

The allergenic yeast *Malassezia furfur* induces maturation of human dendritic cells

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Summary

Background The yeast *Malassezia furfur* (*M. furfur*), present in the normal microflora of human skin, can act as an allergen that incites specific IgE reactivity and T cell proliferation in atopic dermatitis (AD) patients. The role of antigen presenting dendritic cells (DCs) in the onset and maintenance of AD is not well established.

Objective The objective of the present study was to assess whether the interaction of *M. furfur* with human DCs will result in DC maturation, cytokine production and lymphocyte proliferation.

Methods Monocyte-derived dendritic cells (MDDCs) were generated from human peripheral blood. Immature MDDCs were cultured with or without *M. furfur* or plastic beads, and with or without CD40L stimulation. Interaction of yeast cells by MDDCs was studied by time-lapse photography and cytokines were detected in culture supernatants with ELISA. The ability of MDDCs pre-incubated with *M. furfur* to induce proliferation in autologous lymphocytes was measured by [³H]-thymidine incorporation.

Results Time-lapse photography showed that the majority of immature MDDCs internalized whole *M. furfur* yeast cells within 1 h. The presence of *M. furfur* induced maturation (CD83 expression) of MDDCs, and up-regulation of the costimulatory molecules CD80 and CD86. Production of TNF- α , IL-1 β and IL-18 by MDDCs increased significantly ($P < 0.05$ for TNF- α and IL-1 β , and $P < 0.01$ for IL-18) after the addition of *M. furfur*, while IL-10 and IL-12p70 levels remained unaltered. The CD40L-stimulated IL12p70 production by MDDCs was decreased in the presence of *M. furfur* ($P < 0.05$). Finally, immature MDDCs pre-incubated with *M. furfur* induced a proliferative response in autologous CD14-depleted peripheral blood mononuclear cells, in a dose-dependent manner.

Conclusion The data indicate that immature MDDCs can internalize the opportunistic yeast *M. furfur*. This process was associated with MDDC maturation, production of pro-inflammatory and immunoregulatory cytokines, which might favour induction of a Th2-type immune response, and a capacity to stimulate lymphocyte proliferation. This chain of events most likely contributes to the inflammatory reaction in AD.

Keywords: atopic dermatitis, cytokines, dendritic cells, *Malassezia furfur*, time-lapse photography

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Introduction

A complex interplay between genetic predisposition, immune dysregulation, barrier dysfunction in the skin and

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environmental factors is thought to be of importance in the development of atopic dermatitis (AD) [1–3]. It has been suggested that the skin microflora, in an IgE-dependent manner, might act as sufficient stimulus to maintain an allergic reaction in the skin [4].

Malassezia furfur (*M. furfur*), formerly known as *Pityrosporum orbiculare/ovale*, is a uni-cellular lipophilic yeast that is part of the normal microflora of human skin [5]. *Malassezia* species can, under the influence of predisposing factors, become pathogenic and are implicated to play a role in pityriasis versicolor, *Pityrosporum* folliculitis and seborrhoeic dermatitis [5]. Evidence is accumulating that *M. furfur* also contributes to the inflammatory reaction in AD. Specific IgE reactivity has been reported in 28–84% of AD patients [6–8], and positive atopy patch test reactions to *M. furfur* extract have been demonstrated in this group of AD patients [9].

Dendritic cells (DCs) are known as the most potent antigen-presenting cells (APCs) [10]. In their immature state, DCs take up antigens in the periphery. If the DCs are activated to differentiate into mature professional APCs, they efficiently present antigen-derived peptides on major histocompatibility complex (MHC) molecules to T cells in the regional lymph nodes [10]. Signalling through CD40, on the DCs, and CD40L, on the T cell, will lead to maturation of DCs, as shown by up-regulation of CD83 and the costimulatory molecules CD80 and CD86 [11]. Depending on how and where APCs are activated, they can direct the immune system towards a Th1- or a Th2-type of immune response [12,13].

Several studies have addressed activation of the DCs by micro-organisms. The effects of bacteria, and bacterial products, such as lipopolysaccharide (LPS) and cholera toxin, as well as viruses and mycoplasma, have been studied intensely [14–19]. Few studies have, however, been focused on the effect of yeast on DCs. Previously, we found that human immature monocyte-derived DCs (MDDCs) could take up whole *M. furfur* yeast cells and that uptake of allergenic, IgE-binding components from *M. furfur* occurred via the macrophage mannose receptor (MR) and pinocytosis [20].

In this study, the interaction of *M. furfur* yeast cells with human immature MDDCs was first investigated using time-lapse photography. We then assessed if this interaction with *M. furfur* induced maturation and cytokine production in the immature MDDCs, and if the MDDCs acquired an ability to induce proliferation in autologous CD14-depleted peripheral blood mononuclear cell (PBMC) cultures.

Materials and methods

Culturing of *M. furfur*

M. furfur strain no. 42132, obtained from American Type

Culture Collection, was cultured at 37 °C for 4 days [21] on Dixon solid phase medium [22] with some modifications; i.e. addition of mycological peptone (Oxoid AB, Sollentuna, Sweden) and use of Tween 60 and chloramphenicol (Sigma Chemical company, St Louis, MO, USA) instead of Tween 40 and streptomycin sulphate. The yeast was harvested in sterile water and counted under a light microscope before incubation with MDDCs. The *M. furfur* strain was also cultured on blood agar and Sabouraud dextrose agar plates as a control to ensure that the yeast was not contaminated with bacteria or other yeasts.

Cell lines

L cells, a fibroblast cell line, and L cells transfected with CD40L ([23], kindly provided by Dr P. Garrone, Schering-Plough, Dardilly, France), were cultured in 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, Paisley, UK) and 10% (v/v) heat-inactivated fetal calf serum (FCS, Hyclone, Logan, UT, USA), denoted RPMI 1640 with 10% FCS. The expression of CD40L was measured regularly by flow cytometry during culture of the cell lines (data not shown).

Generation of MDDC cultures from peripheral blood

Buffy coats were obtained from 17 healthy donors from the Karolinska Hospital blood bank. They were all RAST-negative to *M. furfur* (= 0.35 kU/L, M70, Pharmacia & Upjohn CAP systems RAST FEIA, Pharmacia AB Diagnostics, Uppsala, Sweden). Fourteen of the donors were Phadiatop negative, i.e. did not show IgE reactivity to 11 common airborne allergens, and had a median total serum IgE of 8.4 kU/L (range 2–130 kU/L, Pharmacia & Upjohn CAP systems IgE FEIA). Three donors were Phadiatop-positive with total serum IgE values of 39, 130 and 350 kU/L, respectively. The reference ranges for non-atopic individuals are 1.6–122 kU/L of total serum IgE.

MDDCs were prepared according to Romani et al. [24]. Briefly, buffy coats were diluted in phosphate-buffered saline (PBS, pH 7.4, Ca²⁺- and Mg²⁺-free) and layered on Ficoll Paque (Pharmacia Biotech, Uppsala, Sweden). After centrifugation, the interfaces, containing PBMCs, were collected and washed in PBS. The CD14⁺ monocytes were enriched by positive selection using MACS CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. In short, the mononuclear cell fraction was incubated with CD14 MicroBead mAbs (Miltenyi Biotec) for 1 h, followed by separation on a MidiMACS LS⁺ column (Miltenyi Biotec). The PBMCs, depleted of CD14⁺ cells, were frozen in RPMI 1640 supplemented with 50% FCS and 10%

dimethyl sulfoxide (Merck KGaA, Darmstadt, Germany) at -150°C for later use in proliferation studies. The CD14⁺ cells were diluted to 4×10^6 cells/mL in complete culture medium, RPMIc (RPMI 1640 with 10% FCS, supplemented with $50\text{ }\mu\text{M}$ 2- β -mercaptoethanol, Sigma), and cultured in 25 or 75 cm² culture flasks (Costar Corporation, Cambridge, MA, USA). The RPMIc medium was supplemented with 550 U/mL GM-CSF (Schering Plough, Kenilworth, NJ, USA) and 800 U/mL of recombinant human IL-4 (rIL-4, Nordic BioSite, Täby, Sweden). The cells were cultured at 37°C in a humidified incubator with 6% CO₂ in air for 6 days, re-fed on day three, to become immature MDDCs. The MDDCs were harvested by gentle resuspension and cell viability was assessed by trypan blue exclusion. This work was approved by the local ethics committee.

Co-culture of MDDCs with M. furfur

Immature MDDCs, at day 6 of culture, were incubated in RPMIc medium supplemented with cytokines (550 U/mL GM-CSF and 800 U/mL rIL-4), at a cell density of 4×10^5 cells per mL, with or without addition of *M. furfur* or plastic beads in the same size as the yeast (SPHEROTM, 1.8–2.2 μm , PharMingen, San Diego, CA, USA). The cells were co-cultured in 25 cm² culture flasks (Costar Corporation) at 37°C in a humidified incubator with 6% CO₂ in air, at a 1 : 5 MDDC : *M. furfur* or plastic bead ratio, for 46 h.

Immature MDDCs were also co-cultured with L cells or CD40L-expressing L cells, while incubated with or without *M. furfur*. Briefly, 1.5×10^5 L cells transfected or not with CD40L, were irradiated (8000 rad) and cultured in 1 mL of the RPMI 1640 with 10% FCS media in 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) overnight to attach. Non-attached L cells were washed away with PBS. Approximately 1×10^6 immature MDDCs were then co-cultured with the CD40L-expressing L cells or the non-transfected L cells in 1 mL RPMIc supplemented with cytokines (550 U/mL GM-CSF and 800 U/mL rIL-4), with or without the addition of *M. furfur* at a 1 : 5 ratio, for 18 h.

Time-lapse study

Immature MDDCs were seeded on glass coverslips in a 24-well plate (Becton Dickinson Labware) at a cell density of 2×10^5 mL in RPMIc supplemented with cytokines (550 U/mL GM-CSF and 800 U/mL rIL-4). The cells were allowed to adhere to the coverslips at 37°C during overnight incubation. Each coverslip was transferred to an inert aluminium chamber with RPMIc medium, and *M. furfur* was added in a 1 : 5 ratio just before the chamber was sealed with silicon, and the time-lapse photography

was started. The chamber was transferred to an inverted microscope (Leitz Labovet, Leica AB, Stockholm, Sweden) with an interference contrast lens giving a 6.3×25 -fold magnification. The temperature inside the chamber was kept at 37°C by circulating water [25]. The images were captured with a 3CCD video camera (Sony DXC-750 P, Sony Sverige AB, Spånga, Sweden) with a 80-mm relay lens and a resolution of 710 lines. The signal was recorded by a Perception video recorder card (DPS, Ontario, Canada). The time-lapse procedure was controlled by a computer-controlled device (JoRoTech HB, Uppsala, Sweden) with one frame take every 3 s for varying time periods. The same computer controlled an X–Y table so cells in motion could be followed.

Monoclonal antibodies (mAbs)

The following fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse mAbs were used for flow cytometric analysis: anti-CD1a PE (T6-RD1, Coulter Hialeah, FL, USA), anti-CD14 FITC (Leu-M3, Becton Dickinson), 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), and anti-CD154 PE (anti-CD40L, 89–76, Becton Dickinson). Isotype-matched antibodies were used as negative controls. The mAbs anti-CD80 (MAB104, Immunotech), anti-CD83 (HB15e, PharMingen), and anti-CD86 (2331, FUN-1, PharMingen) were used for immunocytochemical staining, and mouse IgG₁ (Dakopatts, Copenhagen, Denmark) was used as a control.

Flow cytometry

Approximately 5×10^4 cells were incubated for 30 min on ice with either FITC- or PE-conjugated mAbs diluted in PBS. A minimum of 10^4 cells was acquired on a FACSCalibur flow cytometer (Becton Dickinson) and analysed using CellQuest software (Becton Dickinson). A proper gate was set according to a characteristic forward-scatter pattern [26]. Cells considered as positive cells had a higher mean fluorescence index (MFI) value than cells incubated with an irrelevant isotype matched antibody. Only gated cells were considered when calculating the percentage of positive cells. The MFI values reflect all the positive cells. The flow cytometer was calibrated according to the manufacturer's instructions before each acquisition.

Immunocytochemical stainings of MDDCs

MDDCs were washed in PBS and air-dried onto three-well ($\emptyset$ int. 14 mm) microscope glass-slides ($\approx 2 \times 10^4$ cells per well, Novakemi, Enskede, Sweden), and stored at -80°C until use. The cells were fixed in acetone before staining for CD80, CD83 and CD86 using the Vectastain ABC-elite kit according to the manufacturer (Vector Laboratories Inc., Burlingame, CA, USA), and developed with 3-amino-9-ethylcarbazole (AEC, 0.02% w/v, Aldrich-Chemie, Steinheim, Germany) dissolved in acetate buffered (pH 5.5) hydrogen peroxide (0.01%) for 15 min followed by counterstaining with Mayer's haematoxylin (Sigma) for 30 s. A minimum of 500 MDDCs were counted, omitting the lymphocytes, with a Leiz microscope with a $\times 40/0.70$ PL Fluotar objective and a $\times 10$ eyepice (Laborlux K, Nürnberg, Germany). A CD83⁺ cell was defined as one with a definite brownish-red staining and a visible nucleus. For CD80 and CD86 the cells were also considered as having a weak or strong expression of the antigen. The results are expressed as the percentage of positive cells of the total number of counted cells for CD83, and percentage of strongly positive cells of the total number of counted cells for CD80 and CD86. Staining was not found when cells were incubated with the isotype-matched control antibody.

Cytokine detection in culture supernatants

Culture supernatants were kept at -20°C until analysed, and repeated freezing and thawing was avoided. Production of TNF- α , IL-1 β , IL-10, IL-12p70 and IL-18 by MDDCs was assayed by Quantikine[®] ELISA kits (R & D Systems, London, UK) according to the manufacturer's protocol. Samples were run in duplicates. Lower detection limits were 16 pg/mL for TNF- α , 4 pg/mL for IL-1 β , 8 pg/mL for IL-10 and IL-12p70, respectively, and 31 pg/mL for IL-18.

PBMC proliferation after co-culture with MDDCs pre-incubated with *M. furfur*

PBMCs, depleted of CD14⁺ cells, were incubated in the RPMI 1640 with 10% FCS medium, in 96-well plates (Costar), at a density of 2×10^5 cells per well. MDDCs, pre-incubated in medium, with *M. furfur* (1 : 5 ratio) or with plastic beads (1 : 5 ratio) for 46 h, were added at different cell densities (10 , 10^2 , 10^3 and 10^4 MDDCs per well), to autologous CD14-depleted PBMCs. Phytohaemagglutinin (PHA, 10 $\mu\text{g/mL}$, Pharmacia Biotech) was used to ascertain that the PBMCs could proliferate. To detect the background proliferation, CD14-depleted PBMCs were incubated in medium alone. The cells were

cultured in triplicates for 4 days and 1 μCi [^3H]-thymidine per well (specific activity 25 Ci/mmol (925 GBq/mmol), Amersham Laboratories, Buckinghamshire, UK) was added to the cultures for an additional 18 h. The plates were kept at -20°C until [^3H]-thymidine incorporation was determined by scintillation counting.

Statistical analysis

Data were compared using the non-parametric Wilcoxon matched pairs test, except for proliferation values that were compared with the Student's *t*-test for dependent samples. $P < 0.05$ was considered statistically significant. When the levels of cytokines were below the detection limit of the ELISA, the value of the detection limit was used in the statistical calculations. In cases where samples were not finally titrated, the highest value the sample was detected to be above, was used.

Results

Characterization of the MDDCs

A typical immature DC phenotype was present, at days 7–8, in the MDDCs cultured without *M. furfur* (Table 1) [24,26]. The median purity of the MDDC cultures, as determined by cell size and cell granularity using flow cytometry, was 95% (range: 79–99%, $n = 16$). The median viability of MDDCs incubated with *M. furfur* for 46 h was 94% (range: 86–100%, $n = 17$), and of MDDCs incubated

Table 1. Phenotype of the generated immature monocyte-derived dendritic cells (MDDCs)

Phenotypic marker	Immature MDDCs, days 7–8 ($n = 6–17$)*	
	% [†]	MFI [‡]
CD1a	94 (72–99) [†]	266 (75–1185)
CD14	0.4 (0.0–2.2)	32 (12–179)
CD40	92 (86–96)	42 (25–83)
CD54	50 (12–96)	26 (10–40)
CD80	80 (40–95)	25 (16–59)
CD83	2.7 (0.0–7.9)	25 (11–337)
CD86	46 (21–60)	49 (22–101)
HLA-DR	99 (98–100)	239 (133–726)

*Number of experiments using cells from different healthy blood donors. [†]Positively stained MDDCs as analysed by flow cytometry. Results are given as median percentage (range). At least 10^4 cells per sample were analysed. [‡]MFI values presented as median (range).

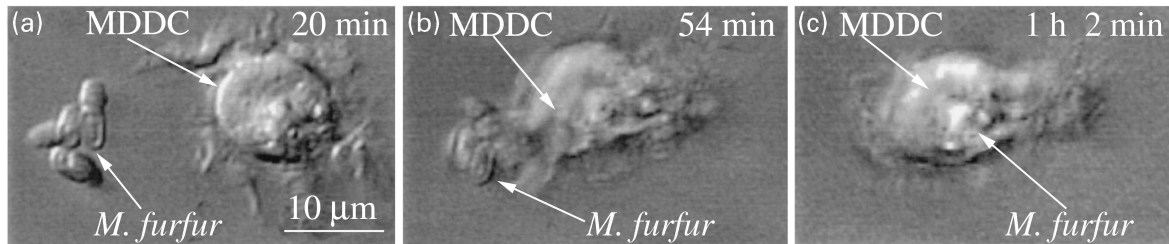


Fig. 1. Immature MDDCs internalize *M. furfur* within 1 h. Time-lapse photography was used to study the kinetics of uptake of the yeast *M. furfur* by MDDCs. One picture was taken every third second. Three time-points are shown: 20 min (a), 54 min (b), and 1 h and 2 min (c), after co-incubation of 2×10^5 immature MDDCs per mL with *M. furfur* at a 1 : 5 ratio. The scale bar is 10 μ m.

in only medium 96% (range: 91–99%, $n = 17$), as determined by trypan blue exclusion.

M. furfur yeast cells are rapidly internalized by immature MDDCs

Time-lapse photography documented the kinetics of *M. furfur* internalization by immature MDDCs. Within 50–60 min, 3–5 yeast cells were usually taken up by the majority of the MDDCs (Fig. 1). Subsequently, the yeast cells were found to be transported from the periphery of the MDDC towards the central part of the cell.

M. furfur induces maturation in immature MDDCs

To assess if interaction of immature MDDCs with the yeast *M. furfur* would induce maturation, MDDCs incubated in medium or co-cultured with either *M. furfur*, plastic beads, CD40L transfected L cells or non-transfected L cells, were stained for the maturation marker CD83 [27] and the co-stimulatory molecules CD80 and CD86. Immunocytochemistry was chosen for evaluation of the CD80, CD83 and CD86 expression, since autofluorescence of the yeast cells hampers the use of flow cytometry. Our results show that *M. furfur* significantly increased the expression of CD83 ($P < 0.05$) and, hence, induced maturation of MDDCs, but not to the same degree as the commonly used maturation stimuli CD40L (Fig. 2 [11]). MDDCs with a strong expression of the co-stimulatory molecules CD80 and CD86 were also significantly increased ($P < 0.05$) after co-culture with *M. furfur*, compared with expression in cells cultured in medium alone (Figs 2a and c). The median CD80 expression induced by non-transfected L cells was 14% (range 9–25%, $n = 4$), CD83 expression 28% (12–54%, $n = 4$), and CD86 expression 36% (12–37%, $n = 4$). Although the plastic beads were taken up by the MDDCs, as observed by examination under a light microscope (data not shown), the CD80, CD83 and CD86 expression did not differ from that in the control cells incubated in culture medium only (Figs 2a–c).

M. furfur induces cytokine production in MDDCs

To understand the early events in the initiation of an immune response to *M. furfur*, the effect on cytokine production by MDDCs after interaction with the yeast was investigated. On the basis of pilot studies, culture supernatants were analysed after 46 h of co-culture. By that time, *M. furfur* significantly increased the production of TNF- α , IL-1 β ($P < 0.05$, respectively) and IL-18 ($P < 0.01$) by the MDDCs compared with production by MDDCs incubated in medium alone (Figs 3a–c). However, neither IL-10 nor IL-12p70 levels were significantly altered by the addition of *M. furfur* (Figs 3d and e). Cytokine production from MDDCs incubated with plastic beads were at background levels (Figs 3a–e). None of the cytokines investigated were detected in culture supernatants from only *M. furfur* (data not shown).

The results demonstrate that the opportunistic yeast *M. furfur* can induce a cytokine response in immature MDDCs, rendering them able to activate cells of the immune system.

M. furfur decreases CD40L-stimulated IL-12p70 production by MDDCs

Co-culture of immature MDDCs with CD40L expressing L cells stimulated the MDDCs to produce increased levels of IL-1 β , IL-18, IL-10 and IL-12p70, compared with MDDCs incubated in medium only (Fig. 4). Addition of *M. furfur* to the cocultures increased the levels of IL-18 ($P < 0.05$) and there was a tendency for enhanced production of IL-1 β ($P = 0.068$), compared with only CD40L stimulation (Figs 4a and b). In contrast, the IL-12p70 production was significantly decreased ($P < 0.05$) and there was a tendency for decreased production of IL-10 ($P = 0.075$) in the presence of the yeast (Figs 4c and d). The TNF- α production by CD40L-activated MDDCs was above 25 ng/mL both with and without addition of *M. furfur*. The cytokine levels in the supernatants from MDDCs co-cultured with non-transfected L cells for 18 h were 1111 (< 16–2052) pg/mL (median and range) for TNF- α , and

similar to the levels of MDDCs cultured in medium only for IL-1 β , IL-18, IL-10 and IL-12p70 ($n = 3-5$). The corresponding figures with addition of *M. furfur* were: $> 10\,000$ ($829->10\,000$) pg/mL for TNF- α ; > 250 ($< 4->250$) pg/mL for IL-1 β ; 239 ($< 31-365$)

pg/mL for IL-18; 442 ($< 8-696$) pg/mL for IL-10; and < 8 ($< 8 - < 8$) pg/mL for IL-12, p70.

Autologous MDDCs exposed to M. furfur induce lymphocyte proliferation

The ability of the MDDCs to induce a proliferative response in autologous lymphocytes after interaction with the yeast *M. furfur* was then investigated. MDDCs pre-incubated with *M. furfur* induced a proliferative response in autologous CD14-depleted PBMCs, in a dose-dependent manner (Fig. 5).

Discussion

We have shown here that human immature MDDCs can internalize the opportunistic yeast *M. furfur*, and that this event induces the MDDCs to mature and produce TNF- α , IL-1 β and IL-18, but not IL-10 and IL-12p70. CD40L-activation of MDDCs results in IL-12p70 production, but the presence of *M. furfur* during CD40L-activation significantly decreased this production. MDDCs pre-incubated with *M. furfur* induced proliferation of autologous lymphocytes.

Because the opportunistic yeast *M. furfur* can give rise to specific IgE antibodies in AD patients [6-8], and induce a significantly higher lymphocyte proliferation in AD patients than in healthy individuals [28,29], we wished to investigate the encounter of this yeast with DCs of the immune system. DCs, the most potent APCs known, act as conductors of the immune response by being a link between the innate and acquired immune systems [30]. We have chosen to generate DCs from peripheral blood monocytes for our studies.

We found that whole *M. furfur* yeast cells were internalized by immature MDDCs within 1 h. It has previously been reported that *M. furfur* is taken up by immature but not mature human MDDCs using confocal laser scanning microscopy [20], and that a human

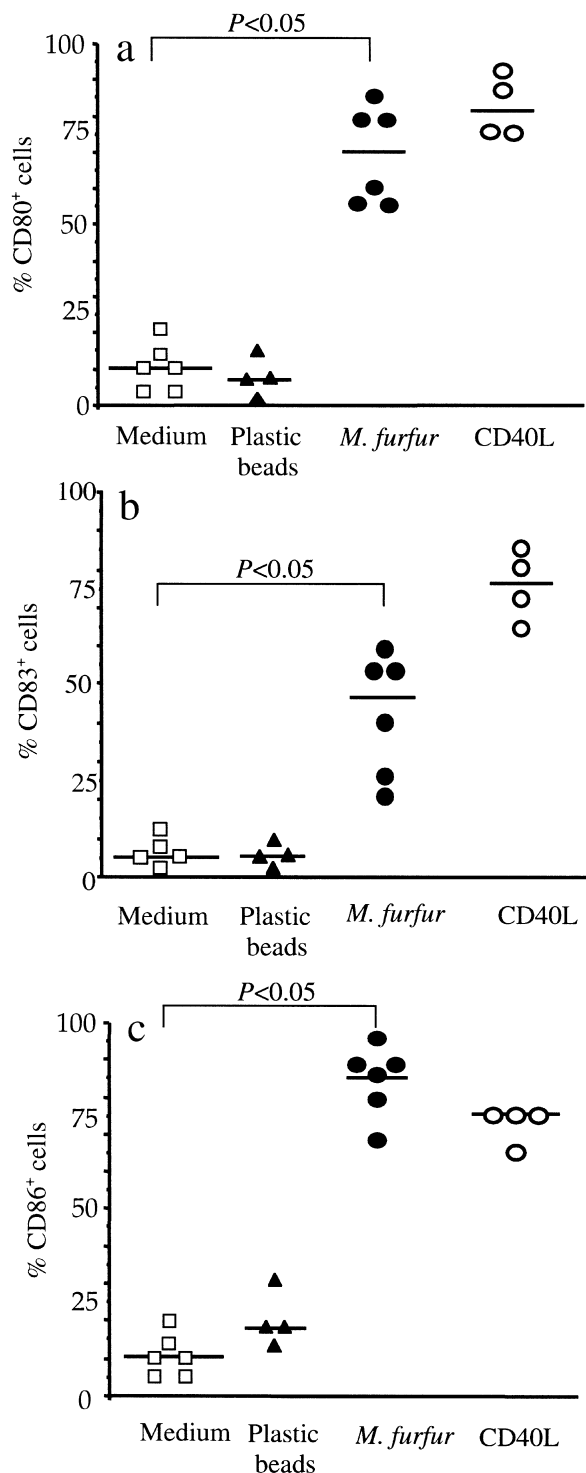


Fig. 2. *M. furfur* yeast cells induce MDDCs maturation, as judged by up-regulation of CD83 expression. Immunocytochemical staining for CD80 (a), CD83 (b) and CD86 (c) expression was performed after 4×10^5 immature MDDCs per mL had been incubated in medium ($\square$), with addition of plastic beads ($\blacktriangle$) or *M. furfur* ($\bullet$) (at a 1 : 5 MDDC : plastic bead or *M. furfur* ratio) for 46 h, or after coculture of MDDCs with CD40L expressing L cells for 18 h ($\circ$). Results are expressed as the percentage of positive cells of the total number of counted cells for CD83, and percentage of strongly positive cells of the total number of counted cells for CD80 and CD86. The horizontal lines indicate the median values. The results shown are from four to six independent experiments.

monocytic cell line takes up *M. furfur* within 30 min [31]. Uptake of another yeast, *Candida albicans*, by human MDDCs has been reported to occur within 2 h [32]. DCs express a number of receptors, such as the macrophage mannose receptor (MR), DEC-205, DC-SIGN, Langerin, Toll-like receptors and Fc-receptors, that are involved in the interaction or uptake of micro-organisms [33–38]. Uptake of *M. furfur* by a human monocytic cell line [31] and of *M. furfur* extract by human immature MDDCs, was previously shown to be mediated by the MR [20].

M. furfur induced activation of MDDCs with regard to CD80, CD83 and CD86 expression. Compared with CD40L-stimulated MDDCs, *M. furfur* induced lower levels of CD83 expression, suggesting a weaker activation

stimulus by the yeast, or slower kinetics in the induction of maturation. The CD83-expression found in the MDDC population after CD40L activation is similar to an earlier report [11]. Evidently, individual micro-organisms, such as *Listeria monocytogenes*, mycoplasma, measles virus and mycobacteria, or products from micro-organisms, such as CpG DNA and LPS, differ in their ability to induce CD83, as well as CD80 and CD86, expression in human DCs, and the extent of CD80, CD83 and CD86 expression we found in MDDCs after co-culture with *M. furfur* is in agreement with these observations [11,14,19,39,40]. The fact that *M. furfur* induced up-regulation of CD83 in MDDCs suggests that the yeast is fully capable of maturing DCs in the skin and thereby inducing their cytokine production and

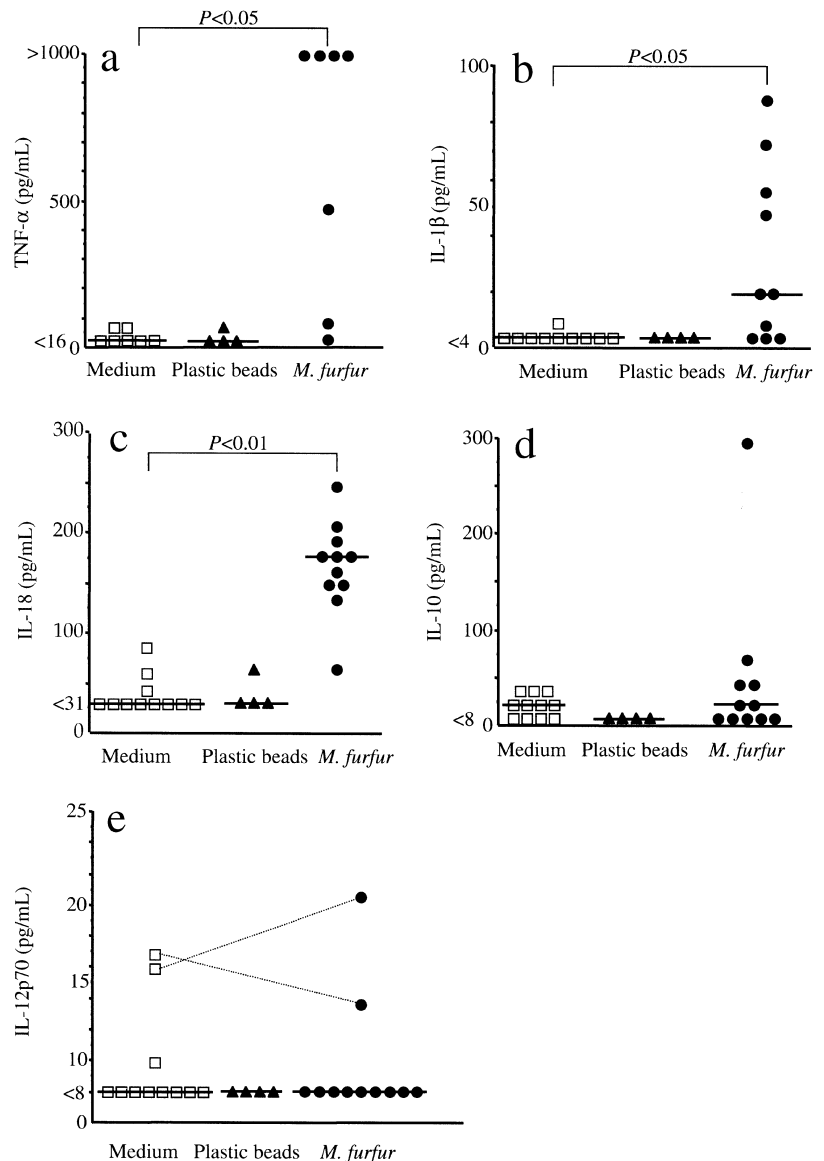


Fig. 3. *M. furfur* yeast cells induce production of TNF- α , IL-1 β and IL-18 in MDDCs. The cytokine production from 4×10^5 MDDCs per mL was measured in culture supernatants by ELISA after incubation in medium (□), with plastic beads (▲) or with addition of the yeast *M. furfur* (●) at a 1 : 5 ratio for 46 h. The results shown are TNF- α (a), IL-1 β (b), IL-18 (c), IL-10 (d) and IL-12p70 (e) levels from four to 11 independent experiments using cells from different blood donors. The horizontal lines indicate the median values. The detection limit for the different cytokines is indicated on the y-axis.

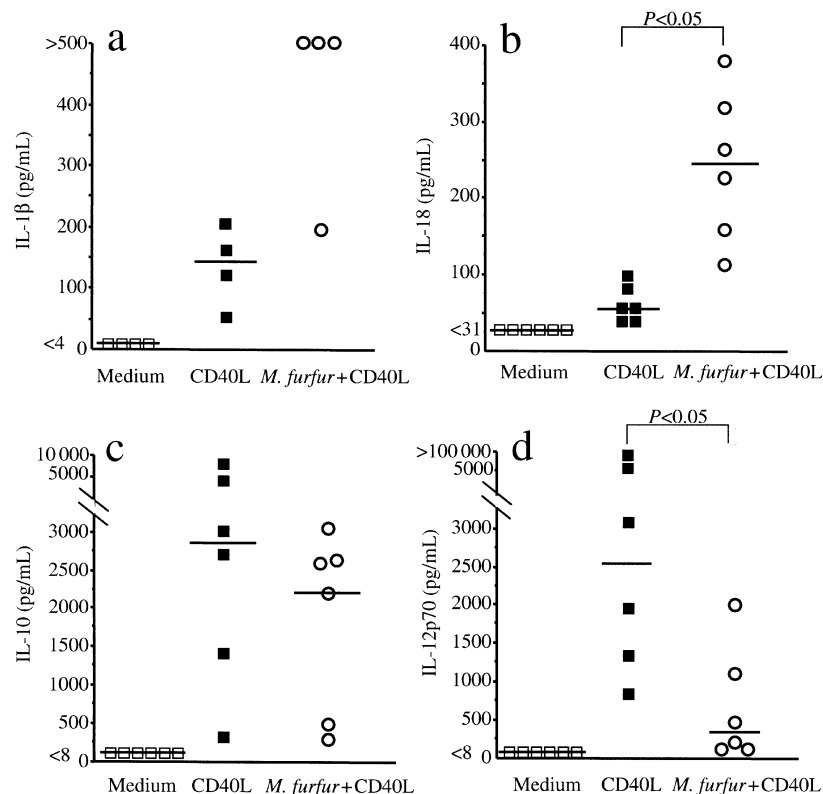


Fig. 4. CD40L-stimulated production of IL-1 β and IL-18 by MDDCs is increased, whereas production of IL-12p70 is decreased when co-cultured with *M. furfur*. MDDCs were cultured in medium ($\square$), with CD40L expressing L cells ($\blacksquare$) or with CD40L expressing L cells and the yeast *M. furfur* ($\circ$) (at a 1 : 5 MDDC : *M. furfur* ratio) for 18 h. The cytokine production from 1×10^6 MDDCs per mL was measured using ELISA. The results shown are IL-1 β (a), IL-18 (b), IL-10 (c) and IL-12p70 (d) levels from four to six independent experiments using cells from different donors. The horizontal lines indicate the median values. The detection limit for the different cytokines is indicated on the y-axis.

migration to regional lymph nodes for further activation of T cells. Indeed, we found that *M. furfur* had a positive effect on the MDDC's capacity to induce lymphocyte proliferation, and that the degree of proliferation was dependent on the number of MDDCs present. It has previously been reported that DCs are very efficient at stimulating both naive and memory T cells [10].

The cytokine profile expressed by human DCs is dependent on the mode of activation [41]. We chose to study the production of the pro-inflammatory cytokines TNF- α and IL-1 β by MDDCs after co-culture with *M. furfur*, since these two cytokines have been shown to be of major importance for DC activation and migration in the skin [42]. When immature MDDCs were co-cultured with *M. furfur*, we found significantly elevated levels of TNF- α and IL-1 β , suggesting that the MDDCs in this way could be induced to migrate from the skin. Our results are in agreement with a study where three other *Malassezia* species were reported to induce production of TNF- α and IL-1 β when co-cultured with human blood mononuclear cells [43]. After stimulation of the MDDCs by *M. furfur* and CD40L expressing L cells the levels of TNF- α and IL-1 β were further increased, suggesting that activation through CD40-CD40L together with *M. furfur* stimulation, induces a stronger signal for production of TNF- α and IL-1 β . IL-1 β has also been reported to be one of the first

substances in a chain of events leading to recruitment of cutaneous lymphocyte-associated antigen (CLA $^+$) T cells to the skin [44]. Infiltration of T cells has been well documented in lesional AD skin and in positive patch test reactions [1,9,45].

Several cytokines strongly influence the commitment of naive T cells towards distinct effector functions [46]. We decided to study IL-10, IL-12 and IL-18 production by the MDDCs since these cytokines can polarize the immune response towards a Th1- or Th2-type of response. IL-12 produced by DCs upon microbial stimuli, such as mycoplasma and *Mycobacterium tuberculosis* [19,47], favour the differentiation of Th0 cells into Th1 cells [48]. Yet, we did not detect an elevation in the secretion of IL-12p70 by MDDCs after adding *M. furfur*. The MDDCs from two non-atopic donors had higher IL-12p70 production than the MDDCs from the others, but these levels were not further enhanced by *M. furfur* (Fig. 3e). Our data thus indicate that *M. furfur* activates human immature MDDCs without polarization towards a Th1-type inducing cytokine pattern.

In support of our finding is a report showing that adenoviruses do not induce IL-12 production by MDDCs [49]. After CD40L-induced maturation the MDDCs produced high levels of IL-12p70, as previously reported [50], indicating the importance of a proper activation of the

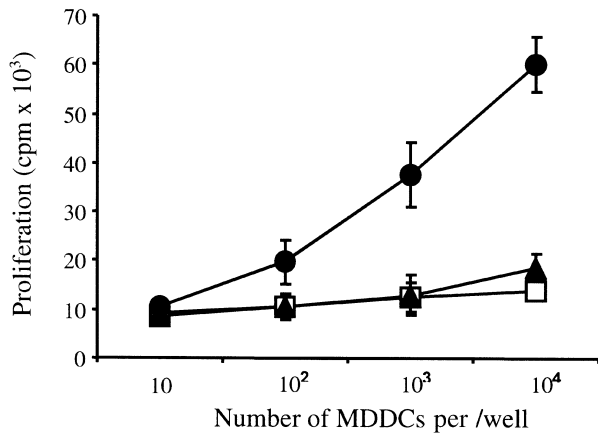


Fig. 5. MDDCs pre-incubated with *M. furfur* induce proliferation in autologous CD14-depleted PBMCs. MDDCs were cultured in only medium (□), or pre-incubated with plastic beads (▲) or *M. furfur* (●) at a 1 : 5 MDDC : plastic bead or *M. furfur* ratio for 46 h. The MDDCs were thereafter co-cultured with autologous CD14-depleted PBMCs (2×10^5 cells per well) in triplicates for 4 days with addition of ^3H -thymidine for the last 18 h. The results are shown as mean $\pm$ SEM of three to four independent experiments. The proliferation induced by 1×10^3 and 1×10^4 MDDCs pre-incubated with *M. furfur* was significantly higher ($P < 0.01$) than the proliferation induced by MDDCs incubated in medium or with plastic beads. The PHA-stimulated proliferation in the CD14-depleted PBMCs was $18 \times 10^4 \pm 3 \times 10^4$ cpm ($n = 3$), and the background proliferation in the CD14-depleted PBMCs was $7 \times 10^3 \pm 1 \times 10^3$ cpm ($n = 3$).

DCs by T cell contact. In the presence of *M. furfur*, however, the production of IL-12p70 was significantly decreased ($P < 0.05$). Such a decrease in IL-12 production has been described also after infection of human DCs with measles virus [11,51] and after treatment with cholera toxin [16]. This suggests that even though the DC receives an activating signal from a T cell through CD40–CD40L interaction, the presence of *M. furfur* would decrease the IL-12 production, and thus perhaps prevent induction of a Th1-type of immune response. IL-10 has been shown to down-regulate IL-12 production and lead to a Th2-type of response [52]. The IL-12p70 production was decreased in the presence of both *M. furfur* and CD40L stimulation, compared with only CD40L stimulation. There was also a tendency for decreased production of IL-10 after *M. furfur* and CD40L stimulation. This indicates that the inhibitory effect of *M. furfur* on IL-12p70 production by MDDCs might not be mediated via IL-10.

IL-18, which is reported to act synergistically with IL-12 [53], was found to be up-regulated in the MDDC cultures in the presence of *M. furfur* for 46 h. Simultaneous *M. furfur* and CD40L stimulation also increased the production of IL-18 compared with only CD40L stimulation. IL-18 administered

together with IL-12 has in mice been shown to inhibit IgE production, whereas IL-18 alone stimulates IL-4 production and histamine release by mast cells, as well as IgE production [54–56]. This might indicate that the cytokine profile produced by MDDCs after co-culture with *M. furfur* would favour a production of Th2-type cytokines like IL-4, leading to an IgE production. Interestingly it has been reported that *M. furfur*-specific T cell clones from the lesional skin of AD patients show a Th2- or Th2/Th0-like cytokine profile [28,29].

In further studies it will be interesting to compare DCs from normal healthy individuals with those from AD patients in their interaction with *M. furfur*, and its allergenic components. So far nine allergens have been cloned and sequenced from *M. furfur*, and four of them have no significant sequence homology to other known proteins in the searched databases [57–60]. Also, the hyphal form of the yeast, which has been reported to be present in several skin manifestations where *M. furfur* is involved, would be interesting to compare with the yeast form [5], since in mice it has been found that DCs presenting peptides derived from unicellular *C. albicans* induce a Th1-type of response, whereas hyphae-derived peptides from the same yeast induce a Th2-response [61].

In conclusion our data indicate that the opportunistic yeast *M. furfur* activates MDDCs to mature and produce cytokines that might favour induction of a Th2-type immune response. It is most likely that these events are of importance for the inflammatory reaction in atopic dermatitis.

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