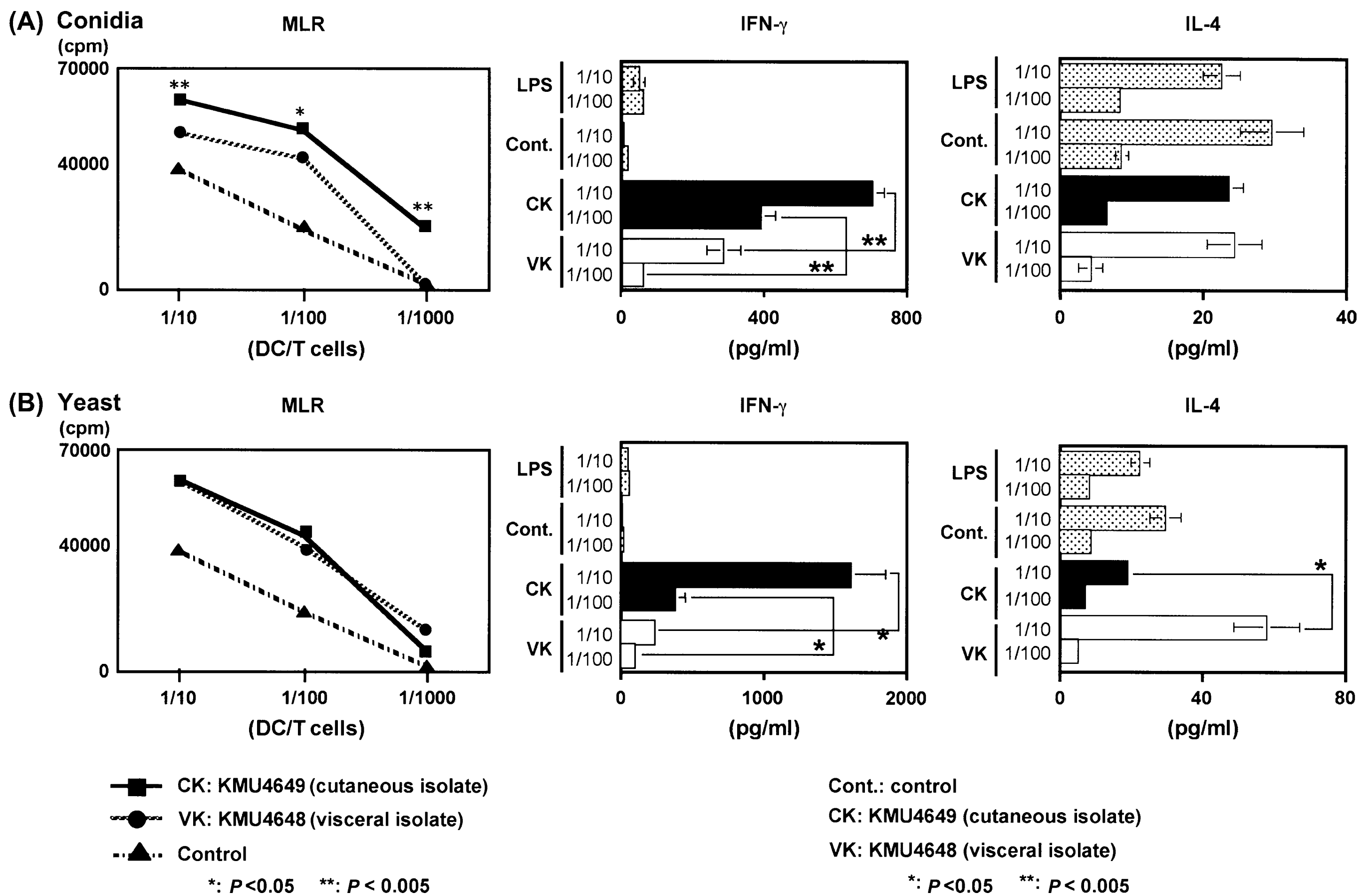

Fig. 4. Allo-T cell stimulatory functions of DCs stimulated with the conidia and the yeasts of cutaneous and visceral isolates of *Sporothrix schenckii.* (A and B, left) Mature DCs stimulated by the conidia and the yeasts of cutaneous and visceral isolates were co-cultured in triplicate with allogenic T cells for 5 days. T cell proliferation was measured by incorporation of [^{3}H]thymidine. Data are analyzed using Fisher's protected least significant difference. The results are the means of three independent experiments. Brackets indicate statistical comparisons; *$P < 0.05$ and **$P < 0.005$. (A and B, middle) Production of IFN-γ in the allogenic MLR by DCs activated with the conidia and the yeasts of cutaneous and visceral isolates of *S. schenckii.* (A and B, right) Production of IL-4 in allogenic MLR with DCs activated with the conidia and the yeasts of cutaneous and visceral isolates of *S. schenckii.* Data are analyzed using Dunn's procedure as a multiple comparison procedure. The results are the means of three independent experiments. Values are presented as means ± SEM. Brackets indicate statistical comparisons; *$P < 0.05$ and **$P < 0.005$.

Discussion

The sporotrichosis can occur in the visceral lesions, as well as in the skin, and impaired immunity of the hosts and/or the different virulence of individual strains could cause these different clinical manifestations. Indeed, many of visceral cases have been observed in immune compromised patients (2, 6, 18). In chronic mucocutaneous candidiasis (CMC), altered cytokine production, such as increased levels of IL-10, is critical for development of candidiasis (19). However, in visceral sporotrichosis, altered cytokine production like CMC has not been reported. In the current study, to assess the possible involvement of different virulence of individual *S. schenckii* strains as a factor for such different manifestations, we examined DC interactions with *S. schenckii* of different origins and found the differential T_h1-inducing potency (though the activated DCs) between *S. schenckii* of cutaneous and visceral origins.

Generally, escaping from the host immune defense is a crucial factor for survival and/or multiplication in the host for micro-organisms, including *S. schenckii*. In the current studies, we demonstrate that *S. schenckii* of cutaneous origin are more potent to activate DCs to induce subsequently stronger T_h1-prone immune response than those of visceral origin as evidenced by (i) higher expressions of HLA-DR and other co-stimulatory molecules and (ii) higher induction of T_h1 cytokines (IFN-γ and TNF-α). We further demonstrate that several reportedly important signaling pathways (such as JNK and p38 MAPK) may be involved in the differential T_h1 induction by *S. schenckii* of different origins.

An association between morphogenesis and virulence has long been presumed for pathogenic dimorphic fungi, including *S. schenckii* (20). Therefore, using selected *S. schenckii* of cutaneous and visceral origins, we also investigated whether there are any different patterns of DC interaction with *S. schenckii* of different origins of different forms. We also found that, in the comparison between the yeasts forms which are the forms to be observed in clinical specimens, *S. schenckii* of visceral origin positively induced T_h2 environment as evidenced by significantly higher IL-4

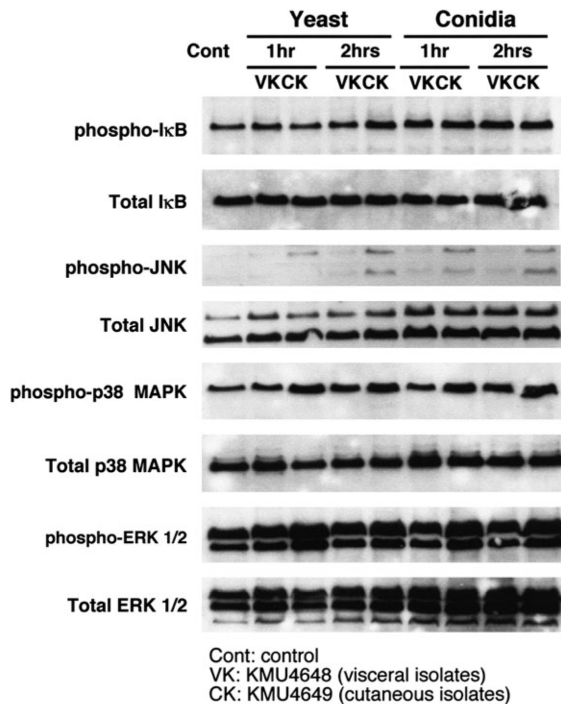

Fig. 5. The phosphorylation of IκB, JNK, p38 MAPK and ERK1/2 was detected by western blotting. Membranes were incubated with antibodies to the phosphorylated form of IκB, JNK, p38 MAPK and ERK1/2 and reprobed with antibodies to total IκB, JNK, p38 MAPK and ERK1/2. Results are representative of three separate experiments.

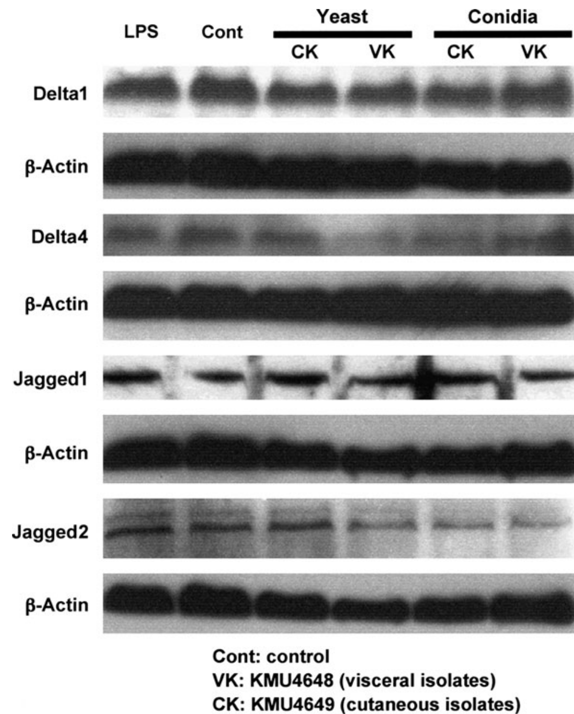

Fig. 6. Expression of Notch ligand Delta1, Delta4, Jagged1 and Jagged2 was detected by western blotting. Membranes were incubated with antibodies to Delta1, Delta2, Jagged1 and Jagged2 and reprobed with antibodies to β-actin. Results are representative of four separate experiments.

production along with the inability to induce strong T_h1 immune responses. Thus, this may further explain their differential clinical manifestation in the context of the factors of the pathogen side. The allo-T cell-proliferating capacity was not different between *S. schenckii* of cutaneous and visceral origins when the yeasts forms were compared. Among the co-stimulatory molecules, OX40 ligand was also significantly up-regulated by co-incubation with *S. schenckii* of cutaneous, as compared with that of visceral origin. The OX40 ligand was originally reported to be a T_h2-related molecule, but the essential contribution of the molecule to T_h2 response has been controversial (21). For a possibility other than $T_h1/2$ balance, the association between the differential thermo-tolerance and the virulence has been frequently discussed, but with poor consensus (22–25).

Together, our data suggest that *S. schenckii* of visceral origin may escape from the host immune defense by inducing little T_h1-prone responses. Thus, the clinical manifestations of sporotrichosis may be regulated by the difference among individual *S. schenckii* strains as DC (or immune) activators as well as immune status of the host.

There could be two possibilities to explain the different interactions of DCs with *S. schenckii* of different origins. First, it has been reported that different efficacy for the internalization of individual *S. shenckii* strains may affect immunostimulatory response of DCs (21, 22). However, in accordance with previous reports (26, 27), *S. schenckii* of both origins appeared equally internalized as shown (Fig. 1).

Differential expressions of surface molecules on *S. schenckii* could be the second possibility. Pattern-recognition receptors (PRRs) are expressed on various APCs, such as DCs and macrophages (28). PRRs play important roles to recognize microbial pathogens, activating innate immune system, releasing pro-inflammatory cytokines and/or modulating acquired immunity by DCs (29). Fungal pathogens are recognized by DCs via PRRs such as Toll-like receptors (TLRs) and lectin (30). Although PRRs that recognize *S. schenckii* have not been identified, TLR2 and TLR4 may be plausible because many fungi such as *Candida albicans*, *Aspergillus niger*, *Aspergillus fumigatus* and *Saccharomyces cerevisiae* are recognized through TLR2 and TLR4 (31). Recognition of fungi via TLR2 and TLR4 activates JNK, ERK, p38 MAPK and NF-κB pathway, and induces release of pro-inflammatory cytokines such as IL-6 and TNF-α (32–34). Results from the current study indicate that *S. schenckii* of cutaneous origin, but not that of visceral origin, induces JNK, ERK and p38 MAPK activation and IL-6 and TNF-α release, as well as other fungi. Thus, both TLR2 and/or TLR4 could be the receptors to recognize *S. schenckii* of cutaneous origin. Although both TLR2 and TLR4 ligation can induce such pro-inflammatory cytokines, TLR2 signals are also known to mediate anti-inflammatory effect by release of IL-10 (35). In our hands, release of IL-10 was not detected. Thus, we speculate that TLR4, but not TLR2, might be the receptor to recognize *S. schenckii* of cutaneous origin and induce strong T_h1 immune response. Identification of PRRs which recognize *S. schenckii* of cutaneous and visceral origins would address these points.

Pathogen recognition by DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and mannose receptor is known to induce T_h2-prone immune response (35).

Thus, DC-SIGN or mannose receptor might be involved in the recognition of *S. schenckii* of visceral origin, although further investigations will obviously be needed to support this idea.

Recent studies have demonstrated that β-glucan is recognized by dectin-1, one of the non-classical C-type lectin, which could induce production of pro-inflammatory cytokines (30). It is true that >50% of fungal cell wall consists of β-glucan and that these carbohydrates could contribute to the immune recognition of fungal pathogens (36). However, since β-glucans are usually shielded by a layer of mannans (except budding scars of yeasts) (35), recognizing fungi through dectin-1 may not be very efficient. Furthermore, since both *S. schenckii* strains (cutaneous and visceral origins) naturally have β-glucans as their cell wall component, the involvement of dectin-1 for their differential T_h1/2-prone immunity is less likely.

Additionally, even in a same *S. schenckii* strain (isolation part was not clarified), it has been reported that the conidia cultured for 7 days are more virulent than those cultured for 12 days. The different virulence is thought to be derived from the different rhamnose: mannose ratio of their surface, which has been changed during the culture (37). Such slight difference in the fungal surface sugar composition might contribute the differential activation of MoDCs. Thus, further investigation of such cell wall components may be of interest to clarify their roles in the differential MoDCs activation by individual *S. schenckii* strains. In addition, the PRRs which recognize cutaneous and visceral isolates of *S. schencki* should be determined.

In the present study, we have demonstrated that *S. schenckii* of cutaneous origin is much more potent to activate DCs, as compared with those of visceral origin, to induce T_h1-prone immune responses. In contrast, the *S. schenckii* of visceral origin are only weak activators for DCs with minimal induction of IFN-γ, and instead, rather positively inducing T_h2 immune responses. Although more precise mechanisms to govern the differential activation of MoDCs by individual *S. schenckii* strains remain to be elucidated, we conclude that the inability of *S. schenckii* of visceral origin to activate DCs for efficient T_h1 immunity induction may be associated with their unique clinical manifestation and the virulence.

Abbreviations

APC	antigen-presenting cell
CMC	chronic mucocutaneous candidiasis
DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule 3-grabbing non-integrin
ERK	extracellular signal-regulated kinase
GM-CSF	granulocyte macrophage colony-stimulating factor
IκB	inhibitory nuclear factor κB
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
2-ME	2-mercaptoethanol
MLR	mixed lymphocyte reaction
MoDC	monocyte-derived dendritic cell
PRR	pattern-recognition receptor
rh	recombinant human
TGF-β	transforming growth factor-β
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α

References

1 Travassos, L. R. 1985. *Sporothrix schenckii.* In Szaniszlo, P. J., ed., *Fungal Dimorphism with Emphasis on Fungi Pathogenic for Humans*, p. 121. Plenum Press, New York.

2 Carvalho, M. T. T., de Castro, A. P., Baby, C., Werner, B., Neto, J. F. and Queiroz-Telles, F. 2002. Disseminated cutaneous sporotrichosis in a patient with AIDS: report of a case. *Rev. Soc. Bras. Med. Trop.* 35:655.

3 Ware, A. J., Cockerell, C. J., Skiest, D. J. and Kussman, H. M. 1999. Disseminated sporotrichosis with extensive cutaneous involvement in a patient with AIDS. *J. Am. Acad. Dermatol.* 40: 350.

4 Hay, R. J. and Moore, M. 1998. Sporotrichosis. In Champion, R. H., Burton, J. L., Burns, D. A. and Breathnach, S. M., eds, *Rook/Wilkinson/Ebling Textbook of Dermatology*, 6th edn. p. 1351. Blackwell Science, UK.

5 Weedon, D. 1997. Sporotrichosis. In Weedon, D., ed., *Skin Pathology*, p. 569. Churchill Livingstone, New York.

6 Bonifaz, A., Peniche, A., Mercadillo, P. and Saul, A. 2001. Successful treatment of AIDS-related disseminated cutaneous sporotrichosis with itraconazole. *AIDS Patient Care STDS* 15:603.

7 Carlos, I. Z., Sgarbi, D. B., Angluster, J., Alviano, C. S. and Silva, C. L. 1992. Detection of cellular immunity with the soluble antigen of the fungus *Sporothrix schenckii* in the systemic form of the disease. *Mycopathologia* 117:139.

8 Tachibana, T., Matsuyama, T. and Mitsuyama, M. 1999. Involvement of CD4+ T cells and macrophages in acquired protection against infection with *Sporothrix schenckii* in mice. *Med. Mycol.* 37:397.

9 Koga, T., Duan, H. and Furue, M. 2002. Immunohistochemical detection of interferon-γ-producing cells in granuloma formation of sporotrichosis. *Med. Mycol.* 40:111.

10 Fujimura, T., Asai, T., Muguruma, K., Masuzawa, M. and Katsuoka, K. 1996. Local expression of migration inhibitory factor and Th1 type cytokine mRNA in sporotrichosis lesions. *Acta Dermato-Venereol. (Stockh.)* 76:321.

11 Nakahara, T., Uchi, H., Urabe, K., Chen, Q., Furue, M. and Moroi, Y. 2004. Role of c-Jun N-terminal kinase on lipopolysaccharide induced maturation of human monocyte-derived dendritic cells. *Int. Immunol.* 16:1701.

12 Koga, T., Duan, H., Urabe, K. and Furue, M. 2001. Immunohistochemical localization of activated and mature CD83+ dendritic cells in granulomas of sporotrichosis. *Eur. J. Dermatol.* 11:527.

13 Romani, N., Reider, D., Heuer, M. *et al.* 1996. Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. *J. Immunol. Methods* 196:137.

14 Kiertscher, S. M. and Roth, M. D. 1996. Human CD14+ leukocytes acquire the phenotype and function of antigen-presenting dendritic cells when cultured in GM-CSF and IL-4. *J. Leukoc. Biol.* 59:208.

15 Liu, Y. J. 2001. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* 106:259.

16 Rutz, S., Mordmuller, B., Sakano, S. and Scheffold, A. 2005. Notch ligands Delta-like 1, Delta-like 4 and Jagged 1 differentially regulate activation of peripheral T helper cells. *Eur. J. Immunol.* 35:2443.

17 Amsen, D., Blander, J. M., Lee, G. R., Tanigaki, K., Honjo, T. and Flavell, R. A. 2004. Instruction of distinct CD4 T helper cell fates by different Notch ligands on antigen-presenting cells. *Cell* 117:515.

18 Morris-Jones, R. 2002. Sporotrichosis. *Clin. Exp. Dermatol.* 27:427.

19 Lilic, D. 2002. New perspectives on the immunology of chronic mucocutaneous candidiasis. *Curr. Opin. Infect. Dis.* 15:143.

20 Romani, L., Francesco, B. and Puccetti, P. 2002. Fungi, dendritic cells and receptors: a host perspective of fungal virulence. *Trends Microbiol.* 10:508.

21 Kim, M. Y., Bekiaris, V., McConnel, F. M., Gaspal, F. M. C., Raykundalia, C. and Lane, P. J. L. 2005. OX40 signals during priming on dendritic cells inhibit CD4 T cell proliferation: IL-4 switches off OX40 signals enabling rapid proliferation of Th2 effectors. *J. Immunol.* 174:1433.

22 Kwon-Chung, K. J. 1979. Comparison of isolates of *Sporothrix schenckii* obtained from fixed cutaneous lesions with isolates from other types of lesions. *J. Infect. Dis.* 139:424.
23 de Albornoz, M. B., Mendoza, M. and Torres, E. D. 1986. Growth temperatures of isolates of *Sporothrix schenckii* from disseminated and fixed cutaneous lesions of sporotrichosis. *Mycopathologia*. 95:81.
24 Bustamante, B. and Campos, P. E. 2001. Endemic sporotrichosis. *Curr. Opin. Infect. Dis*. 14:145.
25 Tachibana, T., Matsuyama, T., Ito, M. and Mitsuyama, M. 2001. *Sporothrix schenckii* thermo-intolerant mutants losing fatal visceral infectivity but retaining high cutaneous infectivity. *Med. Mycol*. 39:295.
26 Locksley, R. M., Heinzel, F. P., Fankhauser, J. E., Nelson, C. S. and Sadick, M. D. 1988. Cutaneous host defense in Leishmaniasis: interaction of isolated dermal macrophages and epidermal Langerhans cells with the insect-stage promastigote. *Infect. Immun*. 56:336.
27 von Stebut, E., Belkaid, Y., Jakob, T., Sacks, D. L. and Udey, M. C. 1998. Uptake of *Leishmania major* amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implication for the initiation of anti-*Leishmania* immunity. *J. Exp. Med*. 188:1547.
28 Buentke, E. and Scheynius, A. 2003. Dendritic cells and fungi. *Acta Pathol. Microbiol. Immunol. Scand*. 111:789.
29 Netea, M. G., Van der Graaf, C., Van der Meer, J. W. M. and Kullberg, B. J. 2004. Recognition of fungal pathogens by Toll-like receptors. *Eur. J. Clin. Microbiol. Infect. Dis*. 23:672.
30 Brown, G. D. 2006. Dectin-1: a signaling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol*. 6:33.
31 Shoham, S. and Lavitz, S. M., The immune response to fungal infections. *Br. J. Haematol.* 129:569.
32 Takeda, K. and Akira, S. 2004. TLR signaling pathways. *Semin. Immunol.* 16:3.
33 Roeder, A., Kirschning, C. J., Rupoc, R. A., Schaller, M., Weindl, G. and Korting, H. C. 2004. Toll-like receptors as key mediators in innate antifungal immunity. *Med. Mycol*. 42:485.
34 Kisho, T. and Akira, S. 2006. Toll-like receptor function and signaling. *J. Allergy Clin. Immunol*. 117:979.
35 Netea, M. G., Van der Meer, J. W. M. and Kullberg, B. J. 2006. Role of the dual interaction of fungal pathogens with pattern recognition receptors in the activation and modulation of host defence. *Clin. Microbiol. Infect*. 12:404.
36 Lopes-Bezerra, L. M., Shubach, A. and Costa, R. O. 2006. *Sporothrix schenckii* and sporotrichosis. *An. Acad. Bras. Cienc.* 78:293.
37 Fernandes, K. S. S., Mathews, H. L. and Lopes-Bezerra, L. M. 1999. Differences in virulence of *Sporothrix schenckii* conidia related to culture conditions and cell-wall composition. *J. Med. Microbiol*. 48:195.