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Immunological characteristics of subjects with asymptomatic skin sensitization to birch and grass pollen

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Summary

Background Asymptomatic skin sensitization (AS) has been shown to be a risk factor for respiratory allergic disease.

Objective We investigated allergen and recall antigen-driven T cell proliferation, cytokine production and T cell expression of the chemokine receptor CCR4, in cultures derived from symptomatic atopics (SA), subjects with AS and healthy controls (HC). Numbers of allergen-specific precursor T cells in all three groups were also estimated.

Methods Peripheral blood mononuclear cells from the three groups were isolated and stimulated with allergen and tetanus toxoid. Proliferation, cytokine production and CCR4 expression were measured by flow cytometry.

Results A significantly increased proportion of CD4⁺ memory T cells proliferated in response to allergen in SA as compared with subjects with AS ($P < 0.001$) and HC ($P < 0.001$). Only in SA was expansion of CD4⁺CCR4⁺ T cells, after allergen stimulation observed. SA had higher frequencies of allergen-specific T cells than subjects with AS and HC ($P = 0.02$, for both). With regard to allergen-induced production of T-helper type 1 (Th1) and Th2 cytokines, subjects with AS and HC resembled each other, while differing significantly from SA.

Conclusion We conclude, that subjects with AS, although clearly IgE sensitized, have significant diminished numbers of allergen-specific T cells as well as decreased allergen-induced CD4⁺ memory T cell proliferation as compared with SA. To a large extent, our findings are capable of explaining the immunological characteristics associated with AS. Our findings may serve as better prognostic markers for subsequent allergic progression, than previously described clinical and paraclinical characteristics.

Keywords asymptomatic skin sensitization, CCR4, cytokines, memory T cells, precursor frequencies, proliferation

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Introduction

Subjects with asymptomatic skin sensitization (AS) are IgE sensitized (positive skin tests) to one or several allergens without exhibiting allergic symptoms. It has been demonstrated that AS, dependent upon certain clinical and paraclinical characteristics, have an increased risk for developing respiratory allergic disease [1, 2].

A rapid and enhanced immune response to antigen is associated with increased proportions of antigen-specific lymphocytes in the memory pool than among naïve lymphocytes. Upon encountering allergen, specific memory T-helper type 2 (Th2) cells secrete IL-4 and IL-5. IL-4

induces Ig class shift in mature B cells resulting in the production of allergen-specific IgE [3]. IL-5 promotes differentiation of eosinophils and facilitates their survival at sites of allergic inflammation [4]. IFN- γ secreted by Th1 cells antagonize IgE production [5], while IL-10, secreted by regulatory T cells (T_{reg}) [6] and Th2 cells [7], inhibits antigen-specific proliferation and cytokine production in Th2 cells [7]. Whereas the mentioned cytokines are responsible for lymphocyte differentiation, a number of chemokines and adhesion molecules direct the activated lymphocytes to the sites of inflammation. The chemokine receptor CCR4 has, in several studies, been associated with allergen-induced lower airway disease [8, 9].

Few studies have described the immune response to allergens in AS. Reduced levels of IL-5 in allergen-stimulated cultures, have been found in AS as compared with symptomatic atopics (SA) [10]. Elevated serum levels of IL-10 in AS during pollination, have been reported [11]. Among AS, investigation of allergen-specific precursor T cell frequencies, allergen-driven CD4⁺ T cell proliferation and CCR4 expression, has not been performed hitherto. The aim of this study was to investigate, whether AS could be discriminated immunologically on the basis of these characteristics.

Methods

Pollen season

Birch pollen symptoms tend to persist weeks after the decline in pollen count and therefore was the season in which symptoms were attributable to birch pollen defined as the period starting on the first day with birch pollen levels > 10 grains/m³ and until grass pollen reached a level of 10 grains/m³ (at this end-point, concomitant birch

pollen counts for the last 10 years were: median: 4.5 grains/m³; 1.0–14.0 grains/m³). Annual accumulated pollen loads during the birch pollen season for the last 10 years were (5732.5, 2244.0–9162.0 grains/m³). The corresponding birch figures in the period from grass pollen levels reaching 10 grains/m³ until birch became unmeasurable low were (49.5, 11.0–92.0 grains/m³). Thus, according to our definition of the birch pollen season, we missed less than 1% (0.8%, 0.2–1.8%) of the total birch season (as measured by pollen load). The grass pollen season started on the first day with grass pollen counts exceeding 10 grains/m³ (with concomitant birch pollen counts below 10 grains/m³) and until the end of July (end of diary).

Patients

Patients were included and attended visits at the times shown in Table 1. During the birch pollen season (April and first half of May) and grass pollen season 2003 (end of May until end of July) all patients completed daily diary cards, where symptoms from eyes, nose and lungs were

Table 1. Clinical and paraclinical characteristics of participating SA and AS

	Autumn 2002 SPT*incl. (> 3 mm)	Winter 2003 SPT*vis.1 (> 3mm)	Autumn 2003 SPT* visit 2 (> 3 mm)	Winter 2003 CAP visit 1 (class)	Autumn 2003 CAP visit 2 (class)	Winter 2003 HR visit 1 (class)	Autumn 2003 HR visit 2 (class)	Winter 2003 CPT visit 1 (neg.) (SQ)	Autumn 2003 Symptoms	LPR (mm)
SA 1 M/B	4.5	7.0	9.0	4	4	3	3	1000	70	62.0
2 F/B	8.0	8.5	5.5	3	3	3	3	1000	7	43.5
3 M/B	7.5	11.0	6.0	3	3	3	3	1000	28	56.0
4 M/B	3.5	5.5	5.5	2	3	2	3	1000	68	44.5
5 M/B	11.5	9.0	8.0	4	4	3	2	1000	48	0.0
6 F/G	16.0	14.5	13.5	3	3	3	3	1000	140	135.0
7 M/G	8.5	19.0	10.5	3	3	3	3	100	176	82.0
8 F/G	10.0	20.0	14.0	4	3	3	3	100	59	100.0
9 M/G	14.5	11.5	12.5	4	4	3	3	100	182	93.0
10 F/G	8.0	5.0	12.0	2	2	0	3	1000	44	43.5
11 F/B	7.0	11.0	7.5	3	3	3	3	1000	40	56.0
AS 1 M/B	6.5	4.5	7.5	0	1	2	3	10000	0	0.0
2 F/B	3.5	5.0	3.5	1	1	3	2	100 000	0	0.0
3 F/B	3.5	3.5	6.0	0	0	2	3	10000	0	0.0
4 F/B	3.5	3.5	4.0	0	0	2	1	100 000	0	0.0
5 F/B	4.0	4.0	6.0	0	0	3	2	100000	0	49.5
6 F/B	4.5	3.5	Neg.	0	0	2	2	10000	0	0.0
7 F/G	5.0	4.5	6.5	0	0	0	0	100000	0	0.0
8 F/G	6.0	Neg.	6.0	2	2	0	1	100 000	0	0.0
9 F/G	3.5	5.5	4.5	0	0	0	0	100 000	0	0.0
10 F/G	5.5	4.0	4.0	0	0	0	0	100 000	0	0.0
11 M/G	4.0	3.5	3.5	1	2	3	2	10000	0	23.5

*The SPT was performed in duplicate (positive: mean weal diameter > 3 mm). The mean diameter of the largest weal is shown. Negative: SPT ≤ 3 mm. For the CPT the highest concentration not eliciting symptoms (itchy and red eyes) is shown. The highest symptom score reached by each individual during the relevant pollen season is shown.

The demographic data (age) on the 10 healthy controls (seven women/three men): median: 23.2 years; range: 21.5–42.1 years.

F, female; M, male; B, sensitized (and for SA also symptoms) to birch and stimulated with birch; G, sensitized (and for SA also symptoms) to grass and stimulated with grass; HR, histamine release; SPT, skin prick test; LPR, late-phase reactions; CPT, conjunctival provocation test; SA, symptomatic atopics; AS, asymptomatic skin sensitisation.

recorded and graded from 0 to 3 points (0 = none, 1 = mild, 2 = moderate, 3 = severe symptoms). In agreement with Bodtger et al. [1], the symptom score was calculated as the sum of daily organ (eyes + nose + lungs)-related scores during the relevant season(s). Symptoms were considered as pollen allergy when lasting ≥ 7 days or when symptoms were repeatedly elicited when pollen counts exceeded a certain (individual) level [1]. The skin prick test (SPT) was performed according to the guidelines of European Academy of Allergy and Clinical Immunology [12]. The first group of participants consisted of 10 healthy controls (HC) without any clinical history of hayfever and asthma and with negative SPT against 10 standard aero-allergens (Soluprick, ALK-Abello, Hørsholm, Copenhagen) comprising birch, grass, mugwort, horse, dog, cat, two house dust mite (HDM) species and two mould species. The 11 AS were mono-sensitized (SPT > 3 mm to birch or grass) and without clinical histories of hayfever and asthma. Opposite to SA, the majority of AS had no detectable allergen-induced late-phase reactions (LPR) (Table 1). The third group, numbering 11 mono- or poly-sensitized SA, all presented with rhinoconjunctivitis during the birch and/or grass pollen season. None of the participants had used corticosteroids within the last 3 months. All persons gave informed consent. The study was approved by the local Ethical Committee.

Specific immunoglobulinE determination, histamine release and late-phase reaction

Allergy class ≥ 2 was considered positive (CAP System, Pharmacia Diagnostics, Uppsala, Sweden). Histamine release (HR) was performed according to Ostergaard et al. [13]. The LPR was performed by injecting 0.05 mL of 1000 (standardized quality) SQ birch or grass pollen extract (Aquagen, ALK-Abello) intradermally. The size of the LPR was outlined after 24 h, by resistance to the movement of sharpened pencil, and the mean weal diameter was calculated.

Challenge tests

Clinical susceptibility to birch and/or grass was assessed using titrated challenges of the conjunctivae with birch or grass pollen extracts (Aquagen, ALK-Abello) in tenfold dilution from 100 to 100 000 SQ or until a positive test. A positive test was defined as red and itchy eyes within 10 min after allergen challenge. Results were expressed as the highest dose not eliciting symptoms.

Reagents and antibodies

Tetanus toxoid (TT) (State Serum Institute, Copenhagen, Denmark) was used at a final concentration of 10 $\mu\text{g/mL}$. Phorbol 12-myristate 13-acetate (PMA) and ionomycin

(both from Sigma Chemical Corp., St Louis, MO, USA) were used at final concentrations of 10 ng/mL and 0.5 $\mu\text{g/mL}$, respectively. In order to simulate natural allergen exposure, grass allergen (*Phleum pratense*) and birch allergen extracts (*Betula verrucosa*) (a kind gift from ALK-Abello) were used at a final concentration of 15 $\mu\text{g/mL}$ (total protein). The lipopolysaccharide (LPS) content in both allergen extracts was less than 7 EU/mg of protein (according to ALK-Abello).

Cell culture and carboxyfluorescein succinimidyl ester staining

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood (drawn from birch atopics during the birch pollen season 2004 and from grass atopics during the grass pollen season 2004 as well as from HC (during the birch and grass pollen season 2004)) by density centrifugation on Lymphoprep (Nyegaard, Oslo, Norway), washed and counted. In brief, 5×10^7 cells/mL were resuspended in PBS and incubated at 37 °C for 10 min with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes Inc., Eugene, OR, USA) at a final concentration of 2 μM , followed by RPMI washing steps. PBMCs (3×10^6) were incubated in six-well plates with antigen in 6 mL of low toxin-containing RPMI-1640 with 25 mM HEPES (Biological Industries, Kibbutz, Beit Haemek, Israel) and supplemented with 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin and 50 μM β -mercaptoethanol (all reagents from Gibco, Copenhagen, Denmark) in 10% heat-inactivated autologous serum. The allergen to which a symptomatic atopic subject had symptoms was used for stimulation. Furthermore, the allergen to which a subject with asymptomatic skin sensitization was sensitized was used for stimulation. Cultures from non-atopic donors were stimulated with both birch and grass extracts (concentrations unaltered). At day 6, PMA and ionomycin were added to some wells. Supernatants from these wells were collected at day 7 for cytokine measurements. Cells from the remaining wells were harvested for further flow cytometric analysis. As a control for LPS-induced activation of monocytes, PBMCs from healthy donors were incubated with both allergen extracts for 24 h.

Cytokine measurements

IFN- γ , TNF- α , IL-2, IL-4, IL-5 and IL-10 were measured using the Cytometric Bead Array (CBA) system (Becton-Dickinson, Rutherford, NJ, USA). BD CBA software version 1.1 (Becton-Dickinson) was used for analysing cytokine responses to antigen. For facilitated detection of Th2 cytokines, we used a protocol developed by Kurtzhals et al. [14]. Changes in cytokine production were calculated by subtracting the cytokine values from 'non-stimulated' cells (no antigen at day 0, but PMA and ionomycin added

at day 6) from the values obtained from antigen-stimulated cells (allergen or TT at day 0, plus PMA and ionomycine added at day 6). Twenty-four hours incubation of both allergens with PBMCs derived from healthy donors resulted in undetectable levels of TNF- α (data not shown).

Flow cytometry analysis

Primary labelled antibodies were used: CCR4-PE was purchased from (Becton-Dickinson). CD4-RPE-Cy5 and CD45R0-PE were obtained from (Dako-Cytomation, Copenhagen, Denmark). Antibodies were used at recommended concentrations. Three-colour flow cytometry was performed on day 0 (in duplicate), and after 7 days. Isotype control cut-off values were set to > 98%. A BD FACS scan flow cytometer (Becton-Dickinson) was used, and 10 000 mononuclear cells per sample were acquired. Forward and side-scatter characteristics identified the

lymphocyte population. For memory ($CD45R0^+$) and naïve ($CD45R0^-$) CD4 T cells, antigen-induced proliferation (% divided cells in response to allergen) was estimated by subtracting the area under curve (AUC = divided cell compartment) in the CFSE histogram obtained from non-stimulated cells, from the AUC in the diluted CFSE histogram obtained from antigen-stimulated cells (Fig. 1). Moreover, based on the CFSE profile of CD4 lymphocytes, antigen-specific precursor frequencies were calculated according to the formula (with included erratum) shown in [15].

Statistics

Differences within groups were compared by Wilcoxon's matched paired test, while differences between SA, AS and HC were compared by the Mann-Whitney *U*-test, using SPSS version 11.0 Inc. (Chicago, IL, USA). Two-tailed *P*-values < 0.05 were considered significant.

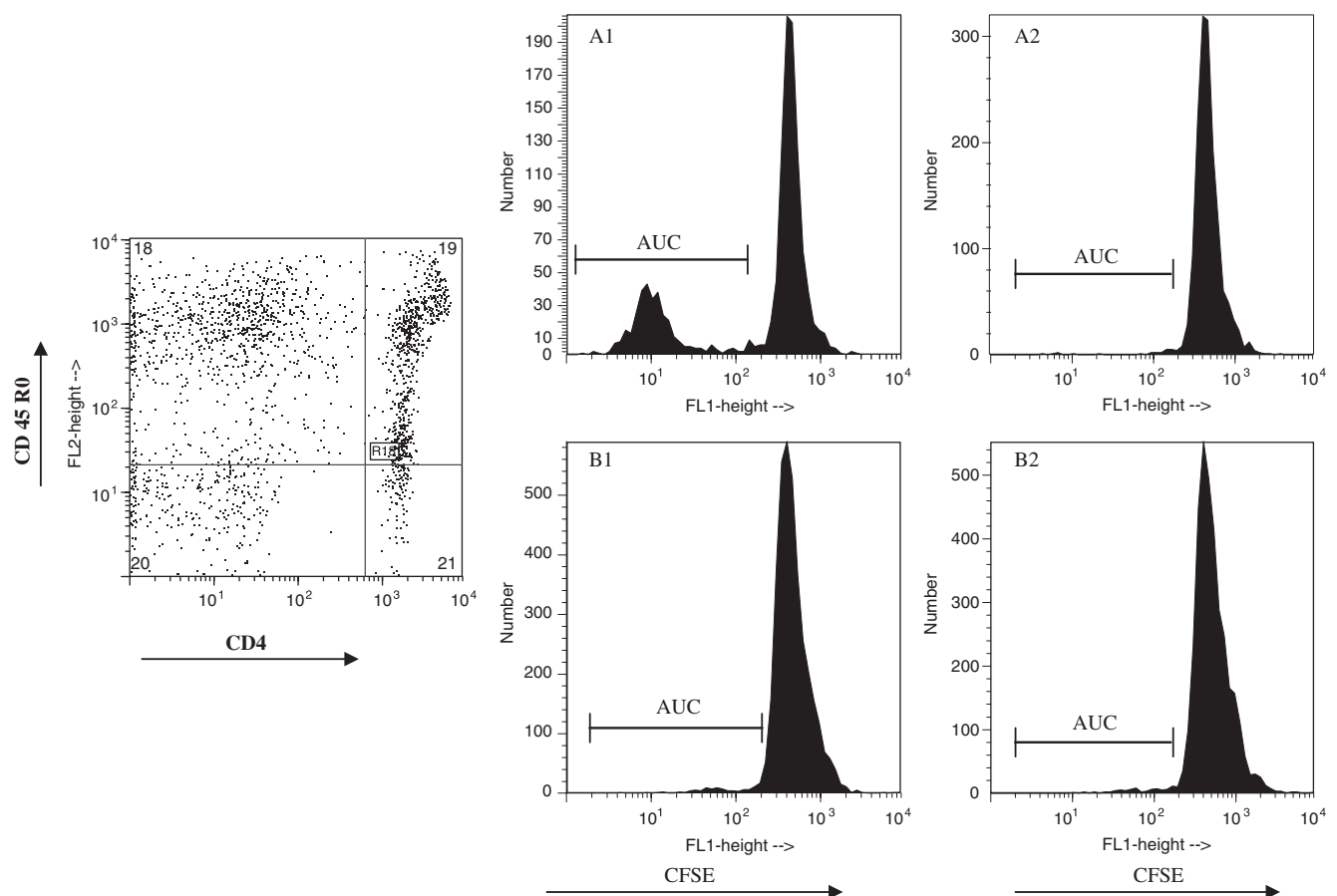


Fig. 1. Memory T cell proliferation in allergen-stimulated and non-stimulated cultures. $CD4^+CD45R0^+$ memory T cells were identified (left panel). (A1) A 7-day allergen-stimulated culture isolated from a symptomatic atopic. The carboxyfluorescein succinimidyl (CFSE) dilution (the area under curve) of more than 1/5 of the initial CFSE fluorescence intensity is consistent with at least five $CD4^+CD45R0^+$ memory T cell divisions. (A2) A CFSE histogram showing the proliferative profile in a 7-day non-stimulated culture from the same donor. (B1) CFSE loaded peripheral blood mononuclear cells were stimulated with allergen for 7 days. The proliferative profile of CFSE loaded memory T cells, derived from a representative subject with asymptomatic skin sensitization, is shown. (B2) A representative proliferative profile of CFSE loaded memory T cells in a 7-day non-stimulated culture from the same donor, is shown.

Results

Characteristics of symptomatic atopics, asymptomatic skin sensitization and healthy controls

Individual clinical and paraclinical characteristics of SA and AS are shown in Table 1, while Table 2 summarizes the clinical and paraclinical data for SA (median: 25.1 years; range: 22.2–42.2 years), AS (24.5 years; 20.5–29.9 years) and HC (23.2 years; 21.5–42.1 years).

Proliferation of CD4⁺CD45RO⁺ and CD4⁺CD45RO⁻ T cells, in response to allergen and tetanus toxoid

In neither of the examined lymphocyte subsets, derived from SA, AS and HC, statistical differences in stimulating potency between grass and birch pollen extracts were observed. Stimulation of CFSE-loaded PBMCs with birch or grass pollen extract, resulted in a pronounced expansion of CD4⁺CD45RO⁺ memory T cells (henceforth memory T cells) in birch or grass SA, as compared with the memory T cell proliferation seen in allergen-stimulated cultures derived from AS and HC (Fig. 1, only data from a representative SA and AS are shown). While allergen-induced memory T cell proliferation in SA differed significantly from that of AS and HC (Fig. 2), we observed no statistical differences, with regard to allergen-induced memory T cell proliferation, between AS and HC (Fig. 2). We noticed no statistical differences in TT-driven memory T cell proliferation between SA, AS and HC (data not shown). Allergen-driven and TT-driven proliferation of naïve CD4⁺CD45RO⁻ T cells was similar in all three groups, and was generally far less pronounced than the corresponding memory T cell responses (Fig. 2, only data for allergen are shown).

Frequency of allergen-specific CD4⁺ T cells

Allergen-specific precursor T cell frequencies were calculated on CD4 gated lymphocytes, entailing that the term precursor cells does not refer to the phenotype (e.g. naïve vs. memory) of the CD4⁺ T cells in question, but simply to their status as being allergen-specific. This definition is in agreement with how the term is used in [16–18]. The frequency of allergen-specific precursor CD4 T cells in cultures isolated from SA ranged from 0.06% to 1.48%, the median value being 0.56% (data not shown). The corresponding values in AS and HC were (0.13%; range: 0.02–0.50%) and (0.12%; range: 0.03–1.11%) respectively (data not shown), – the differences between SA and the two other groups being the only differences of statistical significance ($P=0.02$ for SA vs. AS and SA vs. HC). The median for TT-specific precursor T cell frequencies was 0.38% in SA, 1.70% in AS and 1.20% in HC – these differences being not significant. The percentage of aller-

Table 2. Summary of clinical and para-clinical characteristics of participating atopic and healthy subjects

Patients	Birch pollen						Grass pollen					
	Symptoms median (range)	SPT (mm) median (range)	CAP class median (range)	HR class median (range)	LPR (mm) median (range)	CPT (SQ) median (range)	Symptoms median (range)	SPT (mm) median (range)	CAP class median (range)	HR class median (range)	LPR (mm) median (range)	CPT (SQ) median (range)
SA Birch	44 7–70	7.5 3.5–11.5	3.0 2–4	3.0 2–3	50.3 0.0–62.0	10 ³ 10 ³ –10 ³	0* 0–49	0* 0–20	0* 0–4	1.5* 0–3	0* 0–85	5.1 × 10 ⁴ 10 ² –10 ⁵
SA Grass	0*	0*	0*	0*	ND†	ND†	140	12.5	3	3	93.0	10 ²
AS Birch	0–154	0.0–11.5	0–3	0–3	0.0	5.5 × 10 ⁴	44–182	5.0–20.0	2–4	0–3	43.5–135.0	10 ² –10 ³
AS Grass	0	3.8	0	2	0.0	10 ⁴ –10 ⁵	0	0.0–0.0	0	0	0	ND†
AS Grass	0	3.5–5.0	0–1	1–3	0.0–49.5	10 ⁴ –10 ⁵	0	0.0–0.0	0	0	0.0	10 ⁵
HC	0	0.0	0	0	0	ND†	0	4.5	0	0	0.0	10 ⁴ –10 ⁵
	0–0	0.0–0.0	0–0	0–0	0–0	10 ⁵	0–0	3.5–6.0	0–2	0–3	0.0–23.5	10 ⁴ –10 ⁵
	0	0.0	0	0	0	10 ⁵	0	0.0	0	0	0	10 ⁵
	0–0	0.0–0.0	0–0	0–0	0–0	10 ⁵ –10 ⁵	0–0	0.0–0.0	0–0	0–0	0–0	10 ⁵ –10 ⁵

*Two SA (5M/B and 9M/G) were sensitized to birch and grass pollen and complained from birch and grass pollen related symptoms. Further, one symptomatic subject to birch pollen (SA 2F/B) was asymptotically sensitized to grass pollen. †ND, not determined.

HR, histamine release; SPT, skin prick test; LPR, late-phase reactions; CPT, conjunctival provocation test; SA, symptomatic atopics; AS, asymptomatic skin sensitisation.

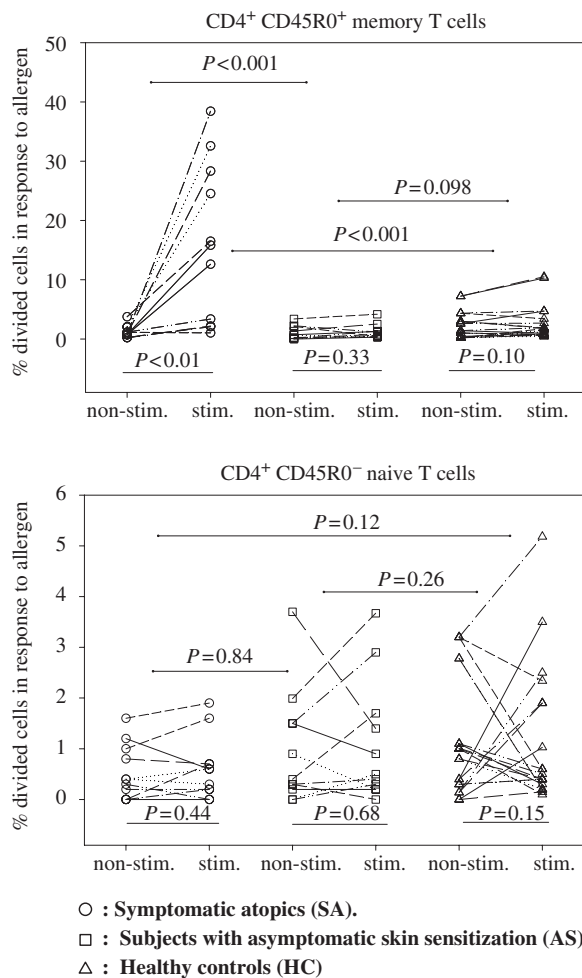


Fig. 2. Allergen-driven proliferation of CD4⁺CD45RO⁺ and CD4⁺CD45RO⁻ T cells. Cultures from symptomatic atopics (SA) ($n = 11$), AS ($n = 11$) and HC ($n = 10$) were stimulated with allergen for 7 days or left non-stimulated. Divided memory T cells (CD4⁺CD45RO⁺) and divided naïve T cells (CD4⁺CD45RO⁻) were identified by flow cytometry. The resulting changes in proliferation (% divided cells) between non-stimulated and allergen-stimulated cultures at day 7 are shown. Wilcoxon's paired test was used to test differences in proliferation between medium and allergen conditions. Mann-Whitney *U*-test was used to test differences between SA, AS and HC.

gen- and TT-specific precursor T cells in cultures derived from SA did not differ significantly. AS and HC displayed more TT-specific than allergen-specific precursor T cells ($P < 0.01$ for both).

Expansion of CD4⁺CCR4⁺ T cells, in cultures from symptomatic atopics, asymptomatic skin sensitization and healthy controls

In CD4⁺ T cell cultures derived from SA, AS and HC, significant differences in CCR4 receptor levels expressed as percentage of positive cells or as mean fluorescence intensity (MFI) were not observed between day 0 and antigen-free cultures at day 7. Among SA, examination of

allergen-driven proliferating cells revealed significant expansions of CD4⁺CCR4⁺ T cells (44.5%; range: 23.7–69.8%) as compared with non-proliferating cells (13.3%; 5.4–15.7%), ($P = 0.02$) (Fig. 3). The numbers of proliferating CD4⁺CCR4⁺ T cells in allergen-stimulated cultures isolated from AS and HC were too few ($< 1\%$), to allow for a consistent comparison with non-stimulated cultures. The expansion of CD4⁺CCR4⁺ T cells in cultures derived from SA was accompanied by increased expression of CCR4 (MFI) (35.8; range: 23.5–47.5) vs. (22.5; range: 19.8–33.1), respectively ($P = 0.02$). We noticed significant expansions of CD4⁺CCR4⁺ T cells as well as increased expression of CCR4 (MFI) among TT-driven proliferating cells, derived from SA ($P = 0.02$ and $P = 0.02$, respectively), AS ($P = 0.03$ and $P = 0.03$, respectively) and HC ($P = 0.01$ and $P = 0.03$, respectively), – without statistical differences between the three groups.

Cytokine responses to allergen and tetanus toxoid in symptomatic atopics, asymptomatic skin sensitization and healthy controls

Allergen-induced levels of Th1 cytokines (IFN- γ , TNF- α) and Th2 cytokines (IL-4, IL-5, IL-10) differed significantly between SA and the two other groups (Fig. 4). Concerning allergen-induced production of Th1 and Th2 cytokines, AS and HC were statistically non-distinguishable (Fig. 4). The IL-5/IFN- γ ratio was significantly higher in SA (median: 0.23%), than in AS (median: 0.00%) ($P = 0.001$) and HC (median: 0.00%) ($P < 0.001$) (data not shown). Apart from differences in net IL-2 levels (allergen-stimulated minus non-stimulated) between SA (median: 2628.4 pg/mL) and AS (median: –3647.2 pg/mL) ($P = 0.01$) and between SA and HC (median: –1011.6 pg/mL) ($P < 0.01$), significant differences in TT-induced Th1 and Th2 cytokine levels, between these three groups, were not observed (data not shown). A strong tendency towards increased levels of IL-5 ($P = 0.05$), but not IL-4 ($P = 0.33$) or IFN- γ ($P = 0.48$), was found in SA after stimulation with allergen as compared with TT. Only in cultures isolated from SA, the IL-5/IFN- γ ratio differed significantly between allergen- and TT-stimulated cells ($P = 0.02$).

Discussion

To our knowledge, we here present the first *in vitro* investigation of allergen-specific precursor T cells combined with a description of allergen-induced memory T cell proliferation and CD4⁺ T cell expression of CCR4, in cultures derived from clinically well characterized AS. As bystander amplification of ryegrass-induced PBMC cytokine responses has been reported in subjects also sensitized to HDMs [19], we aimed at ensuring a minimal pro-inflammatory background in T cell cultures derived from our AS. Thus, AS with positive skin reactions to

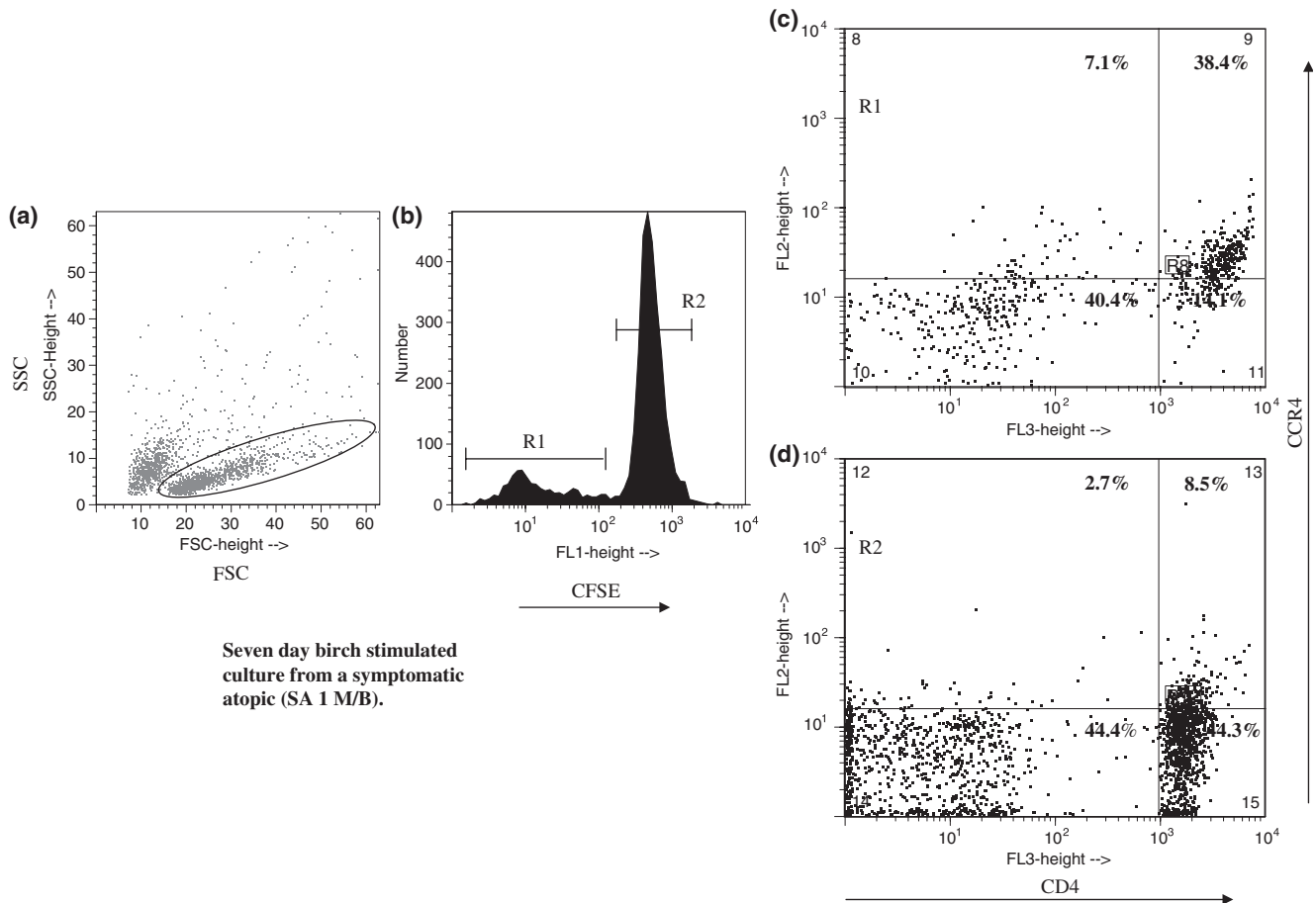


Fig. 3. CCR4 expression among proliferating $CD4^+$ T cells vs. non-proliferating $CD4^+$ T cells. carboxyfluorescein succinimidyl (CFSE) loaded peripheral blood mononuclear cells from a representative symptomatic atopic were stimulated with allergen for 7 days. (a) The lymphocyte population (including proliferating cells) was identified by FCS/SSC characteristics. (b) Histogram showing the CFSE content of the gated lymphocyte population. R1 and R2 represent divided and non-divided cells, respectively. (c) The expression of CCR4 and CD4 by the divided cell population is shown. (d) The corresponding expression of CCR4 and CD4 by the non-divided cell population is shown.

perennial allergens such as pets, HDMs and moulds were not included in this study. It cannot be excluded that the disparity in the numbers of positive skin reactions between SA and AS could contribute to the highly significant differences in allergen-induced proliferation observed between the two groups. However, – as pronounced differences in allergen-induced Th2 cytokine responses between mono-sensitized SA and HC have been demonstrated in a previous study [19], and as our AS resembled HC in all aspects, it seems unlikely that the conclusion of this study would have been altered by the difference in the number of SPT-positive skin reactions between SA and AS.

Atopic subjects with weak but potentially birch- or grass-related symptoms were precluded from further participation, resulting in an overrepresentation of women among AS, as women were much more inclined to complete their symptoms diaries meticulously than men. We would not expect this skewing to distort the value of our observations, since a review of the relevant literature

does not seem to lend support to gender-specific differences in allergen-induced T cell responses [10, 20–22].

As fully differentiated cells, memory Th2 cells have a much lower threshold for activation than naïve T cells [23, 24] and are the principal effector cells after reencountering allergen, mediating their often diverse immunological functions through various cytokines [25]. In our study, only SA were able to mount a substantial allergen-induced memory T cell response, while the naïve T cell responses to both allergen and TT were relatively sparse in all three groups. In analogy, allergen-driven expansion of the $CD4^+CCR4^+$ T cell population and increased expression of CCR4 was only seen in cultures derived from SA. Since approximately 90% of memory Th2 cells express CCR4 [26] and peripheral accumulation of $CCR4^+$ T cells has been associated with allergic symptoms from skin and lungs [8, 9], the clearly diminished allergen driven expansion of memory/ $CCR4^+$ T cells associated with our AS, indicates that these patients either have reduced numbers of allergen-specific memory T cells *per se*, or that

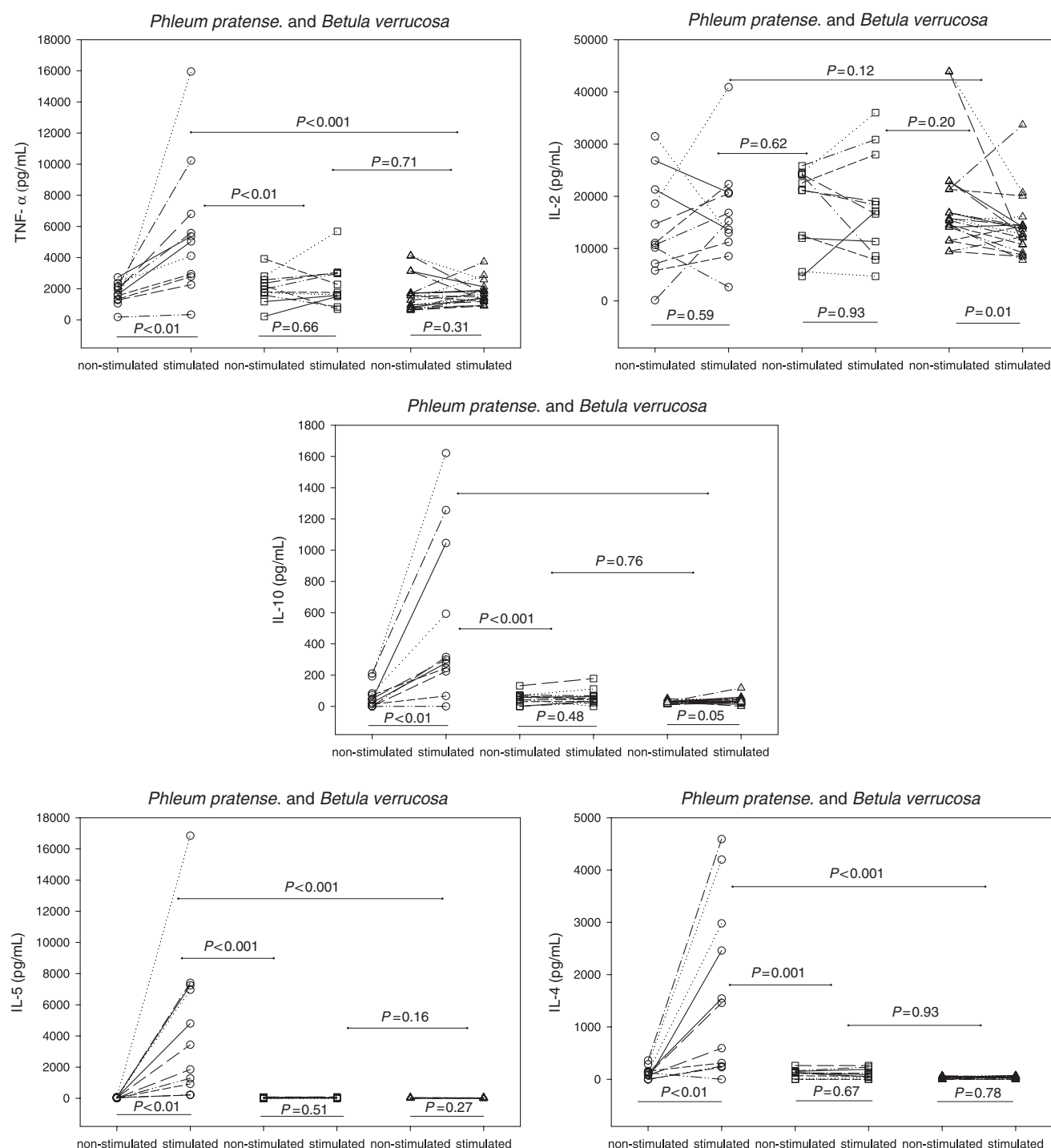


Fig. 4. Allergen-driven cytokine production in peripheral blood mononuclear cell cultures. Cultures were stimulated with allergen from day 0 or left non-stimulated. At day 6, PMA and ionomycin were added, in order to amplify the cytokine response. At day 7, supernatants were harvested and analysed. Changes in the levels of T-helper type 1 (Th1) and Th2 cytokines after 7 days are shown. Wilcoxon's paired test was used to test differences in proliferation between medium and allergen conditions. Mann-Whitney *U*-test was used to test differences between symptomatic atopics, asymptomatic skin sensitization and healthy control.

allergen-induced memory T cell proliferation is impaired because of other mechanisms (as discussed below). In agreement with these considerations, we were not able to detect any significant Th2 cytokine response in 7-day

allergen-stimulated cultures derived from AS. The scant allergen-induced IL-4 response in AS was consistent with their status as being primarily CAP negative (Table 1), while the reduced levels of IL-5 in response to allergen,

correlated with the predominantly non-detectable LPRs in these patients (Tables 1 and 2). In contrast, we observed a pronounced allergen-induced Th1 and Th2 cytokine response among SA, resembling previous findings related to symptomatic atopics, with regard to the IL-5/IFN- γ ratio [27] and the levels of IL-4 and IL-5 [19, 20, 27].

In agreement with Rimaniol et al. [28], we found allergen-specific precursor T cell frequencies up to 1.5% in SA, these frequencies being significantly higher than those found among AS and HC. The median values of TT-specific precursor T cell frequencies for all three groups were similar to those reported by others [15].

Among AS, what could account for the discrepancy between the clear-cut IgE skin sensitization and the lack of an allergen responsive immune system? First, the reduced numbers of peripheral allergen-specific T cells in AS could be a result of T cell homing to allergen-exposed mucosa. Decreased seasonal PBMC proliferation in grass sensitive SA, consistent with seasonal migration of grass-specific T cells has been shown [22]. However, our unpublished data show (paper submitted), that memory T cells from AS, in contrast to those of SA, do not display increased seasonal expression of the chemokine receptors CXCR1, CCR4 as well as L-selectin (CD62L), – receptors which have been associated with mucosal/skin homing in response to allergic inflammation [8, 29, 30]. Thus, our data suggest severely impaired memory T cell recruitment to the respiratory mucosa and skin [8, 31] among AS, during pollination, which is also substantiated by the predominantly negative LPRs seen among AS.

Second, IL-10 produced by allergen-specific T cells could have suppressed allergen-induced proliferation in AS [32]. Contrary to the IgE-positive AS studied by von Bubnoff et al. [11], we were not able to detect elevated levels of IL-10 in allergen-stimulated cultures derived from AS, making it less likely that our findings can be explained by the immunosuppressive effects of IL-10. From a teleological point of view, the less 'aggressive' atopic phenotype associated with our AS, may not require elevated levels of IL-10 in order to suppress allergen-induced proliferation and inflammation.

The most likely cause then, for the clinical features of AS, seems to be the reduced numbers of allergen-specific precursor CD4⁺ T cells, respectively, allergen-specific memory T cells *per se*. As increased apoptosis of antigen-specific memory T cells, because of the absence of continuous stimuli by resident antigen and/or lack of anti-apoptotic molecules, has been documented [33], similar mechanisms could account for the indolent allergen-specific T cell dynamics observed in cultures derived from AS.

A plausible mechanism for the positive skin reactions and the few positive conjunctival provocation tests (< 100 000 SQ) (Table 1) seen among our predominantly CAP-negative AS, is local IgE production (with minimal

'spillover' into the circulation), since IgE-producing plasma cells, resident in the mucosa, have been invoked as an explanation for the discrepancies between sensitization and allergic disease [34]. Interestingly, especially under these conditions, may the lack of a central allergen-specific T cell compartment, capable of generating new Th2 cells, be a prerequisite for maintaining peripheral mucosal immune tolerance.

In situations with low concentrations of total IgE, the extravascular compartment of IgE tends to be high, leading to entrapment in the skin of circulating specific IgE [35], which could contribute to the undetectable serum-specific IgE among AS. For clinical purposes, the cut-off value of the CAP system will classify low serum-specific IgE concentrations (< 0.35 kilo units arbitrary (kUA)/L) as negative.

In conclusion, these clearly IgE-sensitized AS displayed, in allergen-stimulated cultures, immunological characteristics not discernible from those of HC. Our findings raise some interesting questions concerning allergen-specific T cell dynamics within the atopic spectrum. Whether a change in immunological sensitivity, as indicated by allergen-induced memory T cell proliferation, is an early and strong predictor for subsequent development of allergy in AS remains to be established.

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