ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2008) 26: 151-163

Differential Gene Expression Profiles of Human Monocyte-Derived Antigen Presenting Cells in Response to *Penicillium marneffei*: Roles of DC-SIGN (CD209) in Fungal Cell Uptake

Piyaporn Ngaosuwankul[1], Piyapong Pongtanalert[1], Aneke Engering[2] and Sansanee C. Chaiyaroj[1,3]

SUMMARY DNA microarray technology was used to determine the gene expression profile of human monocyte-derived macrophages (hMDMs) after stimulation by *Penicillium marneffei* yeast. The expression levels of 175 macrophage genes were found to be altered by a minimum of two-fold in magnitude following 4 hours of *P. marneffei* exposure. Among those, 41 genes were upregulated in activated hMDMs while 134 genes were downregulated. Real-time PCR and RT-PCR were performed to further examine gene expression associated with the inflammatory response. Increased levels of TNF-α and IL-1β gene expression in both hMDMs and human monocyte–derived dendritic cells (hMoDCs) were observed after stimulation by *P. marneffei* yeast. Furthermore, the genes encoding T-bet, IL-6 and ICAM-1 were also upregulated in hMDMs. Functional analysis of the adhesion of *P. marneffei* to dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN, CD209) was performed in hMoDCs since the microarray data revealed an increased expression of DC-SIGN in activated hMDMs. We found that DC-SIGN-Fc bound preferentially to *P. marneffei* yeast rather than to conidia. Moreover, an anti-DC-SIGN monoclonal antibody inhibited the binding of *P. marneffei* yeast to hMoDCs, but did not inhibit endocytosis of *P. marneffei* yeast. The mannose receptor, on the other hand, was important in both adhesion and phagocytosis. These results suggest that *P. marneffei* may exploit DC-SIGN as a receptor to facilitate the systemic spread of infection. Taken together, our study demonstrates the usefulness of microarray technology in generating valuable expression data to permit conventional immunologic investigations of host-fungal interactions.

Penicillium marneffei is the only identified thermal dimorphic fungus within the genus *Penicillium*.[1] It causes penicilliosis, which is a disseminated opportunistic infection widely spread among immunocompromised individuals, particularly in AIDS patients. There are high incidences of the infection in southern China and areas of Southeast Asia, and these areas include northern Thailand and Vietnam.[2,3] According to the report of the Department of Disease Control, Ministry of Public Health, Thailand, there have been 8,058 AIDS patients (2.43%) who were diagnosed of *P. marneffei* infection from September 1984 to May 2008. In comparison to the other fungal infections, penicilliosis marneffei comes as the fourth opportunistic fungal infection in AIDS patients after *Pneumocystis jirovecii* pneumonia, Cryptococcosis and Candidiasis.[4] *P. marneffei* is capable of switching from a filamentous form at 25°C to a yeast-like morphology at 37°C. There is evidence for the presence of *P. marneffei* fission yeast

From the [1]Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand, [2]Department of Immunology, Armed Forces Research Institute of Medical Science (AFRIMS), Bangkok, Thailand, [3]Laboratory of Pharmacology, Chulabhorn Research Institute, Bangkok, Thailand.
Correspondence: Sansanee C. Chaiyaroj
E-mail: scscy@mahidol.ac.th

in macrophages and other tissue specimens in patients suffering from penicilliosis.[5,6] As a result, *P. marneffei* yeast is often considered the virulent form of the fungus.

Although the underlying mechanisms behind *P. marneffei* infections are unknown, it has been suggested that they occur via inhalation of the airborne fungal spores.[7] Upon entering the host, the innate immune response plays a role in preventing and controlling the infection. Granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and IFN-γ, and polymorphonuclear cells (PMN) can exert fungicidal activities toward *P. marneffei*.[8,9] Human monocytes engulf opsonized and unopsonized *P. marneffei* conidia and produce superoxide anions.[10] Such responses are augmented by macrophage colony-stimulating factor (M-CSF). Peripheral blood mononuclear cells (PBMC) stimulate a respiratory burst and the release of tumor necrosis factor alpha (TNF-α) following *P. marneffei* exposure.[11] This finding corroborates the data from our laboratory where *P. marneffei* conidia was shown to stimulate the production of TNF-α and IL-1β.

In the present work, DNA microarray technology enabling observations of global changes in gene expression was used to examine human monocyte-derived macrophages (hMDMs) following exposure to *P. marneffei* yeast for a period of 4 hours. With this technique, we identified 175 macrophage genes which were expressed differentially, with changes that were greater than 2-fold after stimulation. RT-PCR and real-time PCR were performed to determine the expression levels of selected pro-inflammatory cytokines and cell adhesion molecules. We also demonstrated that DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin; CD209) acts as an adhesion receptor for *P. marneffei* yeast while mannose receptor (MR) is involved in both adhesion and internalization.

MATERIALS AND METHODS

P. marneffei fungal strain

The *P. marneffei* strain RT-72 was used in all experiments. It is a clinical strain that was isolated from the oral cavity of a patient admitted at Prince of Songkla Hospital, Thailand, in 1998. The strain was characterized morphologically and genotypically.[12] It was maintained by subculturing on malt extract agar at 25°C. To obtain *P. marneffei* yeast, 2-week-old conidia were collected by resuspension in 0.01% Tween 20 solution and inoculated into either brain heart infusion (BHI) or 1% peptone broth (Bacto™; Becton, Dickinson and Company, Sparks, MD, USA).[13] The fungal inoculum was cultured with shaking at 37°C for 7-9 days until fission yeast with septum were observed using light microscopy. The yeast were collected by centrifugation and washed with phosphate-buffered saline (PBS) prior to each experiment.

Human monocyte-derived macrophages (hMDMs) and human monocyte-derived dendritic cells (hMoDCs)

Whole blood was acquired from healthy adult volunteers after approval by the Ethical Committee of Ramathibodi Hospital at Mahidol University in Bangkok, Thailand. PBMC was prepared from blood using Ficoll Hypaque gradient centrifugation (Isoprep; Robbins Scientific, Sunnyvale, CA, USA). Monocytes were isolated from PBMC with a MACS® Separation system (Miltenyi Biotec, Germany) and mouse anti-human CD14 antibody-labeled microbeads (Human CD14 MicroBeads, Miltenyi Biotec). The purity of the separated monocytes was determined by staining cells with a FITC-labeled anti-CD14 monoclonal antibody (MAb) followed by analysis with flow cytometry (FACaliber; Becton-Dickinson, CA, USA). Monocyte preparations with purities greater than 95% were retained for further study, from which hMDMs and hMoDCs were then derived. hMDMs were cultured in DMEM (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and penicillin-streptomycin solution for 5 days. hMoDCs were derived from monocytes that were cultured for 5 days in RPMI-1640 medium (Invitrogen) supplemented with 10% heat-inactivated autologous serum, 50 ng/ml of granulocyte macrophage-colony stimulating factor (rhGM-CSF) and recombinant human interleukin-4 (rhIL-4) (R&D Systems, Minneapolis, MN, USA). The spent medium was replaced with fresh medium every 2 days. All cell cultures were incubated at 37°C under an atmosphere of 5% CO_2.

DNA microarray assays

hMDMs were co-cultured with *P. marneffei* yeast for 4 hours at a multiplicity of infection (MOI) of 10. Two independent experiments were performed using hMDMs from two individual donors. Following incubation, cells were washed with PBS and total RNA extracted using an RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturers' recommendations. Total RNA samples were subjected to gene expression profiling using the CodeLink™ Expression Assay Kit (GE Healthcare Bio-Sciences, Chalfont St. Giles, UK). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using a T7 oligo (dT) primer and then purified using an RNeasy® Mini Kit (Qiagen). Biotin-labeled complementary RNA (cRNA) was generated using the cDNA as a template in a reaction containing biotin-11-UTP (Perkin Elmer/Applied Biosystems, Foster City, CA, USA), and recovered with QIAquick PCR purification columns (Qiagen). The cRNA samples were then fragmented and hybridized onto a CodeLink™ UniSet Human 20K I BioArray (GE Healthcare Bio-Sciences). The array slides were stained with Cy5-streptavidin solution (GE Healthcare Bio-Sciences) and washed prior to scanning with an arrayWoRx®e Biochip Reader (Applied Precision, Issaquah, WA, USA). The scanned TIFF file for each array was converted to signal intensities and median normalization was performed using CodeLink™ Expression Analysis software (GE Healthcare Bio-Sciences). GeneSifter® software (GeneSifter.Net, VizX Laboratories) was used for pair-wise statistical comparisons between unstimulated (control) and yeast-stimulated hMDMs (test) gene expression data. Genes exhibiting significant differences in expression levels were categorized into ontological pathways based on GO terms using GeneSifter® software.

RT-PCR and real-time PCR

To measure levels of gene expression by reverse transcription PCR (RT-PCR) and real-time PCR, total RNA was first extracted from unstimulated and yeast-stimulated cells using a MasterPure™ RNA Purification Kit (Epicentre® Biotechnologies, Madison, WI, USA) as per the manufacturers' instructions. DNA contaminants were digested with DNase I, and RNA purities and concentrations were assessed via OD_{260} and OD_{280} measurements. Reverse transcription was then implemented to convert total RNA to cDNA with SuperScript III reverse transcriptase (Invitrogen, Grand Island, NY, USA) and an oligo(dT) primer. The cDNA samples served as templates for RT-PCR and real-time PCR experiments.

PCR was performed with *Taq* DNA Polymerase (Promega, Madison, WI, USA) and gene-specific primers (Table 1) using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Gene expression levels were assessed by electrophoresis of PCR amplification products on 1.2% agarose gels which were then stained with ethidium bromide. The band intensities of PCR products were quantified using a densitometer and BIOPROFIL® image analysis software, BIO-1D (Vilber Lourmat) and were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression levels, which were also determined for each sample. PCR conditions consisted of 25-30 cycles of 95°C for 50 seconds, 50°C for 50 seconds and 72°C for 90 seconds.

Real-time PCR was performed with an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers and probes were designed and purchased based on the Assay-by-Design and Custom Oligo Synthesis Service from Applied Biosystems. Relative quantitative gene expression was analyzed using Sequence Detection Software version 1.2.3 (Applied Biosystems).

ELISA detection of TNF-α

hMDMs and hMoDCs were co-cultured with *P. marneffei* yeast at a MOI of 5 and 10. TNF-α concentrations were determined in hMDM culture supernatants at 4 and 8 hours and in hMoDC supernatants at 24 hours following the initiation of the co-cultures. A human TNF-α ELISA Kit from Pierce Endogen (Rockford, IL, USA) was used for this purpose and was conducted according to the instructions provided by the manufacturer. The sensitivity of the assay for the detection of TNF-α was 15.6 pg/ml.

DC-SIGN-Fc binding assays

DC-SIGN-Fc was a gift from Prof. Y. van Kooyk (VU Medical Center, Amsterdam, The Netherlands). Suspensions containing 1×10^7 *P. marneffei*

Table 1 Primer sequences and expected PCR product fragment sizes for RT-PCR experiments

Target mRNA	Primer sequence	Size (bp)
GAPDH	5'-TCT TCT TTT GCG TCG CCA G-3'	413
	5'-GGG GGC AGA GAT GAT GAC C-3'	
IL-1β	5'-CAG AGA GTC CTG TGC TGA AT-3'	238
	5'-GTA GGA GAG GTC AGA GAG GC-3'	
TNF-α	5'-CGG GAC GTG GAG CTG GCC GAG GAG-3'	355
	5'-CAC CAG CTG GTT ATC TCT CAG CTC-3'	
IL-6	5'-TTC GGT CCA GTT GCC TCT C-3'	495
	5'-TGG CAT TTG TGG TTG GGT CA-3'	
IL-10	5'-TAC TCT TAC CCA CTT CCC CC-3'	312
	5'-TGA GAA ATA ATT GGG TCC CC-3'	
IL-12p35	5'-CAT GCT TTC AGA ATT CGG GC-3'	426
	5'-GTT AGC TCA GAT GCT TTC ATG-3'	
IL-12p40	5'-TCT AAG CGA TTC GCT CCT GC-3'	392
	5'-AAG CTG CTG GTG TAG TTT TG-3'	
ICAM-1	5'-AAA GGA TGG CAC TTT CCC AC-3'	594
	5'-TTC CCC TCT CAT CAG GCT AGA C-3'	
T-bet	5'-GAG GGT CGC GCT CAA CAA C-3'	607
	5'-GGA TGC TGG TGT CAA CAG ATG-3'	

yeast cells or 1×10^8 *P. marneffei* conidia were added to a 96-well plate and allowed to dry overnight at 37°C. Control wells contained 100 μg/ml mannan, 1 mg/ml laminarin or PBS and were incubated at 37°C for 2 hours. DC-SIGN-Fc was diluted in Tris salt medium (TSM) and dispensed into wells supplemented with various ligands and incubated for 2 hours at room temperature with shaking at 30 rpm. The plate was then washed 5 times with TSM containing 0.05% (v/v) Tween 20 (TSM-T). Peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako, Denmark) was added to each well, and the plate was incubated for a further 30 minutes at room temperature. Plates were subjected to 5 washes with TSM-T, after which TMB Peroxidase substrate (SureBlue™, KPL, Gaithersburg, MD, USA) was added to wells and gently oscillated for 10 minutes at room temperature. These reactions were terminated by the addition of H_2SO_4 and color intensities were measured at an emission wavelength of 450 nm using a Multiskan EX microplate photometer (Thermo Electron Corporation, USA).

Adhesion inhibition assays

The inhibition by anti-DC-SIGN and anti-MR MAbs on the binding of hMoDCs to *P. marneffei* yeast was examined. These MAbs are recommended for adhesion blockade assays. hMoDCs (5 x 10^5 cells) were pretreated for 30 minutes at 37°C with 0.01, 0.1 and 1 μg/ml of anti-MR MAb (HyCult Biotechnology, The Netherlands) or 1, 5 and 10 μg/ml of anti DC-SIGN MAb (R&D Systems Inc., Minneapolis, MN). Mouse IgG (Caltag laboratories, Burlingame, CA, USA) was used at a concentration of 10 μg/ml and incorporated as an isotype control. FITC-labeled *P. marneffei* yeast (at an MOI of 5) was then added to pretreated or untreated hMoDCs. Labeled *P. marneffei* yeast bound to hMoDCs were co-incubated for a further 2 hours at 37°C with gentle agitation at 15 minute intervals. After washing, cells were fixed in cold 2% glutaraldehyde and stained with 10% Giemsa solution (MERCK, Germany). Quantitation of yeast cells bound to hMoDCs was achieved using a Nikon EFD-3 epifluorescence

microscope (Nikon, Tokyo, Japan) equipped with a single bandpass filter for FITC.

Internalization inhibition assays

Inhibition by anti-DC-SIGN and anti-MR MAbs on the internalization of live *P. marneffei* yeast was also investigated. hMoDCs (5×10^5 cells) were first treated with 0.1, 0.5 and 1 μg/ml anti-MR MAb or 2.5, 5 and 25 μg/ml anti-DC-SIGN MAb for 30 minutes at 37°C. *P. marneffei* yeast (at an MOI of 5) were then added and the mixtures incubated at 37°C for 3 hours. Amphotericin B was added to a final concentration of 2.5 μg/ml to kill uninternalized extracellular yeast, which was followed by an additional 8-hour incubation. hMoDCs were lysed and the suspension plated on Sabouraud dextrose agar (SDA). The resultant fungal colonies were counted and expressed as colony-forming units per ml (CFU/ml) for each of the cell lysates.

Statistical analysis

Percentage inhibition values were normalized to isotype controls. The Mann-Whitney U test was used to determine statistical differences between test and control groups. A p value < 0.05 was considered significant.

RESULTS

Genes differentially expressed between unstimulated hMDMs and *P. marneffei* yeast-stimulated hMDMs

Total RNA from unstimulated (control) hMDMs and yeast-stimulated hMDMs (test) were used to prepare labeled probes which were hybridized to a CodeLink™ UniSet Human 20K I BioArray slide. The slide contains immobilized DNA oligonucleotides representing approximately 20,000 different genes. The reproducibility of the screening method using microarray was high with the correlation coefficient of > 0.9. Microarray data were analyzed to permit the identification of significant changes in the levels of gene expression between control and test samples. We found that the expression levels of 175 genes in hMDMs were significantly different after exposure to yeast, using a minimum magnitude of change of 2-fold. Among them, 41 genes were upregulated in stimulated hMDMs while the remaining 134 genes were downregulated.

To better illustrate the global changes in the hMDM gene categories that were affected by *P. marneffei* yeast stimulation, the differentially expressed genes were grouped into ontological pathways based on either their involvement in various biological processes or their molecular function. The minimum fold-change used for gene ontology sorting was set at 1.5 in order to obtain a broader array of genes. The most predominant biological roles featuring the altered genes are the following: cellular processes (36.90%), physiological processes (33.37%), regulation of biological processes (14.46%), development (7.40%) and response to stimulus (5.92%) (Fig. 1a). The major groups based on molecular function are: binding (48.46%), catalytic activity (23.85%), signal transducer activity (8.46%) and transcription regulator activity (6.92%) (Fig. 1b).

Table 2 depicts the z-scores of representative ontological pathways that were significantly upregulated or downregulated according to the microarray data obtained in the present study. A z-score greater than 2 indicates significant up-regulation or down-regulation of a pathway in hMDMs following *P. marneffei* stimulation. The cellular defense response pathway yielded the highest z-score (3.99) among the upregulated pathways. Others included system development (3.24), cell surface receptor linked signal transduction (3.13) and cell communication (2.81). Pathways significantly downregulated (z-scores greater than 2) after *P. marneffei* yeast stimulation were those involved in transcription and metabolism. The ontology analysis conducted here suggest that *P. marneffei* yeast stimulation leads to an upregulation of genes engaged in cellular responses and signal transduction with a concomitant down-regulation of genes involved in transcription and cellular metabolism.

Table 3 shows representative upregulated and downregulated genes that possess roles in immune and defense responses, cell adhesion and signal transduction. *P. marneffei* yeast stimulation increases mRNA levels of granulocyte-macrophage colony stimulating factor 2 (GM-CSF 2), interleukin (IL)-4 and the chemokine ligand, CCL16. In contrast, CXCL1, which is another chemokine ligand, was

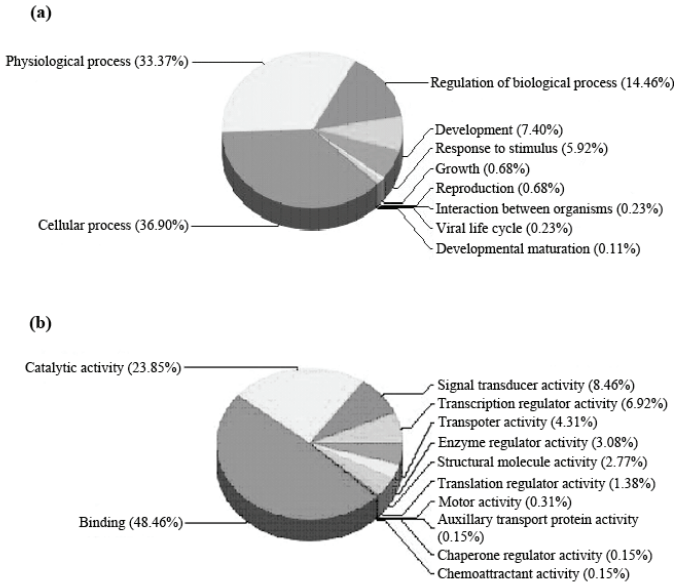

Fig. 1 Ontology-based grouping of differentially expressed hMDM genes. Altered genes were grouped according to (a) biological processes in which they are involved and (b) their molecular function.

Table 2 Upregulated and downregulated pathways in *P. marneffei*-stimulated hMDMs

Ontological pathway	z-score
Up-regulated pathway	
Cellular defense response	3.99
System development	3.24
Cell surface receptor linked signal transduction	3.13
Cell communication	2.81
Intracellular signaling cascade	2.52
G-protein coupled receptor protein signaling pathway	2.51
Physiological defense response	2.45
Down-regulated pathway	
Transcription initiation	4.16
Cellular protein metabolism	3.44
RNA metabolism	3.13
Protein transport	3.08
RNA processing	2.96
Dephosphorylation	2.92
Protein localization	2.86
Ribosome biogenesis and assembly	2.85

Ontological pathway analyses and z-scores were generated using GeneSifter® Software. A z-score greater than 2 indicates a significant upregulation or downregulation of the pathway in *P. marneffei*-stimulated hMDMs.

down-regulated. Increased expression of genes encoding adhesion molecules such as DC-SIGN (CD209) and laminin S were also observed after yeast stimulation, while neuropilin 2 and plexin C1 gene expression decreased. Changes in the expression of genes involved in intracellular signal transduction (e.g. protein kinases) also occurred as a result of *P. marneffei* yeast stimulation. We detected an elevated expression of the TRAF family member-associated NFκB activator (TANK) gene, but a de-

Table 3 Representatives of upregulated and downregulated genes of *P. marneffei* yeast-stimulated hMDMs as compared to unstimulated hMDMs (greater than a cutoff ratio of 1.5-fold)

Gene name	Accession no.	FC
Defense response and immune response		
Colony stimulating factor 2 (granulocyte-macrophage)	NM_000758	3.29
Paired box gene 5 (B-cell lineage specific activator)	NM_016734	1.69
TYRO protein tyrosine kinase binding protein	NM_003332	1.59
Natural cytotoxicity triggering receptor 1	NM_004829	1.54
Chemokine (C-C motif) ligand 16	NM_004590	1.54
Interleukin 4	NM_000589	1.51
Chemokine (C-X-C motif) ligand 1	NM_001511	-2.17
Conserved helix-loop-helix ubiquitous kinase	AF080157	-2.09
Cell adhesion		
Microfibrillar-associated protein 4	L38486	1.78
CD209 molecule	NM_021155	1.61
Integrin-linked kinase	U40282	1.6
Hyaluronan binding protein 2	NM_004132	1.59
laminin, beta 2 (laminin S)	NM_002292	1.52
Neuropilin 2	NM_018534	-4.79
Plexin C1	AF035307	-3.28
Intracellular signaling cascade		
CDC28 protein kinase regulatory subunit 2	NM_001827	2.37
Dynamin binding protein	AB023227	1.83
Growth arrest and DNA-damage-inducible, beta	NM_015675	1.79
Src homology 2 domain containing transforming protein D	AK056268	1.67
TRAF family member-associated NFKB activator	AV682198	1.62
P21/Cdc42/Rac1-activated kinase 1	U24152	1.58
Chondroitin sulfate proteoglycan 4	NM_001897	1.57
Serine/threonine kinase 38	NM_007271	-6
A kinase (PRKA) anchor protein 11	NM_016248	-2.75
Interleukin-1 receptor-associated kinase 1 binding protein 1	AL049321	-1.54
NFKB inhibitor interacting Ras-like 1	NM_020345	-1.51

Accession no. indicates the sequence used as a probe in the CodeLink™ UniSet Human 20K I BioArray system.
FC = Fold change.
Negative sign (-) denotes downregulation.

crease in the expression of interleukin-1 receptor-associated kinase 1 binding protein 1 (IRAK1BP1) and NFκB inhibitor interacting Ras-like 1 (NKIRAS1) was found in stimulated hMDMs.

Confirmation of expression of cytokines and adhesion molecules upon stimulation of hMDMs and hMoDCs by *P. marneffei* yeast

RT-PCR experiments revealed gene expression was upregulated for TNF-α (2.53-fold increase in hMDMs and 4.14-fold increase in hMoDCs) and IL-1β (6.62-fold increase in hMDMs and 9.33-fold increase in hMoDCs) after exposure to *P. marneffei* yeast (Fig. 2). This up-regulation of IL-β and TNF-α gene expression was confirmed by real-time PCR in the stimulated hMDMs (Fig. 3). A 2.52-fold increase in IL-6 expression was also observed by RT-PCR in hMDMs. Increases in T-bet and ICAM-1 gene expression of 3.14- and 1.48-fold, respectively, were observed using RT-PCR (Fig. 3). Although ICAM-1 gene expression levels were found to be less than 2-fold using RT-PCR, the more sensitive, quantitative real-time PCR methodology uncovered

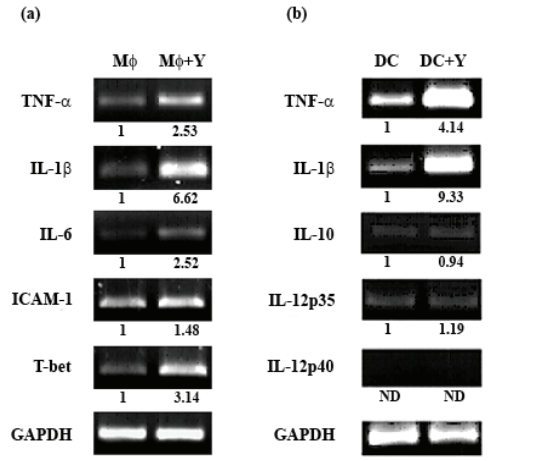

Fig. 2 mRNA expression levels of *P. marneffei*-stimulated APCs detected by RT-PCR. (Mϕ; hMDMs, DC; hMoDCs, Y; *P. marneffei* yeast). hMDMs or hMoDCs were co-cultured with *P. marneffei* yeast for 4 hours. Total RNA from each APC preparation was extracted and subjected to RT-PCR. Band densities arising from *P. marneffei*-stimulated cells is represented as a value relative to the corresponding band densities of unstimulated cells.

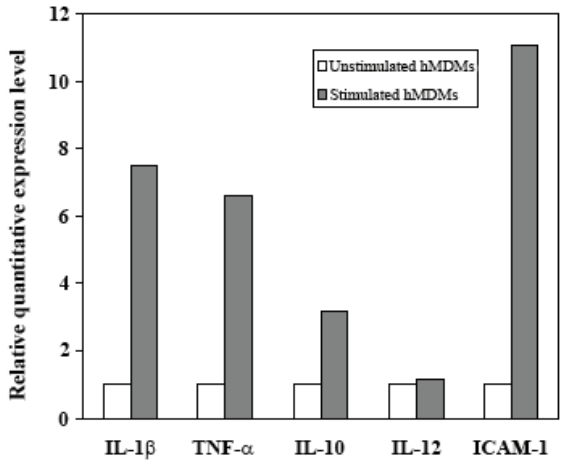

Fig. 3 mRNA expression levels of *P. marneffei*-stimulated hMDMs as determined by real-time PCR. hMDMs were co-cultured with *P. marneffei* yeasts for 4 hours. Total RNA from hMDMs was extracted and subjected to real-time PCR. GAPDH gene expression levels were used to normalize amounts of input RNA. The gene expression value from *P. marneffei*-stimulated hMDMs is presented as a relative value to the gene expression value from unstimulated hMDMs (which was given a value of 1).

an approximate 10-fold increase in levels. IL-12 gene expression levels were unchanged in both hMDMs and hMoDCs. While levels of IL-10 gene expression were comparable in the unstimulated and stimulated hMoDCs as adjudged by RT-PCR, real-time PCR experiments showed a 3-fold increase in expression in stimulated hMDMs (Figs. 2 and 3).

TNF-α protein secretion was examined in the supernatants from hMDMs and hMoDCs that had been co-cultured with *P. marneffei*. The yeast stimulated the TNF-α secretion by hMDMs after 4 hours of incubation, and levels increased in a time-dependent fashion (Fig. 4A). TNF-α secretion was also detected after 24 hours in the culture supernatant from hMoDCs stimulated with *P. marneffei* (Fig. 4B).

Recognition of *P. marneffei* yeast by DC-SIGN (CD209) and the mannose receptor (MR) on hMoDCs

Since the microarray data had revealed that upregulation of DC-SIGN (CD209) occurs upon yeast stimulation, we investigated the possibility of DC-SIGN acting as an adhesive receptor. The mannose receptor was also tested in parallel, prompted by reports that it is a major receptor in several fungi species. Various concentrations of monoclonal antibodies (MAbs) against DC-SIGN and MR were implemented to determine if they prevented the receptors from binding to yeast cells. Indeed, anti-MR and anti-DC-SIGN MAbs inhibited the adhesion of *P. marneffei* yeast to hMoDCs in a dose-dependent manner ($p < 0.05$) (Fig. 5). The anti-MR MAb, employed at a concentration of 1 μg/ml, can inhibit binding by up to 70%. This level of inhibition was similar to that of 10 μg/ml anti-DC-SIGN MAb. Thus, both MR and DC-SIGN are involved in *P. marneffei* adhesion.

The DC-SIGN-Fc was then examined for binding to either *P. marneffei* yeast or conidia. Mannan and laminarin [β(1,3)-glucan] are common cell wall components in most fungi and were included as positive control polysaccharides, whereas PBS was used as the negative control. Fig. 6 shows that DC-SIGN-Fc bound to *P. marneffei* yeast with a maximum optical density (OD) reading of 1.2 at 450 nm, while OD values for binding to conidia, laminarin and mannan were between 0.3 - 0.4. This indicates that DC-SIGN preferentially interacts with *P.*

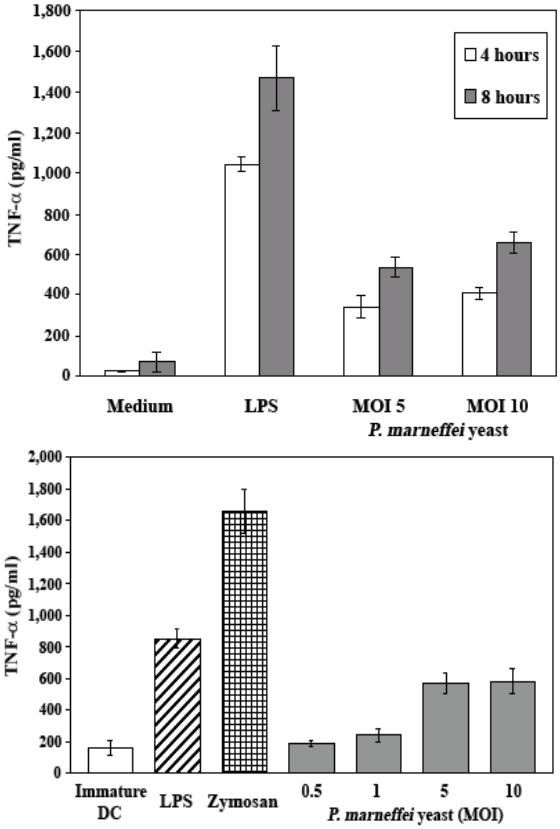

Fig. 4 TNF-α production in *P. marneffei*-stimulated APCs. (a, upper) hMDMs were co-cultured with *P. marneffei* yeast for 4 and 8 hours; (b, lower) hMoDCs were co-cultured with *P. marneffei* yeast for 24 hours. Cell culture supernatants were collected for ELISA cytokine assays. Results are expressed as mean ± standard deviation from duplicate assays.

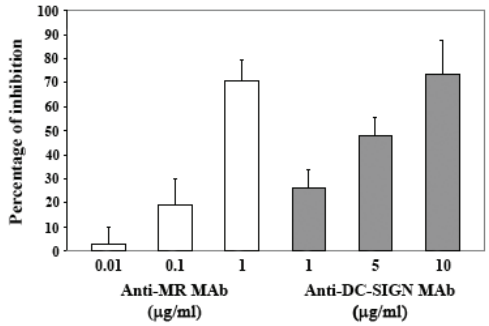

Fig. 5 Inhibition of the adhesion of *P. marneffei* yeast to hMoDCs, using MAbs towards DC-SIGN and MR. Anti-MR and anti-DC-SIGN MAbs were incubated with hMoDCs for 30 minutes prior to the addition of *P. marneffei* yeast. The values represent the mean ± standard deviation of 3 independent experiments.

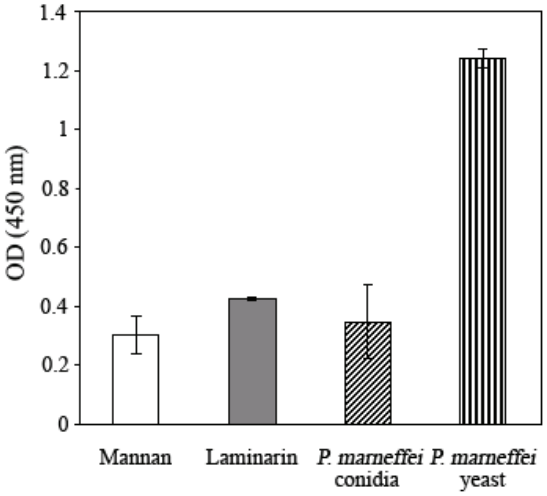

Fig. 6 Adhesion of DC-SIGN-Fc to *P. marneffei* yeast. Mannan, laminarin *P. marneffei* conidia and *P. marneffei* yeast were tested for their ability to adhere to DC-SIGN-Fc. The OD_{450} reading reflects the degree of ligand binding to DC-SIGN-Fc. The values represent the mean ± standard deviation of 3 independent experiments. The OD value of the negative control (PBS) was subtracted from the value obtained for each ligand.

marneffei yeast rather than to the conidia, mannan or laminarin alone.

The mannose receptor, but not DC-SIGN, mediates *P. marneffei* endocytosis into hMoDCs

Phagocytosis is an important and specific form of endocytosis which occurs after cellular adhesion. It is employed by APCs for further processing and presentation of endocytosed antigens. We investigated whether the binding of *P. marneffei* yeast to hMoDCs leads to a subsequent endocytic process. The anti-MR MAb reduced *P. marneffei* yeast internalization to approximately 40% ($p < 0.05$) at an antibody concentration of 1 μg/ml (Fig. 7). Interestingly, no significant reduction in yeast endocytosis arose following treatment of hMoDCs with up to 25 μg/ml anti-DC-SIGN MAb. These findings suggest that dendritic cells utilize MR rather than DC-SIGN for the vesicular internalization of *P. marneffei* yeast.

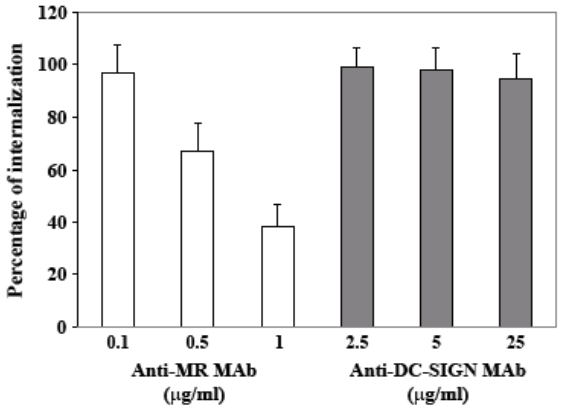

Fig. 7 Inhibition by anti-MR but not anti-DC-SIGN MAb of hMoDC endocytosis of *P. marneffei* yeast. hMoDCs were pretreated with anti-MR and anti-DC-SIGN MAb for 30 minutes prior to the addition of *P. marneffei* yeast. Amphotericin B was added into culture medium to kill uninternalized yeast and incubated for 8 hours. Cells were lysed and plated onto SDA agar. Colony forming units of engulfed yeast were determined. The values are presented as mean ± standard deviation of 3 independent experiments.

DISCUSSION

P. marneffei infections in endemic areas pose a great threat to immunocompromised individuals, particularly AIDS patients. In healthy people, the immune system is capable of removing the fungus that is acquired from the environment. However, the immune system of HIV-positive patients is rendered incapable of doing so, which leads to lethal systemic infections. Previous studies on *P. marneffei* and immunity in mice showed that both innate and adaptive immune responses contribute to protection from *P. marneffei* infections.[14-16] Human studies on innate immune cells, including PMN and macrophages, show that they serve an important role in the clearance of *P. marneffei*. PMN in particular could control and kill the infection in a manner that was enhanced by specific cytokine stimulation.[8-10] Another study demonstrated that stimulation of PBMC by *P. marneffei* yeast produced the pro-inflammatory cytokine, TNF-α,[11] possibly linking the innate immune response and adaptive responses in order to facilitate the elimination of the fungus.

In the present study, we investigated the interaction between *P. marneffei* yeast and APCs. The APCs are the innate immune response cells that act as the first line of defense against infections. DNA microarray technology was used to identify changes in gene expression in hMDMs following exposure to *P. marneffei* yeast. The gene encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) was upregulated in hMDMs. Although the primary function of GM-CSF is the stimulation of hematopoietic cell development, it is well known that GM-CSF released from monocytes may also be involved in the enhancement of monocyte functions towards tumor cells and microbes.[17] GM-CSF augments human monocytes/macrophages fungicidal activities both *in vivo* and *in vitro*.[18] Furthermore, GM-CSF possesses stimulatory effects on other phagocytic cells. Kudeken *et al*. showed that GM-CSF enhances of the fungicidal activity of human PMN toward *P. marneffei*.[8,9] Our result suggests that macrophages and PMN may work in conjunction within the same microenvironment to control and/or eliminate *P. marneffei* infections.

An analysis of ontological pathways demonstrated that a significant upregulation of genes involved in signal transduction pathways occurred, including cell surface receptor linked signal transduction, intracellular signaling cascades and cellular defense response. Dendritic cells (DCs) and macrophages are crucial innate immune cells that play roles in first-line detection of foreign agents and link innate immunity with adaptive immunity.[19,20] Therefore, cytokines were measured in two *P. marneffei* yeast-infected APCs, hMDMs and hMoDCs, since cytokines are involved in signal transduction in APCs. Following *P. marneffei* yeast stimulation, both hMDMs and hMoDCs showed increased IL-1β and TNF-α expression. Concomitant increases in IL-6 mRNA and TNF-α levels were also observed in stimulated hMDMs and thus indicative of cell stimulation. Previous reports have demonstrated that *Aspergillus fumigatus, Candida albicans* and *Scedosporium* stimulate TNF-α and IL-1β production in mouse and human mononuclear cells.[21-23] Additionally, *A. fumigatus* and *Scedosporuim* induce IL-6 expression.[21] *P. marneffei* infections in both mice and humans lead to TNF-α production.[11,16] Together, these findings, along with those reported in the present study, indicate that the TNF-α, IL-β and IL-6 cytokines play important roles in the cellular response to fungal infections that include *P. marneffei*.

We also studied the expression of IL-10 and IL-12, cytokines that link the innate and adaptive immune responses.[24] IL-12 was not detected in our experiments, while increases in the levels of IL-10 were observed in stimulated hMDMs. This suggests that IL-10 cytokine may have a regulatory function in *P. marneffei* yeast infections. Studies on *A. fumigatus* have shown that its fungal conidia can trigger IL-10 production from hMoDCs, but IL-12 could not be found.[25]

P. marneffei yeast stimulation can also lead to increased levels of the T-box transcription factor family member T-bet (also known as Tbx21),[24] which controls the Th1 response. While the function of T-bet as a Th1 regulator has primarily been studied in lymphocytes, other work has shown that T-bet is expressed in hMDMs and hMoDCs, both of which are cells of myeloid lineage, upon cell stimulation by interferon (IFN)-γ.[26] The function of T-bet in non-lymphoid cells remains unclear. It is possible that this transcription factor may be part of the Th1 response in hMDMs and could play a role(s) in the elimination of infection.

Our microarray results provided evidence for the function of the immunoreceptor, DC-SIGN (or CD209), which was upregulated in hMDMs stimulated by *P. marneffei* yeast. The increase DC-SIGN gene expression is concordant with upregulated IL-4 gene expression, and it has been shown that the expression of DC-SIGN in monocytes and macrophages is potentiated by IL-4 and IL-13.[25] hMoDC was therefore used in the subsequent experiments on the involvement of DC-SIGN in the recognition and internalization of *P. marneffei* yeast, as it was previously reported to produce abundant quantities of DC-SIGN.[27]

Fungi such as *P. marneffei* contain mannan as an outer cell wall component. Mannan and β(1,3) glucan (a laminarin) are representative fungal cell wall components, and although the organization of the fungal cell wall is not fully understood, it is composed of the polysaccharides glucan (both α(1,3) and β(1,3) linkages), mannan and chitin.[28] A previous report shows that galactomannan appears to be the main DC-SIGN ligand in the conidial wall of *A. fumigatus*.[25] Interestingly, we observed that DC-SIGN binds to *P. marneffei* yeast to a much greater extent than for *P. marneffei* conidia, mannan and laminarin. Furthermore, DC-SIGN-Fc binding to mannan and laminarin is weaker than binding to *P. marneffei* yeast, indicating that an intact yeast cell wall is required for strong DC-SIGN binding. The effects of the different cell wall components among *P. marneffei* fission yeast and other budding pathogenic yeasts on DC-SIGN binding remain to be seen.

DC-SIGN (CD209) is a type II membrane c-type lectin that contains a mannan-binding lectin domain linked to a transmembrane and cytoplasmic tail. It mediates interactions between dendritic cells and various pathogens such including HIV, Ebola virus, hepatitis C virus, Dengue virus, and the parasites *Leishmania* and *Mycobacterium tuberculosis*.[27] The role of DC-SIGN as an antigen-uptake receptor within fungi has previously been reported in *A. fumigatus* and *C. albicans*.[25,29] DC-SIGN mediates adhesion and internalization of *A. fumigatus* conidia on dendritic cells and macrophages.[25] DC-SIGN acts as a receptor for binding and uptake of *C. albicans* yeast by dendritic cells.[29] An inhibition assay in our study using specific MAbs against DC-SIGN and MR showed that both DC-SIGN and MR are involved in the binding of hMoDCs to *P. marneffei* yeast. This supports a previous report containing evidence for the mannoprotein Mp1p, the ligand for MR, in the cell wall of *P. marneffei* yeast.[30] Indeed, MR is an endocytosis receptor in various fungi, including *P. marneffei*.[25,29,31-33] DC-SIGN had no effect on *P. marneffei* yeast internalization by hMoDCs. These results indicate that DC-SIGN acts as a receptor for *P. marneffei* which could contribute to the mechanism of systemic infection.

Together, the findings in the present study suggest that during the initial phases of the interaction between *P. marneffei* yeast and APCs, MR and DC-SIGN (C-type lectin receptors) mediate the specific recognition of the fungus. MR, but not DC-SIGN, plays an active role in yeast internalization. The interaction between *P. marneffei* yeast and APCs causes the increased expression of IL-1β and TNF-α in hMDMs and hMoDCs, and increases in IL-6, ICAM-1 and T-bet gene expression in hMDMs. This study demonstrates the value in using microarray technology to generate high-throughput expression data, which can generate important immunologic information pertaining to host-fungal interactions.

ACKNOWLEDGEMENTS

Piyaporn Ngaosuwankul is a Ph.D student of Mahidol University supported by The Royal Golden Jubilee Ph.D. Scholarship (PHD/0087/2547) of the Thailand Research Fund (TRF). Piyapong Pongtanalert was supported by the Faculty of Graduate Studies, Mahidol University, under the Research Assistant Program. This work was funded by BIOTEC, National Science and Technology Development Agency (NSTDA). We would like to thank Mr. Rachasak Boonhok for providing additional information for this study.

REFERENCES

1. Cooper CR, Jr., Haycocks NG. *Penicillium marneffei*: an insurgent species among the penicillia. J Eukaryot Microbiol 2000; 47: 24-8.
2. Deng Z, Ribas JL, Gibson DW, Connor DH. Infections caused by *Penicillium marneffei* in China and Southeast Asia: review of eighteen published cases and report of four more Chinese cases. Rev Infect Dis 1988; 10: 640-52.
3. Nittayananta W. Penicilliosis marneffei: another AIDS defining illness in Southeast Asia. Oral Dis 1999; 5: 286-93.
4. Report of the Epidemiological Information Section, Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand. http://epid.moph.go.th. 2008.
5. Ma ES, Ho PL. Disseminated *Penicillium marneffei* infection. Br J Haematol 2005; 130: 2.
6. Lim D, Lee YS, Chang AR. Rapid diagnosis of *Penicillium marneffei* infection by fine needle aspiration cytology. J Clin Pathol 2006; 59: 443-4.
7. Duong TA. Infection due to *Penicillium marneffei*, an emerging pathogen: review of 155 reported cases. Clin Infect Dis 1996; 23: 125-30.
8. Kudeken N, Kawakami K, Saito A. Cytokine-induced fungicidal activity of human polymorphonuclear leukocytes against *Penicillium marneffei*. FEMS Immunol Med Microbiol 1999; 26: 115-24.
9. Kudeken N, Kawakami K, Saito A. Mechanisms of the in vitro fungicidal effects of human neutrophils against *Penicillium marneffei* induced by granulocyte-macrophage colony-stimulating factor (GM-CSF). Clin Exp Immunol 2000; 119: 472-8.
10. Roilides E, Lyman CA, Sein T, Petraitiene R, Walsh TJ. Macrophage colony-stimulating factor enhances phagocytosis and oxidative burst of mononuclear phagocytes against *Penicillium marneffei* conidia. FEMS Immunol Med Microbiol 2003; 36: 19-26.
11. Rongrungruang Y, Levitz SM. Interactions of *Penicillium marneffei* with human leukocytes in vitro. Infect Immun 1999; 67: 4732-6.
12. Trewatcharegon S, Sirisinha S, Romsai A, Eampokalap B, Teanpaisan R, Chaiyaroj SC. Molecular typing of *Penicillium marneffei* isolates from Thailand by *NotI* macrorestriction and pulsed-field gel electrophoresis. J Clin Microbiol 2001; 39: 4544-8.
13. Tongchusak S, Pongtanalert P, Pongsunk S, Chawengkirttikul R, Chaiyaroj SC. Distinct immunologic properties of *Penicillium marneffei* yeasts obtained from different *in vitro* growth conditions. Asian Pac J Allergy Immunol 2004; 22: 229-35.
14. Kudeken N, Kawakami K, Kusano N, Saito A. Cell-mediated immunity in host resistance against infection caused by *Penicillium marneffei*. J Med Vet Mycol 1996; 34: 371-8.
15. Kudeken N, Kawakami K, Saito A. Role of superoxide anion in the fungicidal activity of murine peritoneal exudate macrophages against *Penicillium marneffei*. Microbiol Immunol 1999; 43: 323-30.
16. Sisto F, Miluzio A, Leopardi O, Mirra M, Boelaert JR, Taramelli D. Differential cytokine pattern in the spleens and livers of BALB/c mice infected with *Penicillium marneffei*: protective role of gamma interferon. Infect Immun 2003; 71: 465-73.
17. Epling-Burnette PK, Wei S, Blanchard DK, Spranzi E, Djeu JY. Coinduction of granulocyte-macrophage colony-stimulating factor release and lymphokine-activated killer cell susceptibility in monocytes by interleukin-2 via interleukin-2 receptor β. Blood 1993; 81: 3130-7.
18. Jones TC. Use of granulocyte-macrophage colony stimulating factor (GM-CSF) in prevention and treatment of fungal infections. Eur J Cancer 1999; 35: S8-10.
19. Palucka K, Banchereau J. Dendritic cells: a link between innate and adaptive immunity. J Clin Immunol 1999; 19: 12-25.
20. Banchereau J, Briere F, Caux C, *et al*. Immunobiology of dendritic cells. Annu Rev Immunol 2000; 18: 767-811.
21. Wang JE, Warris A, Ellingsen EA*, et al*. Involvement of CD14 and toll-like receptors in activation of human monocytes by *Aspergillus fumigatus* hyphae. Infect Immun 2001; 69: 2402-6.
22. Mambula SS, Sau K, Henneke P, Golenbock DT, Levitz SM. Toll-like receptor (TLR) signaling in response to *Aspergillus fumigatus*. J Biol Chem 2002; 277: 39320-6.
23. Netea MG, Van Der Graaf CAA, Vonk AG, Verschueren I, Van Der Meer JWM, Kullberg BJ. The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. J Infect Dis 2002; 185: 1483-9.
24. Szabo SJ, Sullivan BM, Peng SL, Glimcher LH. Molecular mechanisms regulating Th1 immune responses. Annu Rev Immunol 2003; 21: 713-58.
25. Serrano-Gomez D, Dominguez-Soto A, Ancochea J, Jimenez-Heffernan JA, Leal JA, Corbi 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.
26. Lighvani AA, Frucht DM, Jankovic D, *et al*. T-bet is rapidly induced by interferon-γ in lymphoid and myeloid cells. PNAS 2001; 98: 15137-42.
27. Geijtenbeek TBH, Torensma R, van Vliet SJ, *et al*. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 2000; 100: 575-85.
28. Bernard M, Latge JP. *Aspergillus fumigatus* cell wall: composition and biosynthesis. Med Mycol 2001; 39: 9-17.
29. Cambi A, Gijzen K, de Vries JM, *et al*. The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for *Can-*

dida albicans on dendritic cells. Eur J Immunol 2003; 33: 532-8.
30. Cao L, Chan CM, Lee C, Wong SS, Yuen KY. MP1 encodes an abundant and highly antigenic cell wall mannoprotein in the pathogenic fungus *Penicillium marneffei*. Infect Immun 1998; 66: 966-73.
31. Buentke E, Scheynius A. Dendritic cells and fungi. APMIS 2003; 111: 789-96.
32. Romani L. Immunity to fungal infections. Nat Rev Immunol 2004; 4: 1-23.
33. Syme RM, Spurrell JC, Amankwah EK, Green FH, Mody CH. Primary dendritic cells phagocytose *Cryptococcus neoformans* via mannose receptors and Fc gamma receptor II for presentation to T lymphocytes. Infect Immun 2002; 70: 5972-81.