transcription initiation site. This region was reported to contain fragments responsible for the transcriptional activity [18]. Finally, two constructs were generated: one with rs2564978T/rs3841376 insertion and the other with rs2564978 C/rs3841376 deletion. The fragments were amplified using the primers 5′-GACTGCTAGCCGAACAAGG CATGAACAA-3′ and 5′-GTCAAAGCTTGCCGGGTTAGAA CAAGGA-3′. The products were digested with *Nhe*I and *Xho*I (New England Biolabs, Beverly, MA, USA) overnight at 37 °C and then subcloned into *Nhe*I- and *Hind* III-digested pGL3-Basic Vector (Promega, Madison, WI, USA). The orientation and accuracy of the inserted fragments were confirmed by direct sequencing. A549 cells (human lung adenocarcinoma epithelial cell line; RIKEN Bio-Resource Center, Tsukuba, Ibaraki, Japan) were cultured in Eagle's minimum essential medium (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich Co.) at 37 °C with 5% CO_2. Approximately 2×10^6 cells were cotransfected with 1 μg of the test construct and 100 ng of pRL-TK (Promega) supplemented with HilyMax (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions. The cells were incubated at 37 °C for 48 h and later disrupted by adding 500 μL of lysis buffer (Promega). Twenty microlitres of each lysate was used for the luciferase assay, which was performed using the Dual-luciferase reporter assay system (Promega). The expression efficiency was measured using a TD 20/20 luminometer (Turner Designs Instruments, Sunnyvale, CA, USA). The firefly luciferase values were normalized to the Renilla luciferase values of pRL-TK, which were determined simultaneously. Reporter activity is presented as the mean of three independent values.

Statistical analysis

Any deviation from the predicted Hardy–Weinberg frequencies and the significance of the differences in the case–control samples (proportion of gender) were determined using a χ^2 test. Continuous variables such as age, total, and specific IgE were compared with Student's *t*-test. Statistical evaluations for testing the genetic effects of the association between the case–control status and each individual SNP were estimated by logistic regression analysis after adjusting for gender and age. Association analyses were performed assuming an additive, dominant, and recessive effect for each polymorphism using SNPassoc software [19]. Haplotype analysis was performed using the additive model with the Haplo.stats software version 1.1.0 [20]. We also performed linear regression analysis to examine the effect of these variants on the quantitative variables of total serum IgE and specific IgE against JC pollen. Multiple comparisons were corrected by Bonferroni corrections, and corrected *P*-values of <0.05 were considered significant.

The differences in transcriptional activity, which were determined by the luciferase assay, were analysed using Student's *t*-test. The expression levels of *DAF* in the Epstein–Barr virus-transformed lymphoblastoid cell lines derived from Chinese and Japanese populations were retrieved from the GENe Expression VARiation database (GENEVAR) [21]. The differences in *DAF* gene expression in each genotype were analysed using Student's *t*-test.

Results

The clinical details of the patients with JC-induced SAR, those with mite-sensitive asthma, and controls are listed in Table 1. Significant differences in the age, sex ratio, and other than the total and specific IgE levels were observed between cases and controls. Therefore, we performed logistic regression analysis after adjusting for gender and age. The LD map of the *DAF* gene region is shown in Fig. 1. *DAF* is located in the tight LD block spanning 62 kb between rs6686201 and rs2782837. Three tag SNPs (rs6691942, rs10746463, and rs2782837) were genotyped, and 1 SNP – rs10746463 – was found to be associated with JC-induced SAR (Table 2). The genotype frequencies of these three SNPs did not show a deviation from the Hardy–Weinberg equilibrium ($P>0.05$).

Next, we tested the association of rs10746463 with the SAR-related quantitative phenotypes (total serum IgE and JC-specific IgE) using linear regression analysis after adjusting for the effects of age and gender. The additive effects of the rs10746463 A allele for total serum IgE and JC-specific IgE were observed, but this effect disappeared after adjusting for the affection status (Table 3). The results of the haplotype analysis conducted after adjusting for age and gender are shown in Table 4. The individual SNP as well as haplotype analyses revealed a significant association of the *DAF* variants with the development of SAR.

In order to evaluate the effects of the *DAF* variant on the development of other allergic diseases, we performed an association analysis with patients with HDM-sensitive adult atopic asthma. Logistic regression analysis performed after adjusting for gender and age revealed that rs10746463 was also associated with mite-sensitive asthma (Table 5; $P=0.043$; recessive model).

The re-sequencing of the *DAF* promoter region identified 3 SNPs (rs2564978, rs3841376, and rs28371583) in 32 individuals (Fig. 2). Among them, rs2564978 and rs3841376 were in complete LD with rs10746463 ($r^2=1$). The haplotypes of rs3841376, rs2564978, and rs10746463 were rs2564978 C/rs3841376 deletion/rs10746463 A (the risk haplotype for allergic respiratory diseases) and rs2564978 T/rs3841376 insertion/rs10746463 G. The SNP rs3841376 is a 21-bp insertion/deletion polymorphism located 333 bp upstream of the transcription initiation site. Therefore, it is possible that rs3841376 can influence

Table 1. Characteristics of patients and controls

	JC-induced SAR ($n = 684$)	Mite-sensitive asthmatics ($n = 188$)	Controls ($n = 346$)
Age	33.0+9.6*	46.8±14.8*	34.5+9.8
Male/female ratio	0.36*	0.97*	0.53
IgE (IU/mL, mean, range)	469 (10–11 000)*	688 (10–8710)*	35 (0–330)
Japanese ceder pollen-specific IgE (UA/mL, mean, range)	30.9 (0.71–>100)*	NA	<0.34
Dermatophagoides pteronyssinus-specific IgE (UA/mL, mean, range)	8.9 (<0.34–>100)*	24.4 (0.71–>100)*	<0.34
Dermatophagoides farina-specific IgE (UA/mL, mean, range)	12.0 (<0.34–>100)*	23.5 (0.71–>100)*	<0.34

*$P < 0.05$ between cases and controls.
NA, data not available; JC, Japanese cedar; SAR, seasonal allergic rhinitis.

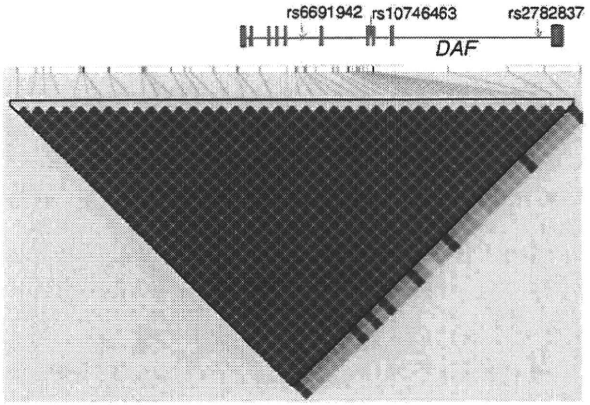

Fig. 1. Pairwise linkage disequilibrium between decay-accelerating factor (*DAF*) polymorphisms in a 62-kb region as measured by r^2 in Asian HapMap subjects. The r^2 values for linkage disequilibrium (LD) are colour-coded by Haploview software, and the extent of red indicates the strength of LD. The positions of the single-nucleotide polymorphisms are indicated with arrows.

the transcriptional activity of the *DAF* gene. We constructed plasmids that contained the 5′ upstream region of *DAF*; this region contained the rs2564978 and rs3841376 polymorphic sites. The plasmid containing the rs2564978 C/rs3841376 deletion (the risk haplotype for allergic respiratory diseases) showed a statistically significantly lower transcriptional activity than that containing rs2564978 T/rs3841376 insertion (Fig. 3, $P = 0.02$). The Sanger Institute GENEVAR expression database [21] shows a strong association between the SNP rs10746463 A, which was in complete LD with the rs2564978 C/rs3841376 deletion haplotype, and the differences in the *DAF* expression levels. Further, the expression levels of *DAF* in the AA ($n = 17$) genotype were found to be lower than that of AG ($n = 53$) and GG ($n = 19$) genotypes ($P < 0.05$, Fig. 4).

Discussion

Our results indicate that the *DAF* SNP is associated with the development of both JC-induced SAR and mite-sensitive asthma. The luciferase transcription assay suggested that the A allele of rs10746463, which is associated with an increased susceptibility to respiratory allergic diseases, decreases the transcriptional activity of *DAF*; this finding is concordant with the pattern of immortalized B cell expression. To our knowledge, this is the first study to show that the *DAF* genotypes and haplotypes were associated with allergic diseases in humans, and our results suggest that decreased levels of *DAF* may be associated with enhanced specific-IgE responses in allergic diseases.

DAF, which is also known as CD55 and is produced by a variety of cells, regulates the complement system by accelerating the decay of the enzyme subunit. It is involved in the pathogenesis of various diseases, including paroxysmal nocturnal haemoglobinuria [22], autoimmune diseases [23], and cancer [24]. DAF also acts as the receptor for pathogens such as the echovirus [25] and *Helicobacter pylori* [26].

Complements play an important role in the innate and adaptive immunity. The complement system has the potential to cause extreme damage to the host tissues; therefore, its activation is tightly regulated by complement regulatory proteins such as DAF, CD46, and CD59. DAF acts by binding to the C3 and C5 convertases of all complement activation pathways. It regulates complement activation at the critical C3 convertase stage by preventing the assembly of C3 convertase and by accelerating the decay of preformed C3 convertases. Therefore, DAF prevents the formation of the anaphylactic cleavage fragments C3a and C5a. It has also been reported that complement activation products such as C3a and C5a contribute to the inflammation of allergic rhinitis. Andersson et al. [27] showed that an allergen challenge test administered to allergic subjects induced nasal symptoms and concomitantly increased the C3a and C5a levels. These levels were also increased in the bronchoalveolar lavage fluid collected after segmental allergen provocation in subjects with allergic asthma [28]. A recent study has reported that the level of C3a receptor expression was significantly higher in the nasal mucosa samples of patients with severe persistent allergy than in the nasal

Table 2. Association of DAF SNPs with SAR

SNP	Location*		Genotype count frequency			Additive *P* (corrected)†	Dominant *P* (corrected)†	Recessive *P* (corrected)†
			AA	AG	GG			
rs28371583	205561302	Case	534 (0.78)	139 (0.20)	11 (0.02)	0.04	0.052	0.27
A>G		Control	284 (0.82)	57 (0.17)	4 (0.01)	(0.48)	(0.62)	(1)
			CC	CT	TT			
rs6691942	205568150	Case	187 (0.28)	338 (0.50)	148 (0.22)	0.87	0.6	0.77
C>T		Control	103 (0.30)	163 (0.48)	77 (0.22)	(1)	(1)	(1)
			AA	AG	GG			
rs10746463	205577219	Case	223 (0.35)	327 (0.51)	90 (0.14)	0.0018	0.096	0.00033
A>G		Control	99 (0.29)	161 (0.47)	81 (0.24)	(0.022)	(1)	(0.004)
			CC	CT	TT			
rs2782837	205597549	Case	469 (0.69)	192 (0.28)	14 (0.02)	0.42	0.94	0.027
C>T		Control	240 (0.69)	89 (0.26)	17 (0.05)	(1)	(1)	(0.32)

*Locations are relative to the contig NT_021877.
†Corrected *P*-values by Bonferroni's correction.
DAF, decay-accelerating factor; SAR, seasonal allergic rhinitis; SNP, single-nucleotide polymorphisms.

Table 3. Association analysis between rs10746463 and total serum IgE and between rs10746463 and JC-specific IgE

Genotype	Mean log (total serum IgE)+SE	*P*-values (*P* adjusted by affection status)
AA	199+0.036	
AG	1.88+0.03	0.006 (0.40)
GG	1.73+0.045	
Mean log (JC-specific IgE)+SE		
AA	0.65+0.05	
AG	0.62+0.041	0.0011 (0.53)
GG	0.35+0.68	

JC, Japanese cedar.

Table 4. Haplotype association test

Haplotype	Haplotype frequency	*P*-value (corrected)
ATAC	0.44	0.058 (0.41)
ACGC	0.24	0.27 (1)
ACGT	0.15	0.8 (1)
GCAC	0.1	0.0066 (0.046)
ACAC	0.021	0.1 (0.7)
ATGC	0.02	0.000006 (0.000042)
ACAT	0.013	0.8 (1)

The SNP order in haplotypes is rs28371583, rs6691942, rs10746463 and rs2782837.
SNP, single-nucleotide polymorphisms.

Table 5. Association of the *DAF* SNP with mite-sensitive asthma

SNP	Genotype count (frequency)			Recessive model *P*
rs10746463	Case	60 (0.32)	99 (0.53)	29 (0.15)	0.044
A>G	Control	99 (0.29)	161 (0.47)	81 (0.24)	

SNP, single-nucleotide polymorphisms; *DAF*, decay-accelerating factor.

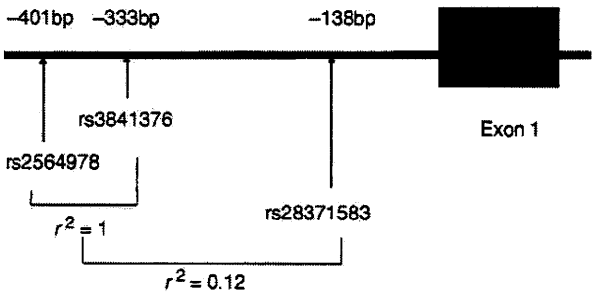

Fig. 2. Locations of the single-nucleotide polymorphisms in the 5′ promoter region of decay-accelerating factor (*DAF*). The distances are relative to the transcription initiation site.

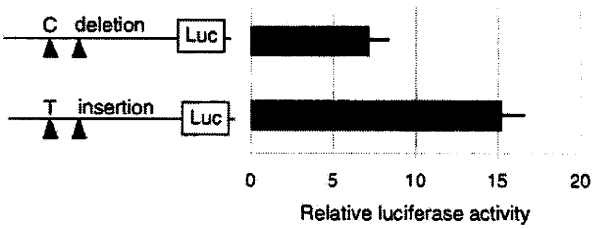

Fig. 3. Comparison of polymorphisms of decay-accelerating factor (*DAF*) analysed by relative luciferase activities in A549 cells. Results are expressed as relative luciferase activity normalized to those of *Renilla* luciferase (pRL-TK). Standard deviation (SD) is indicated by the error bar. The mean relative activities and ±SD were calculated in three independent transfections. Statistical analysis was performed with Student's *t*-test.

mucosa samples of normal subjects and of patients with mild allergy [29]. This suggests that the C3a receptor may mediate mucus secretion and mucosal swelling in the allergic nasal mucosa, especially in cases of severe persistent allergy [29]. Our present data gathered from the Japanese population suggest that subjects possessing

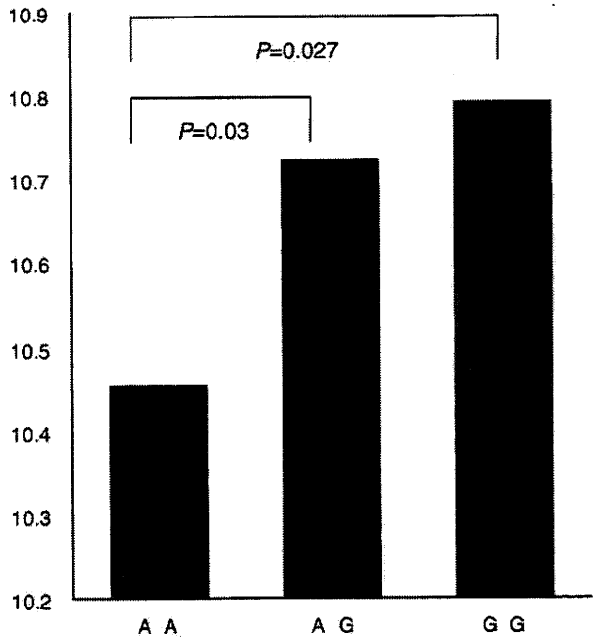

Fig. 4. Gene expression levels of Epstein–Barr virus-transformed lymphoblastoid cell lines from Chinese and Japanese populations according to their genotypes. Normalized expression value of each individual was retrieved from GENEVAR web site [21], and statistical analyses were performed with Student's *t*-test. The bars represent the means of the expression levels of decay-accelerating factor (*DAF*) in the subjects with AA ($n = 17$), AG ($n = 53$), and GG ($n = 19$) genotypes.

genotypes that down-regulate the transcriptional activity of *DAF* were more susceptible to allergic respiratory diseases. Hence, it is likely that decreased levels of DAF contribute to the production of C3a anaphylatoxins, resulting in the heightened susceptibility to allergic respiratory diseases.

Our results indicate that the *DAF* SNP is associated with the development of both JC-induced SAR and mite-sensitive asthma. It should be noted that our control subjects were super-controls, i.e. subjects not reactive to the common inhaled allergens. Therefore, our results can also imply that the rs10746463 GG has a protective effect against allergic diseases.

In summary, our study shows that a genetic variation in *DAF* significantly alters the susceptibility of an individual to allergic respiratory diseases. Our findings further support the role of the complement system in allergic diseases. Thus, our results would facilitate the understanding of the pathogenesis of allergic diseases and be valuable in research for novel treatments.

Acknowledgements

We thank Dr Akihiko Miyatake, Dr Satoru Doi, Dr Masami Taniguchi, and Dr Noritaka Higashi for collecting samples.

We thank all of the participants in this study. This study was supported by a scientific research grants Grant-in-Aid for Scientific Research from the Ministry of Health and Welfare, Japan (H17-Genome-001, H20-Immunology-004) and from the Ministry of Education, Science and Culture of Japan (18591097).

References

1 Okuda M. Epidemiology of Japanese cedar pollinosis throughout Japan. *Ann Allergy Asthma Immunol* 2003; 91:288–96.
2 Nishima S, Chisaka H, Fujiwara T *et al.* Surveys on the Prevalence of Pediatric Bronchial Asthma in Japan: a comparison between the 1982, 1992, and 2002 surveys conducted in the same region using the same methodology. *Allergol Int* 2009; 58:37–53.
3 Kakutani C, Ogino S, Ikeda H, Enomoto T. Impact of allergic rhinitis on work productivity: a pilot study. *Jpn J Allergol* 2005; 54:627–35.
4 Togias A. Rhinitis and asthma: evidence for respiratory system integration. *J Allergy Clin Immunol* 2003; 111:1171–83.
5 Grossman J. One airway, one disease. *Chest* 1997; 111:11S–6S.
6 Braman SS. The global burden of asthma. *Chest* 2006; 130: 4S–12S.
7 Sears MR, Herbison GP, Holdaway MD, Hewitt CJ, Flannery EM, Silva PA. The relative risks of sensitivity to grass pollen, house dust mite and cat dander in the development of childhood asthma. *Clin Exp Allergy* 1989; 19:419–24.
8 Wills-Karp M, Ewart SL. Time to draw breath: asthma-susceptibility genes are identified. *Nat Rev Genet* 2004; 5:376–87.
9 Nystad W, Roysamb E, Magnus P, Tambs K, Harris JR. A comparison of genetic and environmental variance structures for asthma, hay fever and eczema with symptoms of the same diseases: a study of Norwegian twins. *Int J Epidemiol* 2005; 34:1302–9.
10 Gerard NP, Gerard C. Complement in allergy and asthma. *Curr Opin Immunol* 2002; 14:705–8.
11 Wills-Karp M. Complement activation pathways: a bridge between innate and adaptive immune responses in asthma. *Proc Am Thorac Soc* 2007; 4:247–51.
12 Barnes KC, Grant AV, Baltadzhieva D *et al.* Variants in the gene encoding C3 are associated with asthma and related phenotypes among African Caribbean families. *Genes Immun* 2006; 7:27–35.
13 Inoue H, Mashimo Y, Funamizu M *et al.* Association study of the C3 gene with adult and childhood asthma. *J Hum Genet* 2008; 53:728–38.
14 Gasque P. Complement: a unique innate immune sensor for danger signals. *Mol Immunol* 2004; 41:1089–98.
15 Fujita T, Inoue T, Ogawa K, Iida K, Tamura N. The mechanism of action of decay-accelerating factor (DAF). DAF inhibits the assembly of C3 convertases by dissociating C2a and Bb. *J Exp Med* 1987; 166:1221–8.
16 de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. Efficiency and power in genetic association studies. *Nat Genet* 2005; 37:1217–23.
17 Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; 21:263–5.

18 Ewulonu UK, Ravi L, Medof ME. Characterization of the decay-accelerating factor gene promoter region. *Proc Natl Acad Sci USA* 1991; 88:4675–9.
19 Gonzalez JR, Armengol L, Sole X *et al.* SNPassoc: an R package to perform whole genome association studies. *Bioinformatics* 2007; 23:644–5.
20 Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet* 2002; 70:425–34.
21 Stranger BE, Nica AC, Forrest MS *et al.* Population genomics of human gene expression. *Nat Genet* 2007; 39:1217–24.
22 Kinoshita T, Medof ME, Silber R, Nussenzweig V. Distribution of decay-accelerating factor in the peripheral blood of normal individuals and patients with paroxysmal nocturnal hemoglobinuria. *J Exp Med* 1985; 162:75–92.
23 Ruiz-Arguelles A, Llorente L. The role of complement regulatory proteins (CD55 and CD59) in the pathogenesis of autoimmune hemocytopenias. *Autoimmun Rev* 2007; 6:155–61.
24 Spendlove I, Ramage JM, Bradley R, Harris C, Durrant LG. Complement decay accelerating factor (DAF)/CD55 in cancer. *Cancer Immunol Immunother* 2006; 55:987–95.
25 Bergelson JM, Chan M, Solomon KR, St John NF, Lin H, Finberg RW. Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. *Proc Natl Acad Sci USA* 1994; 91: 6245–8.
26 O'Brien DP, Israel DA, Krishna U *et al.* The role of decay-accelerating factor as a receptor for Helicobacter pylori and a mediator of gastric inflammation. *J Biol Chem* 2006; 281:13317–23.
27 Andersson M, Michel L, Llull JB, Pipkorn U. Complement activation on the nasal mucosal surface – a feature of the immediate allergic reaction in the nose. *Allergy* 1994; 49:242–5.
28 Krug N, Tschernig T, Erpenbeck VJ, Hohlfeld JM, Kohl J. Complement factors C3a and C5a are increased in bronchoalveolar lavage fluid after segmental allergen provocation in subjects with asthma. *Am J Respir Crit Care Med* 2001; 164:1841–3.
29 Jun SW, Kim TH, Lee HM *et al.* Overexpression of the anaphylatoxin receptors, complement anaphylatoxin 3a receptor and complement anaphylatoxin 5a receptor, in the nasal mucosa of patients with mild and severe persistent allergic rhinitis. *J Allergy Clin Immunol* 2008; 122:119–25.

厚生労働科学研究費補助金（免疫アレルギー疾患等予防・治療研究事業）
（総合）研究報告書

リアルタイムモニター飛散数の情報の在り方と研究と舌下ペプチド・アジュバント療法の臨床研究

ヒノキ花粉症の病態解明とスギ特異的免疫療法の作用機序の解析

研究分担者　岡野光博　岡山大学大学院医歯薬学総合研究科　耳鼻咽喉・頭頸部外科准教授

研究要旨
舌下免疫療法を施行する上で、投与抗原を吟味することは必須である。これまでのQOL研究で、標準化スギ花粉エキスを用いた特異的免疫療法はスギ花粉飛散期の症状およびQOLを有意に改善する一方、ヒノキ花粉飛散期にはその効果が減弱することが明らかになり、ヒノキ抗原およびヒノキ花粉症の特異性が示唆されている。今回は、末梢血単核細胞応答を指標としたヒノキ花粉症の病態解明とスギ特異的免疫療法の作用機序を検討した。免疫療法群の グループ1抗原（Cry j 1-およびCha o 1）に対するIL-5産生量は、いずれも非免疫療法群に比較して有意に低かった。一方、スギ粗抗原およびグループ2スギ抗原Cry j 2に対するIL-5産生量に関しては、免疫療法群は非免疫療法群と比較して有意に低かったが、ヒノキ粗抗原およびグループ2ヒノキ抗原Cha o 2に対するIL-5産生量に関しては二群間で有意な差を認めなかった。以上の結果より、標準化スギ花粉エキスを用いた免疫療法はグループ1ヒノキ抗原Cha o 1に対する免疫寛容を誘導するものの、グループ2ヒノキ抗原Cha o 2に対する免疫寛容への効果は限定的であることが示唆された。スギ・ヒノキ花粉症の根治を考える上では、Cha o 2のT細胞エピトープペプチドを含む治療用ヒノキ花粉エキスの開発が必要であると思われた。リアルタイム花粉モニターでの情報を基にした研究では、ヒノキ花粉飛散期にはヒノキIgE非依存的なアレルギー性炎症が誘導されうること、スギ・ヒノキ花粉症の増悪には新規Th2サイトカインであるIL-31が関与することを解明した。

A. 研究目的

これまでのQOL研究で、標準化スギ花粉エキスを用いた特異的免疫療法はスギ花粉飛散期の症状およびQOLを有意に改善する一方、ヒノキ花粉飛散期にはその効果が減弱することを明らかにした。今回は、末梢血単核細胞（PBMC）応答を指標とした、スギ特異的免疫療法のヒノキ花粉症に対する作用メカニズムを検討した。またリアルタイム花粉モニターでの情報を基に、ヒノキ感作のヒノキ特異的 IL-5 産生とヒノキ花粉飛散期における鼻眼症状に与える影響や、PBMCのスギおよびヒノキ抗原に対する各種サイトカイン産生とその臨床的意義について検討した。

B. 研究方法

免疫療法施行（SIT群）および非施行（非SIT群）のスギ花粉症患者、あるいは健常人より PBMCを採取した。スギ花粉症患者のヒノキ特異的IgE抗体価をCAPにて測定し、クラス2以上を陽性とした。スギおよびヒノキ粗抗原、グループ1抗原（Cry j 1、Cha o 1）およびグループ2抗原（Cry j 2、Cha o 2）にて刺激し、培養72時間後に上清を回収し IL-5/IL-10/IL-13/IL-17/IL-18/IL-31/IL-33/IFN-γなどのサイトカインをELISAにて測定した。スギおよびヒノキ飛散期の症状およびQOLはJRQLQやアレルギー日記を用いてスコア化した。

（倫理面への配慮）

患者からの検体（末梢血）採取に関しては、学術的な意義について十分な説明を行い、同意・協力が得られた上で採取保存した。

C. 結果

スギ花粉症患者のPBMCは、健常者と比較してスギおよびヒノキ抗原のいずれに対しても IL-5、IL-13 を有意に産生した。IFN-・産生に関しては両群で差を認めなかった。IL-10/IL-17/IL-18/IL-33に関しては両群共に有意な産生を示さなかった。IL-31 産生に関しては、スギ粗抗原においてスギ花粉症患者で有意に亢進した。ヒノキ飛散期においては症状スコアとヒノキ粗抗原特的IL-31産生との間に相関傾向を認め、さらにQOLスコアとヒノキ粗抗原特的IL-31産生との間に有意な正の相関がみられた。SIT群の グループ1抗原（Cry j 1-およびCha o 1）に対する IL-5 産生量は、いずれも非 SIT 群に比較して有意に低かった。一方、スギ粗抗原およびグループ2スギ抗原Cry j 2に対するIL-5産生量に関しては、免疫療法群は非免疫療法群と比較して有意に低かったが、ヒノキ粗抗原およびグループ2ヒノキ抗原Cha o 2に対する IL-5 産生量に関しては二群間で有意な差を認めなかった

D. 考察：

今回の結果は、標準化スギ花粉エキスを用いた免疫療法はグループ1抗原に対する免疫寛容を誘導するものの、グループ2抗原に対しては異なる免疫寛容効果を示すことを示した。最近、Cha o 2のメジャーなT細胞エピトープはCry j 2と交差しないことが報告されている (Sone T, et al. Allergol Int 58: 234, 2009)。今回の検討はこの報告を支持するものであり、スギ花粉エキスを用いた免疫療法はグループ2ヒノキ抗原 Cha o 2に対する免疫寛容を十分に誘導できない可能性が示唆された。粗抗原に対する反応性の違いもグループ2抗原の抗原性の相違から説明できるが、他の抗原についても引き続き検討する必要があると思われる。

E. 結論

スギ花粉症患者のPBMCはスギ抗原に対してTh2タイプのサイトカインである IL-5/IL-13/IL-31 を選択的に産生し、これらのサイトカイン産生は免疫療法で有意に抑制される。さらにヒノキ粗抗原に特異的な IL-31 産生はヒノキ花粉飛散期の症状および QOL を反映するバイオマーカーになりえる可能性が示唆された。標準化スギ花粉エキスを用いた免疫療法は Cry j 2 に対する免疫寛容を誘導するものの、Cha o 2に対する免疫寛容への効果は限定的であることが示された。スギ・ヒノキ花粉症の根治を考える上では、Cha o 2のT細胞エピトープペプチドを含む治療用ヒノキ花粉エキスの開発が必要であると思われた。

F. 研究発表

1．論文発表

1) Nomiya R, Okano M, Fujiwara T, Maeda M, Kimura Y, Kino K, Yokoyama M, Hirai H, Nagata K, Hara T, Nishizaki K, Nakamura M. CRTH2 plays an essential role in the pathophysiology of Cry j 1-induced pollinosis in mice. Journal of Immunology 180; 5680-5688, 2008.
2) Okubo K, Goto M, Fujieda S, Okano M, Yoshida H, Morikawa H, Masuyama K, Okamoto Y, Kobayashi M. A randomized-double-blind comparative study of sublingual immunotherapy for cedar pollinosis. Allergology International 57; 265-275, 2008.
3) Okano M, Otsuki N, Azuma M, Fujiwara T, Kariya S, Sugata Y, Higaki T, Kino K, Tanimoto Y, Okubo K, Nishizaki K. Allergen-specific immunotherapy alters the expression of BTLA, a co-inhibitory molecule, in allergic rhinitis. Clinical and Experimental Allergy 38; 1891-1900, 2008.
4) Ichihara Y, Okano M, Nishioka K, Manabe N, Ichihara N, Jitsunari F, Fujiwara T, Nishizaki K. Aging exacerbates restraint stress-induced inhibition of antigen-specific antibody production in mice. Allergology International 58; 119-124, 2009.
5) Okano M. Mechanisms and clinical implications of glucocorticosteroids in the treatment of allergic rhinitis. Clinical and Experimental Immunology 158; 164-173, 2009.
6) Okano M, Fujiwara T, Higaki T, Makihara S, Haruna T, Noda Y, Kanai K, Kariya S, Yasueda H, Nishizaki K. Characterization of pollen antigen-induced IL-31 production by peripheral blood mononuclear cells in allergic rhinitis. Journal of Allergy and Clinical Immunology 127; 277-279, 2011.
7) Hosoya K, Satoh T, Yamamoto Y, Saeki K, Igawa K, Okano M, Moriya T, Imamura O, Nemoto Y, Yokozeki H. Gene silencing of STAT6 with siRNA ameliorates contact hypersensitivity and allergic rhinitis. Allergy 66; 124-31, 2011.
8) 前田　恵、岡野光博、木村吉伸、大槻剛巳. スギおよびヒノキ花粉アレルゲンに結合する糖タンパク糖鎖の構造特性と免疫活性. 職業・環境アレルギー誌 15: 24-29, 2008.
9) 岡野光博. アレルギー性鼻炎の新しい治療薬開発の現状-免疫療法薬を中心に-. アレルギー・免疫 15: 358-365, 2008.
10) 岡野光博. 通年性アレルギー性鼻炎に対する新規アプローチ. アレルギーの臨床 28: 840-845, 2008.
11) 岡野光博. アレルギー性上気道炎. アレルギーの臨床 29: 24-30, 2009.
12) 岡野光博. アレルギー性鼻炎の薬物治療 プロスタグランジンD2・トロンボキサンA2受容体拮抗薬. 医薬ジャーナル 44: 903-909, 2009.
13) 岡野光博: 花粉症. ガイドライン外来診療. 2009. 364-372.(日経メディカル開発).
14) 岡野光博: 好酸球性炎症におけるエンテロトキシンの作用と PGE_2 による制御. 日鼻誌. 48（1）: 15-17, 2009.
15) 松本亮典, 小川晃弘, 牧野琢丸, 岡野光博: 耳鼻咽喉科で経験した食物依存性運動誘発アナフィラキシーＦＤＥＩＡ症例の検討.

アレルギー. 58（5）: 548-553, 2009.

16) 岡野光博:アレルギー性鼻炎におけるT細胞, 肥満細胞,好酸球の役割について教えてください. JOHNS. 25（3）: 287-291, 2009.

17) 岡野光博: プロスタグラジン D_2 代謝からみたアレルギー性鼻炎の病態と治療戦略. 臨床免疫・アレルギー科. 51(5): 487-493, 2009.

18) 岡野光博: 抗ヒスタミン薬. MB ENT. 2009. .104: 40-48.

19) 岡野光博: アレルギー性上気道炎症(アレルギー性鼻炎)の病態機序.アレルギーの臨床. 29（1）: 24-29, 2009.

20) 岡野光博: 発症のメカニズムと鼻炎におけるアレルギー性鼻炎の位置づけ. アレルギーの臨床. 2009. 29(4): 297-301.

21) 野宮理恵, 岡野光博, 藤原田鶴子, 西崎和則:マウススギ花粉症モデルにおけるCRTH2の役割. 岡山医学会雑誌. 121（2）: 85-90, 2009.

22) 岡野光博: スギ花粉症に対する皮下免疫療法の作用メカニズム - 共抑制分子ＢＴＬＡの関与-. 耳鼻免疫アレルギー. 27(3): 243-248, 2009.

23) 岡野光博. アレルギー性鼻炎（花粉症）のQOL障害と治療による改善～免疫療法～. アレルギー・免疫. 16 (12): 97-105, 2009.

24) 岡野光博. 花粉症に対する初期療法のEBMとは？ 池田勝久、武田憲昭、井之口　明、原渕保明、丹生健一編. EBM　耳鼻咽喉科・頭頸部外科の治療（第1版）. 東京. 中外医学社. 2010. 20-22 頁.

25) 岡野光博. 抗アレルギー薬　トロンボキサン A_2 合成阻害薬・拮抗薬. 永倉俊和、森田寛、足立　満編. アレルギー疾患イラストレイテッド（第2版）. 東京. メディカルビュー社. 2010. 214-219 頁.

26) 岡野光博. 上気道からみた One airway, one disease. 喘息 23: 643-652, 2010.

27) 岡野光博. 親がアレルギー性鼻炎ですが、子どもにも遺伝しますか？ JOHNS 26: 1402-1405, 2010.

28) 岡野光博. DP受容体. 鼻アレルギーフロンティア 10: 34-38, 2010.

29) 岡野光博. ヒノキ花粉のスギ・ヒノキ花粉症に与える影響. メディカルビューポイント 32: 2, 2011.

30) 岡野光博、難波弘行、佐橋紀男. 2011年ヒノキ科花粉飛散予測. アレルギーの臨床 31: 21-26, 2011.

○○○○○○○○○○○○○○○○○○○○

2．学会発表

1) 岡野光博. 好酸球性炎症におけるエンテロトキシンの作用と PGE_2 による制御. 第47回日本鼻科学会学術講演会. 名古屋. 2008年（第44回鼻科学基礎問題研究会シンポジウム）.

2) 岡野光博. スギ花粉症に対する免疫療法の現状と限界、そして未来. **第58回日本アレルギー学会秋季学術大会. 東京. 2008年(シンポジウム).**

3) 岡野光博.スギ花粉症に対する免疫療法の作用メカニズム. **第27回日本耳鼻咽喉科免疫アレルギー学会. 千葉. 2009年(シンポジウム).**

4) 岡野光博、檜垣貴哉、牧原靖一郎、野宮理恵、春名威範、西崎和則.スギ花粉症患者のヒノキ抗原に対する末梢血単核細胞応答. **第27回日本耳鼻咽喉科免疫アレルギー学会. 千葉. 2009年.**

5) 岡野光博、檜垣貴哉、牧原靖一郎、野宮理恵、春名威範、西崎和則. スギ花粉症患者のヒノキ抗原に対する末梢血単核細胞応答-ヒノキ感作の影響-.**第21回日本アレルギー学会春季臨床大会. 岐阜. 2009年.**

6) 岡野光博. 春季花粉症の病態と治療におけるヒノキ花粉のインパクト. 第59回日本アレルギー学会秋季学術大会. **秋田. 2009年(シンポジウム).**

7) 岡野光博、檜垣貴哉、牧原靖一郎、假谷伸、野田洋平、西崎和則. スギ・ヒノキ抗原に対する末梢血単核細胞のサイトカイン産生と免疫療法による制御. 第111回日本耳鼻咽喉科学会学術講演会. 仙台. 2010年.

8) 岡野光博. アレルギー性鼻炎の感作・発症・増悪因子としてのTh2型サイトカインの関与とその制御. 第49回日本鼻科学会学術講演会. 札幌. 2010年（シンポジウム）.

9) 岡野光博、檜垣貴哉、牧原靖一郎、仮谷伸、金井健吾、松山祐子、西崎和則. Cry j 1に対する末梢血単核細胞のIL-13/IL-17/IL-18/IL-31/IL-33産生と免疫療法による制御. 第28回日本耳鼻咽喉科免疫アレルギー学会. 福井. 2010年.

10) 岡野光博. アレルギー性鼻炎に対する免疫療法のABC. 第22回日本アレルギー学会春季臨床大会. 京都.　2010年.

11) 岡野光博. 上気道慢性炎症における真菌の役割-鼻茸分離細胞を用いた検討-.第60回日本アレルギー学会秋季学術大会. 東

京. 2010 年（シンポジウム）.

G. 知的財産権の出願・登録状況(予定を含む)

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

doi: 10.1111/j.1365-2222.2008.03116.x

Clinical and Experimental Allergy, 38, 1891–1900

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
Submitted 18 February 2008; revised 18 August 2008; accepted 3 September 2008

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

Cite this as: M. Okano, N. Otsuki, M. Azuma, T. Fujiwara, S. Kariya, Y. Sugata, T. Higaki, K. Kino, Y. Tanimoto, K. Okubo and K. Nishizaki, *Clinical and Experimental Allergy*, 2008 (38) 1891–1900.

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

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

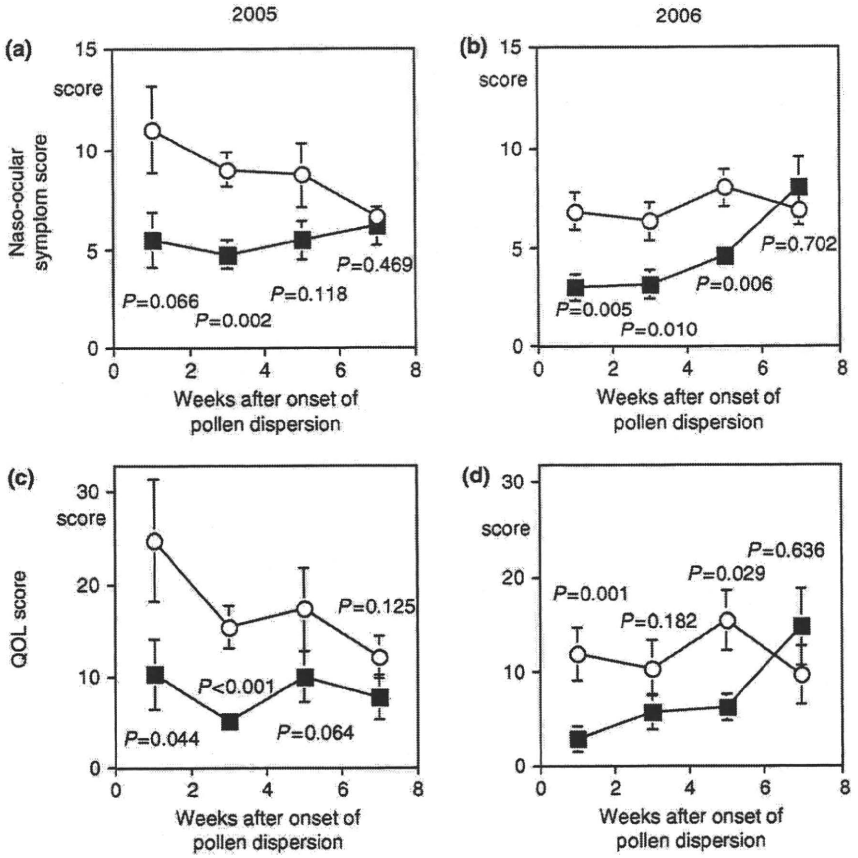

Fig. 1. Effect of cedar immunotherapy on symptom and quality of life (QOL) in Japanese cedar pollinosis (JCP). Naso-ocular symptoms (a, b) and rhinitis-related QOL (c, d) were compared between specific immunotherapy (SIT)-treated patients (closed square) and SIT-untreated patients (open circle) during 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.

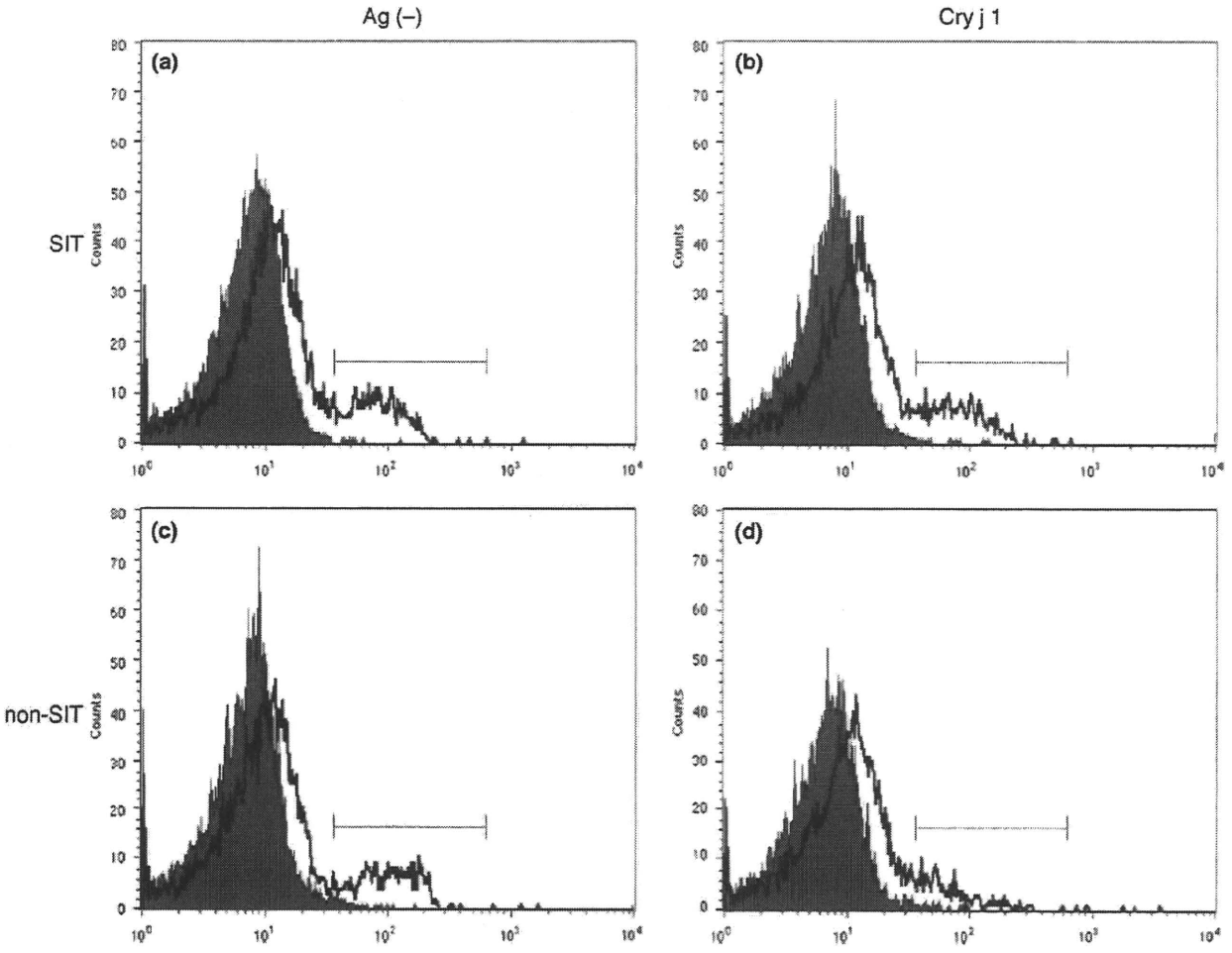

Fig. 2. Typical histogram of B and T lymphocyte attenuator (BTLA) expression. Peripheral blood mononuclear cells (PBMCs) from SIT-treated (a, b) and SIT-untreated (c, d) patients were incubated in the presence or absence of Cry j 1 for 72 h. After incubation, Cry j 1-stimulated (b, d) and unstimulated (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

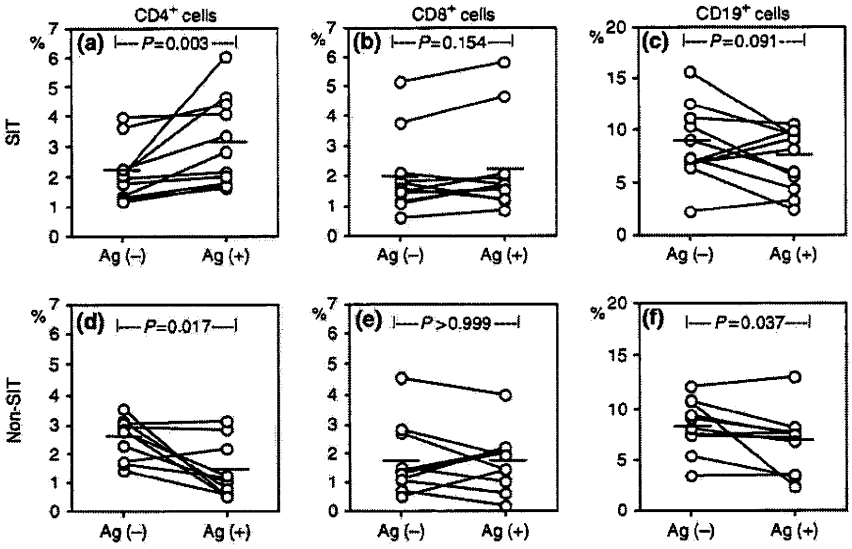

Fig. 3. Phenotype analysis of cells expressing B and T lymphocyte attenuator (BTLA). The peripheral blood mononuclear cells (PBMCs) from patients with (a–c) or without (d–f) specific immunotherapy (SIT) were incubated in the presence or absence of Cry j 1 for 72 h, after which the percentage of CD4+ (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.

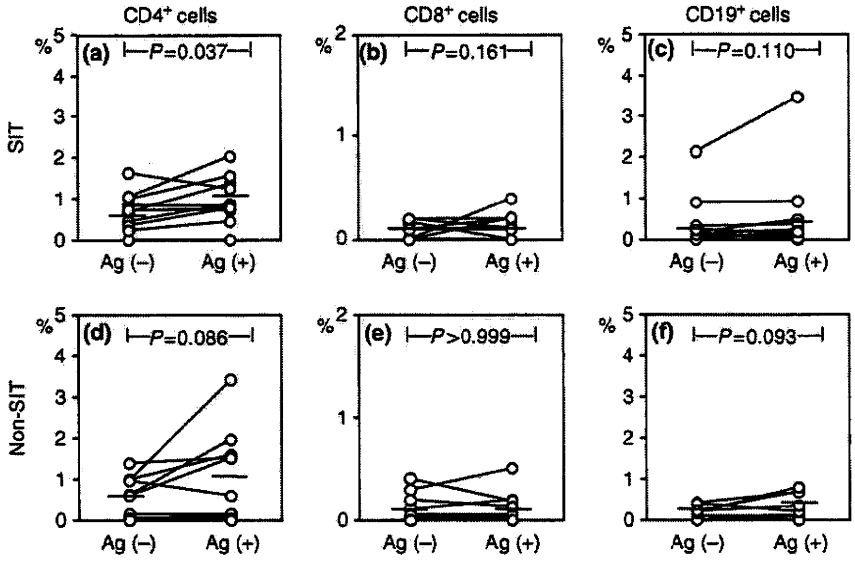

Fig. 4. Phenotype analysis of cells expressing programmed death (PD)-1. The peripheral blood mononuclear cells (PBMCs) from patients with (a–c) or without (d–f) specific immunotherapy (SIT) were incubated in the presence or absence of Cry j 1 for 72 h, after which the percentage of CD4+ (a, d), CD8+ (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

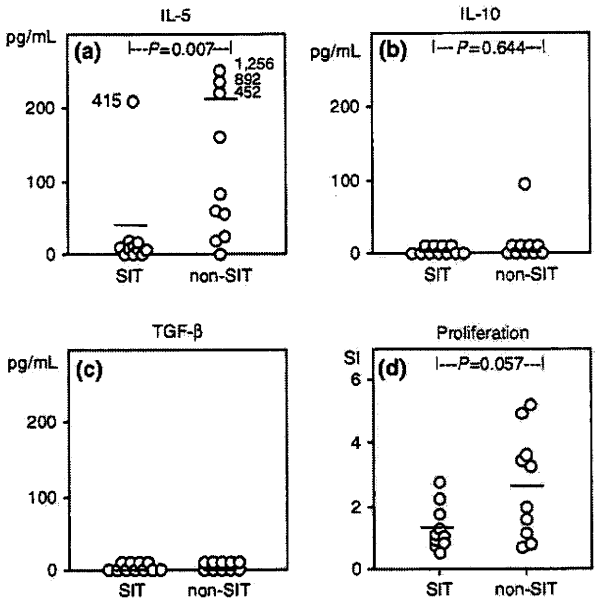

Fig. 5. Comparison of Cry j 1-induced cytokine production and proliferation among specific immunotherapy (SIT)-treated and SIT-untreated Japanese cedar pollinosis (JCP) patients. Peripheral blood mononuclear cells (PBMCs) were incubated in the presence or absence of Cry j 1 for 72 h, after which the levels of IL-5 (a), IL-10 (b) and TGF-β (c) were determined within the supernatant by ELISA. Changes in production were determined by measuring the differences between the levels observed following Cry j 1 stimulation and those observed in the absence of stimulation. In addition, Cry j 1-induced PBMC proliferation was measured by BrdU incorporation (d). *P*-values were determined using the Mann–Whitney's *U*-test. SIT represents the patients treated with SIT. Non-SIT represents SIT-untreated group of patients.

($P = 0.007$, Fig. 5a). On the other hand, IL-10 or TGF-β production in response to Cry j 1 was not detected in either group (Figs 5b and c). IL-10 production was not detected even after 12 h incubation with Cry j 1. Cry j 1-induced IL-5 production was similar between basophil-depleted PBMC (121.9±77.9 pg/mL) and control PBMC (128.9± 59.6 pg/mL, $P = 0.624$), suggesting that basophil responsiveness in IL-5 production is negligible in our culture system. A trend of suppression in Cry j 1-induced proliferation was seen in PBMC from SIT-treated patients as compared with those from SIT-untreated patients ($P = 0.057$; Fig. 5d).

Interestingly, the observed Cry j 1-induced changes in BTLA expression were significantly and negatively correlated with IL-5 production ($\rho = -0.747$, $P < 0.001$; Fig. 6). However, when we analysed the correlation separately, the correlation was not seen in either SIT-treated ($\rho = -0.243$, $P = 0.416$) or SIT-untreated ($\rho = -0.491$, $P = 0.141$) group because Cry j 1-induced IL-5 production was lost in most of SIT-treated patients. The changes in Cry j 1-induced IL-5 production were significantly and positively correlated with both naso-ocular symptom scores ($\rho = 0.616$, $P = 0.024$) and QOL scores ($\rho = 0.719$, $P = 0.008$). The

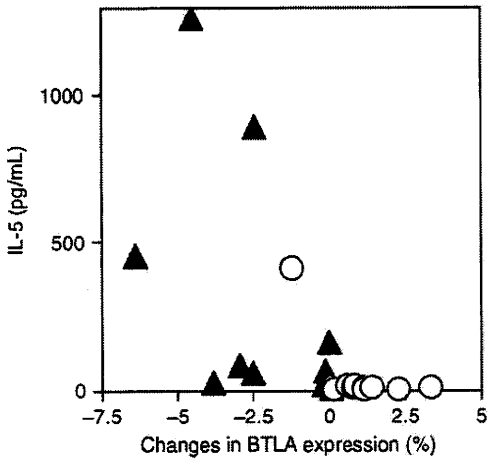

Fig. 6. Relationship between Cry j 1-induced B and T lymphocyte attenuator (BTLA) expression and IL-5 production. Peripheral blood mononuclear cells (PBMCs) were incubated in the presence or absence of Cry j 1 for 72 h, after which the expression of BTLA and IL-5 levels were determined by flow cytometry and ELISA, respectively. Specific immunotherapy (SIT)-treated and SIT-untreated Japanese cedar pollinosis (JCP) patients are represented by the open circles and closed triangles, respectively.

changes in BTLA expression did not correlate with naso-ocular symptom scores ($\rho = -0.063$, $P = 0.760$); however, the changes showed a tendency to inversely correlate with QOL scores ($\rho = -0.450$, $P = 0.067$).

Effect of B and T lymphocyte attenuator cross-linking on Cry j 1-induced interleukin-5 production

Finally, we sought to determine the *in vitro* role of BTLA in Cry j 1-specific PBMC responses. Immobilized anti-BTLA mAb significantly suppressed Cry j 1-induced IL-5 production by PBMC, compared with control mouse IgG2b ($P = 0.016$; Fig. 7a). On the other hand, Cry j 1-induced proliferative responses were not different between immobilized anti-BTLA mAb and the control treatment ($P = 0.879$; Fig. 7b).

Discussion

The key finding of the present study was that BTLA expression is down-regulated after Cry j 1 stimulation in patients not treated with SIT, while it is up-regulated in SIT-treated patients. The up-regulation of BTLA in SIT-treated patients was particularly apparent in a $CD4^+$ T cell subset. Cry j 1-induced changes in BTLA expression were significantly and inversely correlated with IL-5 production by PBMC. Furthermore, cross-linking of BLTA resulted in inhibition of Cry j 1-induced IL-5 production. 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.

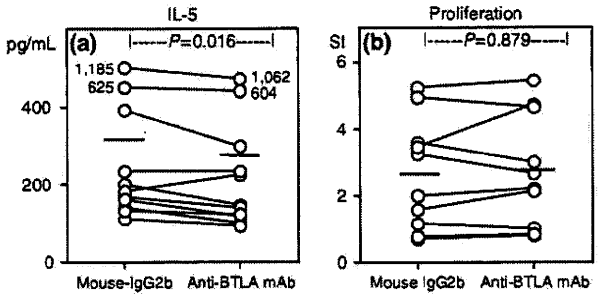

Fig. 7. Effect of cross-linking of B and T lymphocyte attenuator (BTLA) on Cry j 1-induced responses in peripheral blood mononuclear cells (PBMCs). PBMC from patients with Japanese cedar pollinosis (JCP) without immunotherapy were incubated in the presence or absence of Cry j 1, along with either MIH26 or control mouse IgG2b, for 72 h, after which levels of Cry j 1-induced IL-5 (a) and proliferative responses (b) were determined. *P*-values were determined using the Wilcoxon's signed-rank test.

BTLA is a recently identified member of the CD28 family of receptors. Similar to PD-1 and CTLA-4, BTLA contains ITIMs, suggesting that BTLA functions as an inhibitory receptor [17]. In fact, reduced BTLA expression leads to enhanced T cell and B cell responses in mice [17, 25]. However, little is known regarding the potential role of BTLA in human cellular responses. We have previously demonstrated that BTLA ligation inhibits anti-CD3-stimulated proliferation as well as the production of IFN-γ and IL-10 in human $CD4^+$ T cells [22]. Another group has reported suppression of anti-CD3-induced proliferation in response to cross-linking of BTLA, and reduced expression of CD25 and production of IL-2 and IL-4 in addition to IFN-γ and IL-10 in human T cells [26]. In the present study, immobilized anti-BTLA mAb that can cross-link BTLA significantly suppressed Cry j 1-induced IL-5 production by PBMC. This result is consistent with previous reports, and demonstrates for the first time that cross-linking of BTLA also inhibits antigen-specific human PBMC responses. On the other hand, BTLA cross-linking did not alter Cry j 1-induced proliferative responses. We have previously shown that BTLA-induced inhibitory signals depend on the strength of TCR signals [22]. These results suggest that BTLA cross-linking selectively affects Cry j 1-induced IL-5 production in our system. Although, to date, no Ab to block human BTLA is available, the definitive experiment would be to block BTLA (with non-cross-linking Abs or Fab fragments) to see if IL-5 production is restored in SIT patients.

Antigen concentration is one of the key factors regulating *in vitro* cellular responses. We previously reported that PBMC showed proliferative responses to Cry j 1 in a dose-dependent manner, and the samples showed positive responses at 10 μg/mL [20]. Another investigation used Cry j 1 at the concentration of 25–50 μg/mL with substantial results [27]. Thus, we think that the concentration of 10 μg/mL of Cry j 1 is appropriate concentration used in the present study.

Similar to other co-stimulatory molecules, the expression of BTLA is known to change upon activation [22, 25, 26, 28]. The expression of BTLA is up-regulated on T cells and down-regulated on B cells upon activation in mice [25, 28]. In humans, we have demonstrated constitutive BTLA expression on the surface of both CD4 and CD8T cells at high levels, which gradually declines after stimulation with anti-CD3 and anti-CD28 mAb [22]. The relationship between BTLA expression and pathogenesis has been investigated in several human diseases [26, 29, 30]. For example, increased BTLA expression has been demonstrated on $CD4^+$ and $CD8^+$ T cells within pleural fluid in lung cancer patients [26]. An association between a BTLA gene polymorphism and risk of rheumatoid arthritis has also been reported [29]. The baseline expression of BTLA did not differ significantly among SIT-treated and SIT-untreated patients. However, down-regulation of BTLA was only observed in untreated patients in the present study. Although the precise mechanism by which the expression of BTLA is down-regulated remains unknown, signals through the TCR, as well as cytokines produced by Cry j 1-specific T cells and/or pro-inflammatory cytokines/chemokines secreted by monocytes or B cells may all function to regulate BTLA expression. Identification of mechanism regulating BTLA expression must be made in future investigation.

BTLA was originally cloned from murine Th1 cells, and is predominantly expressed by B cells, followed by T cells and APC in mice [17, 25]. In humans, we have recently reported BTLA expression on $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells in freshly isolated human PBMC. Unlike in mice, polarized human Th1 and Th2 cells consist of both BTLA-positive and BTLA-negative populations; however, BTLA expression diminishes with extended length of culture [22]. The present results are consistent with those of previous reports and suggest that BTLA expression persists on human $CD4^+$, $CD8^+$ and $CD19^+$ cells during short-term culture. Furthermore, only $CD4^+$ cells bearing BTLA were significantly increased after recall stimulation with Cry j 1, suggesting that SIT selectively enhances BTLA expression on $CD4^+$ T cells.

A significant difference in Cry j 1-induced IL-5 production by PBMC was seen among SIT-treated and untreated patients. Inhibition of both local and systemic IL-5 production is known to correlate with clinical efficacy [6, 8]. The present result is consistent with a report by Kakinoki et al. [6] demonstrating that the production of IL-5 following Cry j 1-stimulation by PBMC is significantly reduced in good responders, compared with poor responders to SIT in patients with JCP. On the other hand, Cry j 1-induced IL-10 and TGF-β production was not detected in PBMC from SIT-treated patients. One of the reasons why IL-10 production was not induced in our immunotherapy

is that, although our immunotherapy was clinically effective (Fig. 1) and 2000 JAU/mL of JPC extract is maximal concentration commercially available in Japan, the maintenance dose is relatively low (the mean maintenance dose of the extract was 509.0 JAU which is equivalent to 0.37–1.07 μg of Cry j 1) as compared with other reports [1–5]. Moreover, the observed changes in BTLA expression were significantly and negatively correlated with IL-5 production after Cry j 1-stimulation in the present study. This result confirms the result of our cross-linking study and suggests that BTLA has an inhibitory role with regard to Cry j 1-specific IL-5 production in JCP.

In the present study, the expression of co-inhibitory molecules and cytokine production was determined during the pollen season after at least 2 years of SIT treatment. It is interesting to determine the effect of SIT during the first pollen season after starting this therapy. Our preliminary results showed that, as compared with SIT-untreated patients ($n = 10$), PBMC from the newly SIT-treated patients during the first pollen season after starting cedar immunotherapy ($n = 5$) produced significantly less amount of IL-5 (36.7±49.4 vs. 433.6±581.2 pg/mL, $P = 0.020$). On the other hand, Cry j 1-induced changes in BTLA (−1.42±0.80% vs. −3.42±0.97%, $P = 0.178$), PD-1 (0.27±0.38% vs. 0.73±0.33%, $P = 0.390$) and B7-H1 (0.10±0.24% vs. 0.01±0.34%, $P = 0.125$) expression as well as Cry j 1-induced IL-10 (2.4±1.5 vs. 0.2±0.2 pg/mL, $P = 0.137$) and TGF-β (0±0 vs. 0±0 pg/mL) production was not statistically different between the groups. These results suggest that period after reaching of maintenance dose is one of the factors regulating BTLA expression. In addition, the direct effect of SIT on expression of co-inhibitory molecules after reaching the maintenance dose, before the pollen season should be determined.

In conclusion, the present study shows an increase in BTLA-bearing $CD4^+$ T cells in patients treated with SIT. Furthermore, the alterations in BTLA expression were associated with allergen-specific IL-5 production. These results suggest that protection of BTLA down-regulation is a key mechanism of SIT. T cell co-signalling molecules are potential targets in the treatment of allergic airway disease [31]. The present study adds support to this and further suggests that regulation of BTLA expression may be of therapeutic benefit in the treatment of allergic airway disease.

Acknowledgements

There are no conflicts of interests. The authors would like to thank Miki Yamamoto, Rie Nomiya and Hisashi Hattori for their excellent technical assistance, and Yuko Okano for her editorial assistance. This work was supported in part by grants from Research on Allergic disease and Immunology of the Ministry of Health, Labor and Welfare, Japan (14210301).

References

1 Kowalski ML. Systemic and specific treatment for a global disease: allergen immunotherapy revised. *Allergy* 2006; 61:791–5.
2 Purello-D'Ambrosio F, Gangemi S, Merendino RA *et al.* Prevention of new sensitizations in monosensitized subjects submitted to specific immunotherapy or not. A retrospective study. *Clin Exp Allergy* 2001; 31:1295–302.
3 Durham SR, Walker SM, Varga E-M *et al.* Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 1999; 341:468–75.
4 Golden BK, Kagey-Sobotka A, Norman PS, Hamilton RG, Lichtenstein LM. Outcomes of allergy to insect stings in children, with and without venom immunotherapy. *N Engl J Med* 2004; 351:668–74.
5 Jutel M, Akdis M, Blaser K, Akdis CA. Mechanisms of allergen specific immunotherapy – T cell tolerance and more. *Allergy* 2006; 61:796–807.
6 Kakinoki Y, Ohashi Y, Nakai Y, Tanaka A, Washio Y. Pollen immunotherapy inhibits T helper 1 and 2 cell responses, but suppression of T helper 2 cell response is a more important mechanism related to the clinical efficacy. *Arch Otolaryngol Head Neck Surg* 2000; 126:63–70.
7 Gabrielsson S, Soderlund A, Paulie S, van der Pouw Kraan TC, Troye-Blomberg M, Rak S. Specific immunotherapy prevents increased levels of allergen-specific IL-4- and IL-13-producing cells during pollen season. *Allergy* 2001; 56:293–300.
8 Wilson DR, Nouri-Aria KT, Walker SM *et al.* Grass pollen immunotherapy: symptomatic improvement correlates with reductions in eosinophils and IL-5 mRNA expression in the nasal mucosa during the pollen season. *J Allergy Clin Immunol* 2001; 107:971–6.
9 Gardner LM, Spyroglou L, O'Hehir RE, Rolland JM. Increased allergen concentration enhances IFN-γ production by allergic donor T cells expressing a peripheral tissue trafficking phenotype. *Allergy* 2004; 59:1308–17.
10 Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003; 111:1255–61.
11 Schwartz RH. A cell culture model for T lymphocyte clonal anergy. *Science* 1990; 248:1349–56.
12 Greenwald RJ, Freeman GJ, Sharpe AH. B7 family revised. *Annu Rev Immunol* 2005; 23:515–48.
13 Nakada M, Nishizaki K, Yoshino T, Okano M, Masuda Y, Ohta N. CD86 (B7-2) antigen on B cells from atopic patients shows selective, antigen-specific upregulation. *Allergy* 1998; 53:527–31.
14 Hattori H, Okano M, Yoshino T *et al.* Expression of costimulatory CD80/CD86-CD28/CD152 molecules in nasal mucosa of patients with perennial allergic rhinitis. *Clin Exp Allergy* 2001; 31:1242–9.
15 Okano M, Azuma M, Yoshino T *et al.* Differential role of CD80 and CD86 molecules in the induction and the effector phases of allergic rhinitis in mice. *Am J Respir Crit Care Med* 2001; 164:1501–7.
16 Sperling AI, Bluestone JA. ICOS costimulation: it's not just for TH2 cells anymore. *Nat Immunol* 2001; 2:573–4.
17 Watanabe N, Gavrieli M, Sedy JR *et al.* BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol* 2003; 4:670–9.
18 Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based

switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol* 2004; 173:945–54.

19 Hirata H, Arima M, Cheng G *et al.* Effects of CD80 and CD86 on cytokine production in patients with wasp-venom allergy who receive venom immunotherapy. *Cytokine* 2003; 24:1–6.

20 Okano M, Kino K, Takishita T *et al.* Roles of carbohydrates on Cry j1, the major allergen of Japanese cedar pollen, in specific T cell responses. *J Allergy Clin Immunol* 2001; 108:101–8.

21 Youngnak P, Kozono Y, Kozono H *et al.* Differential binding properties of B7-H1 and B7-DC to programmed death-1. *Biochem Biophys Res Commun* 2003; 307:672–7.

22 Otsuki N, Kamimura Y, Hashiguchi M, Azuma M. Expression and function of the B and T lymphocyte attenuator (BTLA/CD272) on human T cells. *Biochem Biophys Res Commun* 2006; 344:1121–7.

23 Okuda M, Ohkubo K, Goto M *et al.* Comparative study of two Japanese rhinoconjunctivitis quality-of-life questionnaires. *Acta Otolaryngol* 2005; 125:736–44.

24 Takahashi K, Takata M, Suwaki T *et al.* New flow cytometric method for surface phenotyping basophils from peripheral blood. *J Immunol Methods* 1993; 162:17–21.

25 Han P, Goularte OD, Rufner K, Wilkinson B, Kaye J. An inhibitory Ig superfamily protein expressed by lymphocytes and APC is also an early marker of thymocyte positive selection. *J Immunol* 2004; 172:5931–9.

26 Wang WF, Chen YJ, Wang Q *et al.* Distinct expression and inhibitory function of B and T lymphocyte attenuator on human T cells. *Tissue Antigens* 2006; 69:145–53.

27 Sone T, Morikubo K, Miyahara M *et al.* T cell epitopes in Japanese cedar (*Cryptomeria japonica*) pollen allergens: choice of major T cell epitopes of Cry j 1 and Cry j 2 toward design of the peptide-based immunotherapeutics for the management of Japanese cedar pollinosis. *J Immunol* 1998; 161:448–57.

28 Hurchla MA, Sedy JR, Garvielli M, Drake CG, Murphy TL, Murphy KM. B and T lymphocytes attenuator exhibits structural and expression polymorphism and is highly induced in anergic CD4+ T cells. *J Immunol* 2005; 174:2277–85.

29 Lin SC, Kou CC, Chan CH. Association of a BTLA gene polymorphism with the risk of rheumatoid arthritis. *J Biomed Sci* 2006; 13:853–60.

30 Tsutsumi Y, Jie X, Ihara K *et al.* Phenotypic and genetic analyses of T-cell-medicated immunoregulation in patients with Type 1 diabetes. *Diabet Med* 2006; 23:1145–50.

31 Kroczek R, Hamelmann E. T-cell costimulatory molecules: optimal targets for the treatment of allergic airway disease with monoclonal antibodies. *J Allergy Clin Immunol* 2005; 116:906–9.

Allergology International. 2009;58:119-124
DOI: 10.2332/allergolint.O-08-535

ORIGINAL ARTICLE

Aging Exacerbates Restraint Stress-Induced Inhibition of Antigen-Specific Antibody Production in Mice

Yumiko Ichihara[1], Mitsuhiro Okano[2], Keiko Nishioka[3], Noriko Manabe[4], Naoto Ichihara[4], Fumihiko Jitsunari[1], Tazuko Fujiwara[2] and Kazunori Nishizaki[2]

ABSTRACT

Background: We have recently found that exposure to acute restraint stress suppresses antigen-specific antibody production, including IgE, in a murine model of allergic rhinitis. Although age-related alterations in immune responses are known, it remains unclear whether aging modulates the antibody production under stressful conditions. In this study, we set out to determine the effects of aging on antibody production under acute restraint stress in mice.

Methods: Both young and aged CBA/J mice were repeatedly sensitized intranasally with phospholipase A2 (PLA2) without adjuvants. Restraint stress was applied using uniform cylinders once a week for a continuous 8 h period, on 5 occasions in total. Blood samples were taken at 0, 20 and 30 days after primary sensitization, and production of PLA2-specific antibodies and levels of IL-4, IFN-γ, IL-10 and IL-1β in sera were determined by ELISA.

Results: Repeated intranasal sensitization with PLA2 induced PLA2-specific IgE, IgG1 and IgG2a production in aged mice. We found that exposure to restraint stress significantly inhibited production of PLA2-specific IgE, IgG1 and IgG2a in aged mice. In addition, antibody production under restraint stress decreased significantly in aged mice when compared with young mice. No IL-4, IFN-γ, IL-10 or IL-1β were detected in sera from non-stressed or stressed aged mice.

Conclusions: Aging exacerbates the immunosuppressive role of acute restraint stress in antigen-specific antibody production in mice.

KEY WORDS

aged mouse, immunosuppression, phospholipase A2, restraint stress, specific antibody

INTRODUCTION

It is known that aging is associated with a reduced immune function, so called immunosenescence, in both humans and animals.[1-4] For example, a shift in lymphocyte population from conventional T cells to NK cells and extrathymic T cells is observed in human centenarians.[1] Changes in the proportion of T cell subsets, in addition to increases in memory T cells, impairment of response to mitogens and other stimuli, and alterations in cytokine production also occur with aging.[2-4]

In terms of humoral immunity, it is known that pro-B cells in old mice are impaired in their capacity to rearrange themselves to both D to J and V to DJ gene segments in mice.[5] In addition, serum IgE levels and antigen-specific IgE production are known to decline with age in humans.[6,7]

Exposure to physical, neurological, or emotional stress can also affect both innate and acquired immune responses.[8-10] For example, exposure to acute stress modulates antigen-specific T cell responses.[11] We have recently reported that inhibition of antigen-specific antibody production was confirmed using a type of restraint stress following intranasal sensitization with phospholipase A2 (PLA2) in mice.[12] How-

[1]Hygiene and Public Health, Social Medicine, Faculty of Medicine, Kagawa University, [3]Department of Otolaryngology, Takinomiya General Hospital, [4]Kagawa Prefectural College of Health Sciences, Kagawa and [2]Department of Otolaryngology, Head and Neck Surgery, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan.
Correspondence: Keiko Nishioka, MD, Department of Otolaryngology, Takinomiya General Hospital, Takinomiya 486, Ayauta, Ayagawa, Kagawa 761-2393, Japan.
Email: Kei@song.ocn.ne.jp
Received 16 January 2008. Accepted for publication 21 August 2008.