

Analysis of T-helper responses and FOXP3 gene expression in patients with Japanese cedar pollinosis

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ABSTRACT

Background: Evidence has been accumulated indicating that regulatory T (T-reg) cells play a crucial role in the maintenance of peripheral T-cell tolerance to allergens. To explore the role of FOXP3, which is required for the development of T-reg cells, in allergen-specific immune responses, we examined the relationship between the alteration of FOXP3 gene expression and *in vitro* immune responses against allergens.

Methods: Peripheral blood mononuclear cells obtained from 19 human histocompatibility leukocyte antigens (HLA)-DPB1*0501 donors, including patients with Japanese cedar pollinosis and nonallergic healthy donors, were stimulated with Cry j 1 p61–75 peptide. On day 7, T cells were tested for peptide-specific reactivity in IFN- γ and interleukin (IL)-5 enzyme-linked immunospot (ELISPOT) assays. Real-time quantitative RT-PCR was performed to assess relative change of FOXP3 gene expression before and after *in vitro* stimulation. Neutralization assays using anti-glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) and anti-IL-10 monoclonal antibody were also performed.

Results: Of 14 patients with allergic pollinosis tested, 10 responders displayed T-helper type 2 (Th2)–polarized reactivity to Cry j 1 p61–75, and 2 donors showed Th0 responses. Notably, the change of FOXP3 gene expression in donors showing peptide-specific T-helper responses was significantly lower than that in nonresponders, regardless of allergic pollinosis.

Conclusion: Our data indicate that FOXP3 is functional in nonallergic healthy donors as well as allergic patients, and FOXP3-expressing T cells may be responsible for the down-regulation of allergen-specific T-helper responses in individuals. A better understanding of the nature and specificity of FOXP3-expressing T cells in a suppressive mechanism is necessary to develop new immunotherapies against allergic rhinitis.

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Key words: Allergic rhinitis, Cry j 1, FOXP3, immunotherapy, Japanese cedar pollinosis, regulatory T cells, Th2, suppressive

Allergic diseases, including rhinitis, are characterized by the presence of specific IgEs to allergens such as pollens, house-dust mites, and animal proteins. T helper 2 (Th2) cells and an associated cytokine secretion profile, including interleukin (IL)-4, IL-5, and IL-13, are thought to play a pivotal role in the initiation and perpetuation of allergic diseases. For example, switching B cells to IgE production and the accumulation of eosinophils are under the control of Th2 cells *via* IL-4 and IL-5, respectively.

A subset of regulatory T (T-reg) cells has been recognized as an immunosuppressive T-cell subset capable of the induction and maintenance of immunologic tolerance to self and nonself antigens.^{1–3} Currently, two different categories of T-reg cells, naturally occurring CD4⁺CD25⁺ T-reg cells and antigen-induced T-reg cells, have been reported.^{4,5} Evidence has been accumulated indicating that these T-reg cells play a crucial role in the prevention of inappropriate Th2 responses

in allergic diseases such as asthma, rhinitis, and dermatitis.^{6,7} Naturally occurring CD4⁺CD25⁺ T-reg cells are able to inhibit proliferation and cytokine production by effector T cells in an antigen-nonspecific, cytokine-independent, but cell–cell contact-dependent, manner. Ling *et al.* reported that allergen-specific Th2 cells from allergic patients were significantly enhanced by the depletion of CD4⁺CD25⁺ T cells.⁸ Bellinghausen *et al.* also indicated that CD4⁺CD25⁺ T cells from both nonatopic donors and atopic patients inhibited the proliferation and Th2 cytokine production of CD4⁺CD25[–] T cells.⁹ On the other hand, antigen-induced T-reg cells are induced in the presence of IL-10, produce high levels of IL-10, and suppress the proliferation and cytokine production of effector T cells in an IL-10-dependent manner. Akdis *et al.* showed that IL-10–producing allergen-specific CD4⁺ T cells represented the predominant subset with significantly high frequency in comparison with IL-4- and IFN- γ -producing T cells in healthy individuals.¹⁰

Recently, it has been shown that FOXP3, which encodes a forkhead/winged-helix transcription repressor termed Scurfin, can be specifically expressed in CD4⁺CD25⁺ T-reg cells.^{11,12} Actually, a mutation in the gene encoding FOXP3 results in a disease called immune dysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX) syndrome. Patients with IPEX have a variety of allergic symptoms including food allergies, increased serum IgE levels, and severe eczema.¹³ Alternatively, FOXP3-transduced CD4⁺ naïve T cells exerted suppression in a cell–cell contact-dependent manner similar to CD4⁺CD25⁺ T-reg cells.¹⁴ Thus, the FOXP3

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gene is a crucial regulatory gene for the development and function of T-reg cells. To explore the role of the FOXP3 gene in allergen-specific immune responses, in particular Th2 response, we examined the relationship between the alteration of FOXP3 gene expression and *in vitro* immune responses against allergens.

In this study, subjects with Japanese cedar pollinosis were the target population. Japanese cedar pollinosis caused by exposure to *Cryptomeria japonica* pollen is one of the most prevalent allergic diseases in Japan, and two major allergens, Cry j 1 and Cry j 2, have been isolated and specific IgE is detected in patients suffering from pollinosis. To date, a number of human histocompatibility leukocyte antigens (HLA) class II-restricted Cry j 1 and Cry j 2 epitopes defined by CD4⁺ T cell have been identified.¹⁵ Among these epitopes, we used an HLA-DPB1*0501-restricted Cry j 1 p61-75 peptide for the induction of allergen-specific T-cell responses. Our findings provide insights into the further elucidation of the mechanism of suppression by T-reg cells as well as the development of novel therapies for allergic diseases.

MATERIALS AND METHODS

Subjects

Peripheral blood was obtained from 5 nonallergic healthy donors and 14 allergic patients suffering from Japanese cedar pollinosis, a diagnosis made based on their case history, clinical symptoms, and the presence of allergen-specific IgE in the sera (radioallergosorbent test class, >2). The study was approved by the Institutional Review Board at Gunma University Hospital. Written informed consent was obtained from each individual. Subject characteristics are summarized in Table 1. Heparinized venous blood (40 mL) was obtained from all subjects, and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll-Hypaque gradients (Amersham Biosciences, Uppsala, Sweden), washed, and counted after staining with trypan blue dye. HLA-DPB1*0501 genotyping was performed using a commercial DP5 typing panel of PCR primers according to the manufacturer's instructions (Dynal, Oslo, Norway).

Cell Line and Peptide

The H0301 cell line, HLA-DPB1*0501 homozygous Epstein-Barr virus-transformed B lymphocytes, used as the peptide-presenting cells in enzyme-linked immunospot (ELISPOT) assays, was kindly provided by Dr. Nishimura (Kumamoto University, Kumamoto, Japan). It was maintained in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin, 2 mM of L-glutamine (all reagents from Invitrogen, Grand Island, NY). HLA-DPB1*0501-restricted Cry j 1 p61-75 peptide (GATRDRPLWI-IFSGN) was synthesized using standard N-(9-fluorenyl) methoxycarbonyl methodology. The peptide was stored as a lyophilized preparation. The amino acid sequence was confirmed by mass spectrometric analysis.¹⁵

IFN-γ and IL-5 ELISPOT Assays for Cry j 1 Peptide-Specific T-Cell Responses

T-helper responses to Cry j 1 peptide were evaluated by both IFN-γ (Th1 type) and IL-5 (Th2 type) ELISPOT assays.

Table 1 Clinical characteristics of human histocompatibility leukocyte antigen-DPB1*0501⁺ donors in this study

	Age/Sex	Japanese Cedar Pollen-Specific IgE (class) (UA/mL)
Pollen allergic		
AR-1	42 M	1.30 (2)
AR-2	32 M	3.67 (3)
AR-3	34 M	16.5 (3)
AR-4	25 F	6.15 (3)
AR-5	23 F	0.85 (2)
AR-6	33 F	10.7 (3)
AR-7	36 M	3.85 (3)
AR-8	33 M	1.39 (2)
AR-9	33 M	2.09 (2)
AR-10	52 F	0.80 (2)
AR-11	49 F	53.5 (5)
AR-12	32 F	>100 (6)
AR-13	24 F	31.7 (4)
AR-14	48 F	13.1 (3)
Healthy control		
HD-1	28 F	<0.34 (0)
HD-2	29 F	<0.34 (0)
HD-3	33 M	<0.34 (0)
HD-4	27 F	<0.34 (0)
HD-5	55 F	<0.34 (0)

AR = allergic rhinitis; HD = healthy donor; F = females; M = males.

PBMC (4×10^6) were cultured in the presence of Cry j 1 peptide (10 µg/mL) in wells of 24-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) in a final volume of 2 mL of AIM-V medium (Invitrogen, Grand Island, NY) containing 10% (v/v) human AB serum. After 7 days of incubation at 37°C, PBMCs were harvested, washed and tested in ELISPOT assay. ELISPOT assays were performed in 96-well flat-bottom plates with nitrocellulose membrane inserts (Millipore, Bedford, MA) as previously described.¹⁶ Briefly, the plates were coated overnight at 4°C with 10 µg/mL of anti-human IFN-γ monoclonal antibody (monoclonal antibody [mAb]; 1-D1K; Mabtech, Nacka Strand, Sweden), or 5 µg/mL of anti-human IL-5 mAb (TRFK5; BD Pharmingen, San Diego, CA) in PBS. H0301 cells used as antigen-presenting cells were pulsed with a Cry j 1 p61-75 peptide (10 µg/mL) and plated in triplicate wells at 1×10^5 cells/well. The responder cells (1×10^5 cells/well) were added to AIM-V medium at a final volume of 200 µL. PBMCs stimulated with 12.5 ng/mL of phorbol 12-myristate 13-acetate and 1 µg/mL of ionomycin were used as a positive control. The plates were incubated at 37°C for 20 hours for IFN-γ assessment and 40 hours for IL-5 assessment. After incubation, the plates were washed with PBS/0.05% Tween 20, and supplemented with biotinylated anti-IFN-γ mAb (7-B6-1; Mabtech) or biotinyl-

ated anti-IL-5 mAb (JES1-5A10; BD Pharmingen). After 2 hours incubation, plates were washed with PBS/0.05% Tween 20 and developed with the avidin-peroxidase complex (Vectastain Elite kit; Vector, Burlingame, CA) for 1 hour. The 3-amino-9-ethyl-carbozole (Sigma, St. Louis, MO) was added and incubated for 5 minutes for the IFN- γ ELISPOT assay and the TMB (3,3',5,5'-tetramethylbenzidine; Vector Laboratories) substrate for peroxidase was added and incubated for 10 minutes for the IL-5 ELISPOT assay. The mean number of spots in control wells (no peptide) was subtracted from the mean number of spots in experimental wells. A T-cell response to a given Cry j 1 peptide was considered to be positive if at least 10 cells per 1×10^5 responder cells secreted IFN- γ or IL-5, as described by Nagorsen *et al.*¹⁷

Real-Time Quantitative RT-PCR

To assess relative FOXP3 gene expression, real-time quantitative RT-PCR was performed. RNA was extracted from isolated PMBCs and 7-day cultured responder cells using the RNeasy mini kit (Qiagen, Valencia, CA). Quantitative RT-PCR was performed using the FullVelocity SYBR Green QRT-PCR Master Mix in a total volume of 25 μ L and the FullVelocity cycling PCR program on an MX 3000P (Stratagene, La Jolla, CA). The melting curve was recorded at the end of every run to assess product specificity. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as an internal control gene. PCR primers used in this study were as follows: FOXP3 forward primer, 5'-GTTCACACGCATGTTTGCCTTC-3'; reverse primer, 5'-GCACAAAGCACTTGTGCAGACTC-3'; GAPDH forward primer, 5'-GCACCGTCAAGGCTGAGAAC-3'; reverse primer, 5'-ATGGTGGTGAAGACGCCAGT-3'. The relative changes in FOXP3 gene expression were determined by the $2^{-(\Delta\Delta Ct)}$ method, where $\Delta\Delta Ct = (Ct_{FOXP3} - Ct_{GAPDH})_{day 7} - (Ct_{FOXP3} - Ct_{GAPDH})_{day 0}$.

Neutralization Assays

Isolated PBMCs were stimulated with Cry j 1 p61-75 (10 μ g/mL) in the presence of anti-IL-10 mAb, anti-GITR mAb, or control IgG (R&D Systems, Minneapolis, MN) at a final concentration of 10 μ g/mL (as suggested by the supplier). Cells were cultured under the same conditions as described previously in "IFN- γ and IL-5 ELISPOT assays for Cry j 1 peptide-specific T-cell responses." After 7 days, cells were harvested and tested for cytokine production in ELISPOT assay.

Statistical Analysis

Mann-Whitney's *U* test was used for statistical analysis of data. The values of $p < 0.05$ were considered significant. Analyses were performed using Statcel2 (OMS Publishing, Tokorozawa, Japan).

RESULTS

T-Helper Responses to Cry j 1 p61-75 of PBMCs Obtained from HLA-DPB1*0501 Donors after *In Vitro* Stimulation

A Cry j 1 p61-75 was tested in *in vitro* stimulation (IVS) for the ability to induce Cry j 1-specific T-helper responses in 19 HLA-DPB1*0501 donors (14 patients with Japanese cedar pol-

Table 2 Analysis of T-cell responses to Cry j 1 p61-75 in human histocompatibility leukocyte antigens-DPB1*0501⁺ donors

	IFN- γ	IL-5
Pollen allergic		
AR-1	0	16
AR-2	6	1
AR-3	0	35
AR-4	0	29
AR-5	0	13
AR-6	0	28
AR-7	0	0
AR-8	17	16
AR-9	10	14
AR-10	2	27
AR-11	0	21
AR-12	0	35
AR-13	0	31
AR-14	7	36
Healthy control		
HD-1	6	0
HD-2	16	17
HD-3	8	0
HD-4	26	0
HD-5	0	1

*Data are the mean number of spots/ 10^5 T cells/well in enzyme-linked immunospot assays.

Bold number indicates positive for T-helper responses to Cry j 1 peptide.

AR = allergic rhinitis; HD = healthy donor; IFN- γ = interferon γ ; IL = interleukin.

linosis and 5 nonallergic healthy donors). PBMCs obtained from these donors were cocultured in the presence of this peptide. After 7-day IVS (1xIVS), T cells were evaluated for peptide-specific reactivity in IFN- γ (Th1 type) and IL-5 (Th2 type) ELISPOT assays. As expected, 12 of 14 pollen-allergic donors tested showed T-helper responses to Cry j 1 p61-75 (Table 2). Of these, 10 of 12 responders displayed Th2-polarized reactivity to Cry j 1 peptide, and the remaining 2 donors showed Th0 responses. On the other hand, 2 of 5 nonallergic healthy donors showed Th0 and Th1 response each and no response was detected in three donors. Next, we examined the possibility that Th2 responses to Cry j 1 peptide correlated with the specific IgE level. Twelve patients showing significant IL-5 production were evaluated. As shown in Fig. 1, there was a significant correlation between the number of IL-5 spots and specific IgE level ($r = 0.70$; $p = 0.02$).

Alteration of FOXP3 Gene Expression and T-Helper Responses

First, we compared the relative expression of FOXP3 gene in PBMCs between pollen-allergic patients and nonallergic healthy donors; however, there was no significant difference (data not shown). Next, FOXP3 gene expression by T cells

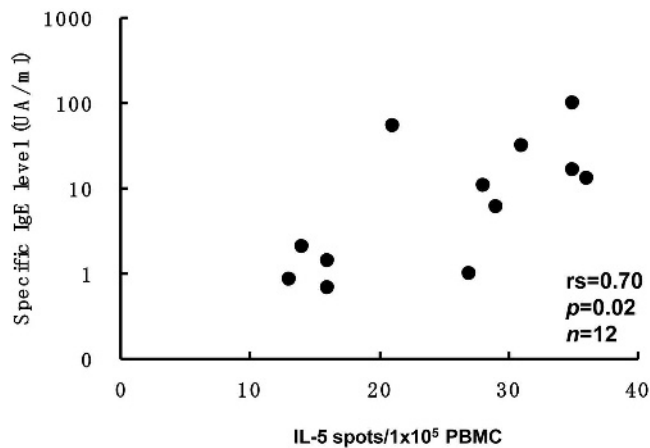


Figure 1. Correlations of T-helper 2 (Th2) responses to Cry j 1 p61–75 with specific IgE level. Peripheral blood mononuclear cells obtained from HLA-DPB1*0501 donors were cocultured in the presence of Cry j 1 p61–75 peptide. After 7-day in vitro stimulation (IVS; 1xIVS), T cells were evaluated for peptide-specific reactivity in IL-5 ELISPOT assays.

before and after IVS was examined, and the relative change in FOXP3 gene expression varied. The relative change in FOXP3 gene expression and the type of T-helper response are summarized in Table 3. Nine of 19 donors showed increased expression of FOXP3, but 10 did not. We next analyzed the relationship between the change of FOXP3 gene expression and several parameters. As shown in Fig. 2, there was no significant difference in the relative change of FOXP3 gene expression between pollen-allergic patients and nonallergic healthy donors. Interestingly, the change in FOXP3 gene expression in donors showing T-helper responses, including Th0, Th1, or Th2, was significantly lower than that in nonresponders ($p < 0.01$). Moreover, there was no significant difference between donors with Th0 and Th2 responses in the changes of FOXP3 gene expression; however, donors with Th0 and Th2 responses showed a smaller change of FOXP3 gene expression compared with those with no response (Th0 versus no response, $p < 0.05$; Th2 versus no response, $p < 0.01$). These results suggested that the FOXP3 gene may affect T-helper responses regardless of the type of responses.

Suppressor Mechanisms of Regulatory Function in T-helper Responses

To investigate the suppression mechanisms in nonresponders, including both pollen-allergic patients and nonallergic healthy donors, a neutralization assay was performed. The addition of anti-IL-10 and anti-glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) to a coculture of PBMCs with Cry j 1 peptide affected T-helper responses, as shown in Fig. 3. The addition of anti-GITR to cocultures of PBMCs with Cry j 1 p61–75 peptide in pollen-allergic patients could induce Th2 responses, whereas that in nonallergic healthy donors it induced Th1 responses. Interestingly, two pollen-allergic patients, AR-2 and AR-7, showed different types of T-helper response by adding anti-IL-10, *viz.*, patient AR-2 and AR-7 revealed enhanced Th2 and Th1 responses, respectively.

Table 3 Summary of T-cell responses to Cry j 1 p61–75 and alteration of mRNA expression of FOXP3 in human histocompatibility leukocyte antigens-DPB1*0501⁺ donors

	T-Helper Type*	Relative Change in FOXP3 Gene Expression
Pollen allergic		
AR-1	Th2	0.34
AR-2	NR	2.43
AR-3	Th2	0.61
AR-4	Th2	1.35
AR-5	Th2	0.80
AR-6	Th2	1.87
AR-7	NR	2.08
AR-8	Th0	0.52
AR-9	Th0	0.93
AR-10	Th2	0.44
AR-11	Th2	1.39
AR-12	Th2	1.41
AR-13	Th2	0.51
AR-14	Th2	0.45
Healthy control		
HD-1	NR	1.92
HD-2	Th0	0.38
HD-3	NR	2.07
HD-4	Th1	0.36
HD-5	NR	2.35

*Th0, Th1, or Th2 assignment for peptide reactivity reflects donor responses of ≥ 10 spots/ 10^5 T cells as determined in interferon γ (IFN- γ) or interleukin (IL)-5 ELISPOT assays, respectively.

AR = allergic rhinitis; HD = healthy donor; NR = no response; Th0 = type 0 response; Th1 = type 1 response; Th2 = type 2 response.

DISCUSSION

In this study, we investigated the role of FOXP3 gene in the suppression mechanism of Th responses, in particular, the Th2 response to Cry j 1–derived peptide. First, we have shown the induction of T-helper responses in Japanese cedar pollinosis using an HLA-DPB1*0501–restricted Cry j 1 p61–75 peptide. To date, a number of Cry j 1–derived epitopes defined by various HLA class II–restricted, CD4⁺ T cells have been identified.¹⁵ In general, it is accepted that PBMCs of allergic patients preferentially induced Th2 rather than Th1 responses under stimulation with certain allergens; however, proteins or longer peptides containing multiple epitopes may induce various T-helper responses, and the extent of T-helper responses to each epitope may also vary. To simplify the evaluation of T-helper responses, we selected HLA-DPB1*0501–restricted Cry j 1 p61–75 as an allergen for this experiments. Hori *et al.* found that the frequency of HLA-DP5 was significantly increased in patients with Japanese cedar

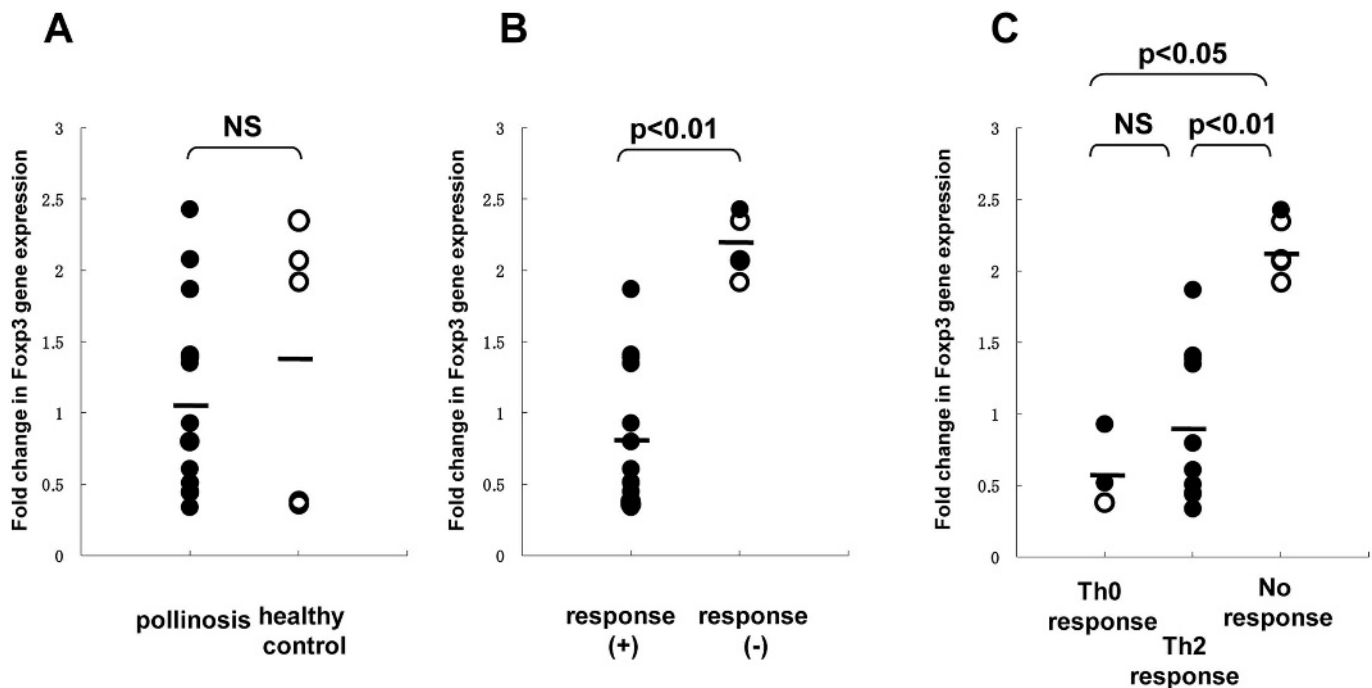


Figure 2. Correlation of alteration of FOXP3 gene expression with T-helper responses. Fold change in FOXP3 gene expression before and after *in vitro* stimulation (IVS) was plotted against (A) pollinosis versus healthy control, (B) positive and negative responsiveness to Cry j 1 p61–75, (C) T-helper 0 (Th0; IFN- γ /IL-5)-type, Th2 (IL-5)-type, and no responses to Cry j 1 p61–75 (closed circles, pollinosis donors; open circles, healthy controls). Th0, Th1, or Th2 assignment for peptide reactivity reflects donor responses of ≥ 10 spots/ 10^5 T cells as determined in IFN- γ or IL-5 ELISPOT assays, respectively. The change of FOXP3 gene expression in donors showing T-helper responses, including Th0 (IFN- γ /IL-5), Th1 (IFN- γ), or Th2 (IL-5) type, was significantly lower than that in nonresponders ($p < 0.01$). Moreover, donors with Th0 and Th2 responses showed lower change of FOXP3 gene expression compared to those with no response, respectively (Th0 versus no response, $p < 0.05$; Th2 versus no response, $p < 0.01$).

pollinosis.¹⁸ Moreover, Sone *et al.* reported that a combination of Cry j 1 p61–75 and DPB1*0501 tended to induce Th2-like cells in T-cell clones.¹⁹ As expected, PBMCs from most allergic patients examined produced IL-5 under stimulation with Cry j 1 p61–75. Meanwhile, those from nonallergic healthy donors did not show the Th2 response. Interestingly, there was a significant correlation between IL-5 production and the specific IgE level. Kimura *et al.* have reported that the production of Th2 cytokines, including IL-4 and IL-13, by PBMCs on stimulation with house-dust mites showed a close positive correlation with house-dust mite IgE radioallergosorbent test.²⁰ Macaubas *et al.* have also shown that the production of IgE antibodies against major house-dust mite allergen Der p 1 was significantly associated with IL-4 and IL-9 mRNA responses and with IL-5 and IL-13 protein responses against crude house-dust mites.²¹ Thus, our findings confirmed that allergen-specific Th2 responses were likely to be the most important in the up-regulation of *in vivo* IgE synthesis in allergic patients.

In the past 10 years, immunologic research regarding T-reg cells has progressed rapidly and has become extremely popular.^{1–5} FOXP3 is considered to be the most specific and reliable marker of T-reg cells.^{11,12} In our study, when stimulated with Cry j 1 p61–75, the FOXP3 gene was up-regulated in donors with undetectable T-helper response. Although FOXP3 is preferentially and stably expressed in naturally occurring T-reg cells, it is controversial whether FOXP3 is

expressed by induced CD4⁺CD25⁺ T-reg cells. Walker *et al.* reported that FOXP3-expressing CD4⁺CD25⁺ T-reg cells were generated by plate-bound anti-CD3/soluble anti-CD28 stimulation,²² whereas Yagi *et al.* have shown that T cell receptor stimulation of CD4⁺CD25[–] naïve T cells failed to elicit FOXP3 expression at the gene or protein level.¹⁴ In addition, it remains unknown whether Cry j 1-specific T-reg cells exist in the periphery in nonallergic healthy donors and/or patients with Japanese cedar pollinosis; therefore, it is unclear which type of T-reg cells are more related to the suppression of Cry j 1-specific T-helper responses; however, our results suggest that the participation of the FOXP3 gene could be required to suppress allergen-specific T-helper responses. Moreover, our results showed no difference between nonallergic healthy donors and patients with Japanese cedar pollinosis in the relative expression of FOXP3 gene in PBMCs, suggesting that FOXP3 function in patients may not be impaired compared with nonallergic healthy donors.

Neutralization assays revealed that two suppressive mechanisms may affect the suppression of Cry j 1-specific T-helper response. GITR is also constitutively expressed by naturally occurring CD4⁺CD25⁺ T-reg cells and cross-linking of GITR by a specific antibody has been shown to abrogate CD4⁺CD25⁺ T-cell-mediated suppression *in vitro*.²³ In our study, two nonresponsive patients to Cry j 1 p61–75 showed increased Th2 response by adding anti-GITR, whereas three nonallergic healthy donors showed increased Th1 response

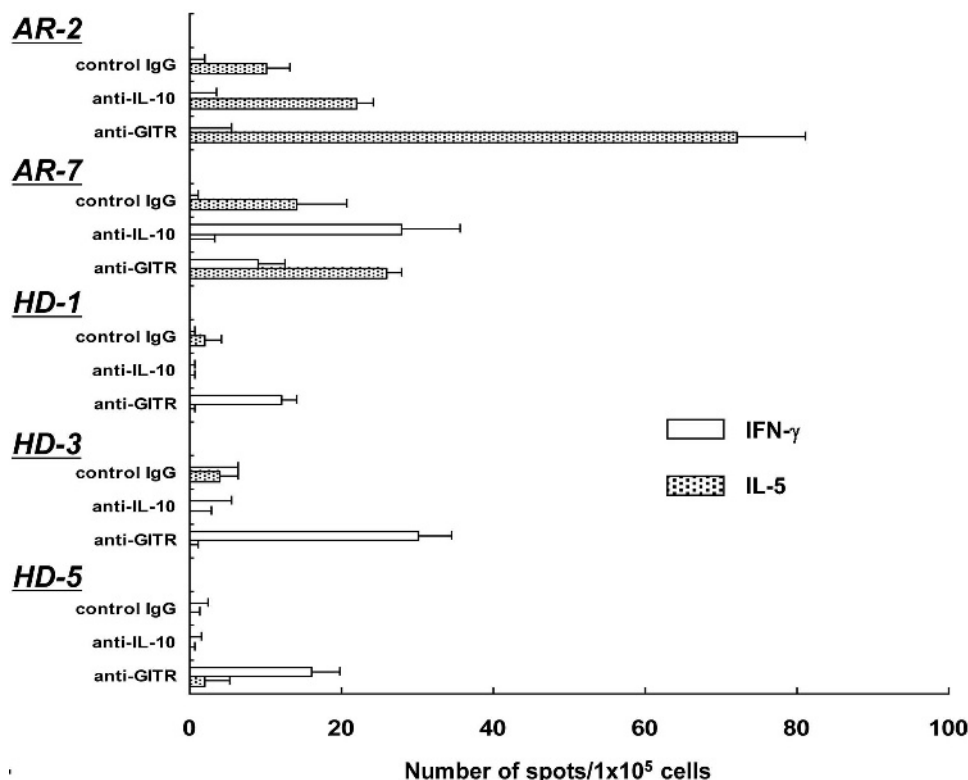


Figure 3. Suppressor mechanisms in donors with no response to Cry j 1 p61–75. Peripheral blood mononuclear cells (PBMCs) were cocultured with Cry j 1 p61–75 peptide in the presence of anti-IL-10 and anti-GITR. T cells were evaluated for peptide-specific reactivity in IFN- γ and IL-5 ELISPOT assays on day 7. The addition of anti-IL-10 and anti-GITR enhanced T-helper responses, including Th1 and/or Th2 responses. Data are expressed as the mean number of spots $\pm$ SD of triplicate cultures.

rather than Th2 response, suggesting that naturally occurring CD4⁺CD25⁺ T-reg cells were able to inhibit both Cry j 1-specific Th1 and Th2 responses. Bellinghausen *et al.* have also shown that CD4⁺CD25⁺ T-reg cells in atopic patients with allergic rhinitis are able to inhibit Th1 as well as Th2 cytokine production.⁹ Moreover, Cosmi *et al.* have reported that Th2 cells were less sensitive than Th1 cells to regulation by CD4⁺CD25⁺ T-reg cells.²⁴ On the other hand, neutralization of IL-10 was also able to induce Cry j 1-specific T-helper responses in patient AR-7 and AR-2. In the experiment shown here, IL-10 ELISPOT assays were also performed under stimulation with Cry j 1 p61–75; however, significant IL-10 production was not detected in our system (data not shown). Minang *et al.* have reported that allergen-induced IL-10 down-regulates Th1- but not Th2-type responses.²⁵ In contrast, Akdis *et al.* have revealed that IL-10-producing allergen-specific CD4⁺ T cells suppressed allergen-specific Th2 cells.¹⁰ Currently, not only the nature of different T-reg cell populations, but also the relationship between naturally occurring CD4⁺CD25⁺ T-reg cells and antigen-induced T-reg cells and the different susceptibility of Th1 and Th2 cells to T-reg cells remain controversial. In addition, other factors such as HLA class II allele, the allergic status of the donor, and type of antigens and their concentration might also influence suppressive mechanisms in individuals.^{26,27} Thus, suppression of allergen-specific T-helper responses appears to be regulated by complex interaction between various factors. Additional

investigation is required to elucidate the exact suppressive mechanism.

Taken together, our data indicate that FOXP3 is functional in nonallergic healthy donors as well as allergic patients, and FOXP3-expressing T cells may be, in part, responsible for the down-regulation of allergen-specific T-helper responses in individuals; therefore, the induction of FOXP3-expressing T-reg cells appears crucial for the maintenance of immunosuppression or the effectiveness of allergen immunotherapy. A better understanding of the nature and specificity of FOXP3-expressing T cells in the suppressive mechanism is necessary to design better and develop more effective immunotherapy in the future.

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