

Uptake of the yeast *Malassezia furfur* and its allergenic components by human immature CD1a⁺ dendritic cells

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Summary

Background Atopic dermatitis (AD) is a chronic inflammatory skin disease with increasing prevalence, though still little is known of the pathomechanisms and the causes of the disease. Patients with AD often have specific IgE reactivity to the yeast *Malassezia furfur* (*M. furfur*), present in the normal microflora on human skin.

Objective To investigate the possible interaction of immature and mature antigen-presenting dendritic cells with the yeast *M. furfur* and its allergenic components.

Methods Monocyte-derived dendritic cells (MDDCs) generated from human peripheral blood were allowed to interact with FITC-labelled whole *M. furfur* yeast cells, *M. furfur* extract, a recombinant allergen from *M. furfur* designated rMal f 5 and *M. furfur* mannan, in the absence of IgE antibodies. Interaction and uptake were detected using flow cytometry and confocal laser scanning microscopy.

Results Internalization of *M. furfur* yeast cells and yeast components by immature MDDCs was found using confocal laser scanning microscopy. Results from flow cytometric studies showed that a median of 94% (range, 65–98%) of the immature CD1a⁺ MDDCs were *M. furfur* extract positive, 81% (75–97%) rMal f 5 positive and 93% (62–98%) mannan positive. Mature CD1a⁺ MDDCs were significantly less efficient in this respect, with the corresponding figures only 26% (6–37%, $P < 0.01$), 6% (2–15%, $P < 0.05$) and 32% (9–50%, $P < 0.01$), respectively. Uptake of the non-glycosylated rMal f 5 by immature CD1a⁺ MDDCs was decreased to 27% (15–38%) by inhibition of pinocytosis. The binding of *M. furfur* extract and mannan was inhibited in a dose-dependent manner by methyl- α -D-mannopyranoside, suggesting uptake via the mannose receptor.

Conclusion Human immature CD1a⁺ MDDCs can efficiently take up *M. furfur* and allergenic components from the yeast in the absence of IgE antibodies, implying that sensitization of AD patients to *M. furfur* can be mediated by immature dendritic cells in the skin.

Keywords: atopic dermatitis, confocal microscopy, dendritic cells, flow cytometry, *Malassezia furfur*, mannose receptor

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Introduction

Dendritic cells (DCs) are recognized as the most potent antigen-presenting cells of the immune system, and are able

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to activate naive T-cells [1]. The Langerhans cells (LC) are a subset of DCs, constitutively expressing CD1a and HLA-DR molecules, that constitute approximately 2% of the epidermal cell population [2]. LCs in the epidermis are so-called immature DCs that are highly efficient at antigen uptake, but less efficient at antigen presentation [1].

Activated LCs migrate to T-cell areas of regional lymph nodes via the lymph vessels [3]. During migration, they differentiate into professional antigen-presenting cells [1]. These so-called mature DCs express CD83 and high levels of co-stimulatory molecules, are poor at antigen uptake but excellent at presenting antigen. DCs with an LC-like phenotype can be generated *in vitro* by culturing the adherent CD14⁺ cell fraction of peripheral blood mononuclear cells [4,5] to gain monocyte-derived dendritic cells (MDDCs).

Atopic dermatitis (AD) is a chronic inflammatory skin disease with increasing prevalence [6]. AD symptoms have been recorded in as many as 10–20% of the children in different regions of the world, according to the recent ISAAC study [7]. Despite this fact, little is known of the pathomechanisms and the causes of the disease. A combination of genetic predisposition, abnormal T-lymphocyte function in the skin and a dysfunction of the skin barrier are thought to be involved [8], as are lifestyle and environmental factors [9,10].

Malassezia furfur (*M. furfur*), also called *Pityrosporum orbiculare/ovale*, is a lipophilic yeast belonging to the normal microflora of human skin, but it can act as an allergen in AD patients [11]. In different studies it has been found that between 28 and 84% of AD patients have a positive skin prick test to *M. furfur* [12–14]. Treatment with ketoconazole is effective in some of the AD patients, where it leads to lower serum IgE levels and improvement of the eczema [15]. It has been shown that the T-cell response to *M. furfur* extract *in vitro* is significantly higher in AD patients compared with healthy controls [16], and that *M. furfur* reactive T-cell clones from lesional skin have a TH₂ or TH₂/TH₀ profile [17]. No evidence for a superantigenic activity in *M. furfur* extract has been found [18], indicating a classical major histocompatibility complex class II restricted allergen presentation.

Several IgE-binding proteins from *M. furfur* have been identified [19–22]. cDNAs encoding *M. furfur* allergens have been cloned and designated Mal f 1 to Mal f 9 [23–25], and some of these allergens have recently been expressed as recombinant proteins, e.g. recombinant Mal f 5 (rMal f 5) [25]. Around 48% of sera from AD patients, RAST positive to *M. furfur* extract, were shown to have IgE antibodies to the 18.2 kDa rMal f 5 allergen when tested in an immunoblot assay [25].

Mannan, a cell wall constituent of many yeast species including *M. furfur*, is a highly immunogenic mannose polymer that can bind IgE [26]. In a group of 40 AD patients with positive *M. furfur* RAST, 77% had IgE antibodies against *M. furfur* mannan [22]. Mannan is recognized by the mannose receptor (MR), first found on macrophages, which mediates both endocytosis and phagocytosis [27,28]. The receptor has a broad specificity

and is involved in host defence [29]. It has recently been shown that a mannose–fucose binding receptor is expressed on freshly isolated human epidermal LCs [30], and on DCs generated both from peripheral blood [31,32] and from cord blood [33]. Uptake of mannosylated antigens has been shown to result in a more efficient presentation to T-cells than presentation of antigens taken up via fluid phase [34].

Given that LCs are immature DCs in the epidermis, where they take up antigens entering the body, and that the entry of the yeast *M. furfur*, or its products, probably occurs through the disrupted skin barrier in AD patients, it is hypothesized that the DCs and the yeast come in contact with each other *in vivo*, and that the DCs hereby play a role in the sensitization to the yeast. The aim was therefore to study the possible interaction of immature and mature MDDCs with whole *M. furfur* yeast cells, *M. furfur* extract, rMal f 5 and *M. furfur* mannan by flow cytometry and confocal laser scanning microscopy (CLSM).

Materials and methods

Isolation and culturing of adherent mononuclear cells from peripheral blood

MDDCs were prepared according to Romani *et al.* [4]. Briefly, peripheral blood mononuclear cells from buffy coats from healthy donors were obtained by separation on Ficoll Paque (Pharmacia Biotech, Uppsala, Sweden). After washing in phosphate-buffered saline (PBS, pH 7.4, Ca²⁺- and Mg²⁺-free), the cells were diluted to 4×10^6 cells/mL in RPMI 1640 supplemented with 25 µg/mL gentamicin (Gibco BRL, Life Technologies Ltd, Paisley, UK), and incubated in a culture flask at 37 °C for 1 h. The non-adhered cells were washed away and complete culture medium, RPMIc [RPMI 1640 medium supplemented with 25 µg/mL gentamicin, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin (Gibco BRL, Life Technologies Ltd), 50 µM 2-β-mercaptoethanol (Sigma Chemical Company, St Louis, MO, USA), and 10% (v/v) heat-inactivated fetal calf serum (FCS, Hyclone, Logan, UT, USA)], was added. The RPMIc medium was supplemented with 550 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, Schering Plough, Kenilworth, NJ, USA) and 800 U/mL human recombinant interleukin 4 (rIL-4, Genzyme, Cambridge, MA, USA). The cells were cultured in a humidified incubator with 6% CO₂ in air for 6 days, to become immature MDDCs. The cells were re-fed on day three. To mature MDDCs, RPMIc supplemented with cytokines (550 U/mL GM-CSF and 800 U/mL rIL-4) and 33% monocyte-conditioned medium [35,36] were added at day 6 of culture. After another 4 days in culture, the cells had become mature MDDC as judged

Table 1. Phenotype of the generated immature and mature MDDCs

Phenotypic marker	Immature MDDCs* (<i>n</i> = 17–20)‡	Mature MDDCs† (<i>n</i> = 5–7)‡
CD1a	90 (77–98)§	81 (75–90)
CD14	0.9 (0.0–9.0)	1.4 (0.3–3.6)
CD40	100 (94–100)	100 (99–100)
CD54	19 (9–46)	37 (8.8–59)
CD80	70 (52–95)	100 (100–100)
CD83	1.6 (0.0–7.0)	83 (64–93)
CD86	63 (33–89)	100 (100–100)
HLA-DR	100 (99–100)	100 (100–100)
MR¶	92 (71–98)	7.0 (1.7–14)

*MDDCs (monocyte-derived dendritic cells) at day 6 of culture.

†MDDCs at day 10 of culture. ‡Number of experiments using cells from different buffy coats. §Positively-stained MDDCs as analysed by flow cytometry. Results are given as median percentage (range). At least 10^4 cells per sample were analysed.

¶MR = the mannose receptor.

by phenotype (Table 1) and morphology. This work was approved by the local ethics committee.

Phenotyping of MDDCs

The phenotype of the cells was determined using either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated mouse monoclonal antibodies (MoAbs). The following MoAbs were used: anti-CD1a PE (T6-RD1, Coulter Hialeah, FL, USA), anti-CD14 FITC (Leu-M3, Becton Dickinson, San Jose, CA, USA), anti-HLA-DR FITC (L243, Becton Dickinson), anti-CD40 FITC (LOB7/6, Serotec Ltd, Oxford, UK), anti-CD54 PE (B-C14, Diaclone Research, Besançon, France), anti-CD80 FITC (MAB104, Immunotech, Marseille, France), anti-CD83 PE (HB15a, Immunotech), anti-CD86 FITC (2331 FUN-1, PharMingen, San Diego, CA, USA), non-conjugated anti-MR antibody number 17 [30] (kindly provided by Dr Schmitt, Hôpital Edouard Herriot, Lyon, France) and non-conjugated anti-IgE MoAb (B3102E8, Southern Biotechnology Associated Inc., Birmingham, AL, USA). For detection of the anti-MR MoAb and the anti-IgE MoAb, a secondary FITC-conjugated rabbit anti-mouse antibody (Dakopatts, Copenhagen, Denmark) was used. Isotype-matched antibodies were used as negative controls. The cells were analysed in a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson). A proper gate was set according to cell size. The gated population constituted 50% (median, range 36–69%, *n* = 18) of the cells in the culture. A minimum of

10^4 cells per sample was analysed. Dead cells were excluded with propidium iodide. Only samples where at least 75% of the MDDCs expressed CD1a were used for experiments, and at least 70% of the immature MDDCs had to express the mannose receptor.

Culturing of *M. furfur* and preparing *M. furfur* extract, rMal f 5 and *M. furfur* mannan

M. furfur strain no. 42132, obtained from the American Type Culture Collection, was cultured on glycerol mono-sterate olive oil (Go) solid phase medium [19], at 37 °C, for 4 days. The yeast extract was prepared according to Zargari *et al.* [20]. rMal f 5 was produced in an *Escherichia coli* expression system and therefore not glycosylated [25]. The *M. furfur* mannan was prepared from *M. furfur* strain CBS 7854, typed and deposited in the Centraalbureau voor Schimmelcultures (Delft, The Netherlands). The yeast was cultured according to Leeming and Notman [37] and the mannan was purified by the Cetavlon method [38].

Separation and culturing of human keratinocytes

Human keratinocytes were isolated from human skin removed during plastic surgery and cultured according to Reinwald and Green [39]. Briefly, primary keratinocytes were prepared with dispase and trypsin (Gibco BRL, Life Technologies Ltd), and co-cultured with irradiated mouse fibroblast 3T3 cells.

FITC-labelling of *M. furfur*, *M. furfur* extract, rMal f 5 and *M. furfur* mannan

M. furfur yeast cells were harvested and suspended in carbonate buffer (pH 9.5) to 10^8 yeast cells/mL; 100 µg FITC (Sigma), dissolved in DMSO, were added per mL buffer. After 2 h incubation in the dark at 37 °C, the yeast cells were washed in 0.9% NaCl. *M. furfur* extract (4 mg/mL), rMal f 5 (3 mg/mL) and mannan (1 mg/mL) were dissolved in carbonate buffer. Then 50 µg FITC/mg yeast extract and rMal f 5, and 100 µg FITC/mg mannan, were added and the solutions were incubated for 2 h at room temperature [40]. The unbound FITC molecules were removed on a PD10-column (Pharmacia Biotech) by eluting the FITC-labelled protein or mannan with PBS. The ratio of fluorescence to protein was measured at 495 nm and 280 nm in a Hewlett Packard spectrophotometer, to check the labelling result. The ratio for *M. furfur* extract and rMal f 5 was between 0.3 and 1.0 [41]. The labelling result of whole yeast cells was analysed by flow cytometry, which showed that 100% of the yeast cells were successfully FITC-labelled in all experiments. The yeast

cells were freshly labelled before each experiment. The FITC-labelled yeast components were kept at -20°C .

Detection of CD1a⁺ MDDCs positive for FITC-labelled M. furfur extract, rMal f 5 and M. furfur mannan using flow cytometry

Immature or mature MDDCs (2.5×10^5 cells/sample) were pre-incubated for 30 min at 37°C . All incubation steps and washing steps were in the presence of 10 mM NaF (sodium fluoride, Merck KGaA, Darmstadt, Germany), an inhibitor of endocytosis [30,32]. The MDDCs were allowed to interact with FITC-labelled *M. furfur* extract (10 $\mu\text{g/mL}$), rMal f 5 (10 $\mu\text{g/mL}$) or mannan (35 $\mu\text{g/mL}$) in a total volume of 1 mL RPMI 1640 for 30 min in the dark, at 37°C . As a control, MDDCs were also incubated with the yeast components in the absence of NaF. Human keratinocytes (2.5×10^5 cells/sample) were used for comparison. As a control for unbound FITC not separated by the column, FITC dissolved in DMSO and carbonate buffer was run through a PD10 column and the eluted fluid was added to the MDDCs. The MDDCs were washed in PBS and stained with PE-conjugated anti-CD1a MoAbs for 30 min, on ice, before analysis with flow cytometry. An isotype-matched MoAb was used as control. The viability of the MDDCs was found to be $>95\%$ routinely, using propidium iodide. To verify the capacity to bind soluble antigen to the MR, the cells were incubated with the 40 kDa soluble antigen dextran, labelled with FITC (Sigma) at 10 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ [31], for 30 min. In parallel, MDDCs were also incubated with FITC-labelled *M. furfur* extract, rMal f 5 or mannan for 30 min, in the dark, at 4°C . The interaction of FITC-labelled *M. furfur* extract, rMal f 5 and mannan with immature and mature CD1a⁺ MDDCs, and with keratinocytes, was evaluated by flow cytometry and expressed as median percentage and range of positive cells in the FL1 channel. A gate was set according to cell size, and a minimum of 10^4 cells per sample was analysed.

Uptake of FITC-labelled M. furfur, M. furfur extract, rMal f 5 and M. furfur mannan analysed by CLSM

Immature and mature MDDCs (2.5×10^5 cells per sample) were incubated for 14 h at 37°C with whole FITC-labelled *M. furfur* yeast cells, at a ratio of 1:1, in a total volume of 1 mL RPMIc. The cells were washed in PBS and labelled for CD1a expression. Trypan blue was added to parallel samples in order to quench the fluorescence from adherent, non-internalized yeast. To study uptake of yeast components, immature and mature MDDCs (2.5×10^5 cells per sample) were pre-incubated for 30 min in the dark, at 4°C , with the same concentrations of FITC-labelled *M. furfur* extract, rMal f 5 or mannan as described for the binding

experiments, in a total volume of 1 mL RPMI 1640, and then incubated for 1 h in the dark at 37°C . The cells were washed in PBS and labelled with anti-CD1a PE MoAb. As a control, MDDCs were incubated in the dark at 4°C during the entire incubation time (90 min). The uptake of yeast, yeast extract, rMal f 5 or mannan by immature and mature MDDCs was evaluated using a SARAstro 2000TM CLSM beam scanning system (Molecular Dynamics, Sunnyvale, CA, USA) equipped with a Nikon Optiphot II upright microscope and an argon-ion laser (maximum laser power 25 mW). Light with a wavelength of 488 nm was used for excitation, together with appropriate filter settings. Oil was used as immersion medium, and paraphenylenediamine in glycerol as mounting medium to reduce fading [42]. ImageSpace software (Molecular Dynamics) was used to acquire and visualize the data. Crosstalk compensation of the double-stained images was done using the ImageSpace software.

Inhibition experiments

To determine the contribution of pinocytosis, inhibition experiments were performed by incubating immature MDDCs with a mix of pinocytosis inhibitors consisting of amiloride hydrochloride hydrate (3 mM, Sigma), 5-(N, N-dimethyl) amiloride hydrochloride (10 $\mu\text{g/mL}$, Sigma), and cytochalasin D from *Zygosporium mansonii* (10 $\mu\text{g/mL}$, Sigma) for 15 min at 37°C [31,43], before addition of yeast cell components. Immature MDDCs were also pre-incubated with 2 mg/mL of unlabelled *M. furfur* extract or with methyl- α -D-mannopyranoside (Sigma), at a concentration of 5, 10, 20 or 40 mg/mL, for 30 min at 37°C in the presence of 10 mM NaF, before adding the FITC-labelled yeast extract, rMal f 5 or mannan. Higher concentrations of methyl- α -D-mannopyranoside than the ones used led to lower cell viability (data not shown). The cells were washed in PBS containing 10 mM NaF and labelled for CD1a expression. A minimum of 10^4 cells per sample was analysed by flow cytometry.

Statistics

The Mann–Whitney *U*-test was used to compare the interaction of immature and mature MDDCs with the three different yeast components. Friedman's test was used to compare the effect of increasing concentration of MR inhibitor on the interaction of immature MDDCs with the yeast components. $P < 0.05$ was considered statistically significant.

Table 2. Immature and mature CD1a⁺ MDDCs positive for FITC-labelled *M. furfur* extract, rMal f 5 or *M. furfur* mannan

Added substance	Immature CD1a ⁺ MDDCs* (n = 9–16)‡	Mature CD1a ⁺ MDDCs† (n = 3–4)‡
FITC-yeast extract	94 (65–98)§, ¶	26 (5.8–37)
FITC-rMal f5	81 (75–97)**	6.0 (2.4–15)
FITC-mannan	93 (62–98)¶	32 (9.2–50)
FITC in PBS††	1.4 (0.4–2.2)	0.5 (0.0–1.8)

*MDDCs (monocyte-derived dendritic cells) at day 6 of culture. †MDDCs at day 10 of culture. ‡Number of experiments using cells from different buffy coats. §Positively-stained MDDCs as analysed by flow cytometry. Results are given as median percentage (range). At least 10⁴ cells per sample were analysed. ¶Significantly higher than the corresponding value for mature MDDCs ($P < 0.01$). **Significantly higher than the corresponding value for mature MDDCs ($P < 0.05$). ††FITC in PBS (run through a PD-10 column) was used as a control, to rule out the possibility that unbound FITC in the samples gave the positive results.

Results

Phenotype of MDDCs

The phenotype of immature and mature MDDCs, cultured for 6 or 10 days, respectively, was determined by flow cytometric analysis (Table 1). The cells differed morphologically in that the mature MDDCs had a lot of short distinct dendrites, as evaluated by light microscopy (data not shown). Both the immature and the mature MDDCs had very low levels of IgE bound to their surfaces (median 1.3%, range 0.8–4.5%, $n = 7$).

Detection of CD1a⁺ MDDCs positive for FITC-labelled *M. furfur* extract, rMal f 5 and *M. furfur* mannan using flow cytometry

The results show that 94% (median) of the immature CD1a⁺ MDDCs were yeast extract-positive (Table 2 and Fig. 1b), 81% were rMal f 5-positive (Table 2 and Fig. 1c) and 93% mannan-positive (Table 2 and Fig. 1d). After maturation, significantly less cells were positive to the yeast components ($P < 0.01$ for extract and mannan, and $P < 0.05$ for rMal f 5). Only 26% of the mature CD1a⁺ MDDCs were extract-positive, 6% rMal f 5-positive and 32% mannan-positive (Table 2). The negative control (FITC in PBS run through a PD10 column) stained the immature and mature MDDCs at background levels (Table 2 and Fig. 1e), whereas incubation with FITC-labelled dextran resulted in a high percentage of positive immature CD1a⁺ MDDCs, using both 10 µg/mL (median 80%, range 52–97%, $n = 3$) and 100 µg/mL (data not shown) of the dextran. When omitting NaF, the percentage of positive cells within a cell sample was approximately the same (data not shown). Incubation of the immature

MDDCs at 4 °C resulted in a 3–9-fold reduction of *M. furfur* extract- and mannan-positive cells, and rMal f 5-positive cells were at background levels (data not shown). Only 5% of the keratinocytes were *M. furfur* extract-positive (0–8%, $n = 4$) and 8% mannan-positive (3–8%, $n = 3$). Less than 0.5% of the keratinocytes were rMal f 5-positive ($n = 2$), which is comparable to the background levels. The MR could not be detected on the keratinocytes (data not shown).

Uptake of FITC-labelled *M. furfur*, *M. furfur* extract, rMal f 5 and *M. furfur* mannan

Uptake of whole yeast cells and yeast components was studied using CLSM. By scanning section series (z step 1 µm) of the immature CD1a⁺ MDDCs, and making 3-D reconstructions, it was clearly shown that the yeast cells had been internalized (Fig. 2A). By adding trypan blue to the samples, and thereby quenching fluorescence from extracellular yeast, it was estimated that the majority of the immature MDDCs had internalized *M. furfur* after 14 h incubation at 37 °C. It was also found that the majority of the immature CD1a⁺ MDDCs internalized FITC-labelled *M. furfur* extract, rMal f 5 and mannan after 30 min incubation at 4 °C, followed by 1 h incubation at 37 °C (Fig. 2B–D). The dextran, added as positive control, was also internalized (data not shown).

Mature MDDCs, on the other hand, showed hardly any uptake of the yeast extract (Fig. 2E), rMal f 5 and mannan (data not shown, $n = 3$). Immature and mature MDDCs incubated with either FITC-labelled yeast components at 4 °C for 90 min, or with the negative control (FITC in PBS run through a PD10 column), displayed no detectable uptake as judged by CLSM analysis (data not shown).

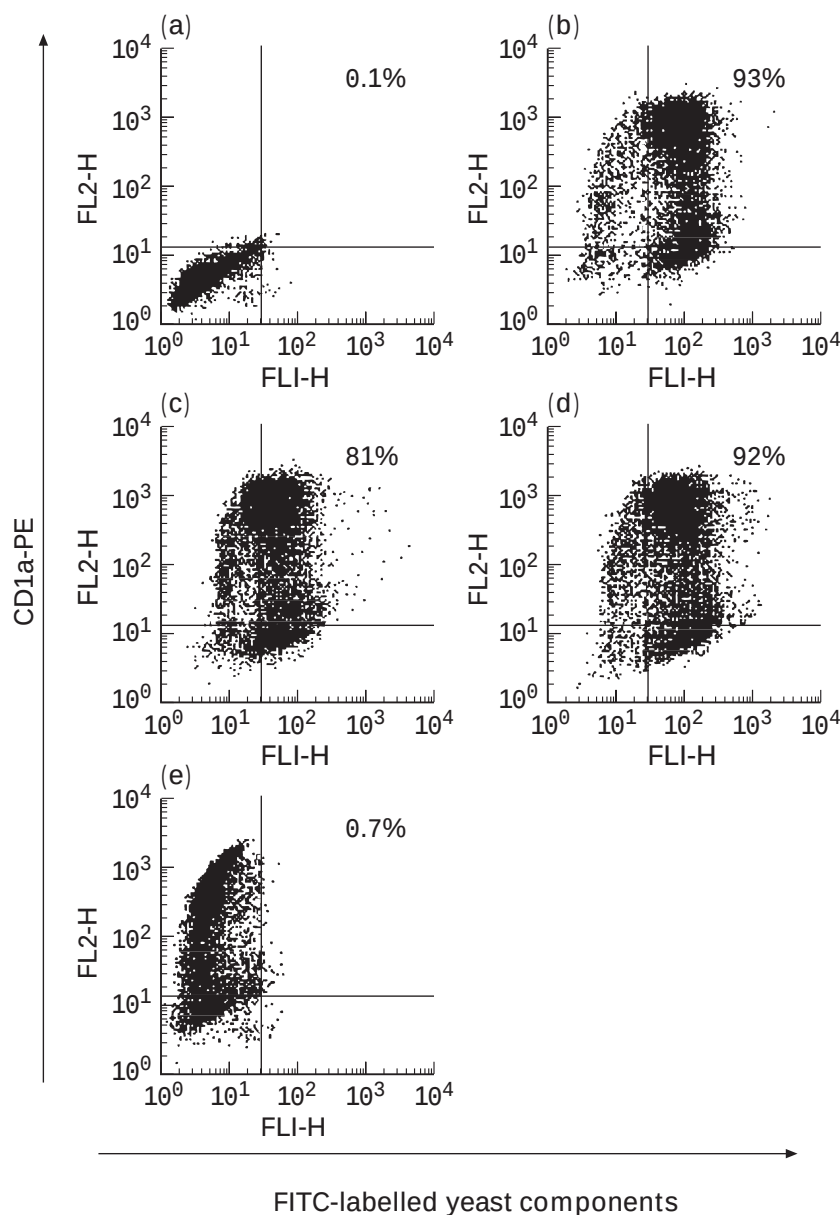


Fig. 1. Immature CD1a⁺ MDDCs positive for FITC-labelled *M. furfur* extract, rMal f 5 or *M. furfur* mannan. Immature CD1a⁺ MDDCs, cultured for 6 days, stained with an isotype-matched control antibody (a), MDDCs positive for FITC-labelled *M. furfur* extract (b), rMal f 5 (c) or mannan (d). Negative control (FITC in PBS run through a PD10 column) (e). The cells were stained for CD1a with a phycoerythrin (PE)-conjugated MoAb, and analysed by flow cytometry. At least 10^4 cells per sample were analysed. The percentage of double-positive cells (a) or percentage of double-positive cells out of the total CD1a⁺ cells (b–e) is written in the upper right corner. Results from one experiment representative of 9–16 experiments, using cells from different buffy coats, are shown.

Inhibition studies

To investigate the mechanisms mediating the interaction between the yeast *M. furfur* and the MDDCs, several inhibition experiments were performed. The binding and uptake of FITC-labelled *M. furfur* extract and mannan to immature CD1a⁺ MDDCs could be inhibited from 92% (median, range 90–97%, $n = 3$) positive cells down to 6% (3–25%), and from 91% (88–96%) to 6% (3–43%), respectively, by adding 2 mg/mL unlabelled *M. furfur* extract to the cells prior to incubation with the yeast components (Fig. 3). Inhibition of pinocytosis had no effect on the percentage of immature MDDCs binding and

taking up the yeast extract, i.e. 88% (median, range 78–98%, $n = 2$) positive cells before inhibition, 88% (88–89%) after inhibition, and a slightly reducing effect on the mannan from 95% (91–98%) to 72% (67–77%), whereas there was a great reduction in the percentage of cells taking up rMal f 5 from 84% (72–97%) to 27% (15–38%) (Fig. 4).

When the MR was blocked by adding different concentrations of methyl- α -D-mannopyranoside, the percentage of immature CD1a⁺ MDDCs positive to FITC-labelled *M. furfur* extract and mannan was inhibited in a dose-dependent manner (Fig. 5a,b, $P < 0.05$). Blocking of the MR also affected the percentage of rMal f 5-positive

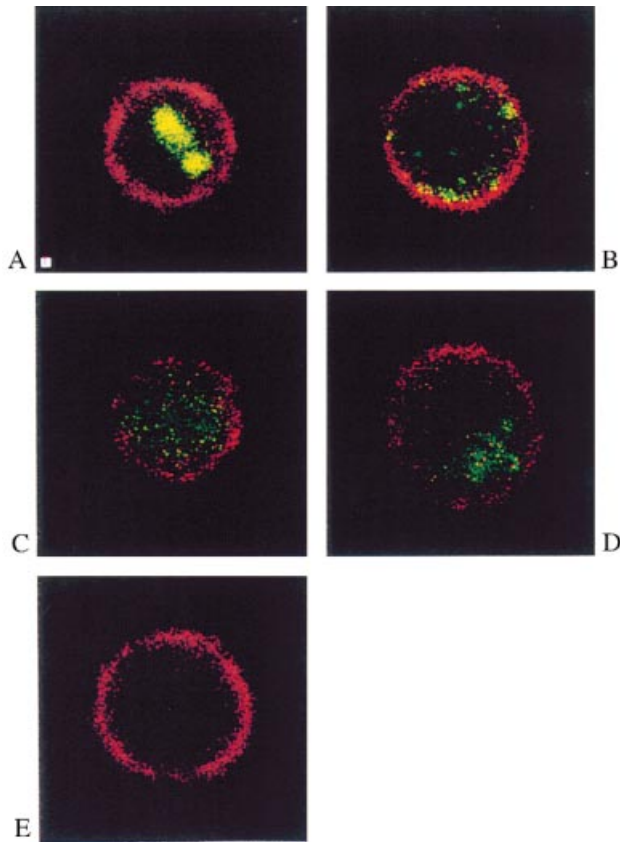


Fig. 2. Uptake of FITC-labelled *M. furfur* yeast cells, yeast extract, rMal f 5 or *M. furfur* mannan by immature CD1a⁺ MDDCs. Internalization of yeast cells by immature CD1a⁺ MDDCs was detected, after 14 h of incubation at 37 °C, by confocal laser scanning microscopy (A). Immature CD1a⁺ MDDCs internalized *M. furfur* extract (B), rMal f 5 (C) and mannan (D) after 30 min incubation at 4 °C followed by 1 h at 37 °C. Internalization of *M. furfur* extract by mature CD1a⁺ MDDCs could not be detected (E). The images show one optical section approximately through the middle of the MDDCs. Red is phycoerythrin-conjugated MoAbs to CD1a, and green is FITC-labelled yeast cells, yeast extract, rMal f 5 or mannan. Compensation for cross-talk between the detection channels has been done using the ImageSpace software. The white box in the lower left corner (A) is 1 × 1 μm, valid also for the images (B–E). The images are representative of three experiments.

MDDCs to some degree, but there was no statistically significant inhibition (Fig. 5c). MR expression could not be detected with the anti-MR MoAb after pre-incubation with methyl- α -D-mannopyranoside (data not shown).

Discussion

In this study, it was shown for the first time that human immature CD1a⁺ MDDCs can interact with, and take up,

whole *M. furfur* yeast cells, as well as different IgE binding *M. furfur* components. Mature MDDCs were found to be significantly less efficient in this respect, as were human keratinocytes. Little is known about the mechanisms underlying AD, but a number of studies indicate a role of the yeast *Malassezia* [11–17,22,44]. The *in vivo* relevance of the yeast has recently been investigated by Tengvall Linder *et al.* [44], with the observation that 53% of the AD patients in the study were patch test positive to *M. furfur* extract, and an infiltration of CD4⁺ T-cells and eosinophils, as well as an upregulation of ICAM-1 and HLA-DR, was detected at the patch test sites. In the *in vivo* situation, it seems likely that LCs come into contact with secreted components and breakdown products from the yeast. *M. furfur* contains a number of allergenic components, some of which are expressed on the cell surface [45] and can be released into the culture media (own unpublished data). In the case of severe barrier dysfunction, it could be assumed that LCs would also encounter whole yeast cells. It was decided to study the interaction of MDDCs with different IgE-binding *M. furfur* components, as well as the interaction with whole yeast, since the skin barrier of AD patients is often disrupted.

In agreement with previous reports, the immature DCs derived from monocytes expressed CD1a, HLA-DR and the MR, moderate levels of CD40, CD80 and CD86, and were low in CD14 expression, while the mature MDDCs had a high expression of CD40, CD80, CD83 and CD86, and a low expression of the MR [1,5,46]. DCs generated from CD34⁺ progenitors in human cord blood according to Caux *et al.* [47], which more closely resemble human LCs, were used in pilot studies and were found to react in a similar way as the MDDCs to the yeast *M. furfur* and its allergenic components. For practical reasons, it was decided to use DCs generated from peripheral blood monocytes. It was found that immature MDDCs could interact with and internalize both whole *M. furfur* yeast cells and yeast components. The mature MDDCs were not as efficient at interacting or taking up the yeast and its components. The somewhat different results on uptake by mature MDDCs, obtained by flow cytometry and CLSM, might be due to different sensitivities of the two methods, and because many more cells and more experiments were analysed by flow cytometry. The present results are in line with the general idea that immature DCs are good at antigen uptake, an ability lost upon maturation [1]. DCs have been shown to be efficient at uptake of a large variety of antigens, including whole bacteria and protozoan parasites [3,48,49], as well as cellular fragments and apoptotic cells [50,51]. There are, to our knowledge, no reports on uptake of allergenic *M. furfur* components by DCs. Uptake of whole *M. furfur*, however, has been reported, but only in a monocytic cell line [52]. Heat-killed yeast cells were

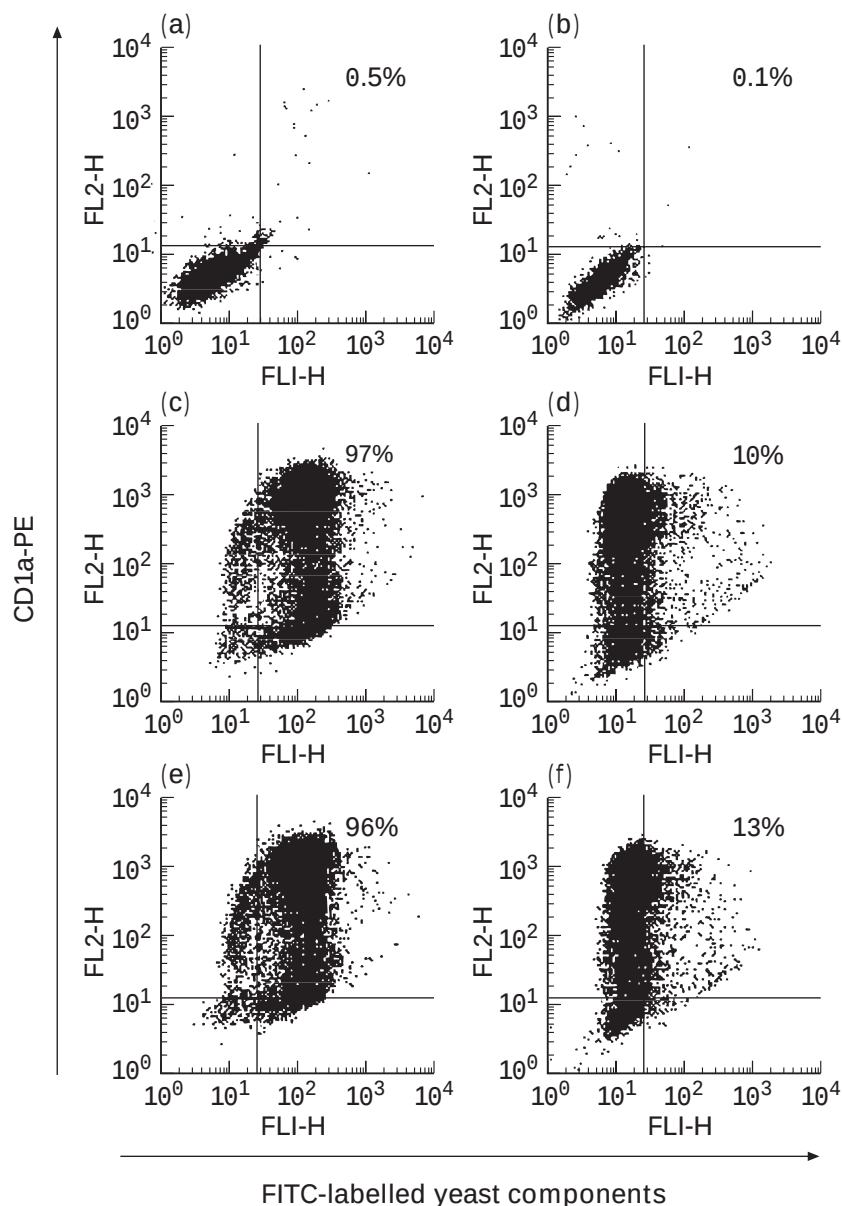


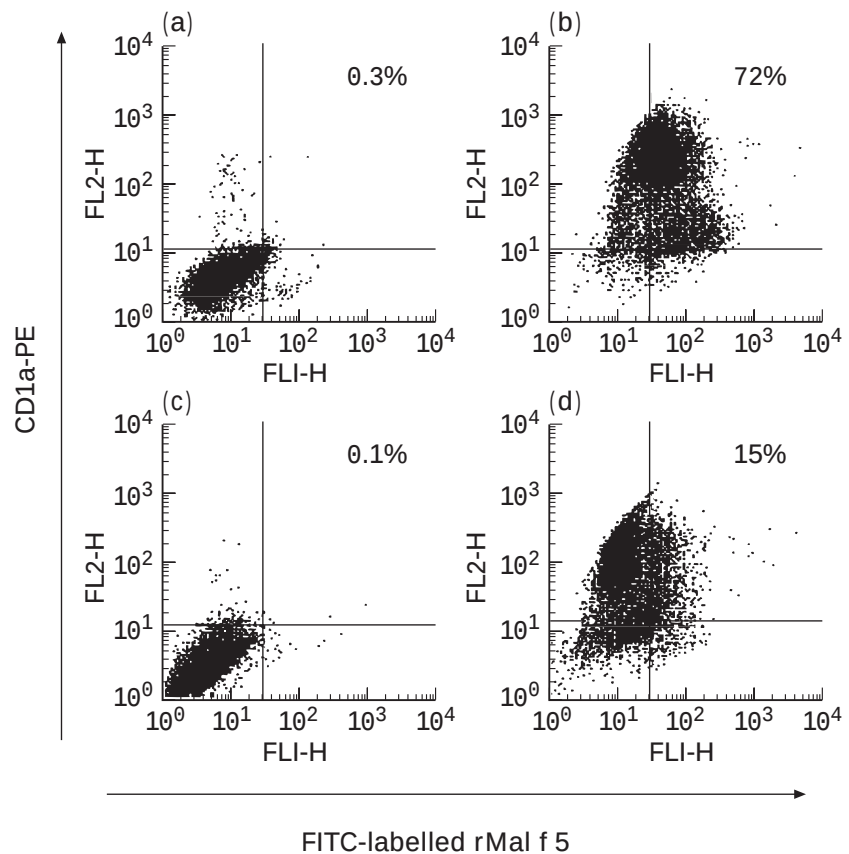
Fig. 3. Inhibition of binding of FITC-labelled *M. furfur* extract and mannan to immature CD1a⁺ MDDCs by unlabelled *M. furfur* extract. CD1a⁺ MDDCs, cultured for 6 days, stained with an isotype-matched control antibody before and after inhibition with unlabelled *M. furfur* extract (a and b). Binding of FITC-labelled *M. furfur* extract before inhibition (c), and after inhibition with unlabelled *M. furfur* extract for 30 min (d), binding of FITC-labelled mannan before inhibition (e) and after inhibition with unlabelled *M. furfur* extract (f) to MDDCs. The cells were stained for CD1a, with a phycoerythrin-conjugated MoAb, and analysed with flow cytometry. At least 10⁴ cells per sample were analysed. The percentage of double-positive cells (a and b) or percentage of double-positive cells out of the total CD1a⁺ cells (c, d, e and f) is written in the upper right corner. The results shown are from one experiment representative of three.

shown to be more readily taken up by the monocytes than live yeast, and opsonization with normal human sera enhanced the uptake. This uptake could be blocked by yeast mannan or β -1,3-glucan, suggesting an involvement of the MR [52]. The yeast cells used in the present study were not opsonized prior to incubation with the immature MDDCs, as no difference between uptake of yeast opsonized by incubation in normal human sera and non-opsonized yeast were found in the pilot studies using DCs generated from cord blood progenitors.

The mechanisms mediating the uptake of yeast cells and yeast components by the MDDCs was investigated. No cell surface-bound IgE could be detected on either immature or

mature MDDCs used in this study, ruling out the possibility of an IgE-mediated antigen uptake. Incubation of the cells at 4 °C resulted in less interaction between the MDDCs and *M. furfur* extract or mannan, as judged by percentage of positive cells, and no rMal f 5-positive CD1a⁺ MDDCs were detected, indicating that the binding and internalization observed after incubation at 37 °C was an active process. DCs have been reported to be very good at antigen uptake via fluid phase, by pinocytosis [1,31]. To determine the contribution of pinocytosis, inhibition experiments were performed using a mix of pinocytosis inhibitors. The proportion of immature CD1a⁺ MDDCs positive to the yeast extract

Fig. 4. Inhibition of uptake of FITC-labelled rMal f 5 by immature CD1a⁺ MDDCs, by inhibiting pinocytosis. Uptake of FITC-labelled rMal f 5 by CD1a⁺ MDDCs cultured for 6 days before inhibition (b), and after inhibition of pinocytosis (d). MDDCs stained with an isotype-matched control antibody before and after inhibition (a and c). The cells were stained for CD1a, with a phycoerythrin-conjugated MoAb, and analysed with flow cytometry. At least 10⁴ cells per sample were analysed. The percentage of double-positive cells (a and c) or percentage of double-positive cells out of the total CD1a⁺ cells (b and d) is written in the upper right corner. The results shown are from one experiment representative of two.



and mannan was approximately the same when the inhibitors were used.

Pre-incubation of the MDDCs with unlabelled *M. furfur* extract inhibited subsequent interaction of FITC-labelled yeast extract and mannan with the MDDCs, pointing to a receptor-mediated uptake. As yeasts are rich in mannosylated proteins and in mannan [26], the MR, recently reported to be expressed by freshly isolated LCs [30] and by immature MDDCs [46], was studied as a possible target receptor for uptake of *M. furfur* and different yeast components. It was found that the interaction of immature MDDCs with *M. furfur* extract and mannan was significantly inhibited by blocking the MR with methyl- α -D-mannopyranoside. The interaction was never completely abrogated, however, which might indicate the presence of additional receptors involved in the uptake of extract and mannan. It is noteworthy that there was a great reduction in the expression of the MR on mature MDDCs, in accordance with Cella *et al.* [46], which might partly explain the poor binding and uptake by these cells, and again, support the idea of the MR being the main route for the uptake of yeast extract and mannan by MDDCs. According to previous studies, there is some controversy as to whether human LCs express the MR or not. Some studies have reported that

neither LCs in the epidermis nor freshly isolated LCs express the MR [53,54], whereas Condaminet *et al.* [30] have reported that the MR could be detected on freshly isolated LCs. Mommaas *et al.* [54] found MR⁺/CD14⁺ and MR⁺/CD68⁺ cells in the dermis, but no MR⁺ cells in the epidermis. They also reported that mannosylation of antigens did not enhance the uptake of the antigens by freshly isolated LCs, indicating again that these cells lack a functional MR. On the other hand, there are reports stating that freshly isolated LCs from mouse and man are very efficient at binding and uptake of mannosylated proteins [30,55]. Immunohistochemical stainings were made on skin biopsy specimens from normal healthy individuals and AD patients with specific serum IgE to *M. furfur*, included in the study by Tengvall Linder *et al.* [44], using the mouse anti-human MR MoAb D547 (kindly provided by Dr A. Lanzavecchia, Basel Institute for Immunology, Basel, Switzerland) used in Mommaas *et al.* [54]. MR⁺ dermal cells were found in normal skin, as previously reported [54]. In lesional skin from three AD patients, and in their positive patch test reactions to *M. furfur* extract at 72 h, there was an increased number of MR⁺ cells, and MR⁺ dendritic cells were also found in the epidermis (own unpublished observations). The DCs used in this study are

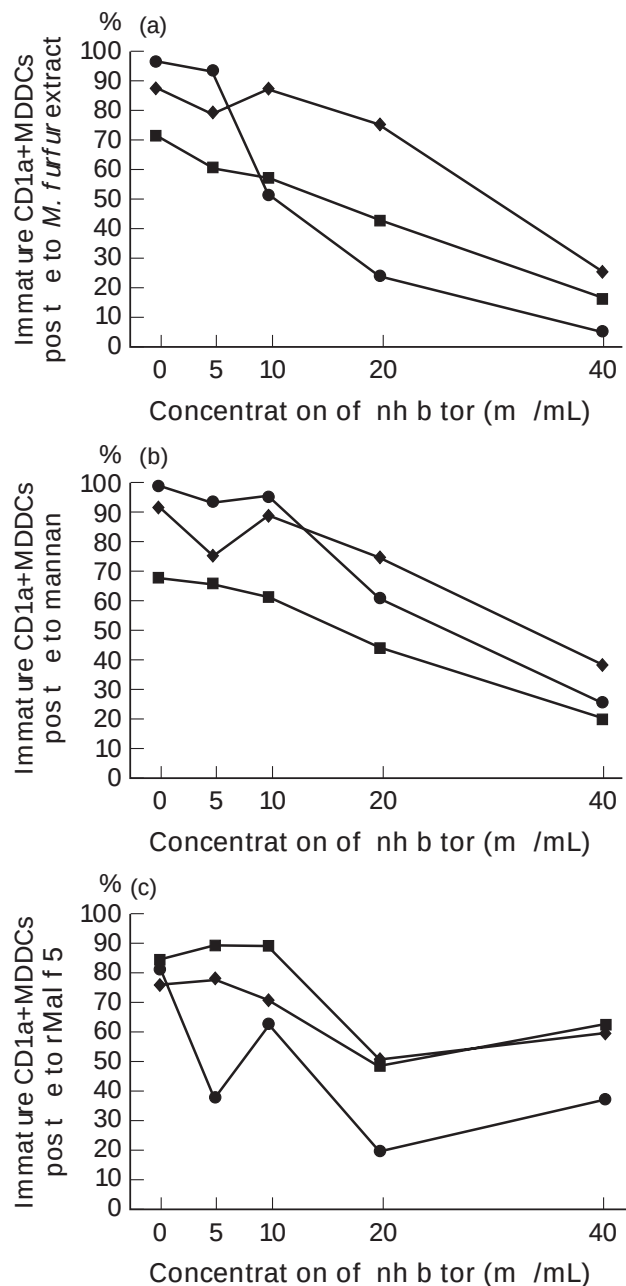


Fig. 5. Immature CD1a⁺ MDDCs positive to *M. furfur* extract, rMal f 5 and *M. furfur* mannann, after pre-incubation with methyl-α-D-mannopyranoside. The percentage of immature CD1a⁺ MDDCs, cultured for 6 days, positive to FITC-labelled *M. furfur* extract (a), mannann (b) or rMal f 5 (c) was significantly reduced in a and b ($P < 0.05$, respectively) by pre-incubation with different concentrations of methyl-α-D-mannopyranoside for 30 min. At least 10^4 cells per sample were analysed by flow cytometry. Results from three different donors are shown in (a) (b), and (c), respectively.

derived from human monocytes, and it has been well established that these MDDCs express the MR [31,46]. The cytokines IL-4 and GM-CSF, used to obtain DCs from monocytes, have been shown to up-regulate and increase MR activity [56,57]. It might be speculated that the cytokine environment in the inflamed eczematous skin of AD patients could contribute to a locally increased number of MR⁺ cells.

The recombinant yeast protein, rMal f 5, expressed in an *E. coli* system [25], was included in the study to determine whether there would be any differences in the uptake pattern of this non-glycosylated yeast product compared with the yeast extract or mannann. No great difference was found in the percentage of cells taking up the different yeast components. In contrast to the *M. furfur* extract and mannann, however, no significant decrease of immature MDDCs positive for rMal f 5 could be seen when methyl-α-D-mannopyranoside was used to inhibit the MR. Inhibition of pinocytosis, on the other hand, greatly reduced the detected uptake of rMal f 5, indicating that uptake of the non-glycosylated allergen rMal f 5 is mainly mediated via non-specific fluid phase pinocytosis.

In conclusion, human immature CD1a⁺ MDDCs can efficiently bind and take up *M. furfur* and different components from the yeast, whereas mature CD1a⁺ MDDCs are poor in that respect. Indications for an involvement of the MR in uptake of yeast extract and mannann were found. These results, showing that *M. furfur* components can be taken up by MDDCs via a non-IgE mediated route, are important in the sensitization context. They imply that the sensitization of AD patients to *M. furfur* can be mediated by immature DCs in the skin. The production of *M. furfur*-specific IgE antibodies might play a role in maintaining the chronic inflammation in the skin in the later stages of the disease.

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