

Elevated Levels of IgG and IgG4 to *Malassezia* Allergens in Atopic Eczema Patients with IgE Reactivity to *Malassezia*

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Key Words

Atopic eczema · Atopy patch test · IgE · IgG4 ·
Malassezia · Recombinant allergens · Skin prick test

Abstract

Background: The opportunistic yeast *Malassezia* is considered to be one of the factors that can contribute to atopic eczema (AE). Elevated serum IgE levels, T-cell proliferation and positive skin prick test (SPT) and atopy patch test (APT) reactions to *Malassezia* are found among AE patients. **Methods:** Sera from 127 AE patients, 14 patients with seborrheic dermatitis (SD) and 33 healthy controls were investigated for IgE and IgG4 to *M. sympodialis* extract and four recombinant *Malassezia* allergens; rMala s 1, rMala s 5, rMala s 6, and rMala s 9. In addition, IgG to the recombinant allergens was analyzed. The IgG and IgG4 levels were compared to IgE levels and in vivo reactions (SPT and APT) to *Malassezia*. **Results:** AE patients with serum IgE levels >0.35 kU/l to *M. sympodialis* extract had significantly higher IgG4 levels to *M. sympodialis* extract than AE patients without detectable serum IgE to *M. sympodialis* extract, SD patients and healthy controls. Among the AE patients with and without detectable serum IgE to *M. sympodialis* extract,

respectively, there were no differences in IgG4 levels between patients with positive or negative in vivo reactions to *M. sympodialis* extract. IgG4 to the rMala s allergens was almost exclusively found among patients with IgE to the same allergen. Within the four tested rMala s allergens, most IgG4 reactions were found to rMala s 6, an allergen with homology to cyclophilin. **Conclusions:** Elevated serum IgG4 to *M. sympodialis* extract accompanies elevated serum IgE to the extract. This is further confirmed by the association between IgG/IgG4 and IgE to recombinant *Malassezia* allergens.

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Introduction

Atopic eczema (AE) is a common inflammatory skin disease characterized by a chronically relapsing course, a distinctive clinical appearance and severe pruritus. The pathogenesis of AE is still not fully understood. AE is believed to be an aggregation of several diseases with certain clinical characteristics in common [1], which was also reflected in the term 'atopic eczema/dermatitis syndrome' recently proposed by the European Academy of Allergology and Clinical Immunology [2]. The majority of AE

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Table 1. Characterization of the subjects

	n	Gender F/M	Age years	SCORAD [14]	Rhino- conjunc- tivitis and/or asthma, %	Total serum IgE ^a		Phadiatop [®] positive ^b %	Serum IgE (<i>M. sympodialis</i> extract) ^c		<i>M. sympodialis</i> extract % positive	
						kU/l	>122 kU/l, %		kU/l	positive %	SPT	APT
AE patients	127	79/48	31 (18–55)	37 (8–86)	78	310 (2–23,800)	70	78	5.5 (0.39–33.5)	54	52	39
SD patients	14	7/7	33 (18–55)	NA	0	9 (2–45)	0	7	<0.35	0	0	0
Healthy controls	33	29/4	38 (21–55)	NA	0	25 (4–255)	18	24	<0.35	0	0	0

Medians and ranges (in parentheses) are given for age, SCORAD and serum IgE. F = Female; M = male; NA = not applicable.

^a ImmunoCAP[™], reference range 1.6–122 kU/l.

^b Phadiatop[®], serum IgE to any of 11 common aeroallergens.

^c Sepharose RAST, reference range <0.35 kU/l.

patients have elevated serum IgE and positive skin prick test (SPT) reactions to a wide variety of allergens. However, approximately 20% of patients with the clinical phenotype of AE have low serum IgE levels and lack detectable environmental allergen-specific serum IgE and positive skin prick test (SPT) reactions [1, 3].

The opportunistic yeast *Malassezia*, previously denoted *Pityrosporum*, has for nearly 20 years been discussed as an allergen possibly triggering AE, especially in adults. Eight *Malassezia* species have been described so far, all being present on human skin [4, 5]. There are several reports that demonstrate specific serum IgE, positive SPT and/or positive atopy patch test (APT) reactions to *Malassezia* [4]. Several recombinant *Malassezia* allergens have become available for in vivo and in vitro tests [4]. Using *M. sympodialis* extract and three recombinant allergens, we have recently shown positive reactions in serum IgE, SPT and/or APT in 67% of a group of 132 adult AE patients [6]. The pattern of reactions divides the patients in subgroups, further suggesting differences in their immune response.

The APT, frequently used for research purposes, has macroscopic and microscopic similarities with the acute AE lesion [7]. In a study on the response of peripheral blood mononuclear cells to *Malassezia* we showed a correlation between a Th2-like T-cell response and positive APT reactions, which was significantly more pronounced than the correlation between serum IgE and positive APT reactions [8]. The association between T-cell-mediated allergen-specific responses and positive APT reactions has also been demonstrated by others [9].

These findings raise the question whether differences in other parts of the immune response may have an

impact on the symptoms in AE. A correlation between *Malassezia*-specific IgG levels in the AE patients and APT reactions to *Malassezia* has not been found [10]. No studies regarding IgG4 responses to *Malassezia* have been done. Apart from IgE, IgG4 is the isotype that has been associated with Th2-like immune responses in humans, but its biological function is poorly understood [11, 12].

In this study, we examined the presence of IgG4 to *M. sympodialis* extract and IgG and IgG4 to four recombinant *Malassezia* allergens in sera from patients with AE or seborrheic dermatitis (SD) and healthy controls.

Materials and Methods

Subjects

The study included 127 adult patients, with mild-to-severe AE according to the UK Working Party Criteria [13], 14 patients with SD and 33 healthy controls, all participants of a previous study [6] (table 1). Exclusion criteria were eczema only on the hands, autoimmune diseases, inflammatory skin diseases other than AE, immunosuppressive treatment or use of systemic corticosteroids or solarium in the previous 2 months. No topical antifungal treatment was permitted for 1 month before the study, and no topical corticosteroid on the skin test sites was allowed the last week before the study. Antihistamine was withdrawn 5 days before the investigation. The healthy controls had no clinical symptoms or history of allergy or skin diseases. The subjects gave their informed consent to the study, which was approved by the regional ethics committee.

M. sympodialis Extract and Recombinant Allergens

Crude yeast extract was prepared from strain No. 42132 (American Type Culture Collection), as previously described [15]. This strain was earlier denoted *Pityrosporum orbiculare* or *Malassezia furfur*, but recent retyping has shown it to be *M. sympodialis* [16]. Recombinant *Malassezia* allergens rMala s 1, rMala s 5, rMala s 6 and rMala s 9 (corresponding to allergens expressed naturally in

strain No. 42132, American Type Culture Collection) [17–20] were produced in the *Escherichia coli* system as described before [17–19]. *Malassezia* allergens have previously been denoted as Mal f/Mal s. However, with increasing numbers of allergens from different sources, the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies (<http://www.allergen.org/list.htm>) has reclassified the *Malassezia* allergens to be denoted Mala (since Mal stands for apple). This new nomenclature will be used here.

SPT and APT to M. sympodialis Extract

In parallel with the present study, all subjects were investigated for SPT and APT reactions to the crude *M. sympodialis* extract. The SPT was performed according to a standard procedure [21] in a concentration of 100 µg protein/ml and evaluated after 15 min. Phosphate-buffered saline (PBS) served as a negative control and histamine dihydrochloride (10 mg/ml, ALK, Hørsholm, Denmark) as a positive control. The reactions were graded as mean diameter in millimeters, a reaction with a mean diameter ≥ 3 mm being considered as positive. The APT was performed as previously described [10]. Briefly, the allergens or the vehicle control PBS were applied on paper discs in Finn chambers (8 mm; Epitest, Tuusula, Finland) on tape-stripped, non-lesioned skin of the back. The *M. sympodialis* extract was tested in a twofold serial dilution of 5.0–0.6 mg/ml. The APTs were removed after 48 h, and the skin reactions were evaluated. The test results were scored from 0 to +++, where 0 = negative reaction; + = erythema, infiltration and possible papules; ++ = erythema, infiltration, papules and small vesicles, and +++ = erythema, infiltration, papules and large vesicles [10, 22]. In the present study, a reaction of + or more in at least one concentration was regarded as APT positive.

IgE and IgG4 RAST

Specific serum IgE and IgG4 to *M. sympodialis* extract and specific serum IgE to rMala s 9 were analyzed with a Sepharose-based RAST method as described before [23]. In brief, the allergens, *M. sympodialis* extract for IgE and IgG4 and rMala s 9 for IgE, were coupled to CNBr-activated Sepharose-4B (Pharmacia Biotech, Uppsala Sweden) according to the manufacturer's instruction. Three-hundred micrograms of extract or 100 µg recombinant allergen, respectively, were coupled per 100 mg Sepharose. The Sepharose concentration was adjusted to 2 mg/ml in PBS/0.3% bovine serum albumin (BSA)/0.2% Tween-20. Two-hundred and fifty microliters (0.5 mg) of allergen-coated Sepharose were incubated overnight with patient serum, 50 µl in a single sample for the IgE RAST or 20 µl in duplicates for the IgG4 RAST at room temperature. After washing 5 times with PBS/0.05% Tween-20, a ^{125}I -radiolabeled sheep anti-human IgE or a ^{125}I -radiolabeled mouse monoclonal anti-human IgG4 (CLB, Amsterdam, The Netherlands) was added. After incubation overnight, rotated end over end, at room temperature, the Sepharose was washed four times with PBS/0.05% Tween-20 and γ -radiation was counted. The results for IgE RAST were converted into kU/l using a chimeric IgE antibody [24] and for the IgG4 RAST into ng/ml using a chimeric IgG4 antibody [25].

IgE Analysis with ImmunoCAP™

For analysis of specific serum IgE to the recombinant *Malassezia* allergens rMala s 1, rMala s 5, and rMala s 6, the allergens were immobilized onto cellulose solid phase (ImmunoCAP) by covalent binding (MIAB, Uppsala, Sweden). Coupling concentration was

adjusted to attain a linear measurement range and a background below 0.35 kU/l. The recombinant ImmunoCAPs were tested in the Pharmacia CAP system™ according to the manufacturer's instructions. For all IgE analyses, a value ≥ 0.35 kU/l was considered as positive.

Measurements of IgG and IgG4 to the Recombinant Malassezia Allergens

The rMala s allergens were radiolabeled by the chloramine-T method with carrier-free ^{125}I (Amersham, Amersham, UK). Unbound ^{125}I was separated from the labeled allergen by passing through an Ultrogel ACA54 column (BioSeptra SA, Cergy-Saint-Christophe, France). The ^{125}I -labeled allergens were diluted in PBS/0.3% BSA, and the volume was adjusted to give an activity of 20,000 cpm/50 µl, except for rMala s 5 where an activity of 2,000 cpm/50 µl was used.

IgG and IgG4 were measured in allergen-binding assays as described before [26, 27]. For IgG measurement, 20 µl patient serum were incubated with Sepharose-coupled protein G (0.75 mg/test) and 50 µl ^{125}I -labeled allergen in a final volume of 800 µl PBS/0.3% BSA/0.2% Tween-20. After incubation overnight at room temperature, rotated end over end, the Sepharose was washed four times with PBS/0.05% Tween-20 before the γ -radiation was counted. Those samples that were IgG positive were further analyzed for IgG4.

The IgG4 assay was performed in two subsequent incubation steps. First 20 µl of patient serum were incubated with Sepharose-coupled mouse monoclonal anti-IgG4 (1.0 mg/test) in a final volume of 750 µl PBS/0.3% BSA/0.2%. After incubation overnight at room temperature, rotated end over end, the Sepharose was washed five times with PBS/0.05% Tween-20, and subsequently 50 µl ^{125}I -labeled allergen and 500 µl PBS/0.3% BSA/0.2% Tween-20 were added. After a second overnight incubation at room temperature, rotated end over end, the Sepharose was washed four times with PBS/0.05% Tween-20 before the γ -radiation was counted.

Both the IgG and the IgG4 assay were performed in duplicate. The results are expressed as percent binding of total counts added (<2% was regarded as negative).

Statistical Analysis

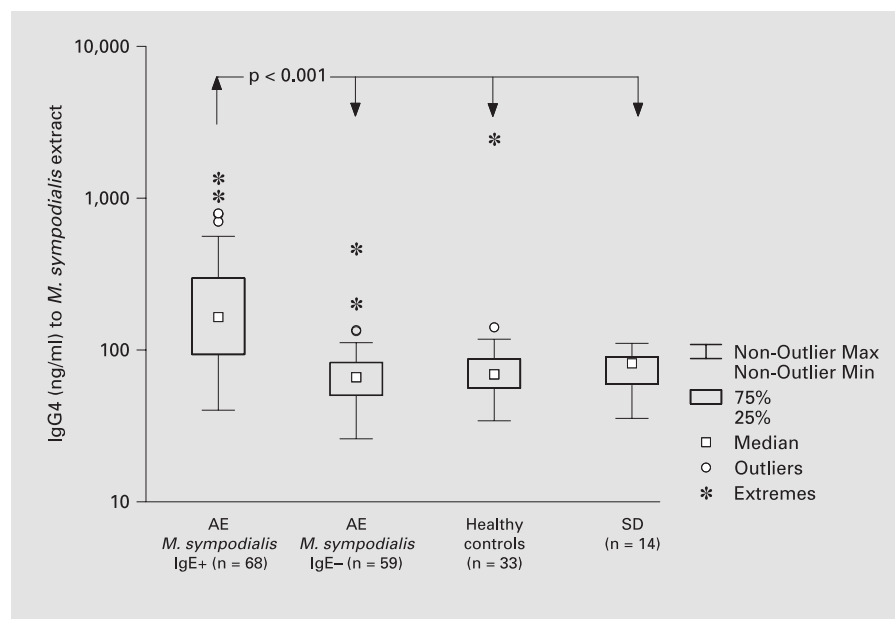
Differences between two groups were analyzed with the Mann-Whitney U test. Differences among more than two groups were first analyzed with Kruskal-Wallis ANOVA by ranks followed by post hoc comparisons with the Mann-Whitney U test. Correction for multiple comparisons was made with the Bonferroni method. The significance tests followed a two-sided alternative hypothesis. Correlations were calculated using Spearman's rank correlation analysis. A p value <0.05 was considered statistically significant.

Results

AE Patients with IgE Antibodies to M. sympodialis Extract Have Elevated Levels of IgG4 to the Yeast Extract

Sixty-eight (54%) of the 127 AE patients had elevated serum IgE levels to *M. sympodialis* extract (table 1). Significantly higher serum IgG4 levels to *M. sympodialis* extract were found in these patients than in the AE

Fig. 1. IgG4 levels to *M. sympodialis* extract in AE patients with and without detectable serum IgE to the extract, healthy controls and SD patients. Serum IgE to *M. sympodialis* extract was analyzed with ImmunoCAP™ (m70, Pharmacia Diagnostics) and serum IgG4 with a RAST method. The arrows indicate statistically significant differences. The differences between the four groups were first analyzed with Kruskal-Wallis ANOVA by ranks ($p < 0.001$) followed by post hoc comparisons with the Mann-Whitney U test. Correction for multiple comparisons was made with the Bonferroni method.



patients without detectable serum IgE to *M. sympodialis* extract (fig. 1). No differences in serum IgG4 levels to *M. sympodialis* extract were found among AE patients without detectable serum IgE to *M. sympodialis* extract, SD patients and healthy controls (fig. 1).

No Indication of a Protective Activity by IgG4 to in vivo Reactivity to M. sympodialis Extract

The AE patients with IgE antibodies to *M. sympodialis* extract had significantly higher IgG4 levels than the IgE-negative patients regardless of their SPT or APT reactivity ($p < 0.01$) except for the IgE-negative/SPT-positive ($n = 7$) and IgE-positive/SPT-negative patients ($n = 9$, $p = 0.104$; fig. 2).

Fifty-nine of the 68 AE patients with serum IgE reactivity to *M. sympodialis* extract also responded with positive SPT reaction (fig. 2a). There were neither significant differences in IgG4 levels to *M. sympodialis* extract between those 59 patients and the 9 SPT-negative ones ($p = 0.17$, fig. 2a) nor in IgG4 levels between the SPT-negative and SPT-positive groups within the *M. sympodialis* extract IgE-negative AE patients (fig. 2a).

Positive APT reactions to *M. sympodialis* extract were more frequent among AE patients with serum IgE reactivity to *M. sympodialis* extract, but were also found in 8 of the 59 AE patients without detectable serum IgE to the extract (fig. 2b). Both APT-positive and APT-negative patients with serum IgE to the extract had significantly higher IgG4 levels to the extract than the serum IgE-nega-

tive APT-positive and APT-negative patients ($p < 0.001$, fig. 2b). No differences in IgG4 levels were found between APT-positive and APT-negative patients within the *M. sympodialis* extract IgE-positive or IgE-negative group, respectively (fig. 2b).

Association between IgE/IgG Antibodies and Separate Malassezia Allergens

Three of the four recombinant allergens, Mala s 5, 6 and 9, bound efficiently (i.e. more than 50% of the added radioactivity was bound) to IgG in at least some of the sera. However, the maximal immunoreactivity of iodinated Mala s 1 was only 15%. IgG to each of the three most immunoreactive allergens was strongly associated with serum IgE reactivity to the same allergen (table 2). The highest number of IgE-positive AE patients was found for rMala s 9 (38%), which was the least efficient in inducing IgG response (7%, i.e. 9 out of 125 patients). The highest proportion of IgG-positive individuals (21%, i.e. 23 + 4 out of 127 patients) was found for rMala s 6, with average IgE induction (25%; table 2). Despite this distinction, the antibody responses to rMala s 6 and rMala s 9 were still highly significantly associated, both for IgE and for IgG.

Non of the SD patients, but 2 of the healthy controls were IgG positive to rMala s 6 and 1 to rMala s 5. Both the SD patients and the healthy controls were negative in IgE and IgG4 to the rMala s allergens (data not shown).

Fig. 2. IgG4 levels to *M. sympodialis* extract in AE patients divided according to serum IgE reactivity and SPT (a) or APT (b) reactivity to the extract. Serum IgE and serum IgG4 to *M. sympodialis* extract were analyzed with RAST methods. The differences among the four groups were first analyzed with Kruskal-Wallis ANOVA by ranks ($p < 0.001$) followed by post hoc comparisons with the Mann-Whitney U test. Correction for multiple comparisons was made with the Bonferroni method. The arrows indicate statistically significant differences.

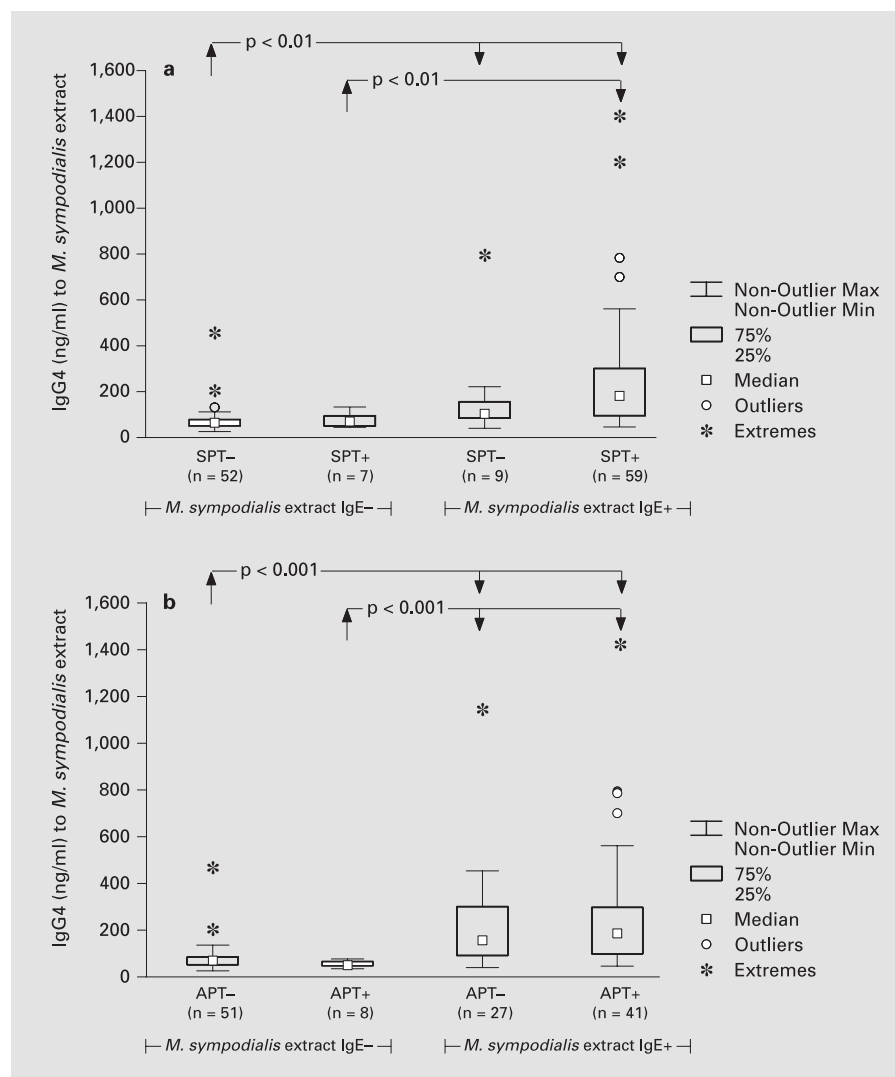


Table 2. Serum levels of IgE, IgG and IgG4 to the recombinant *Malassezia* allergens in the AE patients

Allergens	All AE patients		Serum IgE-positive patients				Serum IgE-negative patients			
	IgE-positive		IgG positive		IgG4 positive		IgG positive		IgG4 positive	
	n	%	n	%	n	%	n	%	n	%
rMala s 1	24/127	19	10/24	42	1/24	4	4/103	4	0/103	
rMala s 5	37/127	29	15/37	41	8/37	22	1/90	1	0/90	
rMala s 6	32/127	25	23/32	74	19/32	59	4/95	4	1/95	1
rMala s 9	47/125	38	9/47	19	4/47	9	0/78		0/78	

Numbers (%) of IgE-positive patients are given in comparison to the number of patients tested for the stated allergen. Serum IgE to rMala 1, 5 and 6 were tested with ImmunoCAP and serum IgE to rMala s 9 was tested with RAST. A serum IgE level >0.35 kU/l to the stated allergen was regarded as positive. Two AE patients were not tested for IgE to rMala s 9 due to lack of serum. None of those 2 patients were positive for IgG to rMala s 9. One of them was positive and 1 was negative in ImmunoCAP for serum IgE to *M. sympodialis* extract. Numbers (%) of IgG- and IgG4-positive patients are given in comparison to the number of patients IgE-positive or IgE-negative, respectively, to the stated allergen. The antigen binding test was used for IgG4. More than 2% binding was regarded as positive.

Relative IgG4 Reactivity of the Individual rMala s Allergens

For rMala s 5 and rMala s 6, the contribution of IgG4 to the IgG response was high compared to that for rMala s 1 and rMala s 9. Among the IgG-positive samples, the IgG4 contribution was more than 20% of the IgG response to rMala s 5 in 6/15 (40%) and to rMala s 6 in 10/24 (42%). In contrast, for rMala s 1 this was the case in 0/10 cases and for rMala s 9 in 2/9 cases.

Discussion

IgE to the yeast *Malassezia* is almost exclusively detected in adult patients with AE [28–31], while in previous reports no difference in IgG levels to *Malassezia* was found between AE patients with or without IgE to *Malassezia*, healthy controls or patients with other *Malassezia*-induced diseases [4]. In this study, we have shown that the serum IgG4 levels to *M. sympodialis* extract were significantly higher in AE patients with IgE reactivity to the extract. This elevated serum IgG4 level was closely associated with the presence of IgE to *M. sympodialis* extract, although no strong linear correlation was observed (Spearman rank $r = 0.4$, $p < 0.001$).

Also, for IgG to individual recombinant allergens, a clear distinction was found between AE patients with IgE to *M. sympodialis* extract and the three other groups (AE patients without IgE to *M. sympodialis* extract, SD patients and healthy controls). The reason for the discrepancy in IgG results obtained with whole *M. sympodialis* extract (i.e. no distinction between AE and controls) [10] might be due to the presence of other, antigenic but not allergenic, antigens in whole extract. These might well be cross-reactive polysaccharides, which are known to induce largely IgG2 antibodies rather than IgG4 antibodies [26].

The contribution of IgG4 to this IgG response was highest for rMala s 5 and rMala s 6, but still modest compared to other situations with chronic high antigen exposure, such as immunotherapy or beekeepers [26], and even lower for rMala s 1 and rMala s 9. These results suggest that additional IgG4-binding antigens are likely to be involved in the response to *M. sympodialis* extract. Since the IgG4 response to whole extract clearly discriminates between AE patients with IgE response to the extract and the other subjects, these additional antigens are likely to be distinct from the polysaccharide-type antigens suggested in the section on the total IgG response.

In a recent study involving school children, Hesselmar et al. [32] has shown that cat keeping was associated with a modified Th2 response, producing IgG4 but not IgE antibodies. However, in agreement with our results, the occurrence of IgG4 antibodies did not directly mediate any protective effect in children producing both IgG4 and IgE to cats. Platts-Mills et al. [33] suggested a modification of the Th2 immune response, rather than a shift towards Th1, as one possible explanation for the development of tolerance. In our study, the Th2 response to *Malassezia* consists of both IgG4 and IgE antibodies. This may be due to the nature of the allergen and/or dose and exposure time [26]. It could also be speculated if the route of exposure is of importance for the development of IgG4 and IgE antibodies to *Malassezia*. Serum IgE to *Malassezia* are rarely found in atopic individuals not suffering from AE [4, 31]. One possibility is that the disrupted skin barrier in the AE patients facilitates the allergen contact and uptake by professional antigen-presenting cells, such as the Langerhans cells, leading to sensitization and perpetuation of the chronic inflammatory eczema [34, 35]. Alternatively, the inhalation route of exposure could also be concerned. The skin flakes produced from the eczema may spread *Malassezia* allergen that could be inhaled as an airborne allergen.

We have previously shown that positive APT reactions to *Malassezia* have a stronger correlation to a Th2-like T-cell response than to elevated levels of *Malassezia*-specific serum IgE [8]. That finding strongly supports that the T-cell response is another crucial factor for allergen-specific eczema reactions in AE in addition to antigen presentation mediated by specific IgE bound to epidermal Langerhans cells [36, 37]. The lack of positive APT reactions to *Malassezia* in certain AE patients with serum IgE reactivity to *Malassezia* may rather be explained by low levels of *Malassezia*-specific Th2-like T cells [8] than by the blocking of IgG4 antibodies.

Within the four rMala s allergens tested, the highest prevalence for IgG reactivity was found for rMala s 6, which also showed the highest ability to induce IgG4. rMala s 6 has sequence similarity to cyclophilin from *Aspergillus fumigatus* and *Schizosaccharomyces pombe* [18] and to human cyclophilin [38]. In our study, IgG to rMala s 6 was preferentially found in AE patients that also had serum IgE reactivity to rMala s 6, but also in a few rMala s 6 IgE-negative AE patients and in a few healthy controls. In a previous study, serum IgG reactivity to rMala s 6 was compared to serum IgG reactivity to human cyclophilin and *Echinococcus granulosus* cyclophilin in patients with cystic echinococcosis, atopic subjects, and

healthy controls [38]. In their study, IgG to rMala s 6 and human cyclophilin was found in all individuals investigated, suggesting cross-reactivity. A probable explanation for the disagreement with our study may be a lower detection level (particularly for low-affinity cross-reactive antibodies) in the study by Ortona et al. [38] together with higher IgG levels to rMala s 6 in the IgE-positive patients.

Both rMala s 5 and rMala s 9 induced IgG4 in about 50% of the AE patients with IgG to the same allergen. However, rMala s 9 only induced IgG4 in 9% of the AE patients with IgE to rMala s 9. The highest number of IgE-positive AE patients was found for rMala s 9, which is in agreement with another study [39] where the specific serum IgE reactivity to *Malassezia* extract was compared with that of rMala s 1, and rMala s 5–9 in 156 adult patients with AE. Furthermore, Mala s 9 has no sequence similarity to any known proteins [18, 19]. This suggests rMala s 9 to be the most important IgE-inducing *Malassezia* allergen of those so far studied.

In conclusion, the IgG4 levels were significantly higher in the AE patients with serum IgE reactivity to *Malassezia*. This suggests that IgG4 rather accompanies the devel-

opment of IgE antibodies than reflects downregulation of the pathogenic process in this subgroup of AE patients. Different patterns in response to the four rMala s allergens tested were found among the patients, therefore recombinant allergens might be useful tools in dissecting pathogenic mechanisms in subgroups of AE patients.

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