Published October 18, 2010

JEM

anti–Ser5-P RNAPII (Abcam), anti–Ser2-P RNAPII (Abcam), anti-MLL (Bethyl Laboratories, Inc.), anti-Bmi1 (Santa Cruz Biotechnology, Inc.), anti-STAT6 (Santa Cruz Biotechnology, Inc.), anti-Menin (Bethyl Laboratories, Inc.), anti-Suz12 (Abcam), and anti-EZH2 (Diagenode). The antibodies used for EMSA were anti-STAT6 (Santa Cruz Biotechnology, Inc.), anti-CBP (Abcam), and anti-p300 (Millipore). The antibodies used for cytoplasmic staining were anti–IFN-γ–FITC, anti–IL-4–PE, and anti-GATA3–Alexa Fluor 647 (BD).

The generation of Th1 and Th2 cells. Th1/Th2 cells were generated as previously described (Yamashita et al., 2006). In brief, splenic CD4 T cells were stimulated with 3 μg/ml of immobilized anti–TCR-β mAb plus 1 μg/ml anti-CD28 mAb under the Th1 or Th2 culture conditions for 5 d in vitro. Th1 conditions were as follows: 25 U/ml IL-2, 10 U/ml IL-12, and anti–IL-4 mAb. Th2 conditions were as follows: 25 U/ml IL-2 and 100 U/ml IL-4. These cells were used as either Th1 or Th2 cells.

Establishment of fully developed Th2 cells. Splenic CD4 T cells from DO11.10 OVA-specific TCR transgenic mice were stimulated with an OVA peptide (1 μM Loh15) plus APC under Th2 culture conditions for 5 d in vitro. The Th2 cells were further cultured in vitro for 2 d in the absence of any exogenous cytokines. The cultured CD4 T cells were then restimulated with OVA peptide (1 μM Loh15) plus APC with IL-2 and anti–IL-4 mAb for 5 d. This cycle was then repeated more than three times.

Quantitative RT-PCR. Total RNA was isolated using the TRIZOL reagent (Invitrogen). cDNA was synthesized using oligo (dT) primer and Superscript II RT (Invitrogen). Quantitative RT-PCR was performed as described previously using a sequence detection system (ABI Prism 7500; Applied Biosystems; Yamashita et al., 2006). The primers and TaqMan probes for the detection of *GATA3*, Bmi1, EZH2, Menin, MLL, and HPRT were purchased from Applied Biosystems and Roche, respectively. The specific primers and Roche Universal probes used are described in Table S1. The expression was normalized by the HPRT signal.

ChIP assay. ChIP was performed using ChIP assay kits (Millipore) as previously described (Yamashita et al., 2006). Quantitative representations of the results are shown as relative band intensities measured by a densitometer (AE6905H [ATTO] and CS Analyzer version 2.08b). The specific primers used are described in Table S1. Real-time quantitative PCR analysis was performed on an ABI Prism 7500 real time PCR machine with TaqMan probes and primers (sequences available in Table S1). To calculate the enrichment of each protein to a particular target DNA, values obtained (via the standard curve method) for each target were divided by the amount of the corresponding target in the input fraction. Enrichments obtained from mock immunoprecipitations performed in parallel with normal IgG were then subtracted from the enrichment values obtained with specific antibodies ([specific antibody ChIP − control Ig ChIP]/input DNA). All the enrichments are expressed as a function of the highest enrichment obtained on the locus (set to 10; Demers et al., 2007).

EMSA. EMSAs were performed using a gel shift assay system (Promega) as described previously (Kimura et al., 2005). In brief, the nuclear extracts were incubated at room temperature with a ^{32}P-labeled, double-stranded oligonucleotide in DNA-binding buffer. In some experiments, the nuclear extracts were preincubated at 4°C with specific antibodies. Electrophoresis was conducted on 4% native polyacrylamide gel (acrylamide/bisacrylamide ratio 29:0.8 in 0.5× Tris-borate-EDTA), and the radioactivity was visualized by autoradiography. The oligonucleotides used in this experiment were normal probe S4, 5′-CTTGGCGTTCAAGAGAATTCTCAA-3′; mutant probe S4, 5′-CTTGGCGTTC***GGTTT***AATTCTCAA-3′; normal probe S7, 5′-AGCCAACTTCCTAGGAAAAAGCTG-3′; and mutant probe S7, 5′-AGCCAACTTC***AGTTT***AAAAAGCTG-3′. The STAT6 consensus motif is underlined, and the mutated nucleic acids are shown in italicized and bold characters.

TSA treatment. STAT6-deficient splenic CD4 cells were cultured under Th2 conditions, and 10 nM TSA (Sigma-Aldrich) was added in the culture on day 2. After another 3-d culture, CD4 T cells were collected for the ChIP assay and RT-PCR.

Retroviral vectors and infection. The pMX-IRES-hNGFR plasmid was generated as previously described (Shinnakasu et al., 2006). Retrovirus vectors containing STAT6VT cDNA (pMXs-STAT6VT-IRES-hNGFR; Daniel et al., 2000) or STAT6VT dDBD cDNA (pMXs-STAT6VT-dDBD-IRES-hNGFR) were used. The infected cells were enriched by magnetic cell sorting with anti-hNGFR (clone C40-1457; BD) and were subjected to a ChIP assay.

A resting and restimulation culture system. Naive CD4 T cells from Menin-deficient mice were cultured under Th2 conditions for 5 d. The Th2 cells were further cultured for 2 d in the absence of cytokines added and then restimulated with anti-TCR mAb in the presence of IL-2 and anti–IL-4 mAb for an additional 5 d. This cycle was then repeated. PcG/TrxG binding and histone modifications were detected by ChIP assays, and the *GATA3* expression was determined using quantitative RT-PCR at the end of the each cycle.

Immunoblot analysis. Cytoplasmic extracts and nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Fisher Scientific). The antibodies used for the immunoblot analysis were anti–histone H3 (Abcam), anti-Erk1 (Santa Cruz Biotechnology, Inc.), anti-MLL (Bethyl Laboratories, Inc.), anti-Bmi1 (Santa Cruz Biotechnology, Inc.), anti-Menin (Bethyl Laboratories, Inc.), and anti-EZH2 (Diagenode).

ChIP-seq and Illumina sequencing. For ChIP-seq analysis, immunoprecipitate and input samples were prepared using ChIP-Seq Sample Prep kit (Illumina, Inc.). Adaptor-ligated DNA fragments were size-fractionated by 12% acrylamide gel, and the 150–250-bp fraction was recovered. DNA thus obtained was amplified by 18 cycles of PCR. 1 ng of the DNA was used for the sequencing reaction of the GAIIx (Illumina, Inc.) according to the manufacturer's instructions. 150,000–250,000 clusters were generated per tile, and 36 cycles of the sequencing reactions were performed. Short-read sequences were aligned to the mouse genome sequences (mm9 as from the University of California, Santa Cruz Genome Browser) using the Eland program. Sequences allowing no more than two mismatches per sequence were used for the analysis.

Online supplemental material. Fig. S1 shows the real PCR product bands of the results shown in Fig. 1 C. Fig. S2 shows expression of Bmi1, EZH2, Menin, and MLL in naive CD4 T cells, Th2 cells, and fully developed Th2 cells. Fig. S3 shows the binding pattern of phosphorylated RNA-PII at the *GATA3* gene locus. Fig. S4 shows that expression of Bmi1, EZH2, Menin, and MLL was not affected by TSA treatment in the STAT6-deficient Th2 cells. Fig. S5 shows phenotypic characterization of peripheral CD4 T cells in Menin-deficient mice. Fig. S6 shows the levels of total H3 at the *GATA3* gene locus in Menin-deficient Th2 cells. Fig. S7 shows IL-4/STAT6-independent maintenance of *GATA3* expression in developed Th2 cells. Fig. S8 shows binding of Menin protein to specific regions around the *GATA3* gene locus. Table S1 shows primers and probes used for ChIP and RT-PCR. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100760/DC1.

We are grateful to Drs. Ralph Kubo and Anjana Rao for their helpful comments and constructive criticisms in the preparation of the manuscript. We thank Ms. Kaoru Sugaya, Hikari K. Asou, Satoko S. Norikane, and Mr. Toshihiro Ito for their excellent technical assistance.

This work was supported by the Global Centers of Excellence Program (Global Center for Education and Research in Immune System Regulation and Treatment), Ministry of Education, Culture, Sports, Science and Technology (MEXT; Japan), and by grants from MEXT (Grants-in-Aid for Scientific Research on Priority Areas #17016010 and #20060003, Scientific Research [B] #21390147, Scientific Research [C] #21591808 and #20590485, and Young Scientists [B] # 20790367, [Start-up] #19890041,

[Start-up] #20890038, and #2056131), the Ministry of Health, Labour and Welfare (Japan), and the Uehara Memorial Foundation.

The authors have no conflicting financial interests.

Submitted: 19 April 2010
Accepted: 20 September 2010

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Current Opinion in Immunology

Role of NKT cells in allergic asthma

Chiaki Iwamura and Toshinori Nakayama

T helper 2 (Th2) cells play crucial roles in the development of allergic asthma, while various distinct cell populations also contribute to the pathogenesis of the disease. Invariant natural killer T (iNKT) cells produce large amounts of cytokines such as IL-4 and IFNγ upon stimulation with a ligand, α-galactosylceramide, and regulate various immune responses. Recently, a critical role of iNKT cells in the mouse model of asthma and also in asthma patients has been reported, while some contradictory results have also been described. Here, we summarize the experimental results in mouse and human systems, and discuss the current understanding of the role of NKT cells in the pathogenesis of asthma, including a possible mechanism by which iNKT cells are activated in asthma patients.

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Current Opinion in Immunology 2010, **22**:807–813

This review comes from a themed issue on
Allergy and hypersensitivity
Edited by Marsha Wills-Karp and Martien Kapsenberg

Available online 4th November 2010

0952-7915/$ – see front matter

DOI 10.1016/j.coi.2010.10.008

Introduction

It is well established that $CD4^+$ T helper 2 (Th2) cells play an important role in allergic disorders by producing Th2 cytokines [1]. IL-4 induces antigen-specific IgE production from B cells, and IgE and antigen activate mast cells to release various chemical mediators. IL-5 induces the development, activation and survival of eosinophils, while IL-13 induces airway hyperreactivity (AHR) and mucus hyper production [2]. Animal studies investigating these processes are very helpful to explain the mechanisms underlying the development of allergic asthma. However, in asthma patients, several types of therapies targeted to these Th2 cytokines were not as effective as many investigators expected [3]. Indeed many patients show a non-allergic form of asthma in which no allergen-specific IgE are detected. Furthermore, it is well known that Th2 cell-independent factors such as viruses, air pollution, and exercise induce and/or exacerbate asthma symptoms. In addition, it has been reported that non-Th2 factors such as IFNγ, IL-17, and neutrophils contribute to severe asthma and steroid-resistant asthma [4,5]. These results indicate that Th2 responses are not obligatory in the development of asthma.

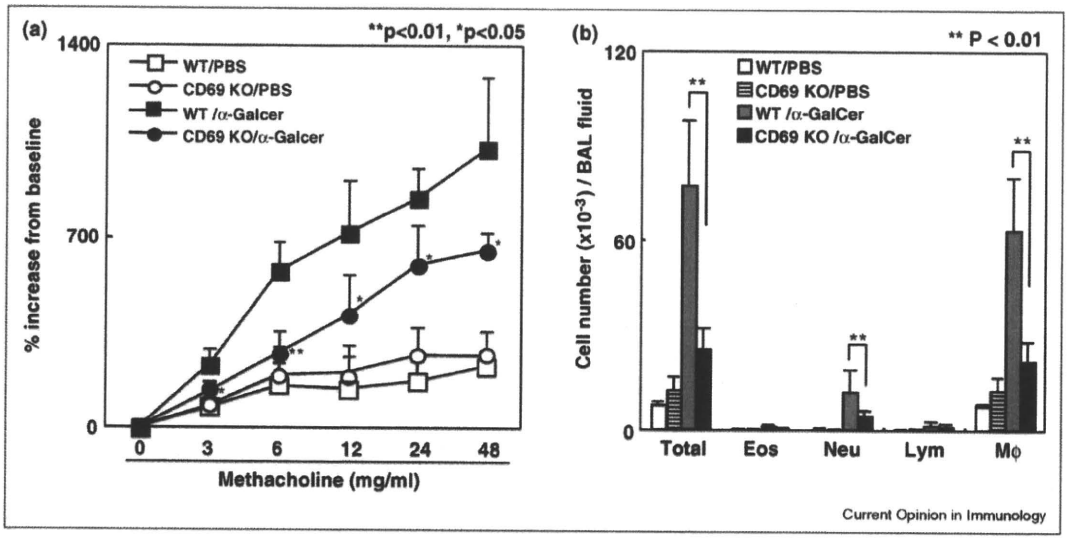
Natural killer T (NKT) cells belong to a novel lymphoid lineage distinct from T cells, B cells or NK cells. NKT cells are characterized by the expression of a restricted repertoire of T cell receptors (TCRs) consisting of Vα14 and Jα18 (in mice) or Vα24 and Jα18 (in humans) [6]. These receptors are associated with a highly skewed set of Vβs, mainly Vβ8.2 (in mice) and Vβ11 (in humans). Since these TCRs are highly restricted, NKT cells having these receptors are called invariant NKT cells (iNKT cells). The most potent and well-analyzed ligand for the iNKT antigen receptor is a glycolipid, α-galactosylceramide (α-GalCer), which is exclusively presented by CD1d, a monomorphic class Ib molecule. Activated iNKT cells play critical roles in the regulation of various immune responses, such as allergic inflammation, anti-tumor immunity and autoimmune responses.

Upon activation, iNKT cells produce a large amount of both type 1 and type 2 cytokines, that is, IFNγ and IL-4/IL-5/IL-13. Therefore, iNKT cells may inhibit or exacerbate allergic responses. The activation of iNKT cells with α-GalCer at the sensitization phase attenuated allergic airway inflammation and Th2 responses via production of IFNγ [7,8,9•]. In these reports, iNKT cells showed a suppressive function rather than promoting disease. On the other hand, many investigators have examined whether experimental allergic asthma is induced in iNKT cell-deficient mice or whether the activation of iNKT cells in the lung results in the development of asthma symptoms in mice. iNKT cells from patients with asthma were also assessed to reinforce the relationship between iNKT cells and allergic disease. In human studies, both positive and negative results regarding the contribution of iNKT cells in asthma were reported [10••,11••]. We herein focus on the role of iNKT cells in the pathogenesis of asthma in animal models and discuss the contribution of iNKT cells to human asthma.

Do NKT cells induce experimental allergic asthma?

Activated iNKT cells may induce allergic asthma symptoms independently from Th2 cells. To address this question, Akbari *et al.* assessed OVA-induced airway heperreactivity (AHR) and airway inflammation in iNKT cell deficient Jα281 KO and CD1d KO mice. This study

Figure 1



iNKT cell-dependent AHR and airway inflammation were attenuated in CD69-deficient mice. To examine the role of CD69$^+$ iNKT cells in NKT cell-dependent asthma, 1.5 μg of α-GalCer was administrated intranasally into wild type mice (WT) and CD69-deficient (KO) mice. **(a)** AHR in WT and CD69 KO mice 24 hours after α-GalCer treatment. Airway resistances were assessed in an invasive system as described previously [46]. **(b)** Decreased infiltrated leukocytes in BALF of CD69 KO mice after intranasal administration of α-GalCer. 24 hours after α-GalCer treatment, BALF of WT and CD69 KO mice were collected. Mean values (5 mice per group) are shown with SDs. *$P < 0.05$ and **$P < 0.01$, student t test.

showed a significant defect in the development of AHR and inflammation in the absence of iNKT cells [12]. The authors concluded that iNKT cells are required for the development of AHR and airway inflammation. The administration of anti-CD1d antibodies or a CD1d-dependent antagonist can also suppress OVA-induced AHR and airway inflammation [13,14•]. The respiratory administration of α-GalCer rapidly induced AHR and inflammation in naïve mice and this effect was seen even in naïve MHC class II-deficient mice, which lack conventional CD4 T cells [15]. In addition, a particular subset of iNKT cells that lack the NK1.1 marker produced IL-17 and induced neutrophilic infiltration following the intranasal administration of α-GalCer [16]. We recently reported that CD69$^+$ Th2 cells play a crucial role in OVA-induced airway inflammation and AHR [17]. As shown in Figure 1, α-GalCer-induced AHR and leukocyte infiltration, including neutrophilic infiltration was attenuated significantly in CD69-deficient mice, indicating that activated iNKT cell-mediated asthmatic responses were dependent on CD69. Thus, CD69$^+$ iNKT cells may play a critical role in the development of AHR and airway inflammation. Matangkasombut *et al.* demonstrated that the direct activation of pulmonary iNKT cells with α-GalCer in non-human primates resulted in the development of AHR, indicating that pulmonary iNKT cells are critical effector cells in this model [18].

However, there are several reports indicating that iNKT cells are dispensable for allergic airway inflammation. Allergic inflammation occurred normally under certain conditions in CD1d-deficient mice and β2-microgloblin (β2m) KO mice that lack iNKT cells [19]. More recently, however, Koh *et al.* found that AHR can develop in β2m KO mice [20]. In this report, they concluded that non-classical NKT cells, which are restricted to a β2m-independent form of CD1d contribute to the development of AHR. Although the reason is not clear, these discrepancies in the results obtained from β2m KO mice may be due to the difference in the experimental systems used.

iNKT cells in asthma patients

In order to identify the possible role of iNKT cells in human asthma, several investigators assessed the number of iNKT cells in asthma patients (Table 1). An initial report was published in 2006 by Akbari *et al.* reporting that more than 60% of CD4 T cells in the bronchoalveolar lavage fluid (BALF) from asthmatic patients were iNKT cells, while NKT cells were not observed in patients with sarcoidosis or in healthy controls [21]. Two other groups published supportive reports in the same year [22–24].

However, four other groups performed similar studies and concluded that iNKT cells did not increase in the patients with asthma [25–29]. Vijayanand *et al.* reported that iNKT cells were found in low numbers in the airways of patients with asthma, COPD or healthy controls with no significant differences between the three groups [26]. Mutalithas *et al.* also reported similar results in the BALF [27]. Moreover, the influx of iNKT cells into the airways

Table 1

iNKT cells in patients with asthma

Year	iNKT cells are			
	pathogenic		not pathogenic	
2006	Akbari	About 60% of $CD4^+CD3^+$ cells in BALF from patients with severe asthma were CD1d-restricted NKT cells [21]	Thomas	Only 0.4–2.1% of lymphocytes in BALF of asthmatic patients were 6B11+ NKT cells [25]
	Pham-ti	The frequency of CD1d-restricted NKT cells in BALF from severe asthmatic children was higher than in BALF from controls [22,23]		
	Hamzaoui	The number of $CD3^+CD56^+$NKT cells in the sputum of severe asthmatic patients is increased as compared to that of controls [24]		
2007			Vijayanand	Fewer than 2% of CD4T cells were CD1d-restricted, $6B11^+$ or $V\alpha24^+$ NKT cells in airway biopsy, BALF, and sputum of mild or moderately severe asthmatic patients and controls [26]
			Mutalithas	The presence of $6B11^+$ NKT cells in BALF of mild asthmatic patients was not significantly higher than that of controls [27]
			Thomas	Approximately 1% of lymphocytes were $6B11^+V\alpha24^+$NKT cells in BALF and this did not increase with an antigen challenge [28]
			Bratke	Less than 1% of T cells were $6B11^+$NKT cells in BALF of mild asthma patients [29]
2009	Matangkasombut	CD1d-restricted NKT cells in BALF of severe or mild asthmatic patients increased (readdressed) [30••]		
	Reynold	CD1d-restricted NKT cells increased in lung specimens from mild asthmatic patients as compared to those from controls [31]		
2010	Koh	$V\alpha24^+$ or $6B11^+$ NKT cells were significantly increased in sputum from patients with asthma as compared to those from controls [32]		

BALF, bronchoalveolar lavage fluid.

was not observed after segmental allergen challenge [28,29].

By contrast, all studies we have identified from 2008 emphasized the existence of, and an increase in iNKT cells in patients with asthma [30••,31,32]. Matangkasombut *et al.* readdressed the issue regarding the number of iNKT cells in BALF from patients with severe asthma [30••]. They confirmed that patients with severe asthma had a significant increase in the number of iNKT cells as compared to healthy controls. In this report, however, the numbers of iNKT cells were 2–7% of total $CD3^+$ cells in BALF of asthmatic patients. Only one patient with severe asthma had 64.5% NKT cells. Reynolds *et al.* supported this idea using lung biopsies with allergen challenge [31]. At present, it is unclear why studies on iNKT cells in asthma patients have provided divergent results. However, as the filed matures it is becoming more evident that iNKT cells likely do play a role in the development and possibly exacerbation of allergic asthma. In addition, the studies of iNKT cells in other asthma etiologies, such as chronic, occupational, steroid-resistant, exercise-induced, and aspirin-induced asthma, where Th2 cells may not play a major role, could provide new insights into these types of diseases.

What activates iNKT cells *in vivo*?

Even if activated iNKT cells can cause and exacerbate allergic asthma, α-GalCer is a component of a marine sponge and it is very rare to be exposed to α-GalCer during daily life. Respiratory organs are continually exposed to environmental stimuli including allergens, pathogens, and air pollution. Several studies indicate that substances naturally occurring in our environment may activate iNKT cells and develop or exacerbate allergic airway inflammation.

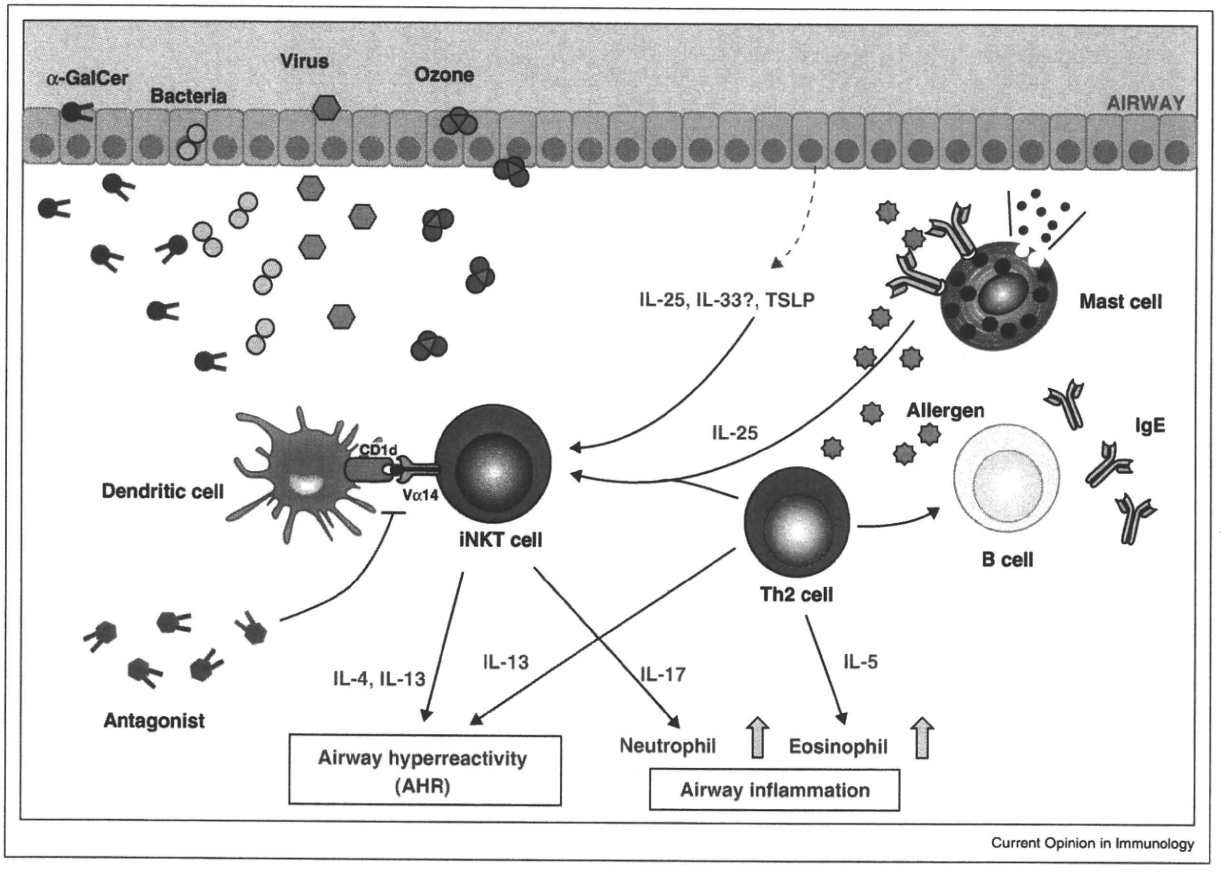
The invariant TCR of iNKT cells recognizes glycolipids from bacteria such as *Sphingomonas*, *Borrelia*, and *Leishmania* species [33]. Glycolipids purified from *Sphingoma-*

nas cell wall induced rapid AHR after respiratory administration in WT mice but not iNKT-deficient mice [15]. Once iNKT cells are activated by virus antigen, they stimulated macrophages to produce IL-13, resulting in the development of AHR and mucus production independently from the adaptive immune response [34]. It is well known that respiratory infection exacerbates the symptoms of allergic diseases under certain conditions. Therefore, iNKT cells activated by glycolipid from pathogens may contribute to the development and exacerbation of asthma symptoms in humans.

Ozone, as an air pollutant, has been reported to be associated with asthma [35,36]. Even in healthy individuals, exposure to ozone resulted in the development of AHR, associated with airway epithelial cell damage and increased numbers of neutrophils. Asthmatic patients appeared to be more susceptible to the adverse effects of this pollutant. Pichavant *et al.* showed that iNKT cells were required for ozone-induced asthma through production of IL-17 in the mouse lung [37]. Although it is not clear how ozone activates iNKT cells, NKT cells activated by ozone can induce a form of asthma that is characterized by neutrophilic infiltration and AHR.

Recently, several cytokines involved in the initiation and amplification of Th2 responses were reported [38]. IL-25 also known as IL-17E, a member of the structurally related IL-17 cytokine family, is produced by activated Th2 cells, epithelial cells, basophils, and mast cells and is capable of enhancing AHR. Administration of recombinant IL-25 induced Th2 type responses, including increased serum IgE levels, eosinophilia, pathological changes in the lung, and AHR. These symptoms induced by IL-25 were not observed in iNKT cell deficient mice [39•,40]. Transfer experiments of iNKT cells showed that iNKT cells expressing IL-17 receptor B (IL-17RB) are essential for IL-25-induced AHR. Thymic

Figure 2



Activated iNKT cells and Th2 cells in the development of AHR and airway inflammation. Th2 cytokines produced by antigen-specific Th2 cells can cause allergic asthma phenotypes through IgE induction, mast cell activation, eosinophilic infiltration into the lung, and AHR. Lung iNKT cells are activated by environmental substances or pro-Th2 cytokines. The activated iNKT cells induce AHR and infiltration of neutrophils predominantly in the airway by producing IL-13 and IL-17, respectively.

stromal lymphoprotein (TSLP) is produced by epithelial cells, mast cells, and basophils, and is considered to play an important role in experimental models of asthma. The targets of TSLP are T cells, mast cells, basophils and dendritic cells (DCs). In addition, Nagata *et al.* showed that TSLP also acts on iNKT cells to enhance AHR by up-regulating their production of IL-13 [41]. IL-33 is an IL-1 family member whose expression is also increased in epithelial cells in asthmatic patients. IL-33 enhanced the production of Th1 and Th2 cytokines in activated iNKT cells [42,43]. Based on these results, natural ligands in the environment may activate iNKT cells to induce allergic asthma, and pro-Th2 cytokines such as IL-25, TSLP and IL-33 may exacerbate the allergic symptoms.

How do iNKT cells control Th2 immune responses?

One enigma is the dynamics of iNKT cell accumulation in the lung during airway inflammation. Since the number of iNKT cells in the lung, lymphoid organs and PBMCs of humans and mice is very low (<0.1%) as compared to that of T cells or NK cells [44], it is curious how iNKT cells contribute to asthmatic symptoms. It is not known whether iNKT cells are activated and proliferated in the asthmatic lung or if activated iNKT cells migrate from lymphoid organs to the lung. We recently visualized the migration of antigen-specific Th2 cells into the lung in living mice after antigen exposure in the airway [45]. Using this *in vivo* live imaging system, visualization of the migration and localization of iNKT cells in the lung may provide important insights into these questions. In addition, possible cell–cell interactions between iNKT cells and various types of APCs, epithelial cells or Th2 cells in the asthmatic lung would be another interesting issue that may be clarified using *in vivo* imaging systems.

Conclusion

Figure 2 illustrates the role of activated iNKT cells and Th2 cells in AHR and airway inflammation. Both activated iNKT cells and Th2 cells produce various cytokines in the lung and induce AHR and airway inflammation. In the murine experimental model of asthma, activated iNKT cells appear to contribute as effector cells and also as an amplifier of allergen-specific Th2 cell responses. Although these mechanisms clarified in mouse models have not been directly investigated in asthmatic patients, NKT cells may yet prove to be a new therapeutic target for allergic diseases.

Acknowledgements

The authors are grateful to Drs. Damon Tumes and Yuki Kinjo for valuable comments during the preparation of this manuscript. This work was supported by Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment), and by grants from the Ministry of Education, Culture, Sports, Science and Technology (Japan) (Grants-in-Aid: for Scientific Research on Priority Areas #17016010; Scientific Research (B) #21390147, Young Scientists (B) #22790452), and the Ministry of Health, Labor and Welfare (Japan).

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Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2010;153:378–387
DOI: 10.1159/000316349

Received: June 20, 2009
Accepted after revision: March 8, 2010
Published online: June 18, 2010

The Induced Regulatory T Cell Level, Defined as the Proportion of IL-10$^+$Foxp3$^+$ Cells among CD25$^+$CD4$^+$ Leukocytes, Is a Potential Therapeutic Biomarker for Sublingual Immunotherapy: A Preliminary Report

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Key Words

Allergic rhinitis · Biomarker · Foxp3 · Immunotherapy · Interleukin-10 · Japanese cedar · Pollinosis · Regulatory T cell · Sublingual immunotherapy

Abstract

Background: Japanese cedar *(Cryptomeria japonica)* pollinosis is one of the most prevalent allergies in Japan. Recently, two reports described the positive effects of sublingual immunotherapy (SLIT) against Japanese cedar pollinosis. However, the therapeutic biomarkers for SLIT are still unclear. We performed this unblinded, nonrandomized, open-label study to identify therapeutic biomarkers for SLIT against Japanese cedar pollinosis. ***Methods:*** We performed an open-label study during one pollinosis season in 2007, enrolling 19 patients from in-house volunteers suffering from Japanese cedar pollinosis. Peripheral blood was obtained from all participants before SLIT treatment as well as before and after the pollen season. The plasma levels of an immunoglobulin specific to a major allergen (Cry j 1) were determined. We analyzed the induction of regulatory T cells (iTregs), namely IL-10$^+$Foxp3$^+$ cells in CD25$^+$CD4$^+$ leukocytes, by flow cytometry. The Th2-type responses were analyzed by cytokine production from peripheral blood mononuclear cells after stimulation with Cry j 1. Clinical symptoms were estimated using a quality of life questionnaire in the middle of the pollen season. ***Results:*** The difference in numbers of iTregs between the medium-only control cell culture and cells stimulated with Cry j 1 was significantly decreased in the non-SLIT group but was unchanged in the SLIT group after the pollen season. The subgroup of the SLIT group with increased iTregs showed more attenuated Th2-type cytokine profiles, and symptom scores in the subgroup with increased iTregs were significantly lower than those in the subgroup with decreased iTregs. ***Conclusion:*** The antigen-specific iTreg level is a potential therapeutic biomarker that correlates with clinical pollinosis symptoms and may be involved in the therapeutic mechanisms of SLIT.

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Introduction

Japanese cedar *(Cryptomeria japonica)* pollinosis is one of the most prevalent allergies in Japan; a nationwide survey in 2008 found a prevalence of 26.5% [1]. Compared with the estimate of 13.1% in 2001, this more recent figure implies that the number of affected patients is rapidly increasing [2].

Recently, two reports have described the positive clinical effects of sublingual immunotherapy (SLIT) against Japanese cedar pollinosis [3, 4]. A randomized double-blind study reported that efficacy variable scores in an active treatment group were significantly lower than those in a placebo group, and the quality of life (QOL) symptom score of the active group was almost half that of the placebo group [3]. We also previously reported that active treatment significantly ameliorated symptom scores and symptom-medication scores compared with placebo in a randomized controlled trial. Furthermore, we reported that SLIT decreased the number of Th2 clones specific to CS712, namely recombinant protein-conjugated T cell epitopes from Cry j 1 and Cry j 2 [4, 5].

Several reports have suggested the involvement of Foxp3-positive regulatory T cells (Tregs) in the therapeutic mechanisms of immunotherapy. It was reported that the number of Tregs, namely $CD25^{bright}$ and/or $Foxp3^+CD4^+$ T cells, was significantly increased during specific immunotherapy against bee venom [6]. It has also been reported that $Foxp3^+CD25^+$ and $Foxp3^+CD4^+$ cell levels were significantly increased in the nasal mucosa of patients receiving immunotherapy treatment with grass pollen [7]. mRNA expression of Foxp3 and IL-10 was reported to be induced after SLIT treatment, and the suppression of effector cell proliferation was IL-10-dependent [8]. The central mechanisms by which Tregs downregulate antigen-specific Th2 responses are suggested to be mediated by the production of the suppressor cytokine IL-10 in a soluble or membrane-bound form [9]. However, the therapeutic mechanisms and the relationship between clinical symptoms and Treg induction remain unclear.

In this study, we analyzed Cry j 1-specific Th2 responses and induced Tregs (iTregs), defined as $CD25^+$ $CD4^+$ leukocytes positive for both IL-10 and Foxp3. We considered that antigen-specific iTregs would produce the suppressor cytokine IL-10 with Cry j 1 activation. These Cry j 1-specific iTregs (Cry j 1-iTregs) from the SLIT group were maintained after pollen season, whereas the Cry j 1-iTregs from the non-SLIT group decreased significantly after pollen season. Furthermore, the subgroup of the SLIT group with increased iTregs showed a tendency for attenuated cytokine profiles compared to both the subgroup with decreased iTregs and the non-SLIT group. The subgroup with increased iTregs also showed lower clinical symptom scores than the subgroup with decreased iTregs. We propose that the level of antigen-specific iTregs is a suitable biomarker for the severity of symptoms and the therapeutic effects of SLIT.

Materials and Methods

Study Population

Nineteen in-house volunteers between 22 and 63 years of age, who were otherwise healthy but who had a clinical history of Japanese cedar pollinosis, were enrolled in this pilot study. The diagnosis of Japanese cedar pollinosis was based on clinical history and IgE specific to Japanese cedar pollen of at least class 2 status (CAP-RAST method, Phadia, Tokyo, Japan). Patients who had a history of any immunotherapy, had a current diagnosis of asthma or were pregnant were excluded. The patients in the non-SLIT group were statistically older than those in the SLIT group; however, there was no statistical difference between the groups with regard to the period of suffering from Japanese cedar pollinosis (3–10 years). All patients had showed moderate or severe symptoms in the previous pollen season [10]. Antigen-specific IgE titers for orchard grass, Japanese cypress and house dust mites were also evaluated by the CAP-RAST method. The protocol was approved by the Ethics Committee of Chiba University; written informed consent was obtained from each of the patients prior to participation in the study.

Clinical Protocols

Standardized Japanese cedar pollen extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) was used for SLIT [11]. The trial was performed from October 2006 to June 2007. The treatment protocol consisted of graded courses of the extract in 50% glycerol, followed by maintenance therapy [4]. The extract was graded in 3 strengths: 20, 200 and 2,000 Japanese Allergy Units (JAU)/ml. The content of Cry j 1 in the 2,000 JAU/ml extract was 1.5–4.2 μg, as determined by enzyme-linked immunosorbent assay (ELISA) and as reported previously [12]. Patients received increasing doses with each vial, beginning with 0.2 ml from the 20 JAU/ml vial and increasing by 0.2 ml a day for 5 days per week; the vaccine was taken sublingually, kept for 2 min without a retention reagent and then spit out. The procedure was then repeated with each vial until the maximum dose (1.0 ml of 2,000 JAU/ml) was reached. The maintenance dose was 1.0 ml of 2,000 JAU/ml once a week until the end of the study. The non-SLIT group was administered neither the vaccine nor a placebo. All participants were allowed to take symptom-reducing drugs.

Blood Samples

Peripheral blood was obtained from each patient before the beginning of treatment, before the pollen season and after the pollen season. Peripheral blood mononuclear cells (PBMCs) were isolated from whole peripheral blood by Ficoll density gradient centrifugation using Lymphocyte Separation Medium (MP Bio-

medicals Inc., Solon, Ohio, USA). Isolated cells were counted and tested for viability by trypan blue exclusion prior to culture. The PBMCs were frozen at –80°C and stored in liquid nitrogen using a cell banker (Nippon Zenyaku Kogyo Co. Ltd., Fukushima, Japan) until use.

Antigens for in vitro Stimulation and ELISA

Cry j 1 was purified from Japanese cedar pollen according to the method of Yasueda et al. [13] with some modifications. The concentration of purified Cry j 1 was determined by the Lowry method using a detergent-compatible protein assay reagent (Bio-Rad Laboratories Inc., Hercules, Calif., USA). CS712, which is a recombinant protein with 7 conjugated regions of T cell epitopes from Cry j 1 and Cry j 2 [4, 5], was kindly provided by Daiichi Sankyo Co. Ltd. (Tokyo, Japan).

Antigen-Specific Immunoglobulin Titer

The Cry j 1-specific IgE titer in the plasma was measured by the method of Yasueda et al. [14]. The Cry j 1-specific IgG4 titer was measured by ELISA as described previously [4].

Flow Cytometric Analysis

For intracellular staining of Foxp3 and IL-10, PBMCs were cultured with or without Cry j 1 for 3 days, followed by culture with 10 ng/ml phorbol 12-myristate 13-acetate, 1 μM ionomycin and 2 μM monensin for 6 h. The PBMCs were stained with phycoerythrin-anti-CD25 (eBioscience, San Diego, Calif., USA) and phycoerythrin-Cy7-anti-CD4 antibody (BD Biosciences, San Diego, Calif., USA) in PBS containing 1% FCS and 0.1% sodium azide for 20 min at 4°C. After surface staining, the PBMCs were stained with FITC-anti-Foxp3 (clone PCH101, eBioscience) and allophycocyanin-anti-IL-10 antibody (BD Biosciences) for 30 min at 4°C using a Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions. The numbers of IL-10$^+$Foxp3$^+$ cells in 10^4 CD25$^+$CD4$^+$ leukocytes were calculated from the percentage of IL-10$^+$Foxp3$^+$ cells in CD25$^+$CD4$^+$ leukocytes.

Clinical Symptoms

The participants were instructed to fill in a QOL questionnaire in the middle of the 2007 pollen season. Japanese cedar pollen scattered from the middle of January to early May in 2007. The Japanese Allergic Rhinitis QOL Standard Questionnaire No. 1 was used for the assessment of QOL symptom scores for allergic rhinitis [15]. The total QOL symptom score was calculated as the sum of each score: none = 0; mild = 1; moderate = 2; severe = 3; very severe = 4. Nasal and ocular symptoms covered by the questionnaire included runny nose, sneezing, nasal congestion, itchy nose, itchy eyes and watery eyes [3].

Enzyme-Linked Immunospot Assay

The numbers of IL-4- and IL-5-producing cells stimulated with Cry j 1 or CS712 were determined by enzyme-linked immunospot assay. A 96-well sterile filter plate (Millipore Corp., Billerica, Mass., USA) was coated with monoclonal antibody to human IL-4 or IL-5 (Mabtech AB, Nacka Strand, Sweden), following preincubation with 35% ethanol. After washing with PBS, the plate was preincubated with AIM-V medium at 37°C for 1 h. The medium was discarded, then 3 × 10^5 PBMCs/well were cultured with fresh medium alone, 10 μg/ml Cry j 1, 20 nM CS712 or 1 μg/ml phytohemagglutinin as a positive control for 17 h at 37°C in AIM-V medium containing 5% human blood plasma fractions. The plates were then washed with PBS, incubated with biotinylated detection monoclonal antibody to human IL-4 or IL-5 for 2 h and then incubated with streptavidin-conjugated alkaline phosphatase for 1 h at room temperature. After washing with PBS, the plates were incubated with BCIP/NBT^{PLUS} (Mabtech AB) for 5 min at 37°C. The numbers of positive spots were automatically calculated by the ImmunoScan^{TM} (Cellular Technology Ltd., Cleveland, Ohio, USA) using the same parameter settings throughout.

Assay of Cytokine Production from PBMCs

Isolated PBMCs were cultured at 2.5 × 10^6 cells/ml with or without 5 μg/ml Cry j 1 for 3 days at 37°C in AIM-V medium containing 5% human AB serum (Sigma-Aldrich Inc., St Louis, Mo., USA). After centrifugation at 300 *g* for 10 min, the supernatant was divided into aliquots and stored at –20°C until cytokine assay. The concentrations of IL-5, IL-10, IL-13 and IFN-γ cytokines were measured by means of the BD^{TM} Cytometric Beads Assay Flex system (BD Biosciences) according to the manufacturer's instructions.

Data Representation

The Cry j 1-specific cytokine production and the numbers of Cry j 1-iTregs are presented as the difference between the value from Cry j 1-stimulated cells and that from the medium-only control cell culture. Each upregulation after pollen season was represented as the difference between the pre-pollen season and post-pollen season values.

Statistical Analysis

Results are presented as means ± SD. Two-group comparisons were performed using the Wilcoxon t test or Mann-Whitney U test to determine the significance of the difference. p values <0.05 were considered significant.

Results

Study Population and Adverse Events

Nineteen adult patients were recruited from in-house volunteers at Chiba University and Chiba University Hospital on the basis of a history of Japanese cedar pollinosis and positive specific IgE (CAP-RAST score over 2; table 1). The 12 subjects who accepted the vaccine were enrolled in the SLIT group, and the 7 individuals who hesitated to take the vaccine were enrolled in the non-SLIT group. Three patients had mild discomfort and 1 patient complained of mild itching in the mouth; however, these adverse events were not serious and did not present a reason to discontinue SLIT.

Immunoglobulin Production

The Cry j 1-specific IgE production in the SLIT group was not statistically significantly higher after the pollen

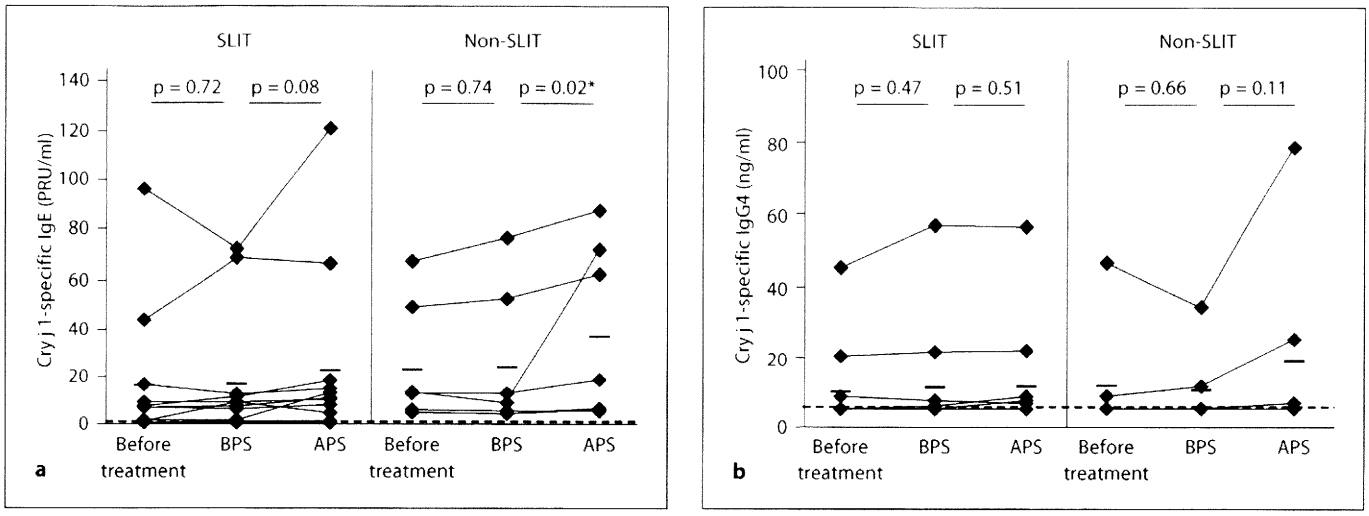

Fig. 1. Cry j 1-specific IgE (**a**) and IgG4 titer (**b**) from the SLIT and non-SLIT groups before treatment and before (BPS) and after the pollen season (APS). Bars show the group averages. The dashed line indicates the threshold for detection. PRU = Phadebas RAST unit. Statistical analysis was performed using the Wilcoxon t test. * $p < 0.05$.

season compared with before the pollen season ($p = 0.08$); in contrast, IgE production in the non-SLIT group was statistically significantly increased after the pollen season compared with before the pollen season ($p = 0.02$; fig. 1a). Cry j 1-specific IgG4 production was not significantly changed after treatment in either the SLIT or non-SLIT group (fig. 1b).

Division of the SLIT Group According to the Change in iTregs

We analyzed a population of IL-10$^+$Foxp3$^+$ cells in CD25$^+$CD4$^+$ leukocytes as a marker of iTregs after stimulation with or without Cry j 1 (fig. 2a). The Cry j 1-iTreg levels, that is, the difference between those stimulated with Cry j 1 and the medium-only control, were significantly increased after treatment, and the difference in numbers of Cry j 1-iTregs before the pollen season was comparable to those after the pollen season in the SLIT group. However, we found that the difference in the non-SLIT group after treatment was comparable with that before treatment and significantly decreased after the pollen season compared to before the pollen season (fig. 2b). The upregulation between before and after the pollen season in the SLIT group (5 ± 42) was higher than that in the non-SLIT group (–24 ± 20), although the difference in the levels between the groups was not statistically significant (fig. 2c).

In all but one participant from the non-SLIT group, the difference in the number of Cry j 1-iTregs was down-regulated after pollen season; in contrast, half of the SLIT group had higher Cry j 1-iTreg levels and the other half had lower levels (fig. 2c). Therefore, we divided the SLIT group into two subgroups according to whether Cry j 1-iTreg levels increased or decreased after pollen season. The total symptom score from the QOL questionnaire in the SLIT group was comparable to that in the non-SLIT group before the division into two groups. After the division, we found that the symptom score in the subgroup

Table 1. The characteristics of participants at the time the study started

	SLIT	Non-SLIT
Participants	12	7
Sex (M/F)	9/3	5/2
Age, years		
Mean ± SD	24.1 ± 2.0	37.5 ± 15.8
Range	22–30	21–63
IgE class[1]	3.5	4.1
Other allergy[2]		
Orchard grass	6	4
Japanese cypress	12	7
House dust mite	3	5

[1] Specific IgE to Japanese cedar pollen; mean CAP allergy class.
[2] Numbers of subjects who had specific IgE of at least class 2.

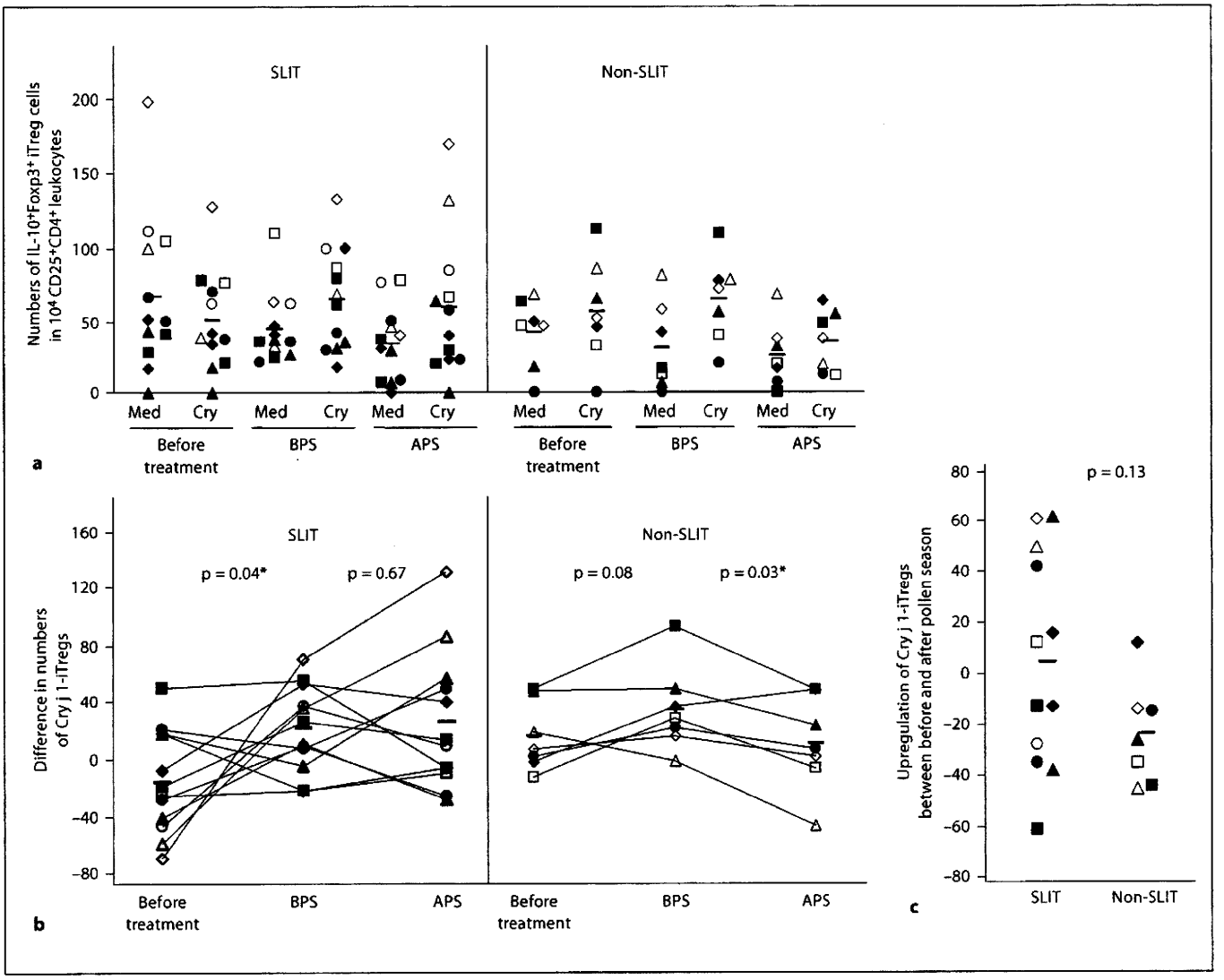

Fig. 2. a The numbers of IL-10$^+$Foxp3$^+$ cells in 10^4 CD25$^+$CD4$^+$ leukocytes (iTregs) cultured with (Cry) or without (Med) Cry j 1 before treatment and before (BPS) and after the pollen season (APS). Each symbol in the SLIT group and the non-SLIT group represents an identical individual. Bars show the group averages. **b** The difference in numbers of Cry j 1-iTregs between medium-only control and cells stimulated with Cry j 1 was plotted for before treatment and before and after the pollen season in the SLIT and non-SLIT groups. Bars show the group averages. Statistical analysis was performed using the Wilcoxon t test. **c** The upregulation of Cry j 1-iTregs between before and after the pollen season in the SLIT and non-SLIT groups. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * $p < 0.05$.

with increased iTregs was significantly lower than that in the subgroup with decreased iTregs ($p = 0.03$; fig. 3).

We also divided the SLIT group into severe and mild subgroups according to their total QOL symptom scores. We found that the upregulation of Cry j 1-iTregs between before and after pollen season in the mild-symptom subgroup was significantly higher than that in both the severe-symptom subgroup and the non-SLIT group (fig. 4).

Th2-Type Cytokine Profiles

We analyzed the numbers of Th2-type cytokine-producing cells and cytokine production after stimulation with native Cry j 1. The numbers of Cry j 1-specific Th2-type cytokine-producing cells were analyzed by enzyme-linked immunospot assay after stimulation with Cry j 1 or CS712 (fig. 5a and data not shown). The upregulation of both IL-4- and IL-5-producing cells in the SLIT group

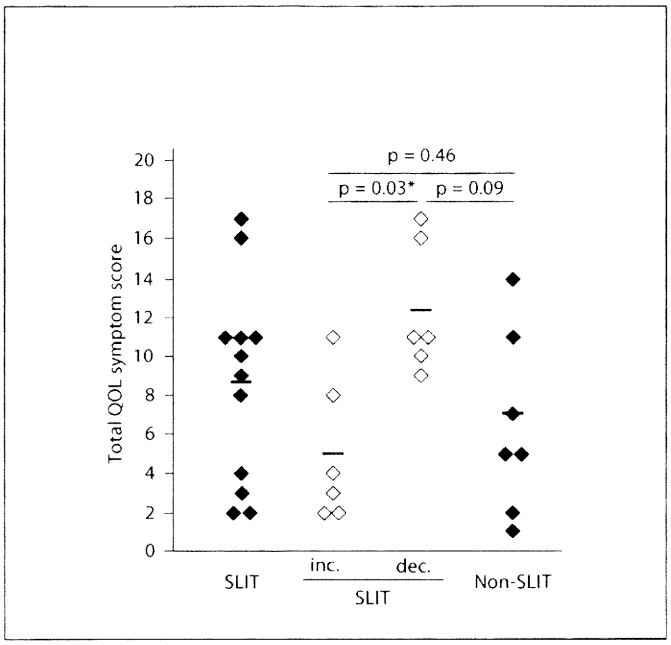

Fig. 3. Total symptom score from the QOL questionnaire was plotted for the SLIT and non-SLIT groups as well as for the subgroups from the SLIT group with increased (inc.) and decreased (dec.) iTregs. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * $p < 0.05$.

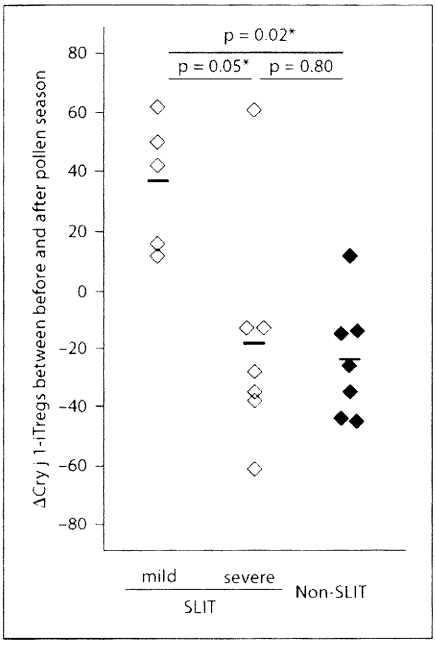

Fig. 4. The differences in numbers of Cry j 1-specific iTregs in 10^4 $CD25^+CD4^+$ leukocytes between before and after pollen season were plotted for the mild and severe subgroups of the SLIT group as well as for the non-SLIT group. The classification into severe and mild subgroups was based on the mean score of the SLIT group. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * $p < 0.05$.

(IL-4: 29 ± 33; IL-5: 23 ± 28) tended to be attenuated compared with that in the non-SLIT group (IL-4: 54 ± 38; IL-5: 43 ± 28). Furthermore, the upregulation in the subgroup with increased iTregs (IL-4: 19 ± 22; IL-5: 10 ± 11) was much lower than that in both the subgroup with decreased iTregs (IL-4: 40 ± 41; IL-5: 36 ± 35) and the non-SLIT group, although the difference in the levels between the groups was not statistically significant (fig. 5b). The same results were obtained using CS712 for stimulation (data not shown).

Cytokine production was analyzed in culture supernatant after 3 days of culture with Cry j 1 (fig. 6a). The upregulation of Th2-type cytokine production (IL-5 and IL-13), i.e. the differences between before and after pollen season, also tended to be attenuated in the SLIT group (IL-5: 94 ± 126; IL-13: 107 ± 134) compared to the non-SLIT group (IL-5: 178 ± 146; IL-13: 248 ± 222). We found that the Th2 cytokine profile in the subgroup with increased iTregs (IL-5: 54 ± 123; IL-13: 47 ± 110) also showed a strong tendency to be attenuated compared with that in the subgroup with decreased iTregs (IL-5: 134 ± 127; IL-13: 167 ± 137) and was significantly lower than that in the non-SLIT group. The upregulation of IL-13 in the subgroup with increased iTregs showed statistically significant suppression compared with that in the non-SLIT group (fig. 6b). Upregulation of both IFN-γ and IL-10 production induced by Cry j 1 was almost the same in the SLIT (IFN-γ: -1.8 ± 22; IL-10: 0.8 ± 2.2) and non-SLIT groups (IFN-γ: -1.2 ± 32; IL-10: 1.2 ± 2.8; data not shown).

Discussion

We performed this pilot study to elucidate clinical biomarkers correlated with clinical symptoms in preparation for a future double-blind, placebo-controlled study of SLIT. Only one commercial standardized extract from Japanese cedar pollen is available for clinical use in Japan [11]. The cumulative dose of this major allergen after using the extract for 4 weeks is comparable to 2,500 SQ-standardized grass allergy immunotherapy tablet (SQ-T) in Europe [16]. In spite of the low dose, SLIT against Japanese cedar pollinosis has still been found to effectively

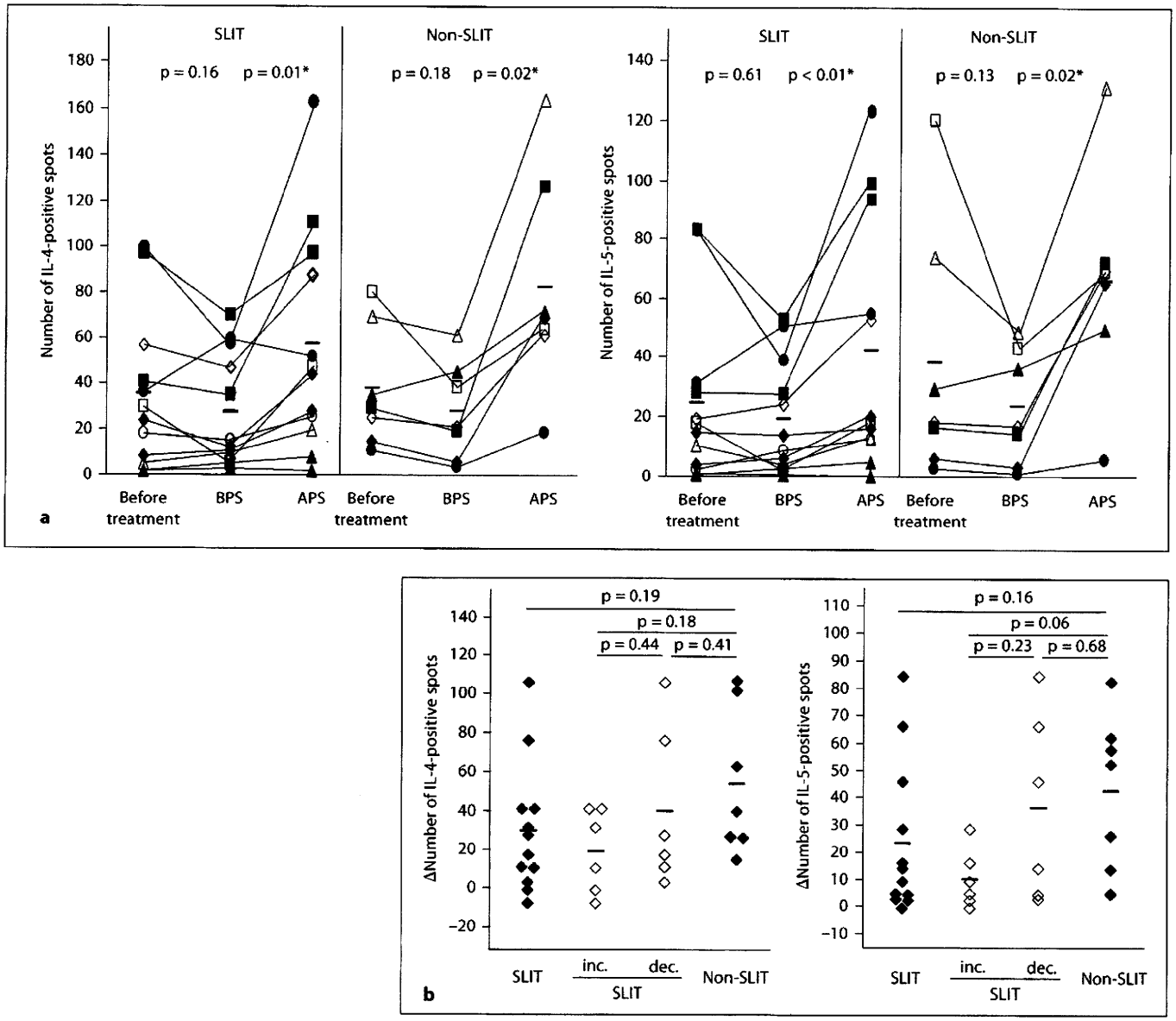

Fig. 5. a The numbers of Cry j 1-specific cytokine-producing cells before treatment and before (BPS) and after pollen season (APS). The numbers of positive cells for medium-only control were 1.3 ± 2.4 (SLIT) and 1.5 ± 3.1 (non-SLIT) for IL-4, and 1.0 ± 1.4 (SLIT) and 0.9 ± 1.6 (non-SLIT) for IL-5. Each symbol in the SLIT group or the non-SLIT group represents an identical individual. Bars show the group averages. Statistical analysis was performed using the Wilcoxon t test. * $p < 0.05$. **b** The difference in numbers of Cry j 1-specific cytokine-producing spots is shown as the difference between values before and after the pollen season for each individual from the SLIT, the Cry j 1-iTreg-increased (inc.), the Cry j 1-iTreg-decreased (dec.) and the non-SLIT groups. The numbers of Cry j 1-specific spots were calculated as the difference between the medium-only control and the culture stimulated with Cry j 1.

ameliorate the QOL symptom score, medication score and symptom-medication score [3, 4]. Furthermore, SLIT attenuated antigen-specific Th2 responses and induced iTregs in some patients; this subgroup with increased iTregs showed greater amelioration of the Th2-type cytokine profile and their clinical symptoms.

In this clinical trial, significant induction of Cry j 1-specific IgG4 was not observed in the SLIT group. Our previous report showed induction of Cry j 1-specific IgG4 production with almost the same protocol; the previous study used a piece of bread to retain extract for sublingual administration [4]. The differences in the participants'

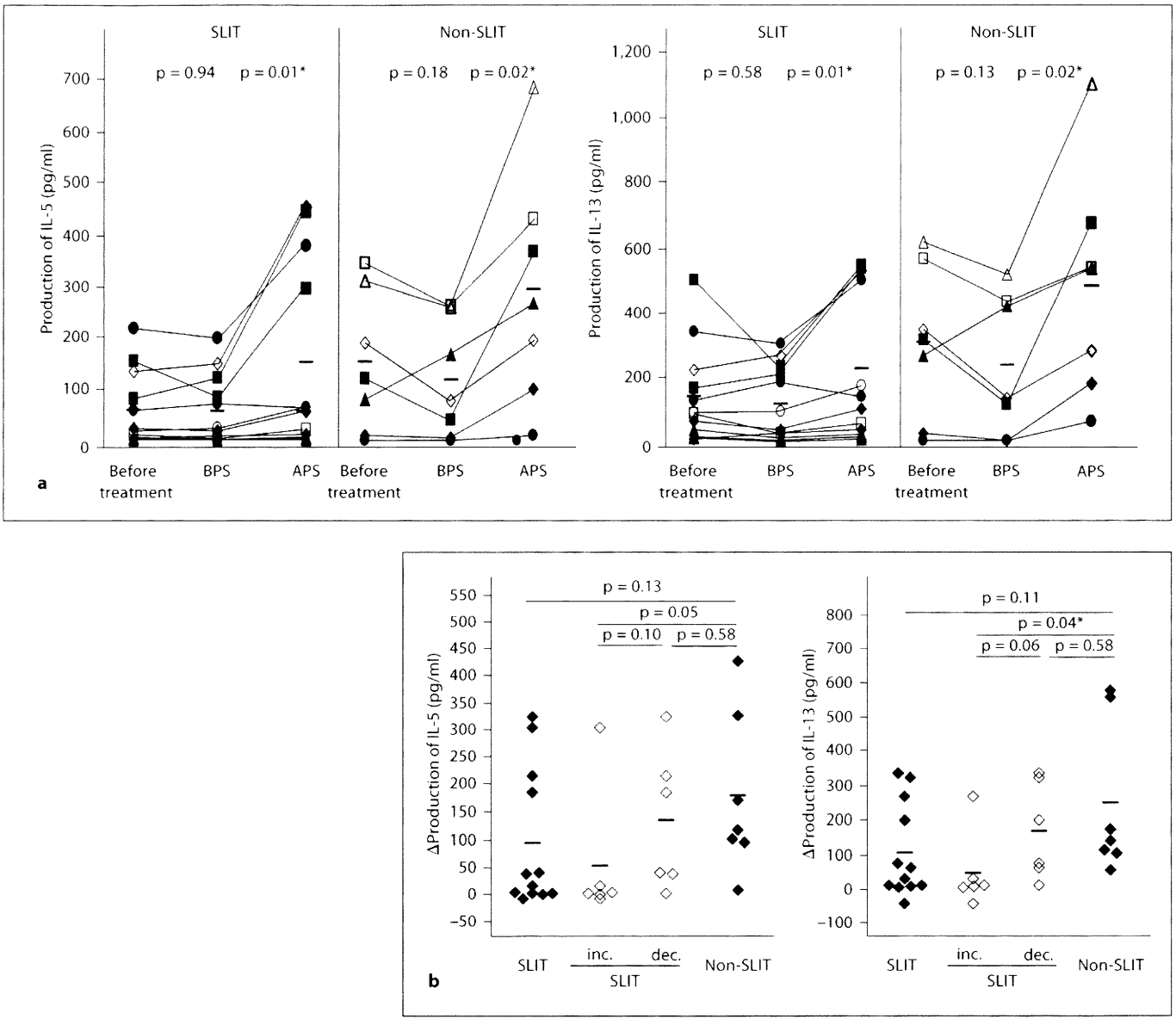

Fig. 6. a Th2-type Cry j 1-specific cytokine production before treatment and before (BPS) and after (APS) the pollen season. The cytokine production levels for medium-only control were 1.3 ± 2.0 (SLIT) and 3.4 ± 4.5 (non-SLIT) for IL-5, and 6.1 ± 9.6 (SLIT) and 10.6 ± 11.6 (non-SLIT) for IL-13. Bars show the group averages. Statistical analysis was performed using the Wilcoxon t test. * $p < 0.05$. **b** The upregulation of cytokine production induced by Cry j 1 between before and after the pollen season from each individual from the SLIT, the Cry j 1-iTreg increased (inc.), the Cry j 1-iTreg decreased (dec.) and the non-SLIT groups is plotted on the y-axis. The cytokine production induced by Cry j 1 was calculated as the difference between medium-only control and the culture stimulated with Cry j 1. Bars show the group averages. Statistical analysis was performed using the Mann-Whitney U test. * $p < 0.05$.

immunological backgrounds, methods of administration, period of administration and/or the amount of antigen absorbed in the oral mucosa may influence IgG4 induction. Antigen-specific IgG production was reported to be induced by high doses of extract, i.e. 25,000 SQ-T for 18 weeks or 75,000 SQ-T for 8 weeks [16]. This report supports the hypothesis that the amount of antigen adsorbed by the oral mucosa affects the induction of antigen-specific IgG4.

We previously reported that SLIT significantly decreases the clone size of IL-4-producing T cells specific to epitopes from Cry j 1 and Cry j 2 [4]. Also, in the current

trial, SLIT attenuated Cry j 1-specific cytokine production, the numbers of IL-4- and IL-5-producing cells and IL-5 and IL-13 production in culture supernatant (fig. 5b, 6b). The difference in downregulation was not statistically significant between the SLIT and non-SLIT groups, but the results showing this tendency were reproducible. No significant difference in Th2-type cytokine production after stimulation with Cry j 1 was observed after sublingual administration of extract alone (i.e. the cytokine levels before and after pollen season in the SLIT group; fig. 5a, 6a). SLIT may attenuate the upregulation of antigen-specific Th2-type responses activated through natural exposure in pollen season. Therefore, the amount of scattering pollen may influence the degree of amelioration of Th2 responses by SLIT.

The upregulation of Cry j 1-specific iTregs, that is, the difference in levels of Cry j 1-iTregs between before and after pollen season, is suggested to be a suitable biomarker for clinical symptoms and therapeutic effects. The average difference in numbers of Cry j 1-iTregs after pollen season, that is, the difference in numbers of IL-10$^+$Foxp3$^+$ cells in CD25$^+$CD4$^+$ leukocytes between those stimulated with Cry j 1 and the medium-only control, was 24.8 in the SLIT group and 9.3 in the non-SLIT group in 10^4 CD25$^+$CD4$^+$ leukocytes after 3 days' culture with Cry j 1 (fig. 2b). The number of antigen-specific Tr1 is reported to be estimated at 0.5–10 in 10^4 whole peripheral CD25$^+$CD4$^+$ Tregs [17]. That estimate suggests the appropriateness of the numbers of Cry j 1-iTregs reported in this study. The subgroup with increased iTregs showed more attenuated Th2 cytokine profiles and a lower total QOL symptom score than the subgroup with decreased iTregs and the non-SLIT group (fig. 3, 5b, 6b). Several reports have shown that Foxp3-positive cells and/or IL-10-producing cells are induced by immunotherapy and that IL-10 is crucial for downregulation of inflammatory Th2 responses [6, 8, 18, 19]. Foxp3-expressing CD4$^+$CD25$^+$ cells are reported to be induced in the nasal mucosa after immunotherapy, and local induction of iTregs is suggested to be important to suppress local inflammation during pollen season [7]. Furthermore, the basal frequencies of Tregs defined as CD4$^+$CD25^{bright}Foxp3$^+$ cells in the peripheral blood of patients with severe allergic reactions to insect stings were reported to be lower than in individuals without a history of allergic diseases, and the Treg population was upregulated to a level comparable to that of nonallergic subjects after immunotherapy against bee venom [6].

The QOL symptom score was higher in the group with decreased Cry j 1-iTregs than in the group with increased Cry j 1-iTregs. IL-17-secreting Foxp3$^+$ Treg cells were recently identified in humans, and IL-17 mRNA expression was significantly correlated with poor clinical outcome after SLIT [20–22]. A low dose of SLIT may induce IL-17-secreting Tregs rather than IL-10-secreting Tregs for nonresponder populations and thereby worsen clinical symptoms.

We divided the SLIT group into two subgroups according to whether IL-10 or Foxp3 single-positive cells increased or decreased after pollen season. However, groups with IL-10 or Foxp3 single-positive cells showed no difference in Th2 cytokine profiles or symptom scores among the increased, decreased and non-SLIT groups (data not shown). We hypothesize that a population of Foxp3 or IL-10 single-positive cells may include many antigen-nonspecific Tregs and effector cells, whereas only antigen-specific iTregs are available as a therapeutic biomarker. These data suggest that IL-10 and Foxp3 double-positive cells rather reflect the antigen-specific iTregs that could be used as therapeutic biomarkers of SLIT.

Soluble IFN-γ and IL-10 production in culture supernatant was not upregulated by the SLIT treatment and did not differ between the SLIT group and the non-SLIT group (data not shown). This suggested that SLIT did not induce Cry j 1-specific Th1-type responses and that the membrane-bound form of IL-10 may be more important than soluble IL-10 for downregulation of Cry j 1-specific Th2 cells. Further investigation is needed to clarify the induction of Th1 cells and IL-10-mediated suppressive mechanisms by iTreg cells. In this trial, we failed to detect other regulatory molecules at the protein level, such as TGF-β from culture supernatant or cytotoxic T lymphocyte-associated protein-4 and glucocorticoid-induced TNF receptor on the surface of CD4$^+$ T cells (data not shown). Further investigations are needed to analyze these regulatory molecules at the mRNA level. We are currently undertaking a transcriptome analysis of CD4$^+$ cells from the SLIT and non-SLIT groups after stimulation with Cry j 1. On the other hand, the regulatory mechanisms of peripheral human Tregs were suggested to occur in a cell contact-dependent but cytokine-independent manner [23]. Furthermore, this report suggested that the regulatory function of human Tregs was independent of CD28, cytotoxic T lymphocyte-associated protein-4, TGF-β and IL-10 [23]. In order to elucidate the therapeutic mechanisms of SLIT, analysis of the regulatory function of iTregs is also important.

In this paper, we investigated antigen-specific iTregs as a therapeutic biomarker for SLIT. However, this study was a preliminary open-label study with a small population; therefore, a randomized, double-blind, placebo-controlled study of a large population will be needed to evaluate iTregs as a therapeutic biomarker for SLIT.

Acknowledgments

We sincerely thank Drs. Takashi Saito and Yasuyuki Ishii (RIKEN, Kanagawa, Japan) for their helpful comments and the fruitful discussions. This work was supported in part by a grant from the Ministry of Health, Labor and Welfare in Japan and in part by the Global Centers of Excellence program entitled Global Center for Education and Research in Immune System Regulation and Treatment (Ministry of Education, Culture, Sports, Science and Technology, Japan).

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