

Positive Atopy Patch Test Reaction to *Malassezia furfur* in Atopic Dermatitis Correlates with a T Helper 2-like Peripheral Blood Mononuclear Cells Response

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The yeast *Malassezia furfur* belongs to the normal cutaneous flora, but is also a triggering allergen that can contribute to atopic dermatitis. To illuminate the effect of circulating allergen-specific T cells in atopic dermatitis, the peripheral mononuclear cell response was correlated with the *in vivo* skin prick test and atopy patch test reactivity to *M. furfur*. None of 16 healthy controls showed any positive *in vivo* reaction. The 40 atopic dermatitis patients, of whom 18 had serum IgE reactivity to *M. furfur*, were subdivided according to their *in vivo* reaction to *M. furfur* extract into three groups: skin prick test positive/atopy patch test positive (n = 12), skin prick test positive/atopy patch test negative (n = 12), and skin prick test negative/atopy patch test negative (n = 16). The skin prick test positive/atopy patch test positive and the skin prick test positive/atopy patch test negative groups had a significantly higher peripheral mononuclear cell stimulation index than the healthy controls. Interestingly, the stimulation index values in the skin prick test positive/atopy patch test positive group were significantly higher

than in the skin prick test positive/atopy patch test negative group. In the *M. furfur* skin prick test positive atopic dermatitis patients (n = 24) a correlation was found between stimulation index and the *M. furfur* atopy patch test reactions, but not between stimulation index and *M. furfur*-specific serum IgE levels. Skin prick test positive and/or atopy patch test positive reactions to the recombinant *M. furfur* allergens rMal f 1, rMal f 5, and rMal f 6 were observed in 7, 14, and 16 of the 40 atopic dermatitis patients, respectively. Further, there was a correlation between production of the T helper 2-related cytokines interleukins 4, 5, and 13 and stimulation index to *M. furfur* extract, but not between the T helper 1-related interferon- γ and stimulation index to *M. furfur* extract. Our data strongly suggest a relationship between circulating specific T cells with a T helper 2-like cytokine profile and positive atopy patch test reactions. **Key words:** allergens/cytokines/ELISPOT/peripheral mononuclear cell proliferation/recombinant proteins. *J Invest Dermatol* 118:1044–1051, 2002

Atopic dermatitis (AD) is a chronic multifactorial inflammatory skin disease, which has increased in prevalence during the past few decades (Rajka, 1989; ISAAC, 1998). Its pathogenesis is still not fully understood. As approximately 80% of AD patients show immediate-type skin reactions to environmental allergens together with elevated serum IgE levels (Öhman and Johansson, 1974; Werfel and Kapp, 1998), it is assumed that allergens are involved in maintenance of the disease. T helper (Th) cells are thought to play an important part in the pathogenesis of AD. Allergen-specific T cells cloned from acute AD lesional skin show a Th2/Th0-like cytokine profile (van der Heijden *et al*, 1991; Tengvall *et al*, 1996), whereas the *in situ* expression of interleukin (IL)-4 in chronic AD lesions is accompanied by expression of

interferon (IFN)- γ (Grewe *et al*, 1994; Hamid *et al*, 1994), suggesting an involvement also of Th1 cytokines in the chronic phase.

The yeast *Malassezia furfur*, earlier denoted *Pityrosporum orbiculare* or *P. ovale*, belongs to the normal cutaneous flora but is also considered to be one of the triggering allergens that can contribute to AD (Faergemann, 1999). A number of IgE-binding components have been found in *Malassezia* (Zargari *et al*, 1994; Lintu *et al*, 1997). The genes for nine *Malassezia* allergens, Mal f 1–9, have hitherto been identified and cloned (Schmidt *et al*, 1997; Yasueda *et al*, 1998; Lindborg *et al*, 1999; Onishi *et al*, 1999; Rasool *et al*, 2000).

Peripheral blood mononuclear cells (PBMC) from AD patients with IgE reactivity to *Malassezia* show elevated proliferation in response to *in vitro* stimulation with *M. furfur* extract (Rokugo *et al*, 1990; Tengvall Linder *et al*, 1996, 1998; Savolainen *et al*, 2001) and also respond with a Th2-like cytokine profile (Tengvall Linder *et al*, 1996, 1998; Savolainen *et al*, 2001). No correlation, however, has been demonstrated between specific serum IgE levels and PBMC proliferation (Tengvall Linder *et al*, 1998, 2000).

The atopy patch test (APT) reaction, an eczematous skin reaction induced by application of aeroallergens on nonlesional skin

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Abbreviations: AD, atopic dermatitis; APT, atopy patch test; ELISPOT, enzyme linked immunospot; PBMC, peripheral blood mononuclear cells; SPT, skin prick test; TT, tetanus toxoid.

Table I. Characterization of the AD patients and healthy controls^a

<i>In vivo</i> reactions to <i>M. furfur</i> extract	n	Gender M/F	Age in years median (range)	Head and neck distribution n	R/A n	Total serum IgE ^b kU per l median (range)	Total serum IgE > 122 kU per liter n	Phadiatop ^d n of positive	Specific serum IgE ^c to <i>M. furfur</i> n of positive
AD ^b (SPT ⁺ /APT ⁺)	12	3/9	27 (21–43)	12	8/6	2900 (59–7000)	10	10	10 ^f
AD ^b (SPT ⁺ /APT ⁻)	12	5/7	38 (24–50)	9	11/4	1110 (130–3700)	12	11	8 ^g
AD ^b (SPT ⁻ /APT ⁻)	16	9/7	38 (19–49)	10	8/8	147 (5–1700)	8	12	0
HC	16	3/13	29 (21–54)	na	0/0	21 (4–220)	1	3	0

^aHC, healthy controls; M/F, male/female; R, rhinoconjunctivitis; A, asthma; na, not appropriate.

^bThe AD patients are grouped according to their *in vivo* SPT and APT reactivity against the crude *M. furfur* extract.

^cImmunoCAP (Pharmacia Diagnostics AB), reference range 1.6–122 kU per liter.

^dPhadiatop (Pharmacia Diagnostics AB), serum IgE to any of 11 common aeroallergens.

^eImmunoCAP (Pharmacia Diagnostics AB), reference range < 0.35 kU per liter.

^fMedian 2.0 kU per liter, range 0.43–22.0 kU per liter.

^gMedian 5.1 kU per liter, range 1.4–17.0 kU per liter.

of AD patients, was first described Mitchell *et al* (1982) and has thereafter been evaluated in many studies (reviewed in Taïeb and Ducombs, 1996). The presence of IgE on epidermal Langerhans cells correlates with positive APT reactions (Mudde *et al*, 1990; Langeveld-Wildschut *et al*, 2000). These reactions are similar to those in acute AD lesions in terms of infiltrating cells and their phenotype, as examined by immunohistochemical techniques (Langeveld-Wildschut *et al*, 1996; Tengvall Linder *et al*, 2000). Positive APT reaction to *M. furfur* has been reported in 64% of the AD patients investigated after chamber scarification (Rokugo *et al*, 1990), 13% when tested on intact skin (Kieffer *et al*, 1990), and 53% on tape-stripped skin (Tengvall Linder *et al*, 2000).

The present aim was to compare the *in vitro* PBMC response with *in vivo* skin prick test (SPT) and APT reactivity and presence of serum IgE to extract of *M. furfur* and recombinant *M. furfur* allergens (rMal f 1, rMal f 5, and rMal f 6) in AD patients and healthy controls. In response to *in vitro* *M. furfur* stimulation both PBMC proliferation and Th2 cytokine production correlated with the APT reactions to *M. furfur*. Thus, these data strongly suggest a relationship between circulating specific T cells with a Th2-like cytokine profile and positive APT reactions.

MATERIALS AND METHODS

Subjects Forty patients, with mild–severe AD according to the clinical criteria of Williams *et al*, 1994, and 16 healthy controls, were included in the study (Table I). Exclusion criteria were eczema only on the hands, autoimmune diseases, inflammatory skin diseases other than atopic dermatitis, immune suppressive treatment, or use of systemic corticosteroids or solarium in the previous 2 mo. On the test sites, no topical antifungal treatment was permitted for 1 mo before the study and no topical corticosteroid for 1 wk before. Antihistamine was withdrawn 5 d before the investigation. The healthy controls had no clinical symptoms or history of allergy or skin diseases. Twenty-six of the 40 investigated AD patients had elevated total serum IgE (> 122 kU per liter, ImmunoCap, Pharmacia Diagnostics AB, Uppsala, Sweden) and positive Phadiatop (serum IgE to any of 11 common aeroallergens, Pharmacia Diagnostics AB) together with rhinoconjunctivitis and/or asthma. Four of the patients had total serum IgE < 122 kU per liter, were negative in Phadiatop and had no rhinoconjunctivitis or asthma, whereas the remaining 10 AD patients had one or two of these conditions. The subjects gave their informed consent for the study, which was approved by the Regional Ethics Committee of Karolinska Institutet, Stockholm, Sweden.

Stimulating antigens Crude *M. furfur* extract (strain no. 42132, American Type Culture Collection) was prepared as previously described (Zargari *et al*, 1994). Recombinant *M. furfur* allergens (rMal f 1, rMal f 5, and rMal f 6) and the recombinant control allergen (rAca s 13, a minor allergen from the dust mite *Acarus siro*; Eriksson *et al*, 1999) were produced in the *Escherichia coli* system as described earlier (Schmidt *et al*, 1997; Eriksson *et al*, 1999; Lindborg *et al*, 1999). For each allergen

(extract and various recombinants) one single batch was prepared and used throughout the whole study in both *in vitro* experiments and *in vivo* testing (SPT and APT). The test batches had, in the concentration of 10 µg protein per ml, a nucleic acid content of less than 60 pg per µl (DNA dipstick, Invitrogen, San Diego, CA) and an endotoxin content of less than 7 EU per ml (Limulus test, performed by Apoteket AB, Stockholm, Sweden). BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL) was used for determining protein concentration in the extract, whereas the protein concentrations in the recombinant allergen preparations were estimated by spectrophotometric absorbance at 280 nm. Protein purity was checked with sodium dodecyl sulfate–polyacrylamide gel electrophoresis. No significant changes, for extract or recombinant proteins, was found in repeated measurement of concentration and purity checking after the study. Phytohemagglutinin (PHA) was purchased from Amersham Pharmacia Biotech, and tetanus toxoid (TT) was obtained from SBL Vaccine Distribution, Stockholm, Sweden.

In vivo* tests and serum IgE to *M. furfur All subjects were tested for serum IgE, SPT, and APT reactions to the crude *M. furfur* extract, the recombinant *M. furfur* allergens rMal f 1, rMal f 5, and rMal f 6, and the recombinant control allergen rAca s 13. The SPT was performed according to standard procedure (Dreborg, 1989) in a concentration of 100 µg allergen per ml and evaluated after 15 min. PBS served as negative control and histamine dihydrochloride (10 mg per ml; ALK, Hørsholm, Denmark) as positive control. The reactions were graded as mean diameter in millimeters, a reaction with mean diameter of 3 mm or more being considered as positive. The APT was performed as previously described (Tengvall Linder *et al*, 2000). Briefly, the allergens or the vehicle control PBS were applied on paper discs in Finn chambers (8 mm; Epitest Ltd Oy, Tuusula, Finland) on tape-stripped, nonlesional skin of the back. The recombinant allergens were tested at a concentration of 4 mg per ml and the *M. furfur* extract in a 2-fold serial dilution of 5.0–0.6 mg per ml. The APT were removed after 48 h and the skin reactions were evaluated. The test results were scored as: 0 = negative reaction; + = erythema, infiltration, and possible papules; ++ = erythema, infiltration, papules, and small vesicles; and +++ = erythema, infiltration, papules, and large vesicles (Rietschel and Fowler, 1995; Tengvall Linder *et al*, 2000). For the *M. furfur* extract, the highest APT score in each individual, regardless of concentration, was used in further comparisons.

Specific serum IgE for *M. furfur* was analyzed with ImmunoCAP “m70” (Pharmacia Diagnostics AB, prepared from *M. furfur* extract according to Zargari *et al*, 1994; strain no. 42132, American Type Culture Collection). For analysis of serum IgE specific to the recombinant *M. furfur* allergens rMal f 1, rMal f 5, and rMal f 6, the allergens were immobilized on to cellulose solid phase (ImmunoCAP) by covalent binding (MIAB, Uppsala, Sweden). The coupling concentration was adjusted to attain a linear measurement range and a background below 0.35 kU per liter. The recombinant ImmunoCAPs were tested in the Pharmacia CAP system according to the manufacturer’s instructions.

Preparation of PBMC and culture conditions PBMC were isolated from heparinized blood by gradient centrifugation on Ficoll-paque (Amersham Pharmacia Biotech). As culture medium we used RPMI

Table II. Comparison between *in vivo* reactivity to *M. furfur* extract and the recombinant allergens in the AD patients

<i>M. furfur</i> extract ^a	n	rMal f 1 ^b			rMal f 5 ^b			rMal f 6 ^b			rAca s 13 ^b		
		SPT ⁺ /APT ⁺	SPT ⁺ /APT ⁻	SPT ⁻ /APT ⁺	SPT ⁺ /APT ⁺	SPT ⁺ /APT ⁻	SPT ⁻ /APT ⁺	SPT ⁺ /APT ⁺	SPT ⁺ /APT ⁻	SPT ⁻ /APT ⁺	SPT ⁺ /APT ⁺	SPT ⁺ /APT ⁻	SPT ⁻ /APT ⁺
AD ^a	12	0	1	2	6	1	0	2	5	1	0	1	0
(SPT ⁺ /APT ⁺)													
AD ^a	12	0	4	0	1	6	0	1	4	1	1	0	0
(SPT ⁺ /APT ⁻)													
AD ^a	16	0	0	0	0	0	0	0	2	0	0	0	0
(SPT ⁻ /APT ⁻)													
Total AD	40	0	5	2	7	7	0	3	11	2	1	1	0

^aThe AD patients are grouped according to their *in vivo* reactivity against the crude *M. furfur* extract.

^bNumber of individuals positive in SPT and/or APT against rMal f 1, rMal f 5, rMal f 6 or rAca s 13, respectively.

1640 supplemented with 25 µg gentamicin per ml, 100 IU penicillin per ml, 100 µg streptomycin per ml, 2 mM L-glutamine (Gibco BRL, Life Technologies Ltd, Paisley, U.K.) and 50 µM 2-mercaptoethanol (Merck, Darmstadt, Germany). Further 10% pooled, heat-inactivated human ABRh + serum with IgE content < 10 kU per liter, negative Phadiatop, and no specific *M. furfur* IgE (ImmunoCAPTM) was added. The cells were cultured at 37°C and 6% CO₂ in air.

PBMC proliferation assay Freshly isolated PBMC (2 × 10⁵ cells in 200 µl culture medium per well) were cultured in 96-well round-bottomed plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) with or without antigen stimulation. The proliferation after stimulation with *M. furfur* extract (50, 10, 1, and 0.1 µg per ml) was measured after 3 and 7 d incubation. The recombinant *M. furfur* allergens and rAca s 13 (10, 1, and 0.1 µg per ml) were tested after 7 d incubation only, due to limited number of cells. As proliferation controls the mitogen PHA (10 µg per ml) was used for 3 d incubation and the protein antigen TT (10 µg per ml) for 3 and 7 d incubation. All tests were set up in triplicate. During the last 18 h of incubation 1 µCi [³H]thymidine (25 Ci per mmol, Amersham Pharmacia Biotech) was added to each well and the thymidine incorporation was determined by scintillation counting. The results are expressed as mean cpm for triplicates and as stimulation index (SI) calculated as mean cpm for stimulated cells divided by mean cpm for unstimulated cells.

Analysis of cytokine-producing cells in PBMC The number of cytokine producing cells was estimated in 35 of the individuals, from whom sufficient numbers of cells were obtainable, selected regardless of their *in vivo* and *in vitro* reactivity to *M. furfur*. The reverse ELISPOT assay was used on freshly prepared or frozen PBMC, mainly as described before (Gabrielsson *et al*, 2001). Briefly, pretreated sterile Multiscreen Immobilon-P Membrane plates (Millipore, Bedford, MA) were coated overnight at +4°C (15 µg per ml, 100 µl per well) with anti-IL-4 monoclonal antibody (MoAb) (IL-4-I) or anti-IL-13 MoAb (IL13-I) (MabTech AB, Stockholm, Sweden). The unbound MoAb was washed away with PBS, followed by the culture medium, whereupon 100 µl of PBMC (2 or 3 × 10⁵ cells per well, prestimulated with 10 µg *M. furfur* per ml, 10 µg PHA per ml, or medium alone for 4 h) was added to the wells. The cells were incubated for 44 h at 37°C in 6% CO₂, before being washed away. Produced cytokines were detected by incubation with 1 mg per ml of biotin-labeled anti-IL-4 (IL-4-II) or anti-IL-13 (IL-13-II) MoAb (Mabtech AB), followed by several washes and incubation with streptavidin-alkaline phosphatase (Mabtech AB, dilution 1/1000). Unbound conjugate was removed with another series of washing and finally 100 µl of BCIP/NBT (Alkaline Phosphatase Conjugate Substrate Kit obtained from Bio-Rad Laboratories, Hercules, CA) was added and incubated at room temperature until dark spots emerged. The spots were counted in a dissection microscope (×40). All assays were set up in triplicate wells and the results are presented as the mean number of cytokine-producing cells in 10⁵ PBMC.

Measurement of cytokines secreted into the culture media Freshly isolated PBMC (2 × 10⁶ cells per 2 ml culture medium) were incubated in 48-well, flat-bottomed culture plates (Falcon) with or without *M. furfur* extract (10 µg per ml). The culture supernatants were collected at day 8, which was found in preliminary investigations to be optimal for both IL-5 and IFN-γ. The supernatants were centrifuged at 300 × g to remove the cells and kept at -20°C until assessed for the presence of

IL-5 and IFN-γ by enzyme-linked immunosorbent assay technique according to the manufacturer's instructions (IL-5, PharMingen Research Products, San Diego, CA; IFN-γ, Mabtech AB). Briefly, 96-well, flat-bottomed plates (Nunc, Roskilde, Denmark) were coated with the MoAb: anti-IL-5 MoAb (TRFK5; 4 mg per ml, 50 ml per well) or anti-IFN-γ MoAb (1-D1K; 2 mg per ml, 100 ml per well) overnight at room temperature followed by treatment with 200 ml of PBS containing 1% bovine serum albumin (Sigma, St Louis, MO). Next, samples or standards (100 ml) were added to the wells in duplicate followed by addition of 1 mg per ml of biotin-labeled anti-IL-5 (JES15A10), or anti-IFN-γ (7-B6-1) MoAb. After incubation with streptavidin-alkaline phosphatase (Mabtech AB, dilution 1/1000), the substrate (p-nitrophenyl-phosphatase disodium, Sigma), diluted in 1 M diethanolamine buffer, pH 9.8 was added. The absorbance at 405 nm was measured with an enzyme-linked immunosorbent assay plate reader (Labsystem Multiscan, Finland). As standards, recombinant human IL-5 (PharMingen) and recombinant human IFN-γ (Bender MedSystems, Vienna, Austria) were used. The detection limit for IL-5 was 35 pg per ml and for IFN-γ 30 pg per ml.

Statistical methods Differences between the groups were analyzed with Kruskal-Wallis ANOVA by ranks and *post-hoc* comparisons were made with the Mann-Whitney U test. Friedman ANOVA was used for comparison of responses with the different recombinant allergens within the groups and *post-hoc* comparisons were made with the Wilcoxon matched pair test. Correction for multiple comparisons was made with the Bonferroni method. All tests followed a two-sided alternative hypothesis. Correlations were calculated using Spearman rank correlation analysis. A p < 0.05 was considered as statistically significant.

RESULTS

***In vivo* response and specific serum IgE** Twenty-four of the AD patients (60%) responded with positive SPT reaction to *M. furfur* extract and 50% of these also responded with a positive APT reaction to the *M. furfur* extract (Table I). Fewer AD patients responded *in vivo* to the recombinant *M. furfur* allergens than to the extract (Tables II and III). The pattern of *in vivo* SPT/APT response to rMal f 5 was most similar to the *in vivo* response to *M. furfur* extract (Table II). As expected, only a few individuals responded with *in vivo* reactions to the irrelevant control allergen rAca s 13 (Table II). None of the healthy controls reacted with a positive SPT or APT reaction to *M. furfur* extract or any of the recombinant allergens.

The SPT for *M. furfur* extract seemed to be more sensitive than ImmunoCap as all patients who had measurable serum IgE to the extract also responded with a positive SPT, and an additional six patients who had *M. furfur*-specific serum IgE below the detection limit had a positive SPT, two of them together with a positive APT (Table I). The two SPT⁺/APT⁺ AD patients that were negative in ImmunoCap for *M. furfur*-specific IgE were also negative in Phadiatop and had relatively low total serum IgE levels (98 and 160 kU per liter, respectively). These patients stated not to have had symptoms of rhinoconjunctivitis or asthma.

A statistically significant correlation was found between *M. furfur*-specific serum IgE and total serum IgE ($r_s = 0.70$, $p < 0.001$) but not between *M. furfur*-specific serum IgE and the SPT reaction ($r_s = 0.19$, $p = 0.20$) in the 24 AD patients with positive SPT reaction to *M. furfur* extract. In this group no correlations were found between the APT reaction to *M. furfur* extract and *M. furfur*-specific serum IgE or the SPT reaction to *M. furfur* ($r_s = 0.24$, $p = 0.27$ and $r_s = 0.31$, $p = 0.14$, respectively); however, in the whole group of AD patients ($n = 40$), there were correlations between the APT reaction to *M. furfur* extract and *M. furfur*-specific serum IgE ($r_s = 0.52$, $p < 0.001$) or SPT to *M. furfur* ($r_s = 0.58$, $p < 0.001$).

In contrast to the results with the *M. furfur* extract, a few individuals with specific serum IgE for any of the recombinant allergens had negative SPT reaction to the same allergen (Table III). Specific serum IgE for *M. furfur* extract or any of the

recombinant *M. furfur* allergens was not found in any of the healthy controls.

PBMC response to *M. furfur* extract correlated with APT reaction The general pattern, at both time points and at all concentrations investigated, was a significantly higher SI to *M. furfur* extract in the AD patients with positive SPT to *M. furfur* extract ($p < 0.01$ compared with healthy controls and $p < 0.05$ compared with the SPT⁻ patients). The SPT⁻ AD patients and the healthy controls showed equally low proliferation (data not shown). The AD patients with positive SPT to *M. furfur* extract were further subdivided according to their APT reaction to the extract, and median SI was six times higher in the APT⁺ group than in the SPT⁺/APT⁻ patients after 3 d stimulation with *M. furfur* extract (Fig 1). In the AD patients with positive SPT reaction to *M. furfur* extract, a significant correlation was found between SI and APT reaction to the extract (Fig 2).

Table III Comparison between *in vivo* reactivity and specific serum IgE to the recombinant *M. furfur* allergens

	rMal f 1					rMal f 5					rMal f 6				
	negative < 0.35 kU per liter ^c					negative < 0.35 kU per liter ^c					negative < 0.35 kU per liter ^c				
	Σn^b	n	class 1–2 ^c n	class 3 ^c n	class 4–5 ^c n	Σn^b	n	class 1–2 ^c n	class 3 ^c n	class 4–5 ^c n	Σn^b	n	class 1–2 ^c n	class 3 ^c n	class 4–5 ^c n
AD ^a (SPT ⁺ /APT ⁺)	0	0	0	0	0	7	0	0	4	3	3	2	0	0	1
AD ^a (SPT ⁺ /APT ⁻)	5	0	0	3	2	7	2	3	1	1	11	1	0	3	7
AD ^a (SPT ⁻ /APT ⁺)	2	2	0	0	0	0	0	0	0	0	2	1	1	0	0
AD ^a (SPT ⁻ /APT ⁻)	33	32	0	0	1	26	24	2	0	0	24	24	0	0	0

^aThe AD patients are divided according to their *in vivo* reactivity to rMal f 1, rMal f 5, or rMal f 6, respectively.

^bTotal number of individuals with the stated pattern of *in vivo* reactivity to rMal f 1, rMal f 5, or rMal f 6, respectively.

^cNumber of individuals with specific serum IgE (ImmunoCAP) to rMal f 1, rMal f 5, or rMal f 6, respectively, divided according to the RAST-class system; class 1–2 = 0.35–3.5 kU per liter, class 3 = > 3.5–17.5 kU per liter, class 4–5 = > 17.5–100.0 kU per liter. < 0.35 kU per liter was considered as negative.

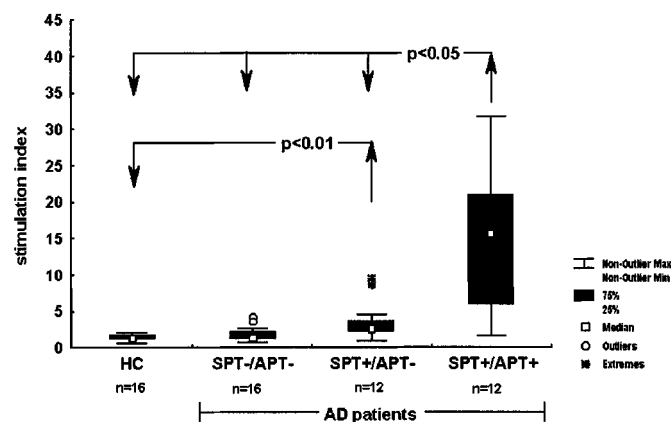


Figure 1. Elevated PBMC proliferation to *M. furfur* extract in the *in vivo* *M. furfur* reactive AD patients. PBMC proliferation to *M. furfur* extract in healthy controls (HC) and AD patients, grouped according to their *in vivo* reactivity to the *M. furfur* extract. The proliferation was measured as [³H]thymidine incorporation in triplicate cultures, after 3 d stimulation with *M. furfur* extract (10 µg per ml). The result are presented as SI calculated as counts per minute for stimulated cells divided by cpm for unstimulated cells. Median for unstimulated cells was 406 cpm, range 176–1690 cpm ($n = 56$). Data are analyzed with Kruskal–Wallis ANOVA by ranks ($p < 0.001$), *post-hoc* comparisons made with the Mann–Whitney U test corrected for multiple comparisons using the Bonferroni method. Arrows indicate statistically significant differences in pairs.

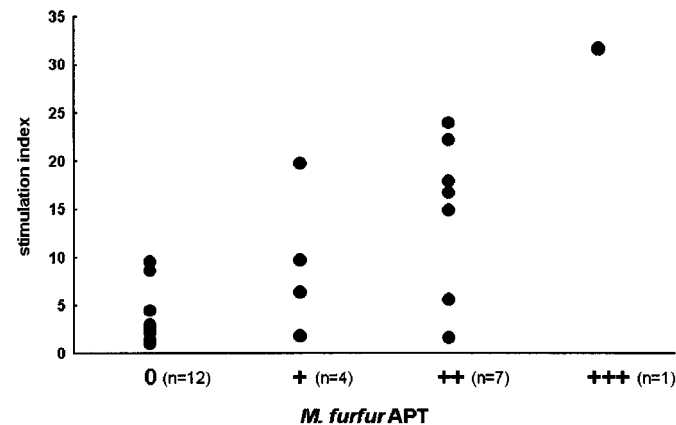


Figure 2. Positive correlation between SI and APT reaction to *M. furfur* extract. The correlation ($r_s = 0.62$, $p < 0.01$) was calculated between SI for *M. furfur* extract (10 µg per ml) and APT reaction to *M. furfur* extract in AD patients with positive SPT to the extract ($n = 24$). The proliferation was measured as [³H]thymidine incorporation in triplicate cultures, after 3 d stimulation with 10 µg per ml *M. furfur* extract. The SI was calculated as counts per minute for stimulated cells divided by cpm for unstimulated cells. The median for unstimulated cells was 399 cpm, range 190–1690 cpm ($n = 24$). The APT was performed in a 2-fold serial dilution of 5.0–0.6 mg per ml *M. furfur* extract. The APT reactions were evaluated after 48 h and scored as 0 to +++ (see Materials and Methods). The highest scores, regardless of concentration, are presented.

PBMC proliferation to *M. furfur* extract was measured on both days 3 and 7 and the same pattern of differences between the groups

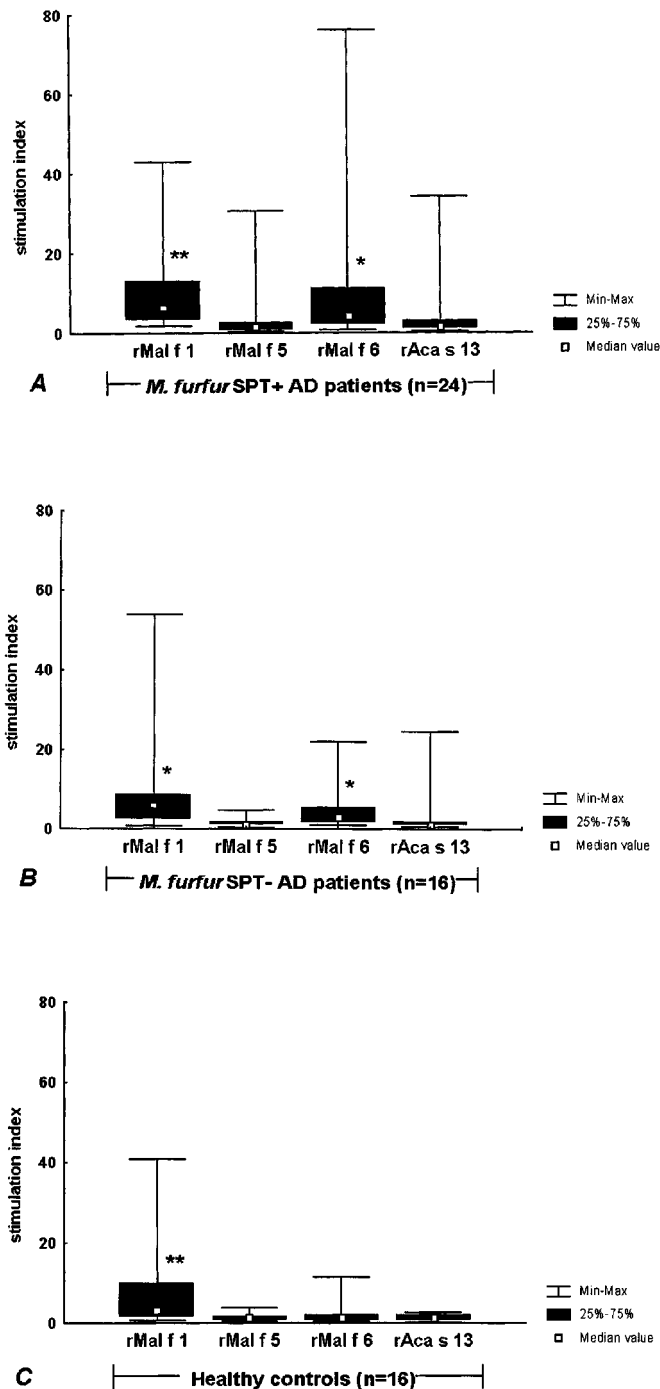


Figure 3. PBMC proliferation to the recombinant *M. furfur* allergens (rMal f 1, rMal f 5 and rMal f 6) compared with the control allergen rAca s 13. PBMC proliferation was measured as [3 H]thymidine incorporation in triplicate cultures, after 7 d stimulation with 10 μ g per ml of the recombinant allergens, in (A) AD patients SPT⁺ to *M. furfur* extract, (B) AD patients SPT⁻ to *M. furfur* extract, and in (C) healthy controls. The result is presented as counts per minute for stimulated cells divided by cpm for unstimulated cells. The median for unstimulated cells was 1464 cpm, range 272–13,886 cpm (n = 56). Data are analyzed with Friedman ANOVA by ranks ($p < 0.001$ for all three groups of individuals), *post-hoc* comparisons made with the Wilcoxon matched pairs test corrected for multiple comparisons using the Bonferroni method. Statistically significant higher SI than for Aca s 13 are indicated with ** $p < 0.01$ and * $p < 0.05$.

was found at both time points. Prolonged incubation time only resulted in larger variations, both within triplicates and between individuals within the groups. This confirms the relevance of measurement of PBMC proliferation to *M. furfur* extract on day 3, the time-point used in earlier studies (Tengvall Linder *et al*, 1996, 1998, 2000 Johansson *et al*, 1999).

No differences between the three patient groups and the healthy controls were found in cpm for the mitogen PHA after 3 d stimulation (median 183,029 cpm, range 87,208–389,161, n = 56), or in SI for the control antigen TT on day 3 (median 6.2, range 1.3–47, n = 56) or day 7 (median 12.1, range 0.4–192, n = 56).

PBMC response to the recombinant *M. furfur* allergens was not connected to the *in vivo* response When the PBMC proliferation in response to the recombinant *M. furfur* allergens was compared with that to the recombinant control allergen rAca s 13 (Fig 3), a significantly higher response to rMal f 1 was found in both healthy controls and AD patients with or without SPT⁺ to *M. furfur* extract ($p < 0.01$, $p < 0.001$, $p < 0.05$, respectively). For

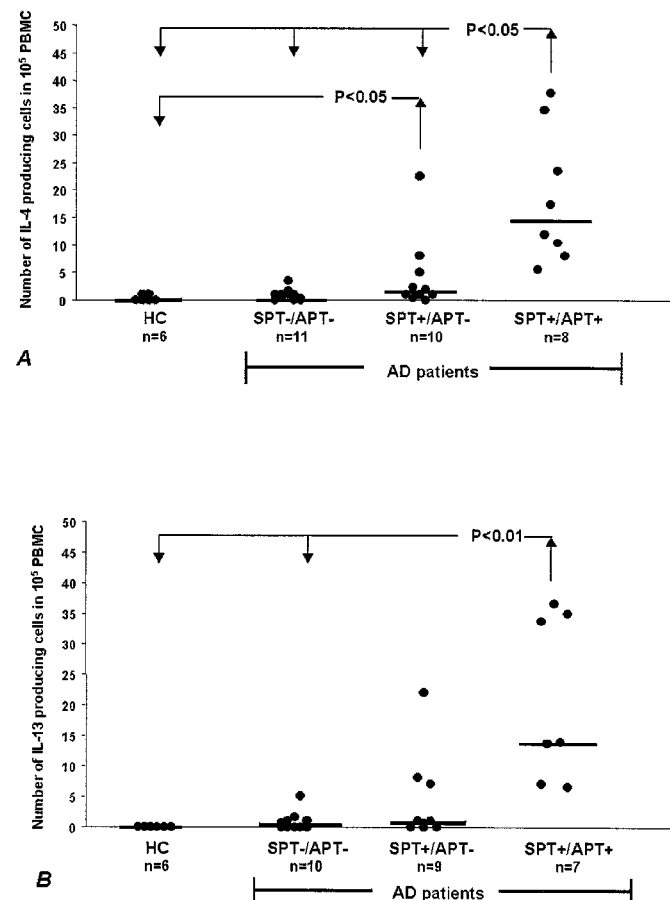


Figure 4. Elevated number of IL-4- and IL-13-producing cells in response to *M. furfur* extract in *in vivo* reactive AD patients. The frequency of (A) IL-4-producing cells and (B) IL-13-producing cells in response to *in vitro* stimulation with *M. furfur* extract (10 μ g per ml) was measured with the ELISPOT method in triplicate measurements and presented as mean number of cytokine-producing cells in 10^5 PBMC. The results are presented in healthy controls (HC) and AD patients, grouped according to their *in vivo* reactivity to the *M. furfur* extract. IL-4- and IL-13-producing cells in unstimulated PBMC were always fewer than 2 in 10^5 PBMC. Data are analyzed with the Kruskal–Wallis ANOVA by ranks ($p < 0.001$ for IL-4 and IL-13, respectively), *post-hoc* comparisons made with the Mann–Whitney U test corrected for multiple comparisons using the Bonferroni method. Horizontal bars indicate median values and arrows indicate statistically significant differences.

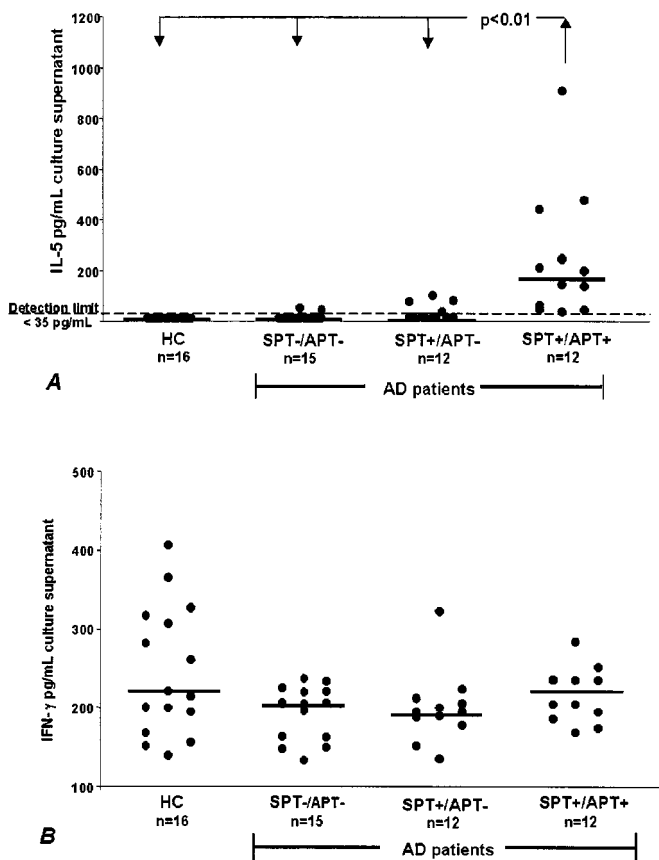


Figure 5. Elevated IL-5 but unchanged IFN- γ production in response to *M. furfur* extract in *in vivo* reactive AD patients. *In vitro* IL-5 (A) and IFN- γ (B) production from PBMC (2×10^6 cells per ml), in response to stimulation with *M. furfur* extract (10 μ g per ml), was measured with enzyme-linked immunosorbent assay as secretion into the cell culture medium on day 8. The results are presented as mean of duplicates. Data are analyzed with the Kruskal-Wallis ANOVA by ranks ($p < 0.001$ for IL-5 and $p = 0.390$ for IFN- γ), *post-hoc* comparisons made with the Mann-Whitney U test corrected for multiple comparisons using the Bonferroni method. Horizontal bars indicate median values and arrows indicate statistically significant differences.

rMal f 6, higher proliferation was found in AD patients with or without SPT⁺ to *M. furfur* extract but not in the healthy controls ($p < 0.05$, $p < 0.05$, $p = 0.68$, respectively). rMal f 5 gave no significant PBMC stimulation in any of the groups (Fig 3). The PBMC reactivity to the control allergen rAca s 13 was generally low (median SI 1.3) and only five individuals showed a SI of more than 3.2.

In contrast to the *M. furfur* extract, no correlation between PBMC proliferation in response to the recombinant allergens and *in vivo* response was seen for any of the recombinant *M. furfur* allergens. No significant differences in PBMC proliferation for any of the recombinant allergens were observed between the groups of AD patients, either when the patients were subdivided according to *in vivo* response to the extract or when subdivided by *in vivo* response to the recombinant allergen being tested (data not shown); however, in the proliferative response to rMal f 6 a significant difference was found between the healthy controls and the whole AD group ($p < 0.001$). The higher proliferative response to Mal f 6 persisted in all AD groups, compared with the healthy controls, when the AD patients were further subdivided according to *in vivo* reactivity to rMal f 6 ($p < 0.05$) or *M. furfur* extract ($p < 0.05$), although there were no differences between the AD groups (data not shown).

Th2 cytokine production in response to *M. furfur* extract correlated with SI and APT reactions The AD patients with SPT⁺/APT⁺ to the extract responded with significantly higher numbers of IL-4 producing cells ($p < 0.05$) than the other groups of AD patients and the healthy controls (Fig 4A). Also the SPT⁺/APT⁺ group responded with significantly higher numbers of IL-4-producing cells ($p < 0.05$) than the healthy controls. In addition, the SPT⁺/APT⁺ AD patients responded with higher numbers of IL-13 producing cells ($p < 0.01$) than the SPT⁺/APT⁺ group and the healthy controls (Fig 4B). The response to PHA analyzed in parallel was >90 IL-4 and >150 IL-13 producing cells in 10^5 PBMC.

A significantly higher IL-5 production was found in the SPT⁺/APT⁺ AD patients than in the other groups of AD patients and healthy controls ($p < 0.01$, Fig 5A), where the IL-5 production was below the detection limit (35 ng per ml) for several individuals. For the unstimulated control PBMC cultures, IL-5 levels above the detection limit were found in three individuals only (38–44 ng per ml). IFN- γ production was detected in both *M. furfur* stimulated and unstimulated cell cultures from all individuals; however, no significant differences were found between the healthy controls and AD groups either in unstimulated (data not shown) or in *M. furfur*-stimulated cell cultures (Fig 5B). In the *M. furfur* SPT⁺ AD patients the degree of APT reaction, PBMC proliferation expressed as SI and Th2 cytokine production in response to *M. furfur* all correlated with each other (Table IV).

DISCUSSION

This study strongly suggests a relationship between circulating allergen-specific T cells and positive APT reactions. We have for the first time shown significantly higher PBMC proliferation to an allergen (*M. furfur*) in AD patients with positive APT reaction to this allergen, than in AD patients with specific IgE but negative APT. Although the SPT⁺/APT⁺ patients responded with significantly higher PBMC proliferation to *M. furfur* than healthy individuals did, considerably higher proliferation was found in the patients also positive in the APT reaction. The high PBMC proliferation in the APT⁺ patients correlated with a high number of Th2 cytokine-producing cells, further supporting their ability to contribute to the eczematous reaction.

Association between specific serum IgE and positive APT reactions has previously been reported (Langeveld-Wildschut *et al*, 1996, 2000; Wistok-Wülfing *et al*, 1999; Tengvall Linder *et al*, 2000). Our study supports this association, as all patients with positive APT reaction to *M. furfur* extract also had a positive SPT reaction and all but two had specific serum IgE to *M. furfur* extract. Membrane-bound IgE improves the allergen-presenting capacity of Langerhans cells (Mudde *et al*, 1990) and monocytes (Maurer *et al*, 1995). In a recently published study Langeveld-Wildschut *et al* (2000) suggest that a positive APT reaction requires the presence of epidermal IgE⁺ Langerhans cells in clinically noninvolved skin, but that other as yet unknown, factors are also involved. Such a factor could be the presence of allergen-specific Th2 cells, which can release cytokines that promote an inflammatory reaction; however, in their study Langeveld-Wildschut *et al* (2000) could establish house dust mite allergen-specific T cell lines from uninvolved skin from only half of the patients with positive APT reaction. If this reflects the true situation in the skin, there may also be an early recruitment of allergen-specific T cells from the circulation. Although we found a clear association between the presence of specific IgE and positive APT reaction to *M. furfur*, the Th2-like PBMC response to *M. furfur* showed stronger correlation with the APT reaction than did the specific IgE levels or SPT reaction. This supports the hypothesis that the Th2-like T cell response is one of the crucial factors for allergen-specific eczema reactions in AD.

The fact that we observed a strong PBMC proliferation to *M. furfur* extract only in sensitized AD patients speaks against the possibility of a mitogenic effect. The antigenicity in the extract in combination with preactivated allergen-specific T cells in the

Table IV. Correlations between *in vivo* and *in vitro* test results in the *M. furfur* SPT positive AD patients

	APT to <i>M. furfur</i> (0 - + + +) n = 24 ^a	SI to <i>M. furfur</i> (3 d, 10 µg per ml) n = 24	<i>M. furfur</i> induced IL-4 producing cells n = 18	<i>M. furfur</i> induced IL-13 producing cells n = 16
SI to <i>M. furfur</i> (3 d, 10 µg per ml) n = 24	0.62 ^b ***			
<i>M. furfur</i> induced IL-4 producing cells n = 18	0.76 ***	0.80 ***		
<i>M. furfur</i> induced IL-13 producing cells n = 16	0.70 **	0.81 ***	0.95 ***	
<i>M. furfur</i> induced IL-5 production n = 24	0.75 ***	0.58 **	0.62 ***	0.57 *

^aCorrelations were calculated in the whole group of *M. furfur* SPT⁺ AD patients (n = 24) for all parameters, but IL-4 and IL-13-producing cells (due to lack of cells from six or eight individuals, respectively), as indicated in the table.

^bCorrelations were calculated using Spearman rank correlation analysis and r_s-values obtained are given in the table.

***p < 0.001,

**p < 0.01,

*p < 0.05.

sensitized individuals is a more likely explanation of the early proliferation. Wistokat-Wülfing *et al* (1999) also showed significantly higher PBMC proliferation in AD patients with positive APT reactions against cat, grass pollen, and house dust mite allergens. A weakness in their study design was that the APT⁺ AD patients were compared with a heterogeneous group of AD patients, without subgrouping for specific IgE to the allergen in question. Nevertheless, both the study by Wistokat-Wülfing *et al* (1999) and our data support an association between T cell-mediated specific immune response and the APT reaction to an allergen.

The difference between the antigen-stimulating capacity of *M. furfur* extract and the recombinant *M. furfur* allergens may be due to several things. First, a few different allergens, randomly selected from several Mal f allergens and used one by one, can only be expected to mimic partly the extract. Another important difference between the crude extract and the pure recombinant allergens may be that they have a different ability to stimulate antigen-presenting cells. Full extracts of allergenic substances contain not just the actual allergens, but also substances that can probably serve as adjuvants and enhance antigen-specific reactions. This may also explain the lack of concordance between *in vitro* PBMC and *in vivo* APT reactions to the recombinant allergens. In this study it was found that all three recombinant *M. furfur* allergens tested, rMal f 1, rMal f 5, and rMal f 6, could cause positive SPT and APT reactions in AD patients. Recombinant allergens may in the future become useful diagnostic tools for AD, in that a mix of relevant recombinant allergens will provide a standardized and more stable preparation than crude allergen extracts; however, at present, the *M. furfur* extract still provides the best tool.

The Th2-like cytokine production in response to *M. furfur* in AD patients agrees with previous findings (Kröger *et al*, 1995; Tengvall Linder *et al*, 1996, 1998; Savolainen *et al*, 2001). This is, to our knowledge, the first time the ELISPOT method has been used for detecting allergen-induced cytokine production in AD patients, although it has been used in investigations of respiratory atopic manifestations (Gabrielsson *et al*, 2001). Measurement of the total amount of produced cytokine gives general information, but the cytokine may be secreted by only a few highly productive cells, or the levels may be falsely low due to consumption within the cell culture. The ELISPOT method, on the other hand, makes it possible to estimate the frequency of cytokine-producing cells in a population. We found a high frequency of IL-4- and IL-13-producing cells,

both cytokines supporting IgE production (Leung, 2000), in response to *M. furfur* stimulation in the *M. furfur* APT⁺ AD patients. In addition, those patients produced large amounts of IL-5, not detected in most individuals in the other groups. IL-5 promotes growth and differentiation of eosinophils (Leung, 2000), one of the early skin-infiltrating cells in the APT reaction (Bruynzeel-Koomen *et al*, 1988; Tengvall Linder *et al*, 2000). The IFN-γ production in response to *M. furfur* was not elevated in our AD patients or in the healthy controls. In the healthy controls and in the *M. furfur* SPT⁻ AD patients this accords with the low proliferative response to *M. furfur*. Conversely, this lack of elevated IFN-γ production in the *M. furfur* SPT⁺ AD patients with elevated proliferative PBMC response to *M. furfur* is likely to be due to a highly Th2-skewed response. *In vitro* studies of antigen uptake and presentation by human dendritic cells have shown that *M. furfur* uptake directs the dendritic cells to promote a Th2-like response (Buentke *et al*, 2001). The source of the elevated IFN-γ levels seen in chronic AD lesions (Grewe *et al*, 1994; Hamid *et al*, 1994) might not be those allergen-stimulated specific T cells but, rather, nonspecific T cells recruited to the AD lesion (Leung, 2000).

Specific serum IgE to *M. furfur* are preferentially found in AD patients with high total serum IgE levels and other signs of atopic manifestations such as positive Phadiatop and allergic asthma and/or rhinoconjunctivitis (Wessels *et al*, 1991; Nordvall *et al*, 1992). Nevertheless, in our rather small group of *M. furfur* APT⁺ AD patients we found two patients without those criteria and also without measurable levels of specific serum IgE to *M. furfur*. We still do not expect their patch test reactions to be Th1-dominated contact dermatitis reactions, as both patients showed local specific IgE reactivity in the *M. furfur* SPT reaction. These patients also had a clearly elevated Th2-like proliferative PBMC response to *M. furfur*. Thus, the possibility of a worsened AD eczema due to *M. furfur* sensitization should not be forgotten in the subgroup of adult AD patients without atopic symptoms other than AD.

Using *M. furfur* extract we have shown a clear association between positive APT reactions and elevated PBMC proliferation accompanied by Th2 cytokine production in response to allergen stimulation. These data strongly suggest a relationship between circulating allergen-specific Th2 cells and positive APT reactions. Those T cells may either provide a pool for rapid recruitment to the skin, or reflect spillover of cells harbored in AD skin, or both,

and might together with epidermal IgE⁺ Langerhans cells be essential for the APT reaction.

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