



Activation profile of THP-1 derived dendritic cells stimulated by allergen Mal f 1 beyond its IgE-binding ability

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ABSTRACT

Background: Mal f 1, the first allergen cloned from *Malassezia furfur*, has positive IgE reactivity in sera from atopic dermatitis (AD) patients. The mechanism by which Mal f 1 induces the maturation of human dendritic cells (DCs) and maintains the symptoms of AD is not well understood.

Objective: The present study aims to explore the activation profile of THP-1 derived dendritic cells (TDDCs) stimulated by recombinant Mal f 1, as well as to explore the IgE-binding ability of rMal f 1 and its correlation with IgE-binding activity of complete allergens of *M. furfur*.

Methods: rMal f 1 was produced by expression in *E. coli* and purification with affinity chromatography. The ability of rMal f 1 and ImmunoCAP complete allergens of *M. furfur* to bind to serum specific IgE was assayed in parallel by ELISA and immunoblotting. Immature TDDCs were stimulated with rMal f 1 or an enzyme-digested product of rMal f 1. The expression levels of markers, CD83, CD80, CD86, and HLA-DR, were investigated by flow cytometry. The levels of interleukin (IL)-6, IL-10, IL-12p70 and tumor necrosis factor (TNF)- α in culture supernatants were determined by ELISA.

Results: Eighteen patient sera were identified that reacted positively to the complete allergens of *M. furfur* as determined by ImmunoCAP and also showed positive responses to rMal f 1. Five patient sera were identified that had no reaction to ImmunoCAP complete allergens of *M. furfur* and also exhibited negative response to rMal f 1. All sera, except for one, had no reaction to the unrelated allergen Bet v 1. rMal f 1 upregulated the maturation surface marker CD83 on TDDCs. In addition, rMal f 1 also induced high levels of CD80 and CD86. Increased expression of HLA-DR, a first signal for T cell activation, was observed. Secretion of IL-6, TNF- α and IL-10 by TDDCs increased significantly ($P < 0.0001$ for IL-6, $P < 0.01$ for TNF- α and $P < 0.05$ for IL-10) after stimulation by rMal f 1, while the IL-12p70 level was unaltered.

Conclusion: We have shown that rMal f 1 has ideal IgE binding ability and good correlation with binding activity to *M. furfur*. Moreover, we have revealed a hitherto unknown DC activation profile after rMal f 1 stimulation whereby TNF- α , IL-6, and IL-10 were significantly increased and IL-12 was unaltered, suggesting that rMal f 1 can predispose a DC bias toward the T_H22/T_H17 pathway beyond the routine IgE-dependent T_H2 pathway, thus providing intriguing clues for clinical treatment involving both pathways.

1. Introduction

Skin colonizers belonging to the *Malassezia* species are being debated as to their pathogenic role in various skin diseases [1]. A close association has been shown between skin and *Malassezia* IgE binding allergens in atopic eczema, while laboratory data support its role in psoriasis exacerbations [2]. In this regard, > 70% of the *Malassezia* species isolated from psoriatic lesions, was *Malassezia furfur* [3] and *M. furfur* isolates are more drug tolerant than other *Malassezia* species [4].

Therefore, in the past two decades, the physiology, biochemistry, immunology, and metabolomics of *M. furfur* has been a major topic of intense basic research. *M. furfur*, previously known as *Pityrosporum orbiculare* (Ovale), is a natural inhabitant of the human skin microflora. This yeast can grow as an anthropophilic fungus in both a yeast phase and a mycelial phase and, thus, may be involved in the pathogenesis of dozens of diseases, such as pityriasis versicolor, folliculitis, seborrheic dermatitis, psoriasis, papillomatosis, allergic rhinitis, asthma, etc. [5–7]. Various studies have shown that 28–84% of AD patients have

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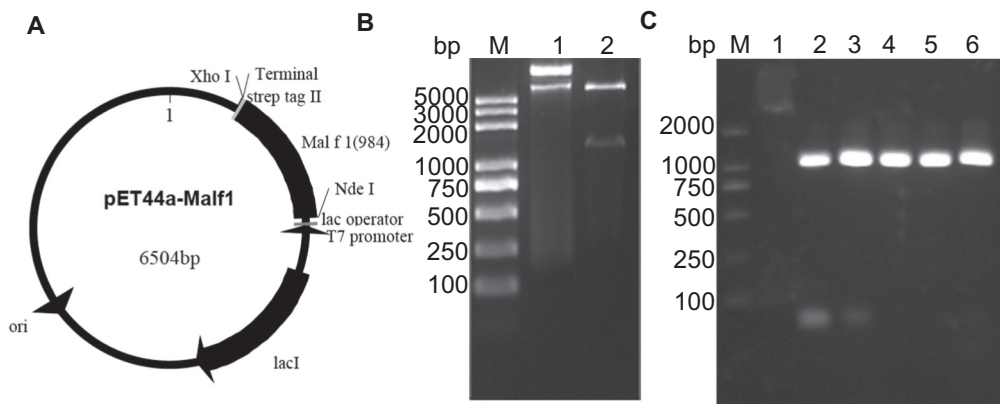


Fig. 1. Cloning and identification of recombinant plasmid pET44a-Mal f 1. A, Schematic map of the recombinant plasmid pET44a-Mal f 1. B, Identification of the pET44a-Mal f 1 by double restrictive enzyme digestion with *Xho* I and *Nde* I. M: DL5000 Marker, Lane 1: the plasmid pET44a-Mal f 1, Lane 2: the product of enzyme digestion. C, Identification of the pET44a-Mal f 1 by PCR. M: DL2000 Marker, Lane 1: negative control, Lane 2–5: PCR products of different colony.

specific IgE reactivity to *M. furfur* [8–10]. Mal f 1 is the first allergen cloned from *M. furfur* and is a cell wall protein that does not show sequence similarity to other proteins [11,12]. Serum IgEs from 43% and 61% of the 95 patients tested showed positive reactivity to rMal f 1 produced in the *E. coli* and baculovirus expression systems, respectively [13]. However, its role in the pathogenesis of the aforementioned diseases, though often displayed by IgE binding results, is inadequately understood.

Dendritic cells (DCs) are undoubtedly the most potent antigen presenting cells and play a critical role in bridging the innate and adaptive immune systems (the initiation of allergic immune response) [14]. DCs recognize and phagocytize exogenous antigens, process them into immunogenic peptides, then express these peptides for presentation on major histocompatibility complex (MHC) class II molecules to immune responses [15]. Buentke E. et al. showed that human monocyte-derived dendritic cells (MDDCs) could efficiently internalize and process yeast and allergenic components from whole live yeast [16,17]. Due to the scarcity of circulating DCs and the limits of availability of PBMC-derived monocytes, methods have been developed to produce quantities of DCs from some acute myeloid leukemia cell lines such as THP-1, KG-1, and CD34⁺ MUTZ3 when they are cultured with GM-CSF and IL-4 [18,19]. These DCs can then be used to study the phenotype and response of DCs to allergen stimuli. A previous study demonstrated that *M. furfur* could induce monocyte-derived DCs to bias toward a T_H2-type immune response [20]. In another study, similar results were obtained when atopic dermatitis patient PBMCs were cultured with the extracts of *M. furfur*, in contrast to PBMCs from patients with psoriasis vulgaris that manifested T_H1-skewed responses to *M. furfur* [21]. However, several issues raise doubts as to whether this individual pathway can explain the complexity of the abovementioned diseases triggered by *M. furfur*. Firstly, some discrepancies are observed in the identification of *Malassezia* species when the results acquired directly from skin samples without further culturing are compared with data obtained using culture-dependent techniques, especially in the results seen from different research institutes and different geographical regions [22,23]. Secondly, certain predisposing topical factors (humidity, sweat, heat), along with individual idiosyncrasies, can influence the conversion of *Malassezia* species from the yeast to the mycelial form and, hence, determine the development of clinical lesions [23–25]. Therefore, single pathogenic molecules like Mal f 1 have long been needed to decipher the mechanism(s) that determine these variations.

In this study, we successfully produced Mal f 1 in an *E. coli* expression system and analyzed its IgE binding ability with patient sera. In addition, we successfully induced DCs from the THP-1 cell line by culturing them with GM-CSF and IL-4. We then investigated the effects of rMal f 1 stimulation on cytokine secretion in these THP-1-derived DCs (TDDCs) in order to determine the various signals sent from DCs to naïve T cells.

2. Materials and methods

2.1. Patients and sera

Written informed consent was obtained from all patients. Sera were collected from 23 allergy patients selected by the State Key Specialty of Allergy of the Second Affiliated Hospital of Guangzhou Medical University based on their reactivity to *Malassezia furfur* when tested by ImmunoCAP[™] (*Malassezia* CAP m70, Pharmacia AB Diagnostics, Uppsala, Sweden) as part of their diagnosis of skin disease and/or airway disease. The positivity of IgE specific to Mal f 1 was further investigated by ELISA and immunoblot assay using recombinant Mal f 1.

2.2. Expression of rMal f 1 in *Escherichia coli*

A full-length sequence of Mal f 1 except the signal-peptide was obtained from UniProtKB (Q01940) and synthesized by Biosune Biology and Technology Company (Shanghai, China) after codon optimization. The *Nde* I/*Xho* I sites were added for construction of the expression plasmid and a Strep II tag was added for purification (Fig. 1A). The target sequence was digested from *PUC57*, inserted into pET44a vector and transformed into *E. coli* strain BL21 DE3 pLysS (Novagen, Darmstadt, Germany). For expression of rMal f 1 in *E. coli*, overnight cultures of BL21 DE3 pLysS containing the recombinant pET plasmid were diluted 50-fold in prewarmed LB media containing 100 µg/mL ampicillin and grown at 37 °C to OD 0.6 at an absorbance of 600 nm. Expression of rMal f 1 was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mmol/L and incubated at 37 °C for another 4 h at which time the cells were harvested.

2.3. Refolding and purification of rMal f 1

The pellet was resuspended in 20 mL of binding buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) and lysed with mild sonication (AMPL 50%, Pulse 5 s, Off 5 s, 30 min). Inclusion bodies were prepared by centrifugation at 10,000 rpm for 30 min at 4 °C, then solubilized in 6 M urea, 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA (pH 8). Protein was refolded by stepwise dialysis against decreasing amounts of urea (4 M, 2 M, no urea) in binding buffer. The supernatant was loaded onto a Strep Trap[™] HR column (1 mL, GE Healthcare, Illinois, USA), elution of the proteins bound to the column was achieved with 2.5 mM desthiobiotin in binding buffer. After successive dialysis against PBS for 4 h, the concentration of recombinant protein was determined by BCA Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA) and rMal f 1 was analyzed by 12% SDS-PAGE and Coomassie brilliant blue staining. The LPS content was analyzed by the Tachypleus Amebocyte Lysate (TAL) method. Samples that contained < 5 EU/mg LPS were deemed to be suitable for use in the

following assays.

2.4. IgE binding activity

The IgE binding assay was conducted as previously described [26]. Ninety-six-well plates were coated with 2.5 µg of rMal f 1 or unrelated allergen rBet v 1 diluted in carbonate buffer (0.1 M Na₂CO₃, 0.1 M NaHCO₃, pH 9.6) overnight at 4 °C. The plates were blocked with PBS containing 0.1% Tween-20 plus 3% bovine serum albumin (BSA). After washing 3 times with PBS plus 0.1% Tween-20, each well was incubated with 100 µL of diluted human patient serum samples (1:10) for 2 h at room temperature. The plate was then washed and incubated with a mouse mAb to human IgE (Abcam, Cambridge, UK) for 1 h at 37 °C. Finally, IgE that bound to the pre-coated allergens was detected with a HRP conjugated horse anti-mouse IgG2a antibody (Cell Signaling Technology, Massachusetts, USA) by measuring the absorbance at 450 nm.

2.5. Immunoblot assay

Protein samples were size-fractionated on 5–12% gradient SDS-polyacrylamide gels and electro-transferred onto PVDF membranes. After incubation with blocking buffer (PBS containing 0.1% Tween 20 and 5% skim milk powder), the blocked membranes were incubated overnight at 4 °C with serum samples from each patient diluted at a ratio of 1:10 in blocking buffer. Then the membranes were washed 3 times with washing buffer (PBS plus 0.1% Tween-20) and subsequently incubated with mouse mAb to human IgE (Abcam, Cambridge, UK) and HRP conjugated horse anti-mouse IgG2a antibody (Cell Signaling Technology, Massachusetts, USA). The IgE-binding components were visualized using an ECL Western Blotting substrate (Thermo Fisher scientific, Massachusetts, USA) and an ImageQuant LAS 4000 mini detection system (PerkinElmer, Massachusetts, USA).

2.6. Digestion of rMal f 1

The purified recombinant Mal f 1 was digested using 200 mg/mL Proteinase K (PK) at 37 °C for 3 h, followed by heating at 95 °C for 1 h to inactivate the PK in the digested samples.

2.7. Cell culture and treatments

THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were subcultured three times per week and maintained at $0.5\text{--}1 \times 10^6$ cells/mL. To induce differentiation into immature DCs, rhIL-4 (1500 IU/mL) and rhGM-CSF (1500 IU/mL) were added. Fresh cytokine-supplemented medium was exchanged every 2–3 days. On day 6, the resulting immature DCs were washed with PBS and further stimulated with rMal f 1 (40 µg/mL) or LPS (1 µg/mL) as control stimuli.

2.8. Flow cytometry analysis of DCs

Flow cytometry was used to analyze the expression of cell surface markers. The following antibodies were used: CD11c-APC, CD209-FITC, CD83-APC, CD86-FITC, HLA-DR-FITC, and relevant IgG isotype controls from BD Bioscience. The cells were harvested with PBA (PBS containing 1% BSA) on day 7 and washed twice. The cells were incubated with antibodies for 1 h at room temperature. After labeling, the cell suspension was washed and resuspended in 500 µL of PBS for flow cytometry. A total of 10,000 cells were analyzed. Expression levels were expressed as mean fluorescence intensity (MFI).

2.9. Enzyme-linked immunosorbent assay

The supernatants from DCs were collected and stored at –80 °C

until assayed for cytokines. The concentrations of IL-6, IL-10, TNF-α and IL12p70 were measured with OptEIA (BD Biosciences, San Diego, CA). Assays were performed using the protocols recommended by the manufacturer. The minimum detectable concentrations of IL-6, IL-10, TNF-α and IL-12p70 were determined to be 2.2 pg/mL, 2 pg/mL, 2 pg/mL and 4 pg/mL.

2.10. Statistical analysis

The correlation between rMal f 1 and ImmunoCAP complete allergens of *M. furfur* was analyzed by Kolmogorov-Smirnov Test [27]. The parameters tested are as follows: D_{n, n_1-n_2} = Maximum $(S_{n_1}|x| - S_{n_2}|x|)$, $D_{n, 0.01} = 1.63/$, $D_{n, 0.05} = 1.36/$, $D_{n, 0.10} = 1.23/$, $n = n_1 * n_2 / (n_1 + n_2)$, where n_1 and n_2 denote the number of patient sera tested by rMal f 1 and ImmunoCAP complete allergens of *M. furfur*, respectively. Other statistical analyses were performed using Prism 5 (version 5.03, GraphPad Software). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression and purification of rMal f 1

The rMal f 1 gene fragment (984 bp) without the signal peptides was cloned in-frame with the strep tag II sequence in the expression vector pET-44a to construct recombinant expression plasmids rMal f 1-pET44a (Fig. 1A). Recombinant Mal f 1 cDNA encoding a protein of 339 amino acid residues was confirmed by PCR screening and restriction endonuclease analysis (Fig. 1B and C).

rMal f 1-pET44a was introduced into *E. coli* BL21. After induction with IPTG at 37 °C for 4 h, the resulting protein was shown to be approximately 35 kDa as visualized on Coomassie blue-stained gel (Fig. 2A). The presence of rMal f 1 was confirmed by Western blot analysis because it was specifically recognized by mouse polyclonal anti-strep tag II antibody, whereas no band was detected in the total protein from *E. coli* BL21/pET-44a-Mal f 1 before induction (Fig. 2B). The refolded rMal f 1 was purified by Strep II tag affinity chromatography and showed only one band of interest in SDS-PAGE (Fig. 2C).

3.2. IgE binding ability and correlation of rMal f 1 to complete allergens of *M. furfur*

After screening of sera stored in our serum bank for reactivity to the complete allergens of *M. furfur* (ImmunoCAP), 23 sera, 18 positive and 5 negative, were selected to test for binding ability of specific IgE to rMal f 1 (Table 1). Data from the ELISA assays indicated that all sera with positive reactivity to *M. furfur* had IgE reactivity to rMal f 1, with cut-off value being 0.106 (mean + 2SD from the controls). Kolmogorov-Smirnov Test demonstrated that the IgE binding ability of rMal f 1 as determined by OD values, with correlation coefficient $R = 0.832$, shared the same binding activity with that of ImmunoCAP complete allergens of *M. furfur* (Table 1), suggesting an excellent correlation of IgE binding between rMal f 1 and *M. furfur*.

To confirm the specificity of IgE binding to rMal f 1, the binding ability of the above 23 sera was tested in parallel against an unrelated allergen rBet v 1 by ELISA and immunoblot assay. As shown in Table 1 and Fig. 3A, neither the sera from the 5 healthy controls, nor sera from 17 of the 18 patients with positive rMal f 1 binding, reacted to rBet v 1, with the exception of patient 4 by ELISA. However, reactivity to mite (*Dermatophagoides farinae*, Df) allergens was observed that was in contrast with that seen against rBet v 1. Fourteen of the 18 rMal f 1 positive sera also reacted positively to mite (Df), with correlation coefficient $R = 0.340$. Therefore, the co-sensitization rate between rMal f 1/*M. furfur* and mite (Df) could be as high as 77.8%.

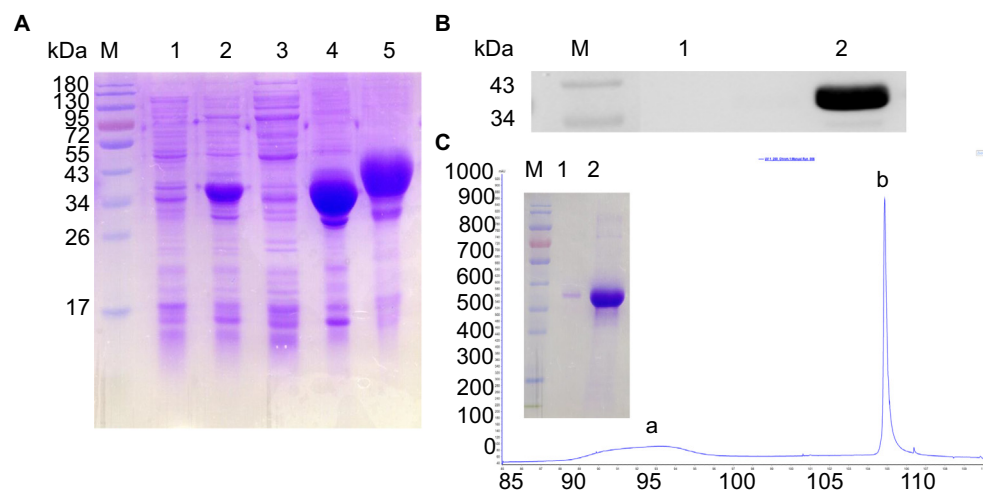


Fig. 2. Expression and purification of the recombinant Mal f 1. The strains BL21-pET44a-rMal f 1 were cultured in LB medium and induced for 4 h by 0.5 mM IPTG. The total extracts were analyzed by SDS-PAGE(A) and Western blot(B), then purified the target protein using a Strep Trap HR column(B). (A). SDS-PAGE identification of the expression of rMal f 1. M, prestained protein ladder, Lane 1 and Lane 2, the total extracts of BL21-pET44a-rMal f 1 before and after IPTG induced. Lane 3, supernatant fraction after sonication, Lane 4, precipitation fraction after sonication. (B). Western blot analysis of the expression of rMal f 1. M, prestained protein ladder, Lane 1 and Lane 2, the total extracts of BL21-pET44a-rMal f 1 before and after IPTG induced. (C). Elution profile of rMal f 1 on a Strep Trap HR column. a, penetration

peak; b, elution peak of the target protein. The inset picture showed the SDS-PAGE analyzed elution fraction before and after ultrafiltration.

3.3. Characterization of immature DCs produced from THP-1 cells

THP-1 cells can be induced to become dendritic cells by culturing with rhGM-CSF (1500 U/mL) and rhIL-4 (1500 U/mL). On day 6 of culture, we harvested these cells and evaluated them for DC surface markers. We observed a significantly increased expression of CD11c and CD209 (DC-SIGN) demonstrating that culturing of THP-1 with rhGM-CSF and rhIL-4 caused cell differentiation that increased the percentage of DC-SIGN⁺ THP-1 cells to > 60% (Fig. 4C). These cells were termed THP-1-derived DCs (TDDCs) and used for further experiments.

3.4. The maturation of TDDCs stimulated with rMal f 1

We checked the TDDCs for surface expression of CD83, a DC maturation marker. As shown in Fig. 5A, we found that unstimulated TDDCs expressed relatively low levels of CD83. After stimulation with LPS, CD83 expression in TDDCs was significantly increased ($P < 0.001$), indicating that LPS could induce TDDC maturation. Stimulation of TDDCs with rMal f 1 also increased the expression of CD83. Furthermore, we confirmed that the protein fraction in the samples of rMal f 1 was responsible for the observed effects by digesting the rMal f 1 with Proteinase K and found that TDDCs treated with rMal f 1 showed

Table 1

Correlation of rMal f 1 to *M. furfur* as determined by the Kolmogorov-Smirnov test.

Patient no.	Sex ^a	Age	ImmunoCAP, KU/L ^b		ELISA, A450 ^c		UniCAP, grade ^d	Kolmogorov-Smirnov test ^e		
			TiGE	sIgE- <i>M.f</i>	rMal f 1	rBet v 1		<i>Fm(aj)-M.f</i>	$\hat{G}m(\hat{aj})$ -rMal f 1	<i>Fm(aj)-\hat{G}m(\hat{aj})</i>
1	M	16	1450.0	24.7	1.302	0.096	6	0.369	0.296	0.073
2	F	41	3852.0	12.8	0.119	0.065	5	0.560	0.323	0.237
3	F	14	497.8	10.10	0.179	0.087	2	0.711	0.364	0.347
4	F	17	29,791.0	6.21	0.648	0.155	6	0.804	0.511	0.293
5	M	9	1278.0	2.69	0.120	0.054	6	0.844	0.538	0.306
6	M	7	10.5	1.63	0.110	0.053	0	0.869	0.563	0.306
7	F	8	321.0	1.10	0.118	0.060	0	0.885	0.590	0.295
8	M	17	77.6	0.99	0.113	0.055	0	0.900	0.616	0.284
9	M	11	13.0	0.85	0.117	0.057	6	0.913	0.642	0.271
10	F	9	919.0	0.82	0.109	0.054	6	0.925	0.667	0.258
11	M	15	153.0	0.79	0.108	0.059	2	0.937	0.691	0.246
12	F	7	2726.0	0.72	0.119	0.062	6	0.948	0.718	0.230
13	M	27	655.0	0.67	0.143	0.078	6	0.958	0.751	0.207
14	F	39	337.0	0.64	0.113	0.058	5	0.967	0.777	0.190
15	F	21	138.8	0.59	0.108	0.056	3	0.976	0.801	0.175
16	F	22	689.0	0.52	0.107	0.050	3	0.984	0.825	0.159
17	F	33	181.0	0.50	0.140	0.072	0	0.991	0.857	0.134
18	M	10	103.8	0.47	0.124	0.059	2	0.998	0.885	0.113
19	M	45	146.8	0.12	0.101	0.078	4	1.000	0.908	0.092
20	F	5	97.8	0	0.100	0.049	4	1.000	0.931	0.069
21	F	32	70.7	0	0.102	0.083	0	1.000	0.954	0.046
22	F	8	15.5	0	0.097	0.059	0	1.000	0.976	0.024
23	F	4	123.0	0	0.104	0.050	0	1.000	1.000	0.000

Notes:

^a M = male; F = female.

^b TiGE, ImmunoCAP total IgE; SiGE, ImmunoCAP specific IgE; *M.f* = *Malassezia furfur*.

^c Recombinant proteins Mal f 1 or Bet v 1 were coated on ELISA plates, cutoff values are 0.106 for rMal f 1, 0.096 for rBet v 1.

^d Phadia UniCAP grades are as follows: Grade 0, < 0.35 kU/L; Grade 1, 0.35–0.75 kU/L; Grade 2, 0.75–3.5 kU/L; Grade 3, 3.5–17.5 kU/L; Grade 4, 17.5–50 kU/L; Grade 5, 50–100 kU/L; Grade 6, > 100 kU/L.

^e The KS-test uses the maximum vertical deviation between the two distribution curves as the statistic D ($D = Fm(aj) - \hat{G}m(\hat{aj})$). For our test, the maximum difference in cumulative fraction is $D = 0.347$, which is less than all the cutoff values, i.e., standards, $D_{12,0.01} = 0.4705$; $D_{12,0.05} = 0.3926$; $D_{12,0.10} = 0.3551$. Hence we should accept the null hypothesis that the two distributions are the same.

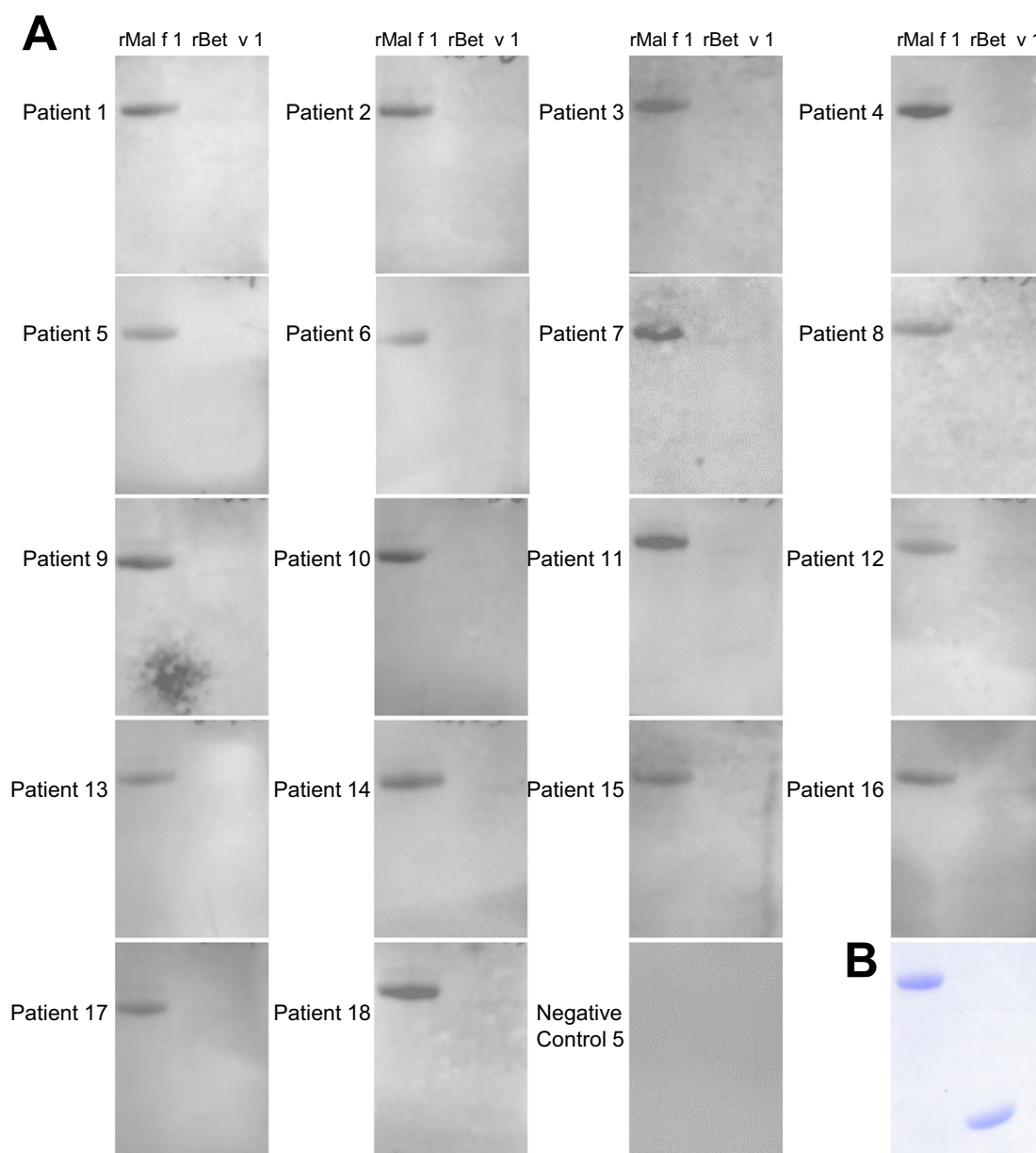


Fig. 3. Immunoblot analysis of purified rMal f 1 and unrelated protein rBet v 1 using sera from *M. furfur* whole extract specific IgE positive patients and SDS-PAGE analysis of this two protein. (A) Immunoblot analysis. (B) SDS-PAGE and Coomassie Brilliant Blue staining of rMal f 1 and rBet v 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a significant increase in CD83 expression when compared with TDDCs treated with digested rMal f 1 ($P < 0.001$) (Fig. 5B). These data indicate that untruncated rMal f 1 proteins are better at inducing TDDC maturation than digested rMal f 1 or LPS that may be present in the samples.

3.5. The expression of co-stimulatory markers and HLA-DR on TDDCs

We next investigated the expression of HLA-DR and two co-stimulatory molecules (CD86 and CD80) to detect antigen presentation, another indicator of DC maturation. Following 24 h of stimulation with the protein fraction containing rMal f 1, expression of CD86, CD80 and HLA-DR was significantly increased, compared to unstimulated cells. Stimulation with rMal f 1 significantly increased the MFI of CD80 ($P < 0.005$) (Fig. 6A) and CD86 ($P < 0.05$) (Fig. 6B) as well as the MFI of HLA-DR ($P < 0.05$) (Fig. 6C). These results suggest that rMal f 1

promotes the maturation of TDDCs.

3.6. Cytokine secretion from TDDCs treated with rMal f 1

Since cytokines secreted by DCs can shape the nature of the adaptive immune response, we further examined the secretion of pro-inflammatory and anti-inflammatory cytokines from rMal f 1-treated TDDCs by ELISA. The results showed that LPS stimulation dramatically increased the production of IL-6, TNF- α and IL-10 by the TDDCs compared with production by the TDDCs incubated in medium alone. Compared to unstimulated TDDCs, treatment with rMal f 1 also led to an extremely significant increase of IL-6 ($P < 0.0001$, Fig. 7A) as well as a large fold increases in TNF- α ($P < 0.01$, Fig. 7B) and IL-10 ($P < 0.05$, Fig. 7C) secretion. IL-12p70 levels were not significantly increased by the addition of either LPS or rMal f 1 (Fig. 7D).

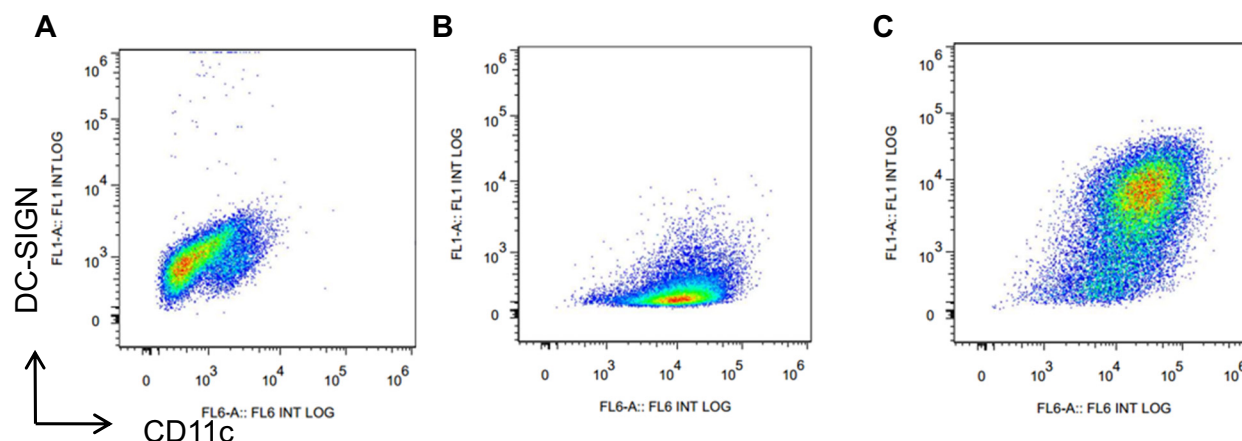


Fig. 4. The expression of DC-SIGN on THP-1 cells induced of GM-CSF and IL-4. THP-1 cells were treated with (B) or without (C) rhGM-CSF (1500 U/mL) and rhIL-4 (1500 U/mL) for 6 days, and then stained with either parallel IgG isotype control antibodies (A) and FITC-anti-DC-SIGN and APC-anti-CD11c.

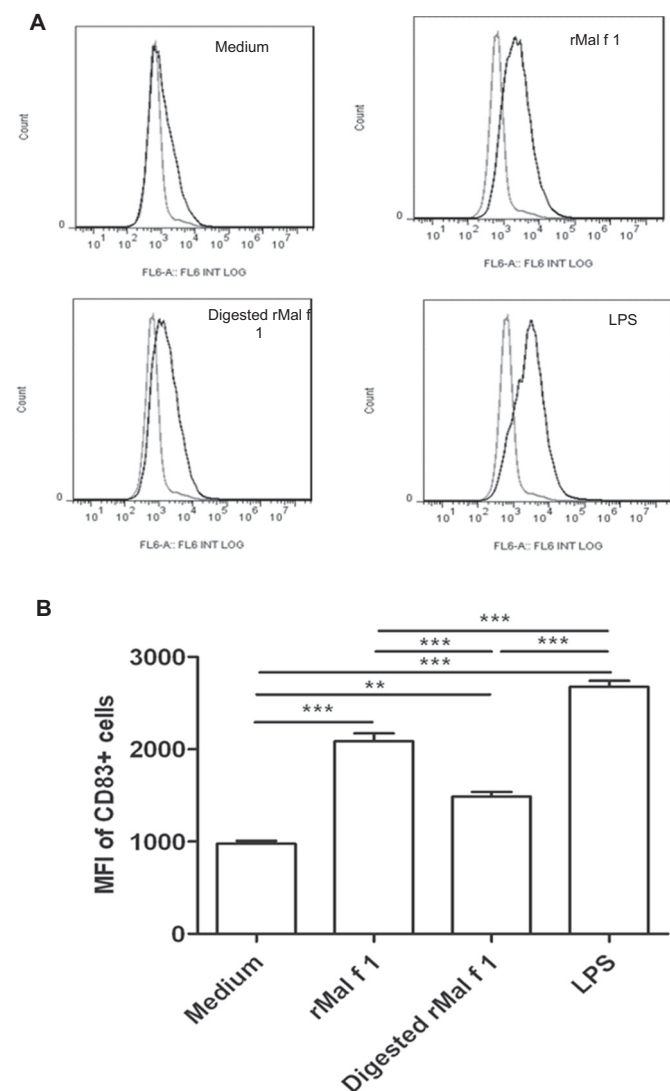


Fig. 5. Flow cytometric analysis of mature marker CD83. THP-1 cells were pulsed with rMal f 1 (40 µg/mL) for 24 h, and then stained with antibodies to CD83. The data of CD83 expression are shown as histograms (A) and as MFI (B). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4. Discussion

In order to characterize the properties of the major allergens of *M. furfur*, Mal f 1 has been produced in different expression systems [13]. Since the Mal f 1 protein does not contain any cysteine residues, we produced rMal f 1 in the *E. coli* expression system and the signal sequence was cleaved off [11]. Several studies have demonstrated that rMal f 1 possesses reasonable IgE binding ability [10]. Our data confirm that rMal f 1 activates the IgE dependent pathway. Additionally, we have shown here that rMal f 1 is recognized by specific IgE in all patient sera positive to complete allergens of *M. furfur* and that patient sera that tested negative to *M. furfur* also showed negative reactivity to rMal f 1. On the other hand, no reactivity was observed to an unrelated allergen rBet v 1 in all, except for one, rMal f 1 positive sera. These data show that reactivity to rMal f 1 correlates well with reactivity to ImmunoCAP complete allergens of *M. furfur* and could have ideal diagnostic potential when discriminating *M. furfur* allergic patients from pollen allergic patients in that rMal f 1 can be used for detecting IgE specifically against *M. furfur*.

Though rMal f 1 has been shown to exhibit the same binding ratio as that seen with ImmunoCAP complete allergens of *M. furfur*, the data of the ELISA assay were somewhat inconsistent with the ImmunoCAP values. For example, the ImmunoCAP value for *M. furfur* of patient 2 (12.80 kU/L) was greater than that of patient 4 (6.21 kU/L), but the ELISA value (OD₄₅₀) for rMal f 1 in patient 2 (0.119) was less than that in patient 4 (0.648). The same trend was observed in the immunoblotting analysis of rMal f 1 for patients 2 and 4 (Fig. 3A).

The possible explanation for the difference between the ImmunoCAP and ELISA data is that *M. furfur* ImmunoCAP extracts contain a wide range of IgE-binding proteins [28–31]. In this study, we used purified rMal f 1 in the ELISA and immunoblot assays in order to eliminate any influence coming from other proteins. Another possibility may be that the serum of patient 2 may have reactivity to other proteins that cross-react with *M. furfur* [30,32]. Nevertheless, there is no evidence for cross-reactive IgE-binding between Mal f 1 and other yeast proteins [29] and it has been documented that Mal f 1 has no homologues with known proteins in other organisms [11]. We have also shown that rMal f 1 and/or complete allergens of *M. furfur* had as high as 77.8% (14/18) co-sensitization with mite allergens. In general, when cross reactivity of allergens is suspected, co-sensitization is the preferred term [33]. However, it is important to explore the underlying mechanism to answer the question whether this co-sensitization is triggered by cross-reactivity or not.

THP-1 is a human monocytic leukemia cell line originating from the blood of a boy with acute monocytic leukemia [34]. THP-1 cells can be differentiated into highly pure, monotypic, and well-defined DCs

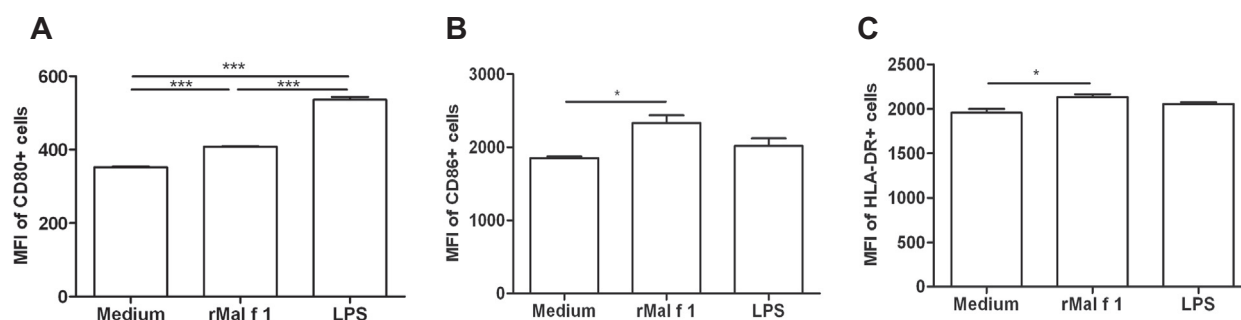


Fig. 6. Flow cytometric analysis of cell surface molecules. TDDCs were pulsed with rMal f 1 (40 μ g/mL) for 24 h, and then stained with antibodies to CD80, CD86 and HLA-DR. The expressions of CD80 (A), CD86 (B) and HLA-DR (C) were shown as MFI. * P < 0.05, ** P < 0.01, *** P < 0.001.

(TDDCs) [18]. In the present study, we successfully induced THP-1 cells to become DCs, i.e., TDDCs, which secreted IL-6, IL-10 and TNF- α but not IL-12 after rMal f 1 activation. This result was corroborated by similar studies. Gao et al. [35] also observed significantly elevated levels of TNF- α and IL-10, but no increase in IL-12p70 expression, from co-cultured pDCs and CD4+ T cells stimulated with cockroach antigen [35]. After stimulation with *Aggregatibacter actinomycetemcomitans* serotype B, elevated levels of IL-6 and TNF- α were detected in DCs, as well as higher levels of IL-22 in T lymphocytes [36]. IL-10, a cytokine belonging to the same family with IL-22, has multiple and pleiotropic effects in immunoregulation and inflammation. Notably, Buentke et al. demonstrated that production of TNF- α by monocyte-derived dendritic cells increased significantly after the addition of *M. furfur*, while IL-12p70 levels remained unaltered [17]. IL-6 is a pleiotropic factor with biological activity and can promote DC generation [37], and thereby initiate TH22 and TH17 polarization [38,39]. IL-10, up-regulated after stimulation by rMal f 1, is a regulatory cytokine which inhibits both antigen presentation and subsequent pro-inflammatory cytokine release [40]. However, IL-12, a key inducer of differentiation toward the TH1 phenotype [41], was unaltered by treatment with rMal f 1, suggesting that rMal f 1 does not induce polarization toward a TH1 phenotype. Therefore, it is tempting to postulate that TDDCs stimulated with Mal f 1 can be directed to the TH22/TH17 pathway, but not the TH1 pathway, under conditions where high-levels of IL-6, IL-10 and TNF- α secreted by TDDCs are present. This deduction has been supported by the very

recent research on psoriatic skin inflammation [42], where the TH17 pathway, but not the TH22 pathway, was shown to be involved in the mechanism [43]. Furthermore, Mal f 1 may be the major representative allergen in *M. furfur*, which could potentially function as a representative allergen to the entire fungi and can differentiate TDDCs toward the TH22/TH17 pathway. Another recent work demonstrated that Asian atopic dermatitis patients are characterized by prominent TH2 and TH22 signatures that correlate with the non-lesional skin profile, suggesting that serum endotyping can serve as a surrogate for assessing disease extent beyond apparent AD lesions [44]. This research is highly consistent with our deduction that Mal f 1-stimulated TDDCs favor a TH2- and TH22-bias. However, the discrepancy between the two above studies urges us to further investigate the communication of DCs and T cells and the subsequent immune response.

In total and based on the results using our expressed and purified rMal f 1, we demonstrated that rMal f 1 has ideal IgE-binding ability and good correlation with binding to *M. furfur*, suggesting that rMal f 1 can be play an important role in the diagnosis of *M. furfur* related diseases. In addition, rMal f 1-activated DCs had a special profile in that TNF- α , IL-6, and IL-10 were significantly increased while IL-12 was unchanged, suggesting that rMal f 1 can switch DCs bias toward the TH22/TH17 pathway rather than just the routine IgE-dependent TH2 pathway and implying that treatment that targets only the IgE-dependent pathway might not produce significant efficacy. Therefore, it is tempting to suggest that both the TH2 and TH22/TH17 pathways should

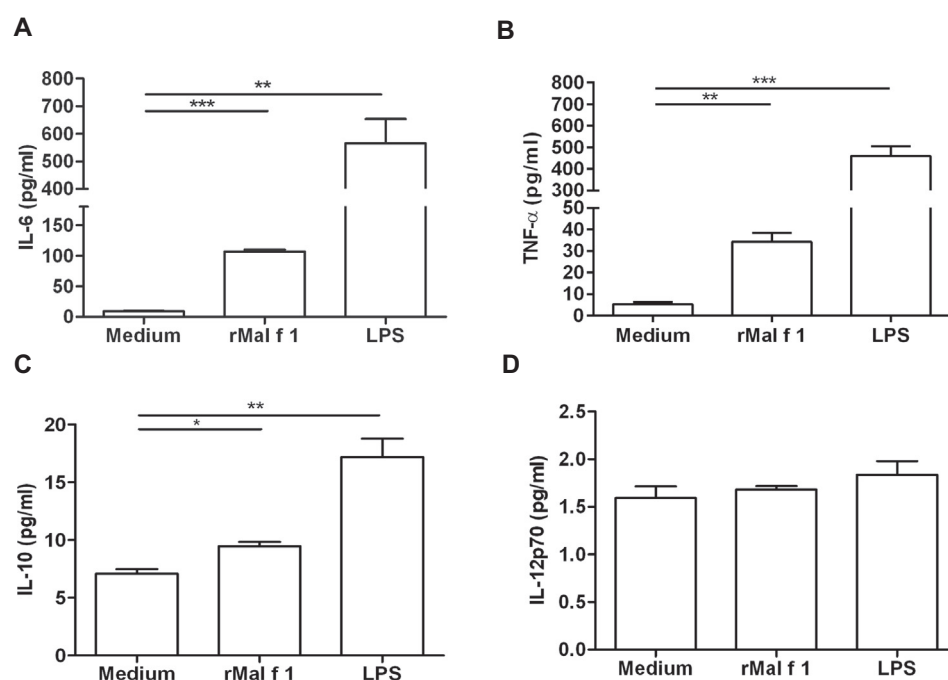


Fig. 7. Changes in secretion profiles of IL-6 (A), TNF- α (B), IL-10 (C) and IL-12p70 (D) in rMal f 1 stimulated TDDCs. TDDCs were treated with rMal f 1 for 24 h. ELISA measured the culture supernatants for IL-6, TNF- α , IL-10 and IL-12p70 secretion. Data are presented as Means $\pm$ SEM of three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001.

be addressed when determining the treatment of *M. furfur* triggered diseases.

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