Immune Regulation by CD4+CD25+ Regulatory T Cells in Patients with Japanese Cedar Pollinosis

Takahiro Yamanishi Kazuaki Chikamatsu Goro Takahashi Shuichiro Endo
Keisuke Masuyama

Department of Otolaryngology-Head and Neck Surgery, University of Yamanashi, Faculty of Medicine, Chuo, Japan

Key Words

Allergic rhinitis · Regulatory T cells · Interleukin-10 · Japanese cedar pollinosis · Cry j 1

Abstract

Background: Evidence indicating that CD4+CD25+ regulatory T (Treg) cells play a crucial role in the maintenance of peripheral T cell tolerance to allergens has been accumulated. To explore the functional role of Treg cells in patients with Japanese cedar pollinosis, we performed an in vitro investigation of the regulation of immune responses to allergens by Treg cells. **Methods:** CD4+ and CD4+CD25– T cells obtained from 12 patients with Japanese cedar pollinosis were stimulated with Cry j 1 protein and Cry j 1-derived peptide. On day 6, T cells were tested for allergen-specific reactivity using a CFSE-based proliferation assay and cytokine ELISA assays. The frequency of Cry j 1-specific interleukin (IL)-10-producing Treg cells was assessed by ELISPOT assays. **Results:** The proportion of proliferated cells induced by allergen stimulation was similar in both CD4+ and CD4+CD25– cell cultures. The production of interferon (IFN)- γ , but not that of IL-5 was significantly enhanced in CD4+CD25– cell cultures compared to that in CD4+ cell cultures. Interestingly, the production of IL-10 was decreased in CD4+CD25– cell cultures. Moreover, Cry j 1-specific IL-10-producing Treg cells were detected in pollen-allergic patients. **Conclusion:** Our

findings suggest that in pollen-allergic patients, Treg cells predominantly suppresses Th1 responses rather than Th2 responses, where allergen-specific IL-10-producing Treg cells may also be responsible for the downregulation of allergen-specific immune responses.

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Introduction

Allergic diseases are characterized by allergen-specific IgE production and the activation of effector cells, including eosinophils, mast cells and basophils. These events are regulated by a distinct subset of T lymphocytes, T helper 2 (Th2) cells, which preferentially produce interleukin (IL)-4, IL-5 and IL-13; therefore, allergic diseases have been defined as the inadequate peripheral regulation of allergen-specific T cells in individuals.

The immune system has developed various mechanisms to prevent or minimize such allergen-specific reactivity. Since a subset of regulatory T (Treg) cells has been recognized as an immunosuppressive T cell subset capable of the induction and maintenance of immunological tolerance to self and nonself antigens [1–3], two major Treg populations, naturally occurring CD4+CD25+ Treg cells and antigen-induced interleukin (IL)-10-secreting Treg cells, have been described so far [4, 5].

Table 1. Clinical characteristics of allergic patients tested in this study

	Age years	Sex	Japanese cedar pollen-specific IgE, UA/ml	RAST score	HLA- DPB1*0501
AR-1	33	F	6.79	3	+
AR-2	38	M	2.68	2	+
AR-3	38	M	0.78	2	+
AR-4	34	M	16.50	3	+
AR-5	25	F	6.15	3	+
AR-6	32	M	3.67	3	+
AR-7	27	M	7.83	3	–
AR-8	26	F	23.70	4	–
AR-9	26	M	2.58	2	–
AR-10	34	F	25.30	4	+
AR-11	36	M	3.85	3	+
AR-12	33	F	10.70	3	+

Naturally occurring CD4+CD25+ Treg 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. Moreover, CD4+CD25+ Treg cells specifically express a transcription factor, Foxp3, which is required for their maintenance of suppressive activity. On the other hand, antigen-induced Treg 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 [6].

To date, the role of CD4+CD25+ Treg cells in allergic diseases has been extensively investigated. Ling et al. [7] reported that allergen-specific Th2 cells from allergic patients were significantly enhanced by the depletion of CD4+CD25+ T cells. Bellinghausen et al. [8] also demonstrated that CD4+CD25+ T cells from both nonatopic donors and atopic patients inhibited the proliferation and Th2 cytokine production of CD4+CD25– T cells. Thus, CD4+CD25+ Treg cells play a crucial role in the prevention of inappropriate Th2 responses in allergic diseases; however, several reports suggest that the suppressive activity of CD4+CD25+ Treg cells appears to be affected by various factors, including the type of allergen, allergen exposure and individual allergic status [9–11]. In addition, recent studies have demonstrated the presence of allergen-specific CD4+CD25+ Treg cells and/or IL-10-producing CD4+CD25+ Treg cells in patients with allergic diseases and/or healthy donors. Maggi et al. [12] have identified circulating Der p 1-specific CD4+CD25+FOXP3+ Treg cells from

nonatopic and atopic individuals. Nagato et al. [13] have also demonstrated that Bet v 1-specific CD4+CD25+FOXP3+ Treg cells are obtained from patients with birch pollen nasal allergy. These findings suggest overlapping allergen-specific subsets within CD4+CD25+ cells and antigen-induced IL-10-secreting Treg cells.

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 detected in sufferers. Currently, it is not known how efficiently naturally occurring Treg cells contribute to immune regulation in Japanese cedar pollinosis. Here we show the functional role of CD4+CD25+ Treg cells in patients with Japanese cedar pollinosis. Moreover, our data demonstrate that Cry j 1-specific IL-10-producing CD4+CD25+ Treg cells exist in the peripheral blood of patients with Japanese cedar pollinosis.

Materials and Methods

Subjects

Peripheral blood was obtained outside the pollen season from 6 nonallergic healthy donors and 12 allergic patients suffering from Japanese cedar pollinosis, a diagnosis made on the basis of their case history, clinical symptoms and the presence of allergen-specific IgE in the sera (RAST class >2). The study was approved by the Institutional Review Board at the University of Yamanashi, University Hospital. Written informed consent was obtained from each individual. Characteristics of 12 allergic patients are summarized in table 1. 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 Culture

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. CD4+ T cells and CD4+CD25– T cells were isolated using a regulatory T cell separation kit according to the manufacturer's protocol (Miltenyi Biotec, Gladbach, Germany). Briefly, cells were washed twice and first CD4+ T cells were negatively selected from the PBMCs, followed by positive selection on anti-CD25 magnetic beads, separating CD4+CD25– and CD4+CD25+ T cells. CD4– cell fraction was irradiated (30 Gy) and used as antigen-presenting cells (APCs). Responder CD4+ and CD4+CD25– cells (1×10^5 per well) were stained with 1.5 μ M CFSE (Molecular Probe/Invitrogen, Grand Island, N.Y., USA), and co-cultured with irradiated APCs (1×10^5 per well) in the presence of Cry j 1 protein (10 μ g/ml; Hayashibara Biochemical Laboratories Inc., Okayama, Japan) or tetanus toxoid (2 μ g/ml; Calbiochem, Darmstadt, Germany) in 96-well round-bottomed plates (Becton Dickinson Labware, Franklin Lakes, N.J., USA) in a final volume of 200 μ l AIM-V medium (Invitrogen)

containing 10% (v/v) human AB serum. For HLA-DPB1*0501 donors, HLA-DPB1*0501-restricted Cry j 1 peptide (position 61–75: GATRDRPLWIIFSGN) was also used as an antigen for cell culture [14, 15]. Following 6 days of incubation at 37°C in a 5% CO₂ atmosphere, responder cells and supernatants were harvested.

Flow Cytometry

The proliferation of CD4+ and CD4+CD25– cells was measured by flow cytometry on the basis of CFSE dilution. Harvested CFSE-labeled responder cells were resuspended, incubated with APC-conjugated anti-CD4 monoclonal antibodies (mAb) (BD Pharmingen) for 30 min at 4°C, and washed twice with phosphate-buffered saline (PBS) containing 0.1% FBS and 0.1% NaN₃. Five microliters of 7-amino actinomycin D was added prior to flow cytometry analysis. Viable cells were gated based on negative 7-amino actinomycin D staining. CFSE data analysis was performed using ModFit LT software provided by Verity Software House (Topsham, Me., USA).

ELISA

Harvest supernatants were stored frozen at –80°C until the cytokine concentrations were determined. IFN- γ , IL-5 and IL-10 ELISA (Endogen, Pierce Biotechnology Inc., Rockford, Ill., USA) were used to quantify cytokines in the supernatants according to the manufacturer's instructions. The detection limit was 2 pg/ml for IFN- γ and IL-5, and 3 pg/ml for IL-10.

CD4+CD25+ Treg Culture and IL-10 ELISPOT Assays

CD4+CD25+ Treg cells (5×10^4) isolated using regulatory T cell separation kit were stimulated with anti-CD3/anti-CD28 expander beads (Miltenyi Biotec) in AIM-V medium containing 5% (v/v) human AB serum and IL-2 (1,000 IU/ml) in 96-well plates (5×10^4 cells/well). On day 7, CD4+CD25+ Treg cells were harvested, washed and tested by ELISPOT, which was performed in 96-well flat-bottomed plates (Millipore, Bedford, Mass., USA). Briefly, the plates were coated overnight at 4°C with 10 μ g/ml anti-human IL-10 mAb (9D7; Mabtech, Nacka Strand, Sweden). Dendritic cells (DCs) were generated by culturing plastic adherent cells in AIM-V medium supplemented with 1,000 u/ml GM-CSF and 10 ng/ml IL-4. On day 7, DCs were harvested and used as APCs. DCs were pulsed with a Cry j 1 protein (10 μ g/ml), irradiated and plated at 2×10^4 cells/well. CD4+CD25+ Treg cells (1×10^5 cells/well) were added to AIM-V medium at a final volume of 200 μ l. The plates were incubated at 37°C for 48 h. After incubation, the plates were washed with PBS/0.05% Tween 20, and supplemented with biotinylated anti-IL-10 mAb (12G8; Mabtech). After 2 h of incubation, plates were washed with PBS/0.05% Tween 20, and developed with streptavidin-alkaline phosphatase (Mabtech) for 1 h. BCIP/NBT substrate (SouthernBiotech, Birmingham, Ala., USA) was added and incubated for 5 min. Spot numbers were automatically determined using a computer-assisted video image analyzer (Zeiss-Kontron, Jena, Germany). The mean number of spots in control wells (no antigen) was subtracted from the mean number of spots in experimental wells. A T cell response to a given Cry j 1 protein was considered to be positive if at least 10 cells per 1×10^5 CD4+CD25+ T cells secreted IL-10. In these cases, Student's t test was performed to determine whether there was a significant difference between the number of IL-10-secreting T cells in Cry j 1-stimulated and unstimulated wells, as described by Nagorsen et al. [16].

Statistical Analysis

The Wilcoxon matched-pairs test and Student's t test were used for statistical analysis of data. $p < 0.05$ were considered significant. Analyses were performed using Stata 9.0 (Stata Corp., College Station, Tex., USA).

Results

Proliferative Responses of CD4+ and CD4+CD25– T Cells to Cry j 1 Protein, Cry j 1-Derived Peptide and Tetanus Toxoid

PBMCs obtained from 12 patients with Japanese cedar pollinosis and 6 nonallergic healthy donors were co-cultured in the presence of Cry j 1 protein. Nine of 12 patients tested were HLA-DPB1*0501 positive; therefore, PBMCs from these patients were also stimulated with HLA-DPB1*0501-restricted Cry j 1-derived peptide. Following 6 days of culture, responder CD4+ and CD4+CD25– T cells were evaluated for proliferative responses by CFSE dilution. The proliferative responses varied among the 12 pollen-allergic donors tested. As expected, some donors showed an increased percentage of proliferated cells by the depletion of CD4+CD25+ Treg cells in response to Cry j 1 protein and/or Cry j 1-derived peptides. A representative dot plot and histogram are shown in figure 1. We next compared the percentage of proliferated cells between CD4+ and CD4+CD25– T cells; however, culture of CD4+CD25– T cells with Cry j 1 did not show a substantially enhanced proliferative response when compared with CD4+ T cells (fig. 2a). With respect to a nonallergic recall protein, tetanus toxoid, proliferative responses were also not increased by the depletion of CD4+CD25+ Treg cells (fig. 2a). On the other hand, no proliferative response to Cry j 1 protein and peptide was found in 6 non-allergic healthy donors tested (data not shown).

Cytokine Production of CD4+ and CD4+CD25– T Cells in Response to Cry j 1 Protein, Cry j 1-Derived Peptide and Tetanus Toxoid

We evaluated cytokine production, including IFN- γ , IL-5 and IL-10, from CD4+ and CD4+CD25– T cells in response to Cry j 1 protein, Cry j 1-derived peptide and tetanus toxoid. Not every patient reacted against Cry j 1 protein and/or Cry j 1-derived peptide; however, if they did respond to a given allergen, CD4+ and CD4+CD25– T cells from pollen-allergic donors preferentially produced IL-5, and cytokine production in response to Cry j 1-derived peptide was less than that to Cry j 1 protein. Notably, depletion of CD4+CD25+ Treg cells resulted in the enhancement of IFN- γ ($p < 0.01$), but not IL-5 ($p = 0.61$) pro-

Fig. 1. Representative results from patient AR-1. CD4+ and CD4+CD25- T cells were isolated, labeled with CFSE, and cultured with irradiated APCs in the presence of Cry j 1 protein or HLA-DPB1*0501-restricted Cry j 1-derived peptide in 96-well round-bottomed plates. On day 6, responder cells were harvested and proliferation was measured by flow cytometry on the basis of CFSE dilution. **a** Background levels of proliferative cells in culture without antigen. **b, c** Percentages of proliferative cells in co-cultures with CD4+ and CD4+CD25- T cells stimulated with Cry j 1 protein and HLA-DPB1*0501-restricted Cry j 1-derived peptide (position 61–75), respectively. The percentages of proliferative cells are given for CD4+CFSE^{low} (left upper quadrant) cells. Corresponding histograms (bottom) show ModFit-generated profiles of proliferative cells of CD4+ T cells.

duction, in response to Cry j 1 protein (fig. 2b, c). Moreover, IL-10 production in response to Cry j 1 protein was significantly decreased by the depletion of CD4+CD25+ Treg cells ($p < 0.01$; fig. 2d). Similarly, Cry j 1-derived peptide-specific IL-10 production also showed a tendency to decrease by the depletion of CD4+CD25+ Treg cells ($p = 0.08$; fig. 2d). On the other hand, no substantial cytokine production, including IFN- γ , IL-5 and IL-10, in response to Cry j 1 protein and Cry j 1-derived peptide was found in nonallergic healthy donors (data not shown). To examine whether the observed difference in IFN- γ production was Cry j 1 specific, cells were stimulated with tetanus toxoid. As shown in figure 2b and c, both IFN- γ and IL-5 were induced by tetanus toxoid, and the amount of IFN- γ , but not IL-5, was increased significantly by depletion of CD4+CD25+ Treg cells. IL-10 production in response to tetanus toxoid was not significantly changed by the depletion of CD4+CD25+ Treg cells (fig. 2d).

Detection of Cry j 1-Specific IL-10-Producing CD4+CD25+ Treg Cells in Patients with Japanese Cedar Pollinosis

Next, we investigated whether Cry j 1-specific CD4+CD25+ Treg cells exist in peripheral blood using PBMCs

from 4 patients (AR-3, AR-7, AR-8 and AR-9). Purified CD4+CD25+ T cells were expanded with anti-CD3/anti-CD28 in the presence of IL-2, and Cry j 1-specific responses were examined using IL-10 ELISPOT assays. Interestingly, Cry j 1-specific IL-10-producing CD4+CD25+ T cells were detected in 2 of 3 patients who showed marked reduction of IL-10 production by depletion of CD4+CD25+ Treg cells in this system (table 2).

Discussion

Currently, Treg cells are a highly heterogeneous population of cells, including the naturally occurring CD4+CD25+ Treg, antigen-induced IL-10-secreting Treg, TGF- β -secreting Treg, some CD8+ T cells, double-negative T cells and $\gamma\delta$ T cells [17]. Moreover, not only the nature of different Treg cell populations, but also the relationship between Treg populations remains unclear. In this study, we focused on the CD4+CD25+ Treg cell subset in Cry j 1-specific CD4+ T cell responses. CD4+ T cells from nonallergic healthy donors did not show any substantial responses to Cry j 1 protein and Cry j 1-derived peptide, while those from pollen-allergic patients showed

Fig. 2. Proliferative responses and cytokine production in co-cultures with CD4+ and CD4+CD25- T cells stimulated with Cry j 1 protein, Cry j 1-derived peptide (p61-75), or tetanus toxoid in pollen allergic donors. Isolated CD4+ and CD4+CD25- T cells were cultured with irradiated APCs in the presence of Cry j 1 protein, Cry j 1-derived peptide or tetanus toxoid. Following 6 days of incubation, responder cells and supernatants were harvested. The percentages of proliferative cells (**a**) were measured by flow cytometry on the basis of CFSE dilution. IFN- γ (**b**), IL-5 (**c**) and IL-10 (**d**) concentrations were measured by ELISA.

Table 2. Frequency of Cry j 1-specific IL-10-producing cells in CD4+CD25+ T cells and alteration of IL-10 production by the depletion of CD4+CD25+ T cells

Pollen-allergic donors	IL-10 spot-forming cells	IL-10 production, pg/ml	
		CD4+	CD4+CD25–
AR-3	17	17.6	11.1
AR-7	18	15.0	0
AR-8	12	9.3	1.5
AR-9	0	0	0

Data are the mean numbers of spots/10⁵ CD4+CD25+ T cells/well in ELISPOT assays. The mean number of spots in control well (no antigen) was subtracted from the mean number of spots in experimental wells.

Isolated CD4+ and CD4+CD25– T cells were cultured with irradiated APCs in the presence of Cry J 1 protein. Following 6 days of incubation, supernatants were harvested and IL-10 production was measured by ELISA. Bold numbers indicate positivity for IL-10-producing CD4+CD25+ T cell responses to Cry j 1 protein.

various patterns of responses. First, we assessed the alteration of proliferative response and cytokine production by depletion of CD4+CD25+ Treg cells. Some patients showed enhanced immune responses to Cry j 1 by depletion of CD4+CD25+ T cells. This finding indicates that CD4+CD25+ Treg cells may be, in part, responsible for the downregulation of Cry j 1-specific CD4+ T cell responses in certain individuals, whereas there was no significant difference between CD4+ and CD4+CD25– cells in proliferative responses and IL-5 (Th2-type) production in all pollen-allergic patients. To date, in pollen-allergic patients, the functional deficiency of CD4+CD25+ Treg cells is still controversial. Thunberg et al. [18] have demonstrated that CD4+CD25+ Treg cells from allergic patients failed to suppress birch pollen-stimulated proliferation. Similarly, Skrinido et al. [19] have demonstrated that no increase in proliferation or Th2 cytokine production was observed in cell cultures depleted of CD4+CD25+ CD127^{lo} cells compared with undepleted PBMCs in allergic patients. In contrast, Bellinghausen et al. [8] have shown that CD4+CD25+ T cells from the majority of allergic donors inhibited the proliferation and Th1 (IFN- γ) and Th2 (IL-4 and IL-5) cytokine production of CD4+CD25– T cells. Interestingly, several reports have suggested that allergen exposure affected the regulatory function of CD4+CD25+ Treg cells in pollen-allergic patients, namely, CD4+CD25+ Treg cells from allergic patients were defective in downregulating pollen-induced proliferation and/or IL-5 production during the pollen season [7, 11]. In our experimental system, PBMCs were obtained outside the pollen season. Nevertheless, no increase in proliferation responses and IL-5 production was observed, suggesting that the suppressive function of

CD4+CD25+ Treg cells in patients with Japanese cedar pollinosis may not be affected by the pollen season. On the other hand, IFN- γ (Th1-type) production was significantly upregulated by the depletion of CD4+CD25+ T cells. Similar phenomena are observed in cytokine production upon stimulation with tetanus toxoid; therefore, the lack of augmented Th2 cytokine production in cell cultures depleted of CD4+CD25+ T cells could not be explained by the strongly Th2-skewed immune response to Cry j 1 in pollen-allergic patients. It has been shown that Th2 cells have lower susceptibility than Th1 cells to the suppressive activity of CD4+CD25+ Treg cells [20]. Indeed, Thunberg et al. [18] have shown that the production of Th1, but not Th2 cytokines, was suppressed by CD4+CD25+ cells from both allergic patients and nonallergic controls upon stimulation with birch pollen extract. Likewise, Grindebacke et al. [11] have demonstrated that in allergic patients, the capacity of CD4+CD25+ Treg cells to suppress IFN- γ , but not IL-5 production is retained. Additionally, more recently, several studies have revealed that Treg cells can be divided into several subsets based on their differential expression of transcription factors [21, 22]. The transcription factor IFN-regulatory factor 4 was shown to be required for Treg cells with the ability to suppress Th2 responses, whereas the induction of Th1-specifying transcription factor T-bet expression by Treg cells resulted in Treg cells with suppressive properties to Th1 responses. Although it remains unclear how each Treg subset contributes to suppress Cry j 1-specific CD4+ T-cell responses, not only the different susceptibility of Th cells to Treg cells, but also the phenotypic and/or functional diversity of Treg cells, may affect the suppression of Cry j 1-specific immune responses in pollen-allergic patients.

Another important finding of this study is that Cry j 1-specific IL-10-producing CD4+CD25+ Treg cells existed in pollen-allergic donors. Depletion of CD4+CD25+ Treg cells resulted in the reduction of IL-10 production upon stimulation with Cry j 1; therefore, we investigated the presence of Cry j 1-specific IL-10-producing CD4+CD25+ Treg cells using IL-10 ELISPOT assays. In general, the suppressive effect of CD4+CD25+ Treg cells is known to be dose dependent, cell-contact dependent, cytokine independent and antigen nonspecific; however, several reports have demonstrated that allergen-specific IL-10-producing CD4+CD25+ T cells exist in peripheral blood in allergic patients [12, 13]. Furthermore, such T cells have also been shown to be induced and increased in patients undergoing immunotherapy [23, 24]. Data from our study and others suggest that IL-10-producing CD4+CD25+ T cells exist and play an important role in the regulation of CD4+ T cell responses to allergens in certain allergic patients. The possibility to induce and increase such T cells in vitro and/or in vivo may be of great interest for the development of a novel immunotherapy.

Taken together, at least in a subpopulation of pollen-allergic patients, CD4+CD25+ Treg cells are sufficiently able to suppress immune responses to Cry j 1; however, their inhibitory activities appear to be regulated by a variety of factors, including allergen exposure, type of Th response and allergic status. Moreover, Cry j 1-specific IL-10-producing CD4+CD25+ Treg cells exist in the peripheral blood of pollen-allergic donors. Development of allergic diseases can result from an inappropriate balance between Treg cells and Th2 cells upon allergen stimulation [25]; therefore, a better understanding of IL-10-producing CD4+CD25+ Treg cells in the suppressive mechanism is necessary to develop more effective immunotherapy in the future.

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