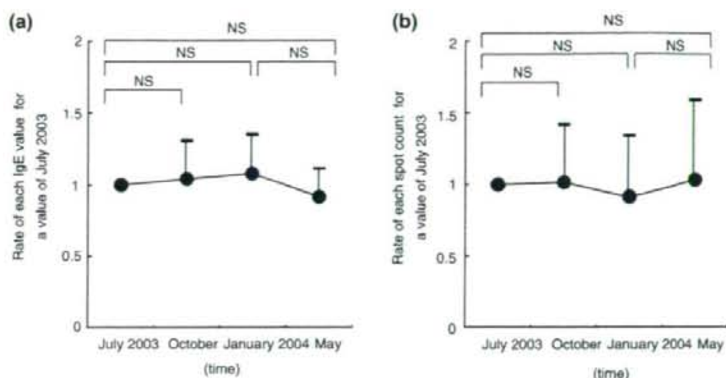

Fig. 5. The samples of each time-point were analysed simultaneously. Mite-specific IgE were analysed using the RAST technique (a). The Der f 1/2 peptide-specific IL-4-producing cells were counted using the ELISPOT technique (b). The rate of each value for the value in July is shown. NS, not significant.

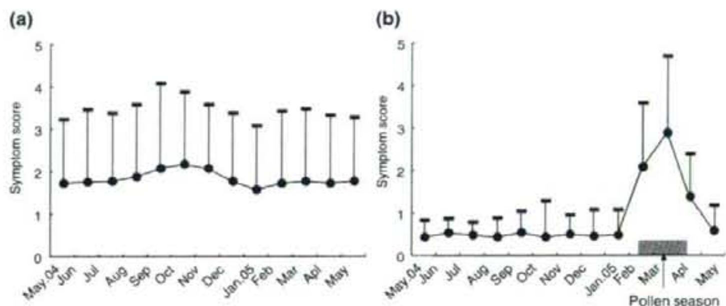

Fig. 6. The mean monthly symptom scores (mean+SD) from May 2004 to May 2005 are shown. (a) Mean nasal symptom scores of 22 patients with mite allergy. (b) Mean nasal symptom scores of 18 patients with Japanese cedar pollinosis.

Japanese cedar pollinosis by an ELISPOT assay using Japanese cedar-specific peptides. Although the number of cedar peptide-specific IL-4 T cells was low, the samples from all the patients examined had 10–100 spots/10^5 PBMC. In contrast, the healthy subjects had no positive IL-4 spot. These IL-4 T cells were thought to be cedar-specific Th2. The cedar peptide-specific IFN-γ T cells, which were thought to be cedar-specific Th1 cells, were detected in several patients. The cedar-specific peptides used in the ELISPOT assay were hybrid peptides that would only react with the T cells of those patients with Japanese cedar pollinosis, but not with the T cells of subjects not suffering from cedar pollinosis. Consequently, these findings could explain the low incidence and detection rates of cedar peptide-specific Th1 cells. The number of cedar peptide-specific IL-4 T cells (Th2 cells) did not correlate with the numbers of specific IFN-γ T cells (Th1 cells). More specifically, no relationship was observed between the cell types, and the cell numbers appeared to be independent of each other (although cedar-specific serum IgE levels did not correlate with the number of cedar-specific IL-4 T cells, the patients who had specific IFN-γ T cells had lower specific IgE levels than those without specific IFN-γ T cells).

IgE production is likely to be controlled by many factors. IFN-γ T cells inhibit IgE production independently of the number of specific IL-4 T cells [21, 22]. Similarly, pollen-specific IgE is known to exhibit seasonal changes [23, 24], thus increasing during the pollen season and decreasing during the off-season. However, the amounts of cedar and cypress pollen in 2004 were extraordinarily low and the pollen counts, which were less than 1/20 of the annual average for the last 10 years, did not contribute to the enhancement of IgE production in the patients enrolled in this study. Conversely, the cedar peptide-specific IL-4 T cells increased by 30% despite the low levels of pollen exposure. The levels of Th2 clone size increased to levels equivalent to that in July 2003.

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Although IL-4 is indispensable for IgE production, the cedar peptide-specific Th cells are thought to be very sensitive to exposure to cedar pollen.

In contrast, because the amount of pollen in 2005 was large, the cedar-specific IgE levels increased to five times after the pollen season. The specific Th2 clone size increased considerably after the pollen season, too, and the clone size increased by 70% after the pollen season. The levels of clone size increased to 1.4 times more than those observed in July 2004. A memory clone size might be affected by allergen exposition considerably. While cedar peptide-specific IL-4 Th2 cells decreased in number during the off-season, 8 months after the cessation of the pollen season, more than 60% of these memory Th2 cells were still found. Such annual drifting was absent in the perennial-allergic subjects. Therefore, pollinosis that undergoes exposition for a very limited period is different from mite perennial allergies.

Indeed, it is difficult to estimate the half-life of allergen-specific Th2. Generally, memory T cells are thought to have a long life-span, considering the duration of the vaccine effect of viral infections. Almost all reports of antigen-specific memory T cell have so far been about infectious diseases, while only a few reports of memory T cell are about type I allergy. The simple theory that an antigen creates a pool of long-lived antigen-specific memory T cells has been surprisingly difficult to prove and it is also still not completely accepted. The issue of longevity is much more controversial for $CD4^+$ than for $CD8^+$ T cells. Regarding $CD4^+$ memory T cells, lymphocytic choriomeningitis virus (LCMV)-specific $CD4^+$ memory T cells [25] and Sendai virus-specific $CD4^+$ memory T cells [26] at first disappeared dramatically, whereas virus-specific $CD8^+$ T cells were fairly stable in numbers over the same period. In humans, however, there have been several reports of vaccinia virus-specific $CD4^+$ memory T cells with a long life-span. These cells were found to decline with a half-life of approximately 10 years, conferring long-term protection [27]. The question remains, however, as to whether these memory Th cells are maintained in the absence of an antigen or by contact with a persistent antigen, bound in the immune complexes on follicular dendritic cells, or by contact with other environmental cross-reactive antigens. Based on our results on memory Th2 cells from cedar pollinosis in comparison with those from perennial mite allergy, the clone size maintenance requires antigen irritation of repetition and clonal mitosis by it. Therefore, the half-life of allergen-specific Th2 might be estimated to be less than 1 year.

The mechanism wherein a daughter Th2 cell discharges Th2 cytokines such as IL-4 in the same way after mitosis from the original Th2 has not been well understood. The opening and closing of chromatin helps to determine the cellular characteristics. It is believed that acetylation and methylation of the chromatin are very stable. Therefore, it is thought that the characteristics of a parent cell are transferred to the daughter cells after mitosis. However, a splitting enzyme for the acetylation and methylation has been discovered in a recent study, and it does not always seem to be stable [28]. It has recently been proposed that the opening and closing of chromatin is induced by an antigen, and that this is due to the Th cells' response [29, 30]. These changes in chromatin may therefore play an important role in retaining the memory functions of Th cells. Further studies need to be conducted on memory retention in order to clarify how these mechanisms could be applied to the development of an effective and fundamental solution for the treatment of allergic diseases.

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ORIGINAL ARTICLE

Association study of the *C3* gene with adult and childhood asthma

Hiroki Inoue · Yoichi Mashimo · Makiko Funamizu · Naoki Shimojo · Koichi Hasegawa · Tomomitsu Hirota · Satoru Doi · Makoto Kameda · Akihiko Miyatake · Yoichi Kohno · Yoshitaka Okamoto · Mayumi Tamari · Akira Hata · Yoichi Suzuki

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Abstract Bronchial asthma (BA) is a multifactorial disorder, the development of which is affected by both environmental and genetic factors. The complement system plays an important role in immunological response against invading microorganisms. It has been shown that complement-C3-deficient mice have reduced inflammation of asthmatic airways. Previously, we reported the association of four single nuclear proteins (SNPs) in the exons of the *C3* gene with childhood and adult BA. The *C3* gene, however, is a large gene, and functional SNPs associated with susceptibility to BA have not yet been identified. We analyzed 26 SNPs in the *C3* gene and its promoter region to narrow down the regions showing association with childhood and adult BA. Childhood and adult atopic BA patients and healthy child and adult controls were recruited from urban cities in Japan and genotyped. In SNP analysis, an SNP (SNP24, rs11569562) located in intron 31 of the *C3* gene was associated with adult BA [corrected P (P_{cor}) = 0.030]. In linkage disequilibrium (LD) block 4 spanning exons 24–41, the frequency of the CCC haplotype in adult BA was significantly higher than that in adult controls (P_{cor} = 0.038). Neither the SNP nor the haplotype showing association with adult BA demonstrated a significant association with serum total immunoglobulin E (IgE) level in BA patients and controls. Our results suggest that LD block 4 confers susceptibility to adult BA with mechanisms relevant to the effector phase of allergic inflammation.

Keywords Complement · *C3* gene · Asthma · SNP · Haplotype · Association · Total IgE

H. Inoue · Y. Mashimo · M. Funamizu · A. Hata · Y. Suzuki (✉)
Department of Public Health, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
e-mail: ysuzuki@faculty.chiba-u.jp

K. Hasegawa · T. Hirota · M. Tamari
Laboratory for Genetics of Allergic Diseases, SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan

N. Shimojo · Y. Kohno
Department of Pediatrics, Graduate School of Medicine, Chiba University, Chiba, Japan

Y. Okamoto
Department of Otolaryngology, Graduate School of Medicine, Chiba University, Chiba, Japan

S. Doi · M. Kameda
Department of Pediatrics, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Habikino, Japan

A. Miyatake
Miyatake Asthma Clinic, Osaka, Japan

Introduction

Bronchial asthma (BA) is an inflammatory airway disease, the development of which is affected by both environmental and genetic factors (Barnes and Marsh 1998). The complement system belongs to the groups of ancient pattern-recognition systems and sensing exogenous (microorganisms) and endogenous (altered-self) danger-associated molecular patterns (Kohl and Wills-Karp 2007). The three pathways of complement activation are the classical, alternative, and lectin pathways, and all complement activation pathways converge at C3. On activation, C3 breaks into a large C3b fragment and a small C3a fragment that is called anaphylatoxin. C3b can

form C5 convertase and activate another component of the complement, C5. C5 is cleaved into C5a anaphylatoxin and C5b, and both fragments exert physiological roles. Both anaphylatoxins also show proinflammatory and immunoregulatory actions. It has been shown that complement-C3-deficient mice have reduced inflammation of asthmatic airways (Barrington et al. 2001; Kohl 2001; Muller-Eberhard 1988). Mice and guinea pigs lacking the C3a receptor (C3aR) essential for C3a action were protected against bronchoconstriction, airway hyperresponsiveness (AHR), and airway inflammation developing after allergen challenge (Bautsch et al. 2000; Drouin et al. 2002; Humbles et al. 2000). Compared with wild-type mice, C3-deficient mice also exhibit diminished AHR and lung eosinophilia when challenged with an allergen (Drouin et al. 2001). They also showed decreased numbers of interleukin (IL)-4-producing lung cells and decreased serum-antigen-specific immunoglobulin E (IgE) levels. Dendritic cells (Zhou et al. 2007) and activated T cells express C3a receptor (Werfel et al. 2000), and C3 and C3a have been shown to regulate interactions between dendritic and T cells (Kawamoto et al. 2004; Zhou et al. 2007). These results suggested that the C3 pathway is involved in allergen sensitization. In BA patients, increased levels of C3a (Humbles et al. 2000; Krug et al. 2001) and C5a (Krug et al. 2001) were observed in bronchoalveolar lavage fluid after segmental allergen provocation, suggesting involvement of the complement system in allergen-induced airway inflammation in humans. Elevation of plasma C3a level was observed in BA patients on acute exacerbation (Nakano et al. 2003). These data are consistent with the notion that the *C3* gene plays an important role in allergic sensitization and allergic inflammation. Furthermore, the *C3* gene is located at chromosome 19p, where several studies suggested linkage to BA and related phenotypes (Blumenthal et al. 2004; Lee et al. 2000; Venanzi et al. 2001). Therefore, the *C3* gene is a strong candidate gene for allergic BA.

Our previous analysis of four single nucleotide polymorphisms (SNPs) in exons of the *C3* gene suggested that variations of this gene conferred susceptibility to both childhood and adult BA (Hasegawa et al. 2004). Barnes et al. reported that a haplotype spanning introns 19–23 showed significant association with BA, log (total IgE) and log [interleukin (IL)-13]/log [interferon (IFN)-γ] (Barnes et al. 2006). The *C3* gene, however, is a large gene consisting of 41 exons and containing hundreds of SNPs; thereby, further investigation is necessary to identify functional SNPs conferring susceptibility to BA. In this study, we analyzed 26 SNPs of the *C3* gene to narrow down the regions showing association with childhood and adult BA.

Methods

Subjects

All participants were Japanese. Three hundred and forty-six childhood BA patients, 518 adult BA patients, and 550 healthy adult controls were recruited in Osaka City area, Japan. Details of these patients are described in a previous report (Nakashima et al. 2006). All participants with BA were diagnosed and selected by physicians according to the American Thoracic Society (ATS) criteria and using questionnaires based on the recommendation of the ATS, Division of Lung Disease (ATS-DLD) (Ferris 1978). In brief, patients showed repeated episodes of at least one of the following symptoms: cough, wheezing, shortness of breath, chest tightness, and sputum production. Spirometry was performed in all patients to confirm the obstructive pattern of the lung function and response to a bronchodilator. Improvement of their forced expiratory volume in 1 s (FEV_1) measurement was at least 12% after β_2 agonist inhalation. The diagnosis of atopic BA was based on one or more positive skin-scratch-test responses to a range of seven common allergens in the presence of a positive histamine control and a negative vehicle control. The seven allergens were house dust, *Felis domesticus* dander (Feld), *Canis familiaris* dander, *Dactylis glomerata*, Ambrosia, *Cryptomeria japonica*, and *Alternaria alternata*. The numbers of atopic childhood and adult BA were 304 and 371, respectively (Table 1). In this study, we only analyzed atopic BA patients. As child control subjects, 411 child volunteers with ages between 6 and 12 years (male:female = 1.0:1.04) were recruited in Chiba City, Japan. Total and eight specific IgE levels in serum were measured in this group (*Dermatophagouides pteronyssinus*, *C. familiaris*, *F. domesticus*, *A. alternata*, *C. japonica*, *D. glomerata*, egg white, golden/black bellied/Hungarian hamster). Questionnaires based on the International Study of Asthma and Allergies in Childhood (Asher et al. 1995) were used to exclude children with BA and/or atopic dermatitis. Three

Table 1 Clinical characteristics of patients and controls

	Childhood asthma	Adult asthma	Child control	Adult control
Number	304	371	333	550
Age mean (years)	9.69	45.13	9.22	44.57
Age range (years)	4–15	20–75	6–12	20–75
Gender (male:female)	1.54:1.0	1.11:1.0	1.0:1.04	2.81:1.0
Atopic asthma (%)	100	100		
Mean total IgE [log (IU/ml)]	2.66	2.54	1.90	ND

IgE immunoglobulin E, *ND* not determined

hundred and thirty-three children were used as a child control group in association studies. If atopy was defined as those who showed positive specific IgE (>0.35 IU/ml) to one or more allergens, 71% of the child controls were atopic. Details of the adult controls were described previously (Nakashima et al. 2006). Adult volunteers were interviewed by physicians, and those who were diagnosed as having BA, atopic dermatitis, and/or allergic rhinitis were excluded from the adult control group. Measurement of serum IgE levels and skin-prick tests were not performed in the adult controls. All patients and volunteers provided written informed consent to participate in the study in accordance with the rules of the process committee at the SNP Research Center (RIKEN). This study was approved by the ethics committee of Chiba University Graduate School of Medicine.

Screening and selection of SNPs

We previously investigated four SNPs in the exons of the *C3* gene (Hasegawa et al. 2004): 912G/A, 1692AG, 1936GA, and 4896 CT in our previous paper, which correspond to SNP6, SNP7, SNP13, and SNP25, respectively, in this paper (Table 2). SNP25 showed association with mite-positive childhood BA and adult BA. Because SNP25 is located in exon 41, the last exon of the *C3* gene, SNPs located around this exon were intensively investigated (SNP17–SNP26). We searched the dbSNP database to list up SNPs with the following criteria: minor allele frequency was more than 0.3, and distance of the nearest two SNPs did not exceed 2 kbp. Considering the potential importance of the gene's promoter region for gene expression, where several regulatory elements have been reported, we searched SNPs up to 5 kbp upstream of exon 1. Four SNPs (SNP1–SNP4) were identified. In addition, we included SNP14, SNP15, and SNP16 in this study to confirm the results of Barnes et al. (2006). To estimate the linkage disequilibrium (LD) status of these SNPs, we genotyped them in 96 adult controls. Haploview 3.32 program (Barrett et al. 2005) was used to show an LD map. LD block was defined with the solid spine of the LD method implemented in the Haploview program. Of the 26 SNPs investigated, 24 composed four LD blocks (Fig. 1). We selected tag SNPs from each LD block with the aid of the Tagger routine incorporated in the Haploview program.

Genotyping

Genomic DNA was prepared from whole blood samples using a standard protocol. Whole genome amplification was performed using the illustra GenomiPhi V2 amplification kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's standard protocol. Amplified deoxyribonucleic acid (DNA) was typed by allele-specific polymerase chain reaction (AS-PCR) using either the modified TaqMan AS amplification (TaqMan-ASA) method (Fujii et al. 2000) or SYBR Green detection. The primer and TaqMan probe sequences are shown in Table 3. For the TaqMan-ASA method, 2× Platinum qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA) was used as master mix, whereas 2× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) or 2× SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) (for only SNP24) was used for AS-PCR together with SYBR Green detection. For the ASA method, the PCR mixture contained 5 μl of 2× PCR master mix, 0.4 μM of each PCR

Table 2 Locations and allele frequencies of single nucleotide polymorphisms (SNPs) of the *C3* gene

SNP	Location	Allele (1/2)[a]	Position[b]	Minor allele frequency (%)[c]	dbSNP number
SNP1	Promoter	T/C	6678365	0.330	rs171094
SNP2	Promoter	C/T	6674037	0.157	–
SNP3	Promoter	A/G	6673635	0.298	rs163913
SNP4	Promoter	A/C	6673022	0.306	rs339392
SNP5	Intron 2	A/G	6669534	0.270	rs2250656
SNP6	Exon 9	G/A	6663291	0.426	rs2230201
SNP7	Exon 14	G/A	6669848	0.468	rs2230204
SNP8	Exon 14	G/A	6663704	0.414	rs2230205
SNP9	Intron 14	G/A	6660074	0.148	rs11569429
SNP10	Intron 14	G/A	6660050	0.016	rs4807984
SNP11	Intron 17	A/G	6656246	0.403	rs11672613
SNP12	Intron 19	A/C	6648829	0.134	rs366510
SNP13	Exon 21	C/T	6648406	0.064	rs423490
SNP14	Intron 23	C/T	6647342	0.128	rs2287848
SNP15	Intron 23	C/T	6647178	0.371	rs10410674
SNP16	Intron 23	C/G	6646001	0.435	rs10402876
SNP17	Intron 33	G/A	6634846	0.436	rs344549
SNP18	Intron 33	C/G	6633953	0.441	rs344550
SNP19	Intron 33	T/–	6633534	0.446	rs11569553
SNP20	Intron 35	A/G	6631937	0.468	rs344552
SNP21	Intron 35	C/A	6631928	0.394	rs344553
SNP22	Intron 36	A/G	6630563	0.457	rs2277983
SNP23	Intron 36	G/A	6630511	0.447	rs2277984
SNP24	Intron 38	T/C	6629753	0.456	rs11569562
SNP25	Exon 41	C/T	6628989	0.447	rs4807893
SNP26	3′ Downstream	T/G	6627442	0.414	rs379527

[a] Base expressed in the direction the gene

[b] Based on National Center for Biotechnology Information (NCBI) Build 35.1 reference group label

[c] In 96 adult control subjects

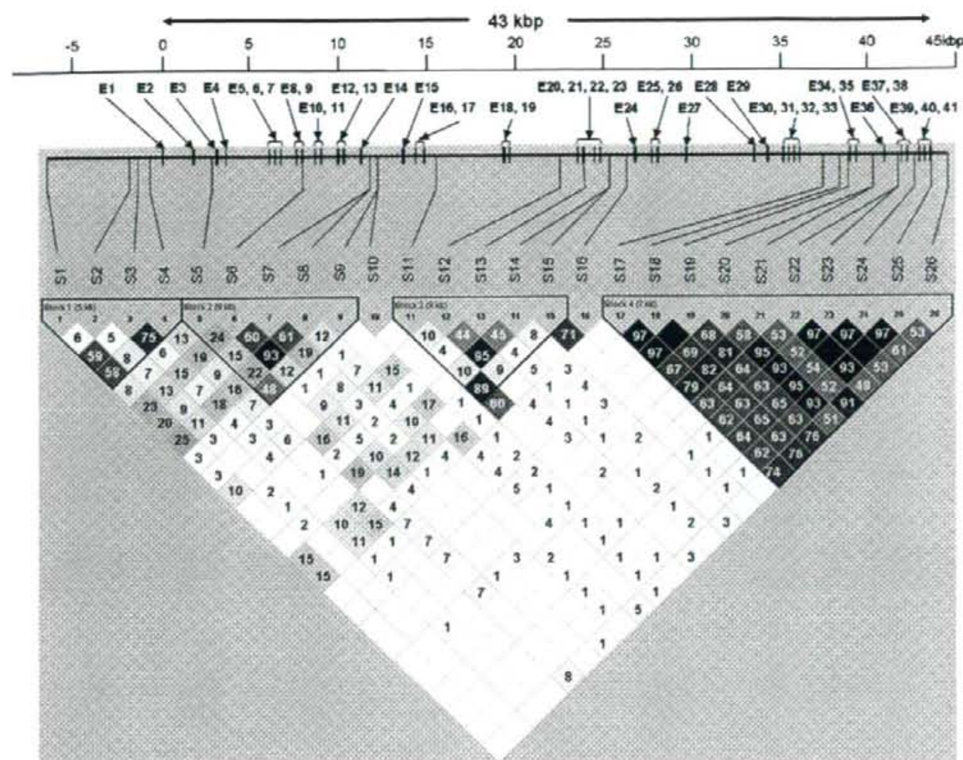

Fig. 1 Structure of the *C3* gene and location of single nucleotide polymorphisms (SNPs) and their linkage equilibrium (LD) map. Exons are indicated by *black boxes*. *Numbers* starting with *E* stand for exons and those starting with *S* for SNPs. Pair-wise LD (r^2) was estimated from 96 control subjects. LD blocks were defined by the solid spine of LD using the Haploview program. *Number* in each cell represents r^2 ($\times 100$); *black cells* $r^2 = 1$; *white cells* $r^2 = 0$. Each cell is colored in a graduated manner according to the strength of LD

primer, 0.12 μM of the TaqMan probe (when needed), and 5 ng of amplified template DNA in a final volume of 10 μl. The samples were analyzed using an ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) and Chromo4 Real-Time System (Bio-Rad). The thermoprofile was 50°C for 2 min, 95°C for 2 min, then 45 cycles of 95°C for 15 s and 60°C for 30 s (for SNP4: 45 cycles of 95°C for 15 s, 55°C for 30 s, and 60°C for 30 s). For SNP2, PCR was initially performed using 0.2 μM of each first PCR primers and *Taq* DNA polymerase (Promega, Madison, WI, USA) according to the manufacturer's standard protocol. The thermoprofile of the first PCR was 95°C for 3 min, followed by 20 cycles at 95°C for 20 s, 60°C for 30 s, and 72°C for 7 min. In the second PCR (TaqMan-ASA method), a reaction mixture of the first PCR diluted tenfold with water was used as a template. Because an SNP was located within the sequence where the AS primer for SNP9 was designed, two primers were mixed and used in the reaction mixture for SNP9 (Table 3).

Statistical analysis

Pairwise LD in SNPs was estimated as r^2 (Hill and Robertson 1968). A 2 × 2 contingency χ^2 test of independence was performed to evaluate the significance of an association between allele frequency and disease status. Haplotype inference and a case–control association study were performed using SNPAlyze ver. 4.1 program (DYNACOM, Mobara, Japan). The effects of genotypes on $\log_{10}$-transformed total serum IgE levels were evaluated using analysis of variance (ANOVA). Statistical analysis was performed with SPSS software (ver. 15.0 J; SPSS Japan, Tokyo, Japan). In SNP association studies, corrected P (P_{cor}) values were calculated by multiplying the number of SNPs tested in this study (15). In haplotype association studies, P_{cor} values were calculated by multiplying the total number of haplotypes tested in the entire gene (27). Association of an SNP or haplotype with the disease was judged as significant if P_{cor} for the test of allele/haplotype frequency was <0.05.

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Table 3 Primers for genotyping single nucleotide polymorphisms (SNPs) in the *C3* gene

SNP genotyped	Sequence
SNP1	
F: T allele	5′-GGCATAAACAAGGGTTAAAATGT
F: C allele	5′-GGCATAAACAAGGGTTAAAATGC
R: common	5′-GCTCACAAACGCCTATGA
TaqMan probe	5′-TGAAATAGATAAGTTGCTGCCACCCG
SNP2	
F: C allele	5′-GCCTGGCCAACATGGCGAACC
F: T allele	5′-GCCTGGCCAACATGGCGAAGT
R: common	5′-TGCCTCCTGGGTTCAAGTGATTCTC
TaqMan probe	5′-TAGTGGCGCATGCCTGTGATCCCAGCTCT
First PCR F:	5′-TTCCAAAGAGTGTGTCGCAA
First PCR R:	5′-CCTGCTTCATAGAGTTGTCGT
SNP3	
F: A allele	5′-TTTGGCAATATCTAGCAAGATTACCTA
F: G allele	5′-TTTGGCAATATCTAGCAAGATTACCTG
R: common	5′-CCTTACCACCTGCTTCATAGAGTTG
TaqMan probe	5′-TGACCCAACATATTTCCTTTCATTGCAACG
SNP4	
F: C allele	5′-CCTGTAACCTGTAAGAATGAGAC
F: A allele	5′-CCTGTAACCTGTAAGAATGAGTA
R: common	5′-CAAAGTGCTGGTGTGAACTACTG
TaqMan probe	5′-TAGTATGTGCTATGTGCTGTCC
SNP5	
R: A allele	5′-GCCTGCCCATTATTCTTGGTCCT
R: G allele	5′-GCCTGCCCATTATTCTTGGTCAC
F: common	5′-CCTTGTGAGCTCTTCTTTTTGAGTTC
SNP6	
R: G allele	5′-CACCCCGTCCAGCAGTACCTAC
R: A allele	5′-CACCCCGTCCAGCAGTACCTAT
F: common	5′-AACAGAGGATTTCCCTGCCTGAA
TaqMan probe	5′-CCCTCAAGCGCATTCCG
SNP7	
F: A allele	5′-AACAGAGGATTTCCCTGCCTGTA
F: G allele	5′-AACAGAGGATTTCCCTGCCTGGG
R: common	5′-CACCCCGTCCAGCAGTACCTTC
TaqMan probe	5′-CCCTCAAGCGCATTCCG
SNP8	
F: G allele	5′-TGCTGAATAAGAAGAACAAACTGAGG
F: A allele	5′-TGCTGAATAAGAAGAACAAACTGAGA
R: common	5′-TGCCTCCGCCTCTTCTCA
SNP9	
F: G allele	5′-TAGGGACGAAGATGGAGATGTG
G allele	5′-TAGGGACGAAGATGGAGATGAG
F: A allele	5′-TAGGGACGAAGATGGAGATGTA
A allele	5′-TAGGGACGAAGATGGAGATGAA
R: common	5′-CTTATCTCCATTTCCCCTCTGATTC
SNP10	
R: G allele	5′-TGATTCCATCTGCATTCCCAAC
R: A allele	5′-TGATTCCATCTGCATTCCCAAT
F: common	5′-TTTCCGGAGTAGGGACGAAGA
SNP11	
F: A allele	5′-AGGGTCACTGGGAAAATTAGACATA
F: G allele	5′-AGGGTCACTGGGAAAATTAGACAGG
R: common	5′-ATGGGCCAAAGGAATTACACAAT
SNP12	
F: A allele	5′-ACTCCCCGACCTTGACACTAA
F: C allele	5′-ACTCCCCGACCTTGACACTAC
R: common	5′-CCTGCATTACTGTGACCTCGAA
TaqMan probe	5′-CCCGAGCAGGGATCTGTGTGGCA
SNP13	
F: C allele	5′-GGAAGTGGAAGTCAAGGCTGGC
F: T allele	5′-GGAAGTGGAAGTCAAGGCTGGT
R: common	5′-GGGTGCCCCAAGCACTCA
TaqMan probe	5′-CCATCATTTCATCAGTGACGGTGTCAGGAA
SNP14	
R: T allele	5′-GAATGAGATGGAATTTGGCTCGA
R: C allele	5′-GAATGAGATGGAATTTGGCTCTG
F: common	5′-CAAGTCCCGGACACCGAGTCT
SNP15	
R: C allele	5′-CAGCGAGCTGAGGTCGGG
R: T allele	5′-CAGCGAGCTGAGGTCGTA
F: common	5′-CATCTGTGATCTGTTTTCCCTCTTTTAC
SNP16	
R: C allele	5′-GAGTGTCTCACTTAATAGTCAACGATG
R: G allele	5′-GAGTGTCTCACTTAATAGTCAACGATC
F: common	5′-TGGTCAGGCTGGTCTTGAACTC
SNP17	
F: G allele	5′-CTGCCAAAGTTTTGGGATCACTG
F: A allele	5′-CTGCCAAAGTTTTGGGATCACTA
R: common	5′-CCACACCCGGCCATTTCC
SNP18	
R: C allele	5′-AATGCCAGAAGTGAACTTCAAAGTG
R: G allele	5′-AATGCCAGAAGTGAACTTCAAAGTC
F: common	5′-CAGCAGGGTCAACATCACCATA
SNP19	
F: T allele	5′-GGCTGCCTGTATTCTTGCCTAT
F: delT allele	5′-GGCTGCCTGTATTCTTGCCTCG
R: common	5′-TGGATTCAAATTCCAGCTCTAAATAAC
SNP20	
F: A allele	5′-ATTCCAAGCATGAGCCACGA
F: G allele	5′-ATTCCAAGCATGAGCCACGG
R: common	5′-GGAGAGGAGAAAGCCCAAATCA
SNP21	
R: A allele	5′-GATGGAGAGAAAATAACAGAAGAGTT
R: C allele	5′-GATGGAGAGAAAATAACAGAAGAGCG
F: common	5′-ATGTTGCTCAAGTTGGTCTCAAACT
SNP22	
R: A allele	5′-GGCCTCCCTCCAAAGACCCT
R: G allele	5′-GGCCTCCCTCCAAAGACCTC
F: common	5′-CGTGTCCCAGGAATCTATGAATTT

Table 3 continued

SNP genotyped	Sequence
SNP23	
R: G allele	5′-ACCGGGTACAGCTTTCCTCTAC
R: A allele	5′-ACCGGGTACAGCTTTCCTCTTT
F: common	5′-GGCTTCTGTGAGTTGAGAGTCTAAGAGA
SNP24	
F: T allele	5′-CATGGCCATGAGGCTACAGTATAT
F: C allele	5′-CATGGCCATGAGGCTACAGTATAC
R: common	5′-CCCATGTCACCATCCACACA
SNP25	
F: T allele	5′-ACACTTGGGTGGAGCACTGGCAT
F: C allele	5′-ACACTTGGGTGGAGCACTGGCTC
R: common	5′-GGTCCTGGCATTGTTTCTGGTTCTC
TaqMan probe	5′-AGGAGGACGAATGCCAAGACG
SNP26	
R: T allele	5′-GGTGAGAATGTGGGCAAGAAGA
R: G allele	5′-GGTGAGAATGTGGGCAAGAAGC
F: common	5′-ACCTACATCCTCTCCGGTGAGTGT

PCR polymerase chain reaction, *F* forward primer, *R* reverse primer. All TaqMan probes were labeled with 6-carboxyfluorescein (FAM, reporter dye) at the 5′ end and 6-carboxy-tetramethyl-rhodamine (TAMRA, quenching dye) at 3′ end

Results

Polymorphisms in the *C3* gene

We selected and characterized 26 SNPs from the *C3* gene to investigate mainly genetic variations of the promoter region, SNPs forming a haplotype that showed significant association with BA in African Caribbean families, and the 3′ end region where a SNP showed significant association with BA in our previous study (Table 2). The location of the SNPs and LD map is shown in Fig. 1. If an LD block was defined by the solid spine of LD, out of the 26 SNPs investigated, 24 composed four LD blocks. SNP10 showed r^2 values <0.033 to any other SNPs investigated in the current study and excluded from any LD block under this definition. Although SNP16 showed moderate LD to SNP15 ($r^2 = 0.71$) and SNP11 ($r^2 = 0.60$), it did not belong to LD block 3, where SNP15 and SNP11 are located. This is because of the characteristic of the "solid spin of LD" definition. When SNP12 and SNP14, both of which showed very low r^2 (<0.01) to SNP16, were omitted from LD block 3, SNP16 was found to be included in LD block 3. As tag SNPs, four SNPs each were selected in LD blocks from 1 to 3 and three SNPs in LD block 4 (Fig. 1).

Association study of childhood and adult BA

We genotyped 15 tag SNPs in 304 childhood BA, 371 adult BA, 333 child controls, and 550 adult controls (Table 1). All loci were in Hardy–Weinberg equilibrium in the control groups. The genotype and allele frequencies of each SNP in the patient and control groups are shown in Table 4. Results of association tests for allele frequency between the patient and control groups are also shown. Allelic frequency of SNP24 was significantly different between adult BA and adult controls ($P = 0.002$). The P value for the difference remained significant ($P_{cor} = 0.030$) after correction for the number of SNPs tested (15). In a recessive model, the odds ratio (OR) and its 95% confidence interval (95% CI) range for this SNP were 1.55 and 1.15–2.09, respectively. In a dominant model, the OR and 95% CI range were 1.37 and 1.01–1.88, respectively. The difference in the allele frequency of SNP24 between all BA and all control showed a similar tendency. However, the P value was not significant ($P = 0.004$, $P_{cor} = 0.060$). None of the other SNPs showed a significant association with either childhood or adult BA.

The frequencies of haplotypes consisting of tag SNPs in four LD blocks are shown in Table 5. A significant difference was observed in LD block 4 (Table 5). In LD block 4 containing exons 24–41, the frequency of the major haplotype CCC in adult BA (54.1%) was significantly higher than that in controls (46.4%) ($P = 0.0014$, $P_{cor} = 0.038$). The frequencies of C alleles of SNP18, SNP19, and SNP24 were higher in adult BA than in adult controls. These increased frequencies were straightforwardly associated with the increased frequency of the CCC haplotype. The frequency of this haplotype in childhood BA (51.0%) was not significantly higher than that in adult controls (48.6%) ($P_{cor} > 1.0$). The OR of the CCC haplotype of LD block 4 was about 1.4 for both childhood and adult BA (Table 6). In LD block 2 containing at least exons 3–14, the frequency of the GGAG haplotype in childhood BA (5.6%) was higher than that in child controls (2.5%) ($P = 0.0044$). This difference, however, did not reach a gene-wide significance ($P_{cor} = 0.12$). In LD blocks 1 and 3, there was no haplotype showing any difference in frequency between patients and controls. LD block 1 contains the promoter region of the *C3* gene; therefore, it is not likely that genetic variations of the promoter region have significant effect on susceptibility to BA in the Japanese population.

Barnes et al. reported that a 3-SNP haplotype consisting of the SNPs identical to SNP14, SNP15, and SNP16 showed significant association with BA, log (total IgE) and log (IL-13)/log (IFN-γ) in the Afro-Caribbean families (Barnes et al. 2006). To assess the reproducibility of their results, we investigated whether this 3-SNP haplotype shows association with either childhood BA, adult BA, or log (total IgE) in our samples. We found that this haplotype showed no significant association with any of these phenotypes.

Table 4 Single nucleotide polymorphism (SNP) association study of the *C3* gene

	Childhood asthma					Adult asthma					Child control					Adult control					Childhood asthma versus child control	Adult asthma versus adult control	All asthma versus all control
	11[a]	12[b]	22[c]	1[d]	2[d]	11[a]	12[b]	22[c]	1[d]	2[d]	11[a]	12[b]	22[c]	1[d]	2[d]	11[a]	12[b]	22[c]	1[d]	2[d]	P_{cor}[e]	P_{cor}[e]	P_{cor}[e]
SNP1	0.382	0.473	0.144	0.619	0.381	0.438	0.432	0.130	0.654	0.346	0.351	0.508	0.141	0.605	0.395	0.395	0.457	0.149	0.623	0.377	>1	>1	>1
SNP2	0.755	0.222	0.024	0.865	0.135	0.785	0.171	0.044	0.871	0.130	0.754	0.192	0.055	0.849	0.151	0.769	0.205	0.027	0.871	0.129	>1	>1	>1
SNP3	0.440	0.443	0.117	0.662	0.339	0.431	0.456	0.113	0.659	0.341	0.375	0.502	0.123	0.626	0.374	0.424	0.467	0.109	0.658	0.343	>1	>1	>1
SNP4	0.433	0.406	0.161	0.636	0.364	0.437	0.439	0.124	0.657	0.344	0.402	0.475	0.123	0.640	0.361	0.426	0.430	0.143	0.642	0.358	>1	>1	>1
SNP5	0.542	0.405	0.054	0.744	0.256	0.616	0.323	0.061	0.778	0.223	0.562	0.369	0.069	0.747	0.254	0.628	0.310	0.062	0.783	0.217	>1	>1	>1
SNP6	0.411	0.461	0.128	0.642	0.359	0.371	0.454	0.175	0.598	0.402	0.381	0.469	0.150	0.616	0.385	0.327	0.485	0.188	0.570	0.431	>1	>1	>1
SNP7	0.201	0.497	0.302	0.450	0.551	0.256	0.497	0.247	0.505	0.496	0.255	0.482	0.264	0.496	0.504	0.285	0.485	0.230	0.528	0.473	>1	>1	>1
SNP9	0.737	0.232	0.030	0.854	0.146	0.750	0.219	0.031	0.860	0.141	0.709	0.273	0.018	0.846	0.155	0.753	0.219	0.028	0.863	0.138	>1	>1	>1
SNP11	0.340	0.505	0.155	0.593	0.408	0.331	0.489	0.180	0.576	0.425	0.324	0.502	0.174	0.575	0.425	0.312	0.478	0.210	0.551	0.449	>1	>1	>1
SNP13	0.862	0.131	0.007	0.928	0.073	0.889	0.106	0.006	0.941	0.059	0.880	0.120	0.000	0.940	0.060	0.890	0.110	0.000	0.945	0.055	>1	>1	>1
SNP14	0.769	0.228	0.003	0.883	0.117	0.779	0.202	0.019	0.880	0.120	0.778	0.213	0.009	0.885	0.116	0.786	0.206	0.008	0.889	0.111	>1	>1	>1
SNP15	0.379	0.480	0.141	0.619	0.381	0.348	0.514	0.138	0.605	0.395	0.363	0.469	0.168	0.598	0.403	0.316	0.503	0.181	0.568	0.433	>1	>1	>1
SNP18	0.305	0.557	0.138	0.584	0.417	0.403	0.442	0.156	0.623	0.377	0.348	0.454	0.198	0.575	0.425	0.328	0.506	0.167	0.580	0.420	>1	>1	>1
SNP21	0.367	0.522	0.111	0.628	0.372	0.433	0.444	0.122	0.656	0.344	0.369	0.471	0.159	0.605	0.395	0.358	0.498	0.144	0.607	0.393	>1	0.540	0.555
SNP24	0.248	0.537	0.215	0.517	0.484	0.225	0.456	0.319	0.453	0.547	0.282	0.450	0.267	0.508	0.492	0.285	0.482	0.233	0.526	0.474	>1	0.030	0.060

[a] Frequency of homozygote for major allele

[b] Frequency of heterozygote

[c] Frequency of homozygote for minor allele

[d] Allele frequency

[e] Corrected *P* value (raw *P* values were multiplied by number of SNPs, 15) for allele frequency difference

Table 5 Haplotype association study of the *C3* gene

Haplotype	Frequency				P_{cor}[a]		
	Childhood asthma	Adult asthma	Child control	Adult control	Childhood asthma vs. control	Adult asthma vs. control	All-asthma vs. control
Block 1[b]							
TCAA	0.441	0.483	0.436	0.467	>1	>1	>1
CCGC	0.295	0.263	0.279	0.280	>1	>1	>1
TTAA	0.129	0.108	0.121	0.103	>1	>1	>1
CCAA	0.051	0.043	0.041	0.042	>1	>1	>1
TCGC	0.032	0.051	0.040	0.021	>1	0.222	>1
CCGA	0.009	0.014	0.032	0.020	0.192	>1	0.297
CCAC	0.019	0.010	0.019	0.016	>1	>1	>1
TCAC	0.018	0.009	0.004	0.017	0.324	>1	>1
Others	0.006	0.022	0.029	0.027			
Block 2[c]							
AAGG	0.322	0.361	0.333	0.403	>1	>1	>1
AGAG	0.337	0.301	0.310	0.291	>1	>1	>1
GGAA	0.124	0.110	0.141	0.115	>1	>1	>1
AGGG	0.058	0.067	0.072	0.063	>1	>1	>1
GGGG	0.049	0.055	0.053	0.051	>1	>1	>1
GGAG	0.056	0.042	0.025	0.037	0.121	>1	0.729
AAAG	0.016	0.018	0.015	0.015	>1	>1	>1
AGAA	0.014	0.024	0.013	0.010	>1	0.567	>1
GAGG	0.019	0.015	0.036	0.009	>1	>1	>1
Others	0.006	0.006	0.002	0.005			
Block 3[d]							
ACCC	0.460	0.446	0.445	0.424	>1	>1	>1
GCCT	0.365	0.382	0.384	0.418	>1	>1	>1
ATTC	0.066	0.058	0.055	0.054	>1	>1	>1
ACTC	0.049	0.059	0.057	0.055	>1	>1	>1
GCCC	0.041	0.041	0.038	0.034	>1	>1	>1
ACCT	0.013	0.012	0.014	0.013	>1	>1	>1
Others	0.006	0.002	0.006	0.003			
Block 4[e]							
CCC	0.510	0.541	0.486	0.464	>1	0.038	0.079
GAT	0.365	0.340	0.387	0.375	>1	>1	>1
CCT	0.066	0.078	0.081	0.098	>1	>1	>19
GCT	0.053	0.033	0.038	0.044	>1	>1	>1
Others	0.007	0.011	0.008	0.019			

[a] Corrected *P* value (raw *P* values were multiplied by the number of haplotypes tested, 27) for haplotype frequency difference

[b] This haplotype is consisted of SNPs 1, 2, 3, and 4

[c] This haplotype is consisted of SNPs 5, 6, 7, and 9

[d] This haplotype is consisted of SNPs 11, 13, 14, and 15

[e] This haplotype is consisted of SNPs 18, 21, and 24

Table 6 Odds ratio (OR) and its 95% confidence interval (CI) of the CCC haplotype for bronchial asthma (BA)

	Diplotype frequency (%)				
	Case		Control		
	CCC/CCC and CCC/other	Other/other	CCC/CCC and CCC/other	Other/other	Odds ratio (95% CI)
Childhood BA	77.8	22.2	71.2	28.8	1.42 (0.99–2.04)
Adult BA	76.9	23.1	71.0	29.0	1.36 (1.00–1.86)
All BA	77.3	22.7	71.1	28.9	1.39 (1.10–1.75)

Diplotype of each individual was inferred with SNPAlyze ver. 4.1. OR and its (95% CI) were determined with SPSS ver. 15.0

Effect of genetic variations of the *C3* gene on serum total IgE level

To examine the effect of genetic variations of the *C3* gene on serum IgE level, 15 tag SNPs and the CCC haplotype were analyzed by analysis of variance (ANOVA) for log-transformed serum total IgE values (Table 7). *P* values <0.05 were observed in SNP14 in adult BA patients ($P = 0.042$) and in SNP13 ($P = 0.018$) and SNP18 (0.041) in child controls. If we want to maintain type 1 error at 0.05 in each patient/control group, a *P* value <0.0031 (0.05/16) should be considered as significant. Thus, we deduced that these SNPs did not significantly affect serum total IgE level. The genotype of SNP24 and the CCC haplotype, both of which showed an association with adult BA, were not significantly associated with the IgE level.

Table 7 Effect of single nucleotide polymorphisms (SNPs) and haplotypes of the *C3* gene on $\log_{10}$-transformed total immunoglobulin E (IgE)

Locus	*P* value in ANOVA test[a]		
	Childhood asthma	Adult asthma	Child control
Block 1			
SNP1	0.347	0.242	0.250
SNP2	0.657	0.960	0.320
SNP3	0.276	0.051	0.720
SNP4	0.329	0.058	0.182
Block 2			
SNP5	0.537	0.794	0.119
SNP6	0.860	0.533	0.805
SNP7	0.952	0.556	0.497
SNP9	0.368	0.795	0.270
Block 3			
SNP11	0.463	0.678	0.937
SNP13	0.917	0.100	0.018
SNP14	0.485	0.042	0.261
SNP15	0.486	0.816	0.688
Block 4			
SNP18	0.197	0.890	0.041
SNP21	0.065	0.280	0.097
SNP24	0.356	0.661	0.526
CCC haplotype	0.175	0.586	0.505

[a] Significance of difference in the mean of $\log_{10}$ (total IgE) among individuals with different genotypes (major allele homozygote, heterozygote, minor allele homozygote) was tested with analysis of variance (ANOVA). Three groups (childhood asthma, adult asthma, and child control) were evaluated separately. Serum IgE level was not determined in the adult control group

Discussion

In this study, we investigated the association of SNPs in the *C3* gene with childhood and adult BA. We observed a significant association of SNP24 and the CCC haplotype in LD block 4 with adult BA. As the CCC haplotype was discriminated from other major haplotypes with a frequency of >1% by the SNP24 allele (C/T), this haplotype association may just be a reflection of the association of SNP24. There was no significant difference in genotype frequency of SNP24 between the adult control group and the child control group, suggesting that the significant association observed in the adult patients–control comparison was not due to skewed genotype frequency of adult controls but to changes in adult BA. The frequency of the T/T genotype in childhood BA was decreased (from 0.282 to 0.248), as in adult BA. However, the change in allele frequency in childhood BA compared with that in child controls was small (T allele: 0.517 vs. 0.508). This SNP (or that with strong LD to this SNP) may not be a risk-modifying variation for childhood BA. Although it was not significant at the gene-wide level, we observed a tendency of the frequency of the GGAG haplotype of LD block 2 to show a difference between childhood BA and child controls ($P = 0.0045$, $P_{cor} = 0.121$). To determine and definitively conclude whether this LD block confers risk for childhood BA, an association study with more childhood samples and functional analyses of this region will be necessary.

LD block 4 is about 20 kb long and contains exon 41 coding the C-terminal end of the C3 protein and 3′ untranslated region as well as upstream 17 exons and introns. Because we could not find SNPs that change the amino acid sequences in exons in this LD block, functional variation(s) in this region should be those affecting either expression of the gene, ribonucleic acid (RNA) splicing, or RNA stability. To the best of our knowledge, there is as yet no study investigating elements affecting gene expression in this region of the *C3* gene. Currently, we do not have sufficient data to discuss the underlying mechanisms of the association between the LD block and adult BA. Further genetic and functional analyses of the LD blocks are necessary to pinpoint genetic variation(s) responsible for differences in susceptibility to BA.

It is possible that some of the control subjects involved in this study will develop asthma. Judging from the prevalence of the asthma in Japan, the chance of developing the disease is expected to be several percent. If we exclude atopic subjects from the control group, the chance may be reduced but not completely eliminated. This issue cannot be thoroughly controlled in a case–control study. The fact that our control group contained presymptomatic BA patients reduces the statistical power to detect an association between genotypes and the disease. We must be aware of this issue

when obtaining negative results. However, the main result (i.e., the significant association of the haplotype with the disease) cannot be changed by this issue. If we could eliminate presymptomatic BA patients, estimated ORs would be higher than the values we presented in this study.

We also investigated whether genetic variations of the *C3* gene affect total serum IgE level using childhood and adult BA patients and child controls. The IgE level was not significantly affected by any SNPs or haplotypes, including those showing a significant association with adult BA. This was true with mite-specific IgE level (data not shown). These results suggest that genetic variations of LD block 4 showing susceptibility to BA have a slight effect on sensitization to allergens but are more relevant to the effector phase of allergic inflammation. The roles of anaphylatoxins in the pathogenesis of BA can be divided into two phases: sensitization to allergens and effector phase of allergic BA (Kohl and Wills-Karp 2007). Several roles of C5a signaling at the interface between dendritic cells and T cells are evident, but those of C3a remain unclear. In a C3aR knockout experiment, different Th2 cytokine production responses were reported in various strains of mice (C57BL/6 and BALB/c) (Drouin et al. 2002; Humbles et al. 2000), suggesting that the effect of C3a is influenced by genetic background. As observed in mice, a difference in genetic background may explain the fact that association of the haplotype consisting of SNP14, SNP15, and SNP16 with total IgE level observed in Afro-Caribbean families was not confirmed in the Japanese population. Discrepancy between our results and those of Barnes et al. may also be due to a difference in the environmental factors of the two study populations. There are a number of studies showing that the effects of genetic variation (−159C/T) of the CD14 gene on allergic sensitization and BA risk differ greatly due to environmental factors such as mite or lipopolysacharide concentration in dust (Ober et al. 2000; Simpson et al. 2006; Vercelli 2003; Zambelli-Weiner et al. 2005). It may be possible that *C3* gene variations also show this type of gene–environmental interaction and cause discrepant results in studies with different populations.

In summary, our results suggest that the LD block containing exons 24–41 of the *C3* gene confer susceptibility to adult BA in the Japanese population. Because this region showed a slight effect on serum IgE level in both BA patients and normal individuals, this region may be involved in the effector phase of allergic inflammation. The effect of variations of the *C3* gene on allergic sensitization and BA susceptibility may differ according to genetic background and environmental factors.

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ORIGINAL PAPER Clinical Mechanisms in Allergic Disease

Allergen-specific immunotherapy alters the expression of B and T lymphocyte attenuator, a co-inhibitory molecule, in allergic rhinitis

M. Okano*, N. Otsuki*[1], M. Azuma†, T. Fujiwara*, S. Kariya*, Y. Sugata*[2], T. Higaki*, K. Kino‡, Y. Tanimoto§, K. Okubo¶ and K. Nishizaki*

*Department of Otolaryngology – Head & Neck Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan, †Department of Molecular Immunology, Tokyo Medical and Dental University, Tokyo, Japan, ‡Division of Research and Development, Meiji Dailies Corporation, Odawara, Japan, §Department of Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan, and ¶Department of Otolaryngology, Nippon Medical School, Tokyo, Japan

Clinical and Experimental Allergy

Summary

Background B7/CD28 family co-signalling molecules play a key role in regulating T cell activation and tolerance. Allergen-specific immunotherapy (SIT) alters allergen-specific T cell responses. However, the effect of SIT on the expression of various co-signalling molecules has not been clarified.

Objective We sought to determine whether SIT might affect the expression of three co-inhibitory molecules, programmed death (PD)-1, B7-H1 and B and T lymphocyte attenuator (BTLA), in Japanese cedar pollinosis (JCP).

Methods Peripheral blood mononuclear cells (PBMCs) were isolated from JCP patients who had or had not received SIT. PBMC were cultured in the presence or absence of Cry j 1, after which the cell surface expression of PD-1, B7-H1 and BTLA, as well as IL-5 production, were determined. In addition, the effect of BTLA cross-linking on IL-5 production was examined.

Results After Cry j 1 stimulation, no significant differences in PD-1 and B7-H1 expression were observed between SIT-treated and SIT-untreated patients. BTLA expression was down-regulated in untreated patients after Cry j 1 stimulation and up-regulated in SIT-treated patients. Up-regulation of BTLA in SIT-treated patients was particularly apparent in a $CD4^+$ T cell subset. IL-5 production was clearly reduced among SIT-treated patients, and the observed changes in BTLA expression correlated negatively with IL-5 production. Moreover, immobilization of BTLA suppressed IL-5 production in JCP patients.

Conclusion These results suggest that both IL-5 production and down-regulation of BTLA in response to allergen are inhibited in SIT-treated patients with JCP. BTLA-mediated co-inhibition of IL-5 production may contribute to the regulation of allergen-specific T cell responses in patients receiving immunotherapy.

Keywords allergen immunotherapy, allergic rhinitis, BTLA, Cry j 1, IL-5

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Correspondence:
Mitsuhiro Okano, Department of Otolaryngology – Head & Neck Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikatacho, Okayama 700-8558, Japan. E-mail: mokano@cc.okayama-u.ac.jp

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Introduction

Allergen-specific immunotherapy (SIT) is an effective treatment for IgE-mediated, type 2 T helper (Th2)-biased allergic diseases, particularly allergic rhinitis [1]. Unlike pharmacotherapy, SIT is unique in that it can alter the natural course of allergic disease by preventing new sensitization/onset and providing long-term remission after discontinuation of treatment [2–4].

A number of studies have shown that SIT alters allergen-specific T cell responses resulting in immune tolerance [1, 5]. For example, SIT suppresses allergen-specific Th2 immunity, including IL-4 and IL-5 production,

[1]Present address: Institute of Medical Science, University of Tokyo, Tokyo, Japan.
[2]Present address: Department of Otolaryngology, Iwakuni Clinical Center, Iwakuni, Japan.

systemically and at local sites [6–8]. SIT alters the immune response to favour Th1 immunity, such as IFN-γ production [9]. In addition, SIT induces immune suppression by activating regulatory T cells and cytokines, such as IL-10 and TGF-β [5, 10].

The activation, proliferation and cytokine production of antigen-specific T cells are regulated by two distinct signals from antigen-presenting cells (APC) [11, 12]. The first signal is provided by interaction of the antigen/major histocompatibility complex with a T cell receptor (TCR). The second signal is delivered by co-signalling molecules. Among these, molecules from the B7/CD28 family play a key role in the regulation of T cell activation and tolerance [12]. B7-1 (CD80) and B7-2 (CD86), as well as their ligands (CD28 and CTLA-4) were the first-discovered B7/CD28 family molecules and therefore have been the most extensively characterized in allergic rhinitis [13–15]. For example, we have previously shown that the expression of both CD80 and CD86 is increased within the nasal mucosa of patients with perennial allergic rhinitis, compared with control subjects following nasal provocation with house dust [14].

In addition to the original B7/CD28 family molecules, new B7 family members, such as inducible co-stimulator (ICOS) L, programmed death (PD)-L1, PD-L2, B7-H3 and B7-H4, have been identified [12]. New CD28 family members, including ICOS, PD-1 and B and T lymphocyte attenuator (BTLA), have also been identified [12]. Among the new CD28 family members, ICOS delivers a range of co-stimulatory signals that augment T cell differentiation and cytokine production and provide critical signals for Ig production [12, 16]. Conversely, both PD-1 and BTLA possess an immunoreceptor tyrosine-based inhibition motif (ITIM) within their cytoplasmic domain, and display co-inhibitory signals that suppress T cell activation [17, 18]. One report has demonstrated enhanced CD86 expression in $CD14^+$ cells after recall stimulation with PLA_2 in patients exposed to bee-venom SIT, as well as suppressed IL-10 production by peripheral blood mononuclear cell (PMBC) following blockade with CD86 in patients exposed to SIT [19]. However, little is known regarding the potential role of co-inhibitory molecules in these responses to SIT.

In the present study, we investigated the expression and characteristics of the co-inhibitory molecules, PD-1 and BTLA, along with B7-H1 (a ligand of PD-1), in allergen-stimulated PBMC from patients with Japanese cedar pollinosis (JCP). We believe that the findings presented here are the first to demonstrate altered BLTA expression in response to Cry j 1, the major allergen of *Cryptomeria japonica* pollen, potentially explaining the beneficial effect of SIT in JCP and providing a basis for future therapeutic approaches aimed at the regulation of BTLA expression to limit allergic diseases.

Materials and methods

Antigens and reagents

Cry j 1 was purified and concentrated from the crude extracts of *C. japonica* pollen, as previously described [20]. The flourescein isothiocyanate (FITC)-labelled anti-human PD-1 (clone MIH4, mouse IgG1), B7-H1 (clone MIH1, mouse IgG1) and BTLA (clone MIH27, mouse IgG2b) were generated as previously described [21, 22]. The purification of anti-human BTLA (clone MIH26, mouse IgG2b) is also described elsewhere [22]. Cy5-labelled anti-human CD4, PE-labelled anti-human CD8 and CD19, as well as their respective control mouse IgG isotypes, were purchased from BD Biosciences (San Jose, CA, USA). Biotinylated anti-human CD203c mAb (FR3-16A11), anti-biotin microbeads and MS columns were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

Patients

Twenty-one patients with JCP (three males and 18 females: aged 21–57, mean age 42.9 years) were enrolled in the study. Written informed consent was obtained from each subject. Sensitization to Japanese cedar pollen was confirmed by the presence of specific-IgE antibodies, as determined by CAP (Pharmacia, Uppsala, Sweden), ranging in concentration from 1.8 to > 100 UA/mL (mean 21.3±27.2). Eleven patients received SIT using a standardized extract of *C. japonica* pollen (Torii Co., Tokyo, Japan) over a period of at least 2 years. A maintenance concentration of 2000 JAU/mL was archived in all the patients treated with SIT. The mean maintenance dose of the extract was 509.0 JAU. None of the patients had used immunosuppressive drugs, including oral steroids, during the pollen season. No significant differences in age or sex existed among the SIT-treated and untreated patients. Clinical characteristics of groups of patients are shown in Table 1. Comparison of naso-ocular symptoms and rhinitis-related quality of life (QOL) during the pollen-dispersed season between SIT-treated patients and SIT-untreated patients using JRQLQ No.1, the Japanese QOL questionnaire for allergic rhinitis [23]. The study was approved by the Human Research Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

Detection of PD-1, B7-H1 and B and T lymphocyte attenuator expression

Heparinized blood was sampled during the season when pollen is dispersed. The PBMCs were then isolated and cultured as previously described [20]. In brief, PBMCs (2×10^6/mL) were incubated in the presence or absence of 10 μg/mL of Cry j 1 at 37 °C in a 5% CO_2/air mixture for

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Table 1. Subject characterization

	SIT patients		Non-SIT patients	
No. of patients	11		10	
Sex (male/female)	0/11		3/7	
Age (years)	47 (36–57)		38 (21–53)	
	At enrollment	2008	At enrollment	2008
CAP titre to JCP (UA/mL)	19.6 (1.8–81.1)	16.6 (0–61.3)	23.1 (1.8–100)	30.5 (6.5–100)
Total IgE (IU/mL)	105 (4–232)	161 (0–458)	145 (27–303)	135 (44–304)
Blood eosinophil (μL^{-1})	306 (43–655)	298 (79–532)	205 (48–725)	218 (69–592)

SIT, specific immunotherapy-treated patients; non-SIT, SIT-untreated patients; JCP, Japanese cedar pollinosis.

72 h. Less than 5% of cultured cells had died as judged by trypan blue exclusion test, indicating high cell viability of cultured cells. After incubation, Cry j 1-stimulated and Cry j 1-unstimulated PBMCs were harvested, blocked and stained with FITC-labelled anti-PD-1, B7-H1 or BTLA, as well as PE-labelled anti-CD8, Cy5-labelled anti-CD4, and CD19, in addition to isotype-matched control Abs [13]. The cells were washed and analysed with FACScan equipment using CellQuest software (BD Biosciences). Lymphocytes were gated according to forward scatter and side scatter and at least 10 000 events were acquired and analysed. Cry j 1-induced expression was determined and the percentage of positive cells in cultured PBMC following Cry j 1 stimulation was subtracted from the percentage observed in unstimulated PBMC. To avoid experimental bias, the laboratory investigators were blinded to the sample origin.

Measurement of cytokines

Supernatant was collected after 12 and 72 h of culture and stored at −80 °C until it was used for assay. Levels of IL-5, IL-10 and TGF-β were measured within each sample of culture supernatant by means of Opt EIA sets (BD Biosciences), in accordance with the manufacturer's instructions. Levels of IL-10 were further measured in supernatant after 12 h of culture. The detection limits of these assays were 5 pg/mL for IL-5, 5 pg/mL for IL-10 and 20 pg/mL for TGF-β. Cry j 1-induced production was determined by subtracting the cytokine levels measured following Cry j 1 stimulation from those measured in the absence of stimulation. In order to determine whether the productions were due to basophil responsiveness, containing basophils were removed from PBMC by immunomagnetic negative selection using biotinylated anti-human CD203c mAb (FR3-16A11), anti-biotin microbeads and MS columns. Complete depletion of basophils was confirmed by Kimura's staining [24].

Proliferative responses

After 72 h of incubation with or without Cry j 1, proliferative responses were measured by means of BrdU incorporation (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions. Proliferation was estimated by the stimulation index calculated as follows: the ratio between mean OD at 450 nm obtained in the culture with Cry j 1 and that obtained in the antigen-free culture.

Cross-linking of B and T lymphocyte attenuator

PBMCs (2×10^6/mL) from JCP patients without SIT were stimulated with 10 μg/mL of Cry j 1 in the presence of immobilized anti-BTLA mAb (MIH26) or control mouse IgG2b. Immobilization was performed by incubation of 20 μg/mL of each Ab diluted in PBS, followed by washing using complete culture medium as previously described [22]. After 72 h of incubation, IL-5 production and proliferation were determined as described above.

Statistical analysis

The non-parametric Wilcoxon's signed-rank test and Mann–Whitney's *U*-test were used. Correlation analysis was performed using Spearman's correlation coefficient by rank. A level of $P<0.05$ was considered statistically significant. Values were given as means±standard deviation. Statistical analysis was performed using StatView™ software (version 4.5; Abacus Inc., Berkeley, CA, USA).

Results

Clinical efficacy of specific immunotherapy in Japanese cedar pollinosis

Analysis using JRQLQ revealed that cedar immunotherapy was clinically effective against not only naso-ocular symptoms but also rhinitis-related QOL in SIT-treated patients in two consecutive seasons (Fig. 1). Levels of serum total IgE ($P=0.360$), JCP-specific IgE titre ($P=0.725$) and blood eosinophil counts ($P=0.205$) were similar between SIT-treated and SIT-untreated patients at the enrollment of the study. And these levels were still

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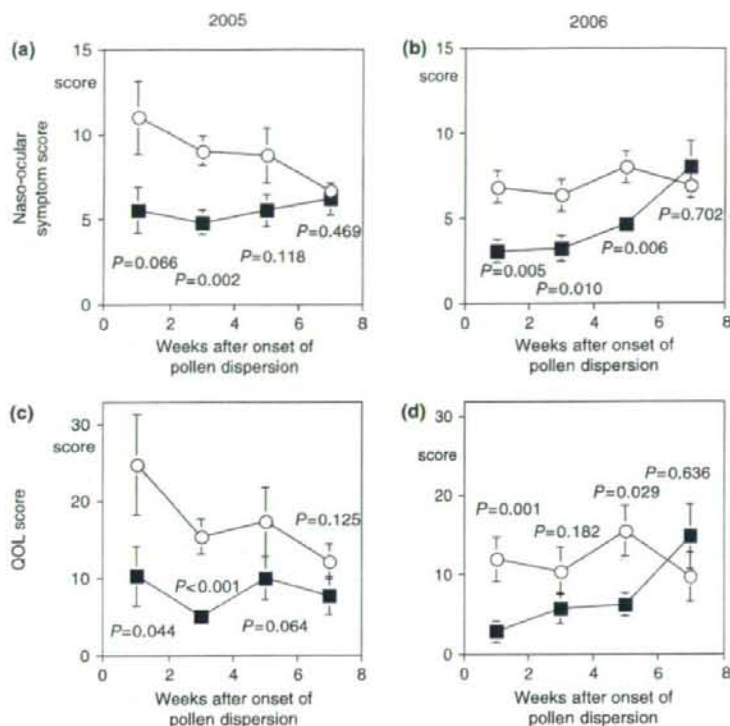

Fig. 1. Effect of cedar immunotherapy on symptom and quality of life (QOL) in Japanese cedar pollinosis (JCP). Naso-ocular symptoms (a, b) and rhinitis-related QOL (c, d) were compared between specific immunotherapy (SIT)-treated patients (closed square) and SIT-untreated patients (open circle) during the pollen-dispersed season in 2005 (a, c) and 2006 (b, d). The x-axis denotes weeks after the onset of cedar and cypress pollen dispersion. The y-axis denotes scores. The P-values were determined using the Mann–Whitney's U-test.

similar between the groups in 2008 (serum total IgE: $P = 0.833$, JCP-specific IgE titre: $P = 0.205$, blood eosinophil counts: $P = 0.181$). However, the levels of JCP-specific IgE titre in 2008 were significantly elevated as compared with those at the enrollment in SIT-untreated group ($P = 0.011$) whereas the levels between before- and after-treatment were not significantly different in SIT-treated group ($P = 0.790$) (Table 1).

Changes in co-inhibitory molecule expression in response to Cry j 1 in peripheral blood mononuclear cells from patients with Japanese cedar pollinosis

The percentage of cells expressing PD-1, B7-H1 and BTLA in PBMC after exposure or no exposure to *in vitro* Cry j 1 stimulation is summarized in Table 2. The baseline expression of these molecules in the absence of Cry j 1 stimulation was similar among SIT-treated and SIT-untreated patients. In addition, the percentage of positive cells observed after stimulation with Cry j 1 did not differ significantly in each molecule. However, when we focused on changes in expression in response to Cry j 1 in each patient, BTLA expression was reduced in SIT-untreated patients, but not in SIT-treated patients (Fig. 2).

Table 2. Percentage of positive cells expressing co-inhibitory molecules with or without Ag stimulation

Molecule	Stimulation	SIT ($n = 11$)	Non-SIT ($n = 10$)	P-value
PD-1	Ag (−)	1.06±0.86	0.86±0.72	0.860
	Ag (+)	1.66±1.47	1.51±1.49	0.888
	Change	0.60±0.74	0.65±1.03	0.805
B7-H1	Ag (−)	1.08±1.07	0.97±0.95	0.778
	Ag (+)	1.55±1.45	1.06±1.04	0.481
	Change	0.47±0.48	0.09±0.90	0.067
BTLA	Ag (−)	2.73±2.50	4.91±4.22	0.460
	Ag (+)	3.70±2.87	2.67±2.33	0.503
	Change	0.97±1.17	−2.24±2.21	<0.001

SIT, specific immunotherapy-treated patients; non-SIT, SIT-untreated patients; PD, programmed death; BTLA, B and T lymphocyte attenuator.

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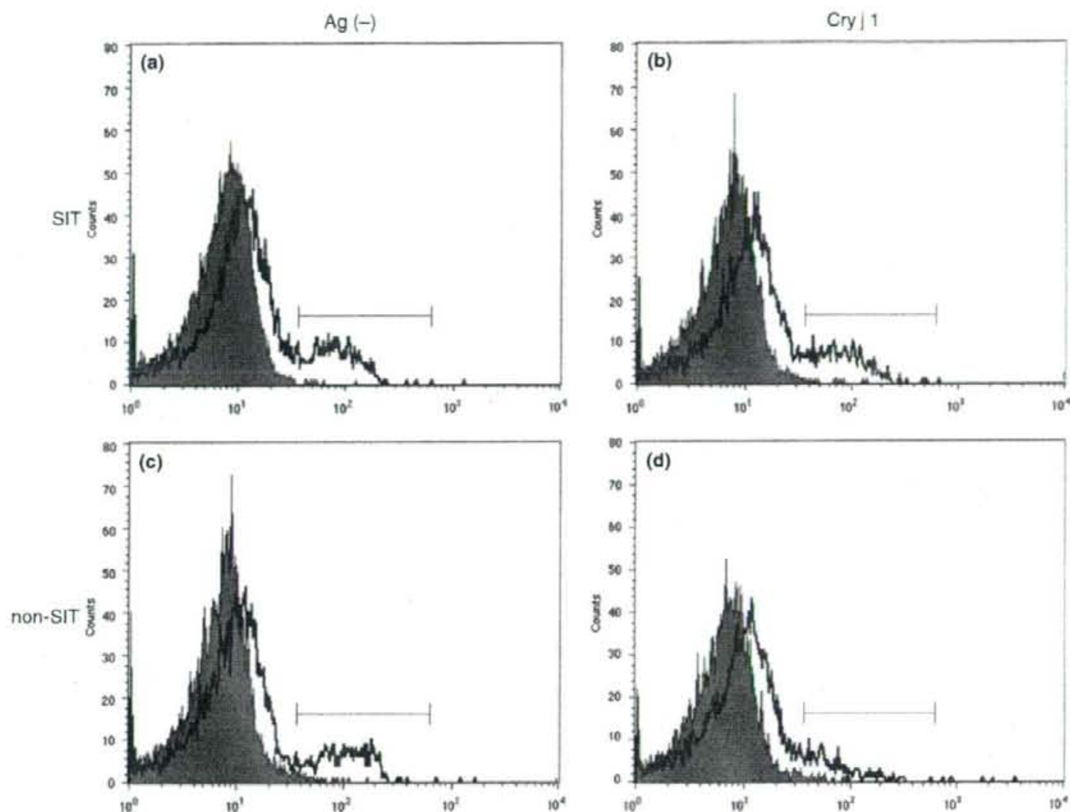

Fig. 2. Typical histogram of B and T lymphocyte attenuator (BTLA) expression. Peripheral blood mononuclear cells (PBMCs) from SIT-treated (a, b) and SIT-untreated (c, d) patients were incubated in the presence or absence of Cry j 1 for 72 h. After incubation, Cry j 1-stimulated (b, d) and unstimulated (a, c) PBMC were harvested, blocked and stained with FITC-labelled anti-BTLA mAb (MIH27, open histogram) or control mouse IgG2b (shaded histogram). Lymphocytes were gated according to forward scatter and side scatter and at least 10 000 events were acquired and analysed. The x-axis (log scale) shows fluorescence intensity and y-axis shows cell counts. SIT, specific immuno therapy-treated patients; non-SIT, SIT-untreated patients, FITC, fluorescein isothiocyanate.

Significantly different responses in terms of BTLA expression following Cry j 1 stimulation were observed among STI-treated and untreated patients ($P<0.001$). A tendency toward enhanced B7-H1 expression was seen in SIT-treated patients, compared with untreated patients ($P=0.067$). On the other hand, changes in PD-1 expression did not differ among patients from both groups ($P=0.805$) (Table 2).

Phenotype analysis of cells expressing co-inhibitory molecules

BTLA is known to be expressed on both B and T cells. In SIT-treated patients, the percentage of $CD4^+$ cells expressing BTLA was significantly increased after recall stimulation with Cry j 1 ($P=0.003$, Fig. 3a). Conversely, the percentage was significantly reduced in SIT-untreated patients ($P=0.017$, Fig. 3d). On the other hand, a significant change in BTLA expression on $CD8^+$ cells was not observed in either SIT-treated ($P=0.154$) or SIT-untreated ($P>0.999$) patients (Figs 3b and e). A reduced expression on $CD19^+$ cells was observed in both SIT-treated ($P=0.091$) and SIT-untreated ($P=0.037$) patients following stimulation (Figs 3c and f).

PD-1 and B7-H1 are also known to be expressed on both B and T cells. The percentage of $CD4^+$ ($P=0.037$) but not $CD8^+$ ($P=0.161$) or $CD19^+$ ($P=0.110$) cells expressing PD-1 was significantly increased in SIT-treated patients after recall stimulation with Cry j 1 (Figs 4a–c). On the other hand, a tendency toward enhanced PD-1 expression on $CD4^+$ ($P=0.086$) and $CD19^+$ ($P=0.093$) but not $CD8^+$ ($P>0.999$) cells were seen in SIT-untreated

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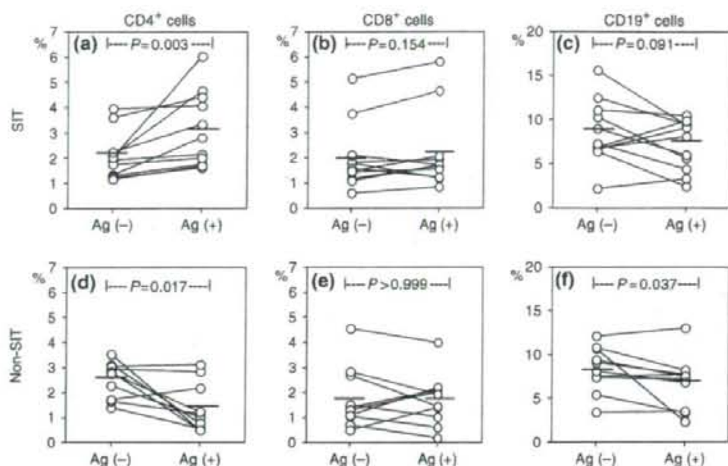

Fig. 3. Phenotype analysis of cells expressing B and T lymphocyte attenuator (BTLA). The peripheral blood mononuclear cells (PBMCs) from patients with (a–c) or without (d–f) specific immunotherapy (SIT) were incubated in the presence or absence of Cry j 1 for 72 h, after which the percentage of CD4+ (a, d), CD8+ (b, e) and CD19+ (c, f) cells expressing BTLA in cultured PBMC was determined by flow cytometry. The *P*-values were determined using the Wilcoxon's signed-rank test.

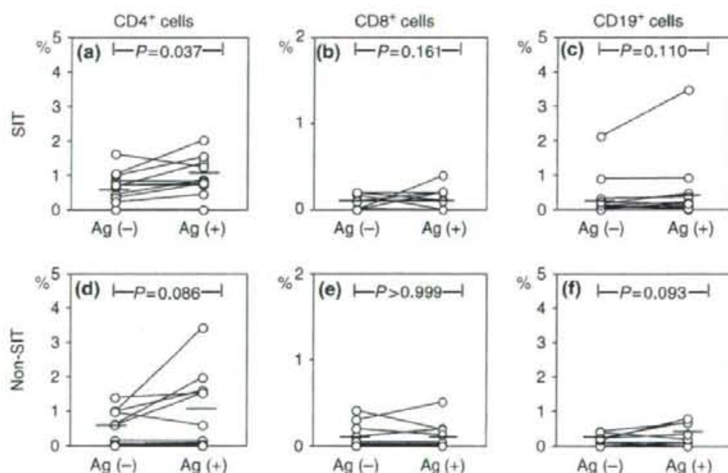

Fig. 4. Phenotype analysis of cells expressing programmed death (PD)-1. The peripheral blood mononuclear cells (PBMCs) from patients with (a–c) or without (d–f) specific immunotherapy (SIT) were incubated in the presence or absence of Cry j 1 for 72 h, after which the percentage of CD4+ (a, d), CD8+ (b, e) and CD19+ (c, f) cells expressing PD-1 in cultured PBMC was determined by flow cytometry. The *P*-values were determined using the Wilcoxon's signed-rank test.

patients (Figs 4d–f). Although a tendency toward overall enhanced B7-H1 expression after recall stimulation with Cry j 1 was seen in SIT-treated patients as compared with untreated patients (Table 2), Cry j 1-induced changes of B7-H1 expression on $CD4^+$, $CD8^+$ or $CD19^+$ cells were not significant in either SIT-treated or SIT-untreated patients (data not shown).

Correlation between Cry j 1-induced interleukin-5 production and changes in B and T lymphocyte attenuator expression

After 72 h of incubation, levels of Cry j 1-induced IL-5 production were significantly reduced in PBMC from SIT-treated patients, compared with untreated patients

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