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REVIEW ARTICLE

Biomarkers for Allergen Immunotherapy in Cedar Pollinosis

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ABSTRACT
To initiate, monitor, and complete effective immunotherapy, biomarkers to predict and visualize the immune responses are needed. First, we need to identify the right candidate for immunotherapy. Secondly, the immune responses induced by immunotherapy should be monitored. For the first objective, analysis of polymorphisms of candidate genes may be helpful, but still be in development. Regarding biomarkers for immune responsese, there are numerous reports that evaluate immunotherapy-induced immune changes such as suppression of effector cells, deviation to Th1 cytokine production, and induction of regulatory T cells. No standardized methods, however, have been established. Among them, a functional assay of blocking IgG activity, the IgE-facilitated allergen binding assay, may be useful. We quantitated induced expression of an activation marker, CD203c, on basophils and found that the assay efficiently predicts sensitivity to particular allergen and severity of the allergen-induced symptoms. In patients who received rush immunotherapy for Japanese cedar pollinosis, reduction in CD203c expression after the therapy was observed, suggesting the utility of the test for monitoring immunotherapy.

KEY WORDS
basophils, CD203c, cedar pollinosis, IgG4, immunotherapy

INTRODUCTION

The incidence of Japanese cedar pollinosis (JCP) is increasing at an astonishing pace, which was first recognized in early 1960s and now affects around one fourth of the population in Japan.[1-3] Effective pharmacotherapy including non-sedating antihistamines, leukotriene receptor antagonists, and topical corticosteroids, has evolved and quality of life of the patients has been improving.[4,5] Yet, the remedies merely control symptoms and do not change natural history of the disease. Further, social burden of the disease is still significant.[6] On the other hand, allergen immunotherapy generally not only alleviate allergic symptoms but has potential to modify the disease since clinical benefits are reported to be maintained at least for 3 years, even for 12 years after discontinuation.[7,8] In children, immunotherapy prevents new sensitizations[9,10] and reduces progression of rhinitis to asthma for up to 10 years.[11] Long-term efficacy of immunotherapy in Japanese cedar pollinosis has also been reported.[12]

Although immunotherapy confers a multitude of benefits, there still exist issues to be addressed; the present form of immunotherapy is still bound to IgE-mediated side effects, some patients may not benefit from the treatment, long periods for treatment are required and the timing of stopping therapy is not well defined. Along with various efforts to improve the therapy, effective biomarkers have to be developed to tailor the existing therapy and to evaluate new forms of the therapy. The markers should identify right patients with favorable therapeutic responses without adverse events, monitor the efficacy based on immunological responses to particular allergen, and identify the right timing of discontinuation. Although "ideal" biomarkers are yet to be established, prospects for the biomarkers in allergen immunotherapy will be discussed in this article. We also describe quantification of allergen-induced CD203c expression

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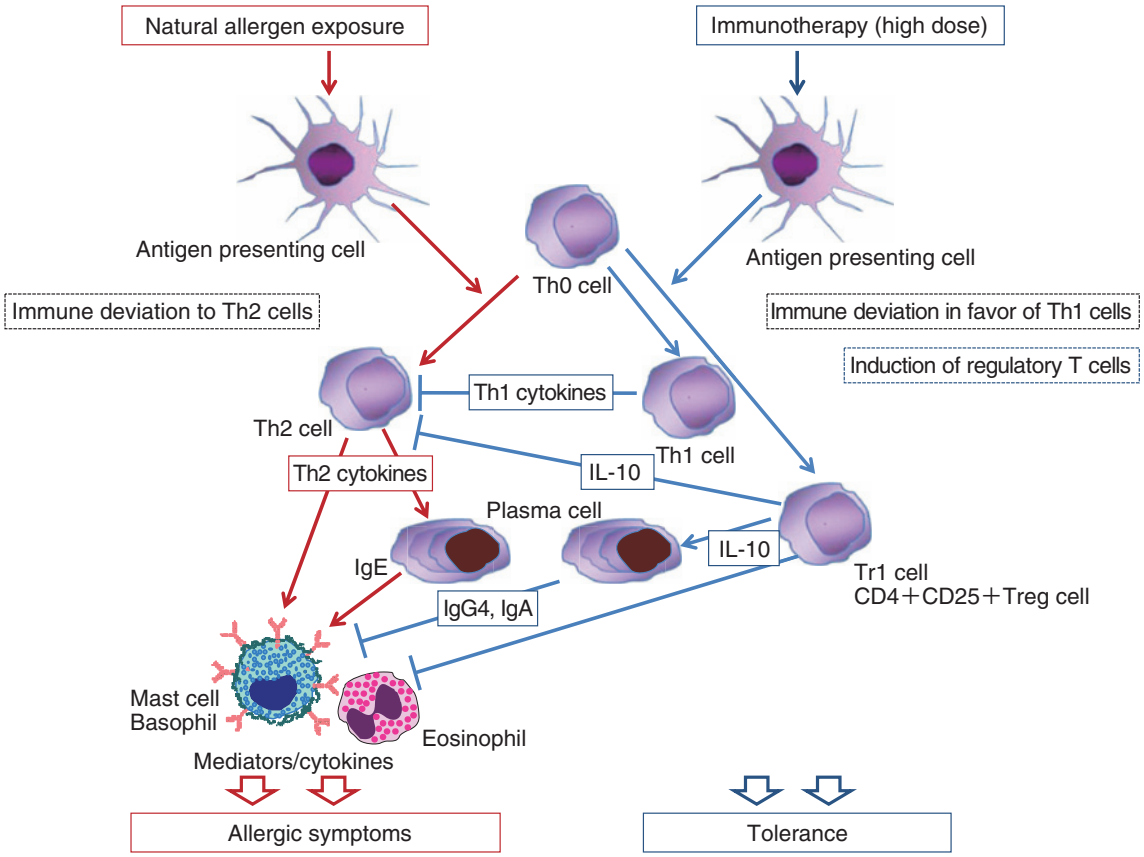

Fig. 1 Mechanisms of allergen immunotherapy.

on basophils as a possible biomarker for Japanese cedar pollinosis. Basophils are important effector cells in the pathogenesis of allergic diseases[13] because they infiltrate in the nasal mucosa of patients with allergic rhinitis[14] and produce a number of mediators and cytokines involved in immediate and late allergic responses.[15] In addition, the fact being circulating cells easily enables us to test the cells *ex vivo* by utilizing a flowcytometry. Here, we show that the basophil activation test utilizing CD203c expression may measure "blocking" activity induced by immunotherapy.

IMMUNOLOGICAL MECHANISMS IN ALLERGEN IMMUNOTHERAPY

THE ALLERGIC RESPONSE

Before discussing biomarkers in allergen immunotherapy, the putative immunological mechanisms are summarized (Fig. 1). The exposure of cedar allergen in the nose, eyes, or bronchi of genetically susceptible individuals causes Th2-deviated immune responses. Cytokines such as IL-4, IL-5, IL-9, and IL-13 derived from Th2 cells are responsible for specific IgE production, differentiation and activation of effector cells such as mast cells, basophils, and eosinophils, and direct stimulation of responder organs including mucus glands and vascular cells in the affected organ. Upon re-exposure to the allergen in the season, IgE-dependent activation of mast cells and basophils results in release of numerous mediators including histamine, cysteinyl leukotrienes, prostaglandins, and platelet activating factor, leading to sneeze, pruritus, waterly discharge, stuffy nose, and sometimes bronchospasm. In addition, mast cells and basophils, are large producers of Th2 and proinflammatory cytokines including IL-4 and TNF-α to potentiate chronic Th2-deviated inflammation in the tissue.

Allergen immunotherapy has potential to inhibit or reverse each step of the above allergic responses and to confer tolerance to the allergen (Fig. 1). Significantly higher amount of allergen is administered in immunotherapy compared to natural exposure. Because it has been shown that deviation to Th2 as expressed by IgE production depends on the allergen dose used to prime the corresponding experimental systems,[16-18] where low allergen doses favor and high allergen dose suppress IgE production. In fact, clinical efficacy is related to the allergen dose,[19,20] higher doses results in better protection.

MECHANISMS OF IMMUNOTHERAPY IN THE EFFECTOR PHASE

Recently, time course analysis of clinical and immunologic measurements during the first year of grass

pollen immunotherapy[21] has been reported, which could substantiate a number of partial information previously observed. The first change was reduction of late phase responses (LPR) to intradermal challenge testing that was observed as early as after the first 2 weeks during up-dosing stage of the conventional injection immunotherapy. Then, elevation of specific IgG4, inhibition of basophil histamine release, and inhibition of binding of allergen-IgE complex to B cells were observed during 6 to 8 weeks at maintenance allergen doses. Reduction of early skin responses, which usually associates with clinical efficacy, was accompanied with these later immunological changes. The investigators also found that allergen-induced IL-10 production from peripheral blood mononuclear cells was a very early event accompanied with LPR suppression. They concluded that IgG responses may be necessary for clinical protection, inhibition of histamine release and allergen/IgE binding to B cells, but that the preceding IL-10 production could contribute to this process.

MECHANISM OF IMMUNOTHERAPY IN T CELL DIFFERENTIATION

The important upstream events that immunotherapy bring about in immune responses to allergen is T cell differentiation, a critical step in regulating downstream effector mechanisms. Cumulative evidence revealed that Th1 cells and T regulatory cells are the key cells in this context.

First, in patients who received grass pollen immunotherapy, increase in cells expressing IFN-γ mRNA were found in the nasal mucosa during allergen-induced late responses and the number of the cells and symptoms scores were inversely correlated.[22] IL-12 is known to be a major cytokine to induce IFN-γ-producing Th1 cells and significant increases in allergen-induced IL-12 mRNA+ cells in cutaneous biopsy specimens was observed in the immunotherapy-treated patients and IL-12+ cells correlated positively with IFN-γ+ cells, inversely with IL-4+ cells.[23] In terms of Th2 cells, seasonal increases in IL-5 and IL-9-expressing cells in the nasal mucosa were significantly inhibited in immunotherapy patients.[24,25] Collectively, Th1 cells are induced and Th1/Th2 balance is altered in favor of Th1 cells by immunotherapy.

There are several subsets of T regulatory cells[26] and there exists inappropriate balance between allergen activation of regulatory T cells and effector Th2 cells in allergy. It was reported that CD4+CD25+ T cells, so-called naturally occurring regulatory T cells (nTreg), from non-allergic donors suppressed proliferation and IL-5 production by their own allergen-stimulated CD4+CD25– cells while the inhibition by CD4+CD25+ T cells from allergy patients were significantly reduced.[27] For these conditions, immunotherapy induces regulatory T cells in the treated patients, so called inducible regulatory T cells (Tr1 cells) and many studies have constantly identified induced expression of IL-10.[21,28-30] One report demonstrated that local increases in IL-10 mRNA and protein-positive cells were observed in the nasal mucosa from patients after 2 years of grass pollen immunotherapy. The changes were observed in treated patients only during the pollen season, not during off-season, nor in placebo-treated subjects and healthy controls.[30] These results suggest that IL-10 responses are allergen-specific, inducible phenomenon. IL-10 acts on B cells to induce production of IgG4.[31] IL-10-induced "blocking" IgG4 inhibits mast cell histamine release and IgE-facilitated allergen-binding to B cells. IL-10 also directly blocks IgE-mediated mast cell activation.[32] Further, IL-10 blocks T cell activation by inhibiting costimulatory molecule CD28 signaling pathway,[33] leading to reduction in cytokines such as IL-5[34] and reduction in inflammatory cell recruitment such as eosinophils.[24]

Table 1 Development of biomarkers for allergen immunotherapy

- Patient selection
  - ✧ Prediction of therapeutic responses
  - ✧ Prediction of serious adverse reactions
  - ✧ Identification of candidates for secondary prevention
- Maintenance
  - ✧ Monitoring of "protective" immune responses
    - "Blocking" antibodies
    - Regulatory T cells, IL-10 and other inhibitory cytokines
    - Suppression of effector cells: mast cells, basophils, eosinophils
  - ✧ Prediction of serious adverse reactions
- Completion
  - ✧ Identification of "normalized" immune responses to allergen
  - ✧ Prediction of recurrence after discontinuation

BIOMARKERS TO MONITOR ALLERGEN IMMUNOTHERAPY

To initiate, monitor, and complete effective immunotherapy, biomarkers to predict and visualize the immune responses are needed (Table 1). First, we need to identify the right candidate for immunotherapy. Although the present form of immunotherapy is effective, some patients may not respond to well the therapy and some may suffer from serious adverse events. We have to select ones who will benefit most. It has been shown that immunotherapy for children with rhinitis prevented "atopic march" from advancing to asthma.[11] We have to select the right child for the intervention since not all children with rhinitis develop asthma. Recent progress in genetics has led to the identification of several candidate genes that are associated with various phenotypes of allergic diseases.[35] It is hopeful in the future that novel genetic

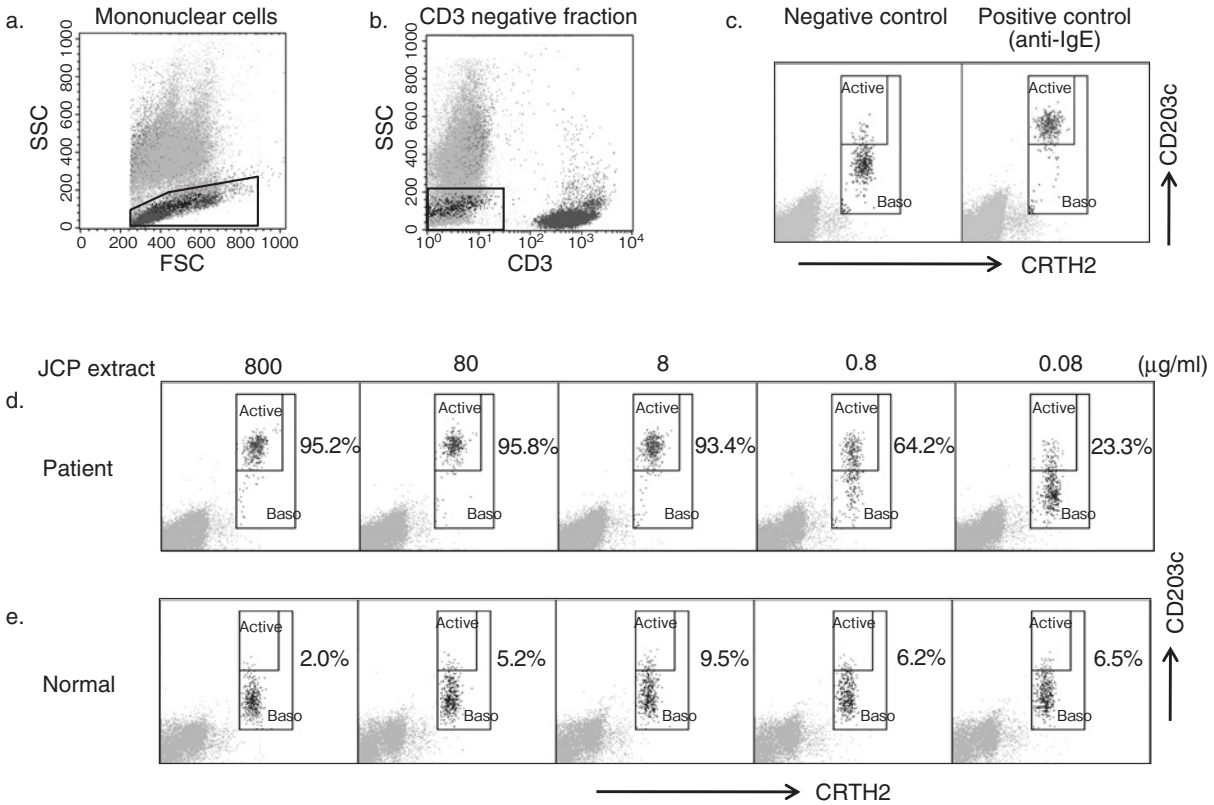

Fig. 2 Flowcytometric analysis of allergen-induced expression of CD203c. EDTA-containing whole blood was incubated with various concentrations of the Japanese cedar pollen (JCP) extract (Torii Pharmaceutical, Tokyo, Japan) for 15 min after addition of sufficient amount of calcium solution to override chelating capacity of EDTA. Anti-IgE antibody as a positive control and PBS as a negative control were also used for stimulation (**c**). PC7-conjugated anti-CD3, FITC-conjugated anti-CRTH2, and PE-conjugated anti-CD203c antibodies were also added during the reaction. The samples were analyzed on a FC500 flow cytometer (Beckman Coulter, CA, USA). Basophils were detected on the basis of forward side scatter characteristics (**a**) and expression of negative CD3 (**b**) and positive CRTH2 (**c**). Up-regulation of CD203c on basophils was determined using a threshold that was defined by the fluorescence of unstimulated cells (negative control) and expressed as $CD203c^{high}$% (**c**). JCP extract induced concentration-dependent enhancement of CD203c expression in a patient with JCP pollinosis (**d**) and no enhancement was observed in a normal control (**e**).

biomarkers identify patients who respond to the therapy without risk of developing side effects.[36]

Secondly, the immune responses induced by immunotherapy need to be evaluated. Based on the knowledge of the mechanisms of immunotherapy, several assays have been reported. Studies of peripheral blood mononuclear cells from patients receiving immunotherapy have identified reductions in proliferative responses to allergen, shifts from Th2 to Th2 cytokine production, and enhanced inhibitory IL-10 production.[25,28,31,37] Some investigators, however, did not reproduce these findings in assays using peripheral blood although changes in the local tissue were demonstrated.[38] Variations in methodology in the peripheral T cell assays may be responsible for the discrepancies and standardization is necessary. Elevation of serum allergen-specific IgG or IgG4 antibodies after immunotherapy have been clearly demonstrated but again correlation between IgG or IgG4 titers and clinical responses to immunotherapy still to be established. Instead, functional assay of blocking IgG activity have been developed. Among them, the IgE-facilitated allergen binding (IgE-FAB) assay is reported to be a validated assay for monitoring allergen immunotherapy.[39] Receptors for IgE, expressed on the surface of antigen presenting cells, B cells in this assay system, facilitate the presentation of allergens in the presence of specific IgE resulting in effective T cell activation at low concentrations of allergen. "Blocking" IgG antibodies interfere with the interaction and the assay simulates the process *in vitro*. Allergen-IgE complexes are incubated with an EBV-transformed B-cell line and complexes bound to CD23 on the surface of cells are detected by flow cytometry. Inhibition of allergen-IgE complex binding to CD23 on B cells by addition of serum from patients who have received allergen-specific immunotherapy is then quantitated. They have demonstrated that the

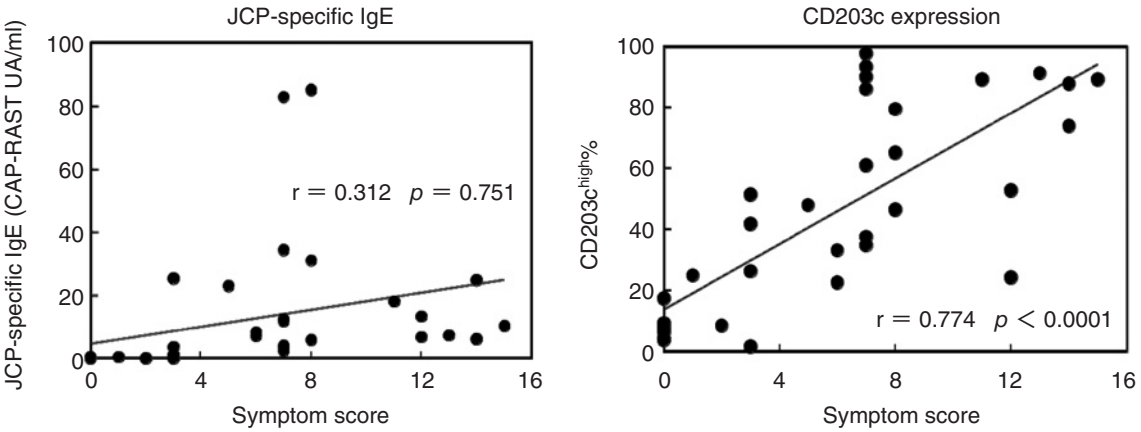

Fig. 3 Correlation of symptom score and JCP-specific IgE levels, CD203c expression by JCP extract. Thirty patients with JCP pollinosis were evaluated. Relationships between symptom score[54] and CAP-RAST titer to JCP, symptom score and JCP allergen-induced CD203c^{high}% were analyzed. Significant correlation was found in the latter.

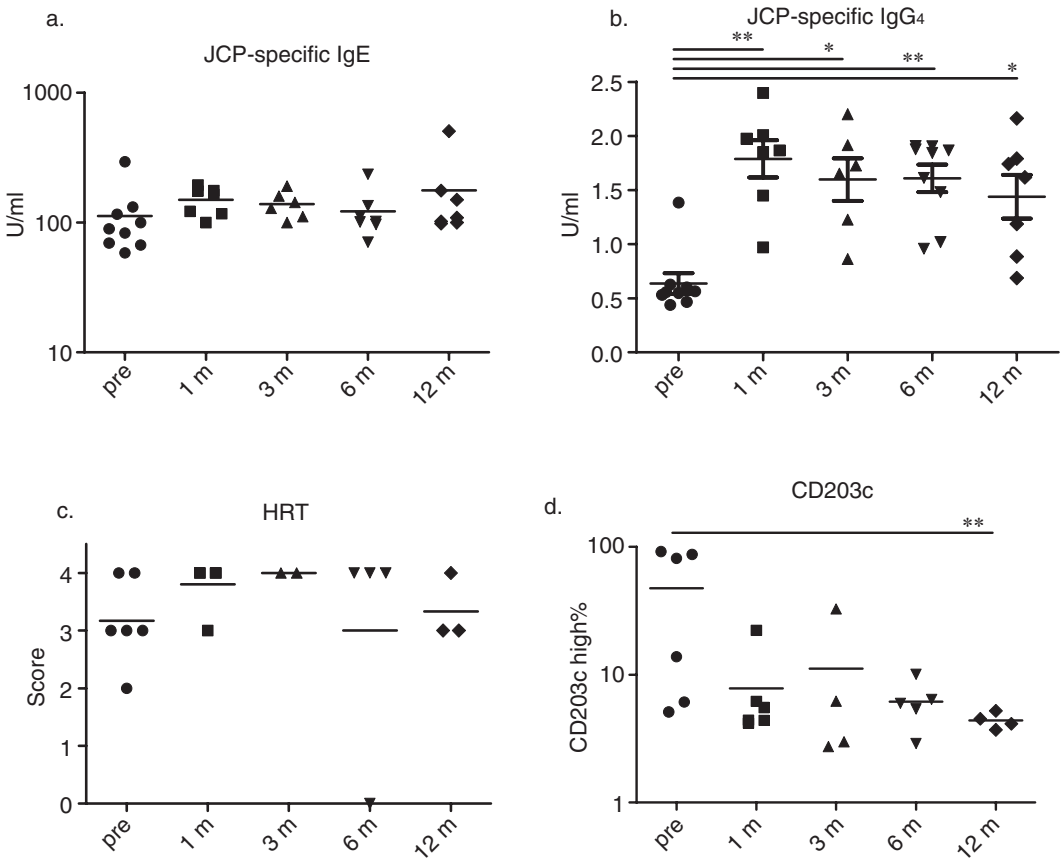

Fig. 4 Changes in JCP-specific IgE levels (**a**), JCP-specific IgG$_4$ levels (**b**), JCP-induced basophil histamine release score in HRT (**c**), and JCP-induced CD203c^{high}% in basophils (**d**) after rush immunotherapy in patients with JCP pollinosis. * $P < 0.05$, ** $P < 0.01$, Dunn's multiple comparison test (adapted from reference 49 with permission). Two subjects in whom basophils did not respond to stimulation with an anti-IgE antibody (non-responders) were excluded from the analysis for HRT and CD203c.

IgE-FAB assay have high specificity and sensitivity to diagnose clinical responses to immunotherapy. Recently, several other studies utilize the method to monitor efficacy of immunotherapy.[21,40]

ALLERGEN-INDUCED EXPRESSION OF CD203c ON BASOPHILS

Basophils play important roles in allergic diseases in effector phase by liberating mediators like histamine as well as in induction phase by producing Th2 cytokines, IL-4 and IL-13.[41] Upon activation through cross-linking of FcεRI by allergen, basophils rapidly express surface molecules such as CD63 and CD203c prior to the mediator and cytokine release. Flowcytometry-based tests for peripheral blood basophils can easily quantify these *in vitro* reactions, which presumably represent their *in vivo* activity. We utilized a commercial kit, Allergenicity Kit (Beckman Coulter, Fullerton, CA, USA), to detect expression of a basophil activation marker, CD203c. CD203c belongs to a family of ecto-nucleotide pyrophoshatase/phosphodiesterases (E-NPPs)[42,43] and has been described as being selectively expressed on basophils, mast cells and their $CD34^+$ progenitors.[44,45] Since CRTH2, a prostaglandinD2 receptor, is selectively expressed on basophils, Th2 cells, and eosinophils,[46,47] the kit identifies basophils as CD3-negative and CRTH2-positive fractions from whole blood samples and measures fluorescent intensity of CD203c that is enhanced by cross-linking of surface-bound IgE molecules (Fig. 2). As CD203c is rapidly up-regulated after allergen challenge in sensitized patients and the levels of up-regulation are well correlated with their symptoms (Fig. 2, 3), it has been proposed as a new tool for allergy diagnosis.[44,48] An important characteristic of the kit is that it employs whole blood during incubation with allergen, which not only detects specific IgE antibodies on basophils but also allows serum and other factors, possibly "inhibitory" factors induced by immunotherapy, in the blood to modify the reaction.

We recently found that induced expression of CD203c by Japanese cedar pollen (JCP) extract decreased after rush immunotherapy (RIT) in patients with JCP pollinosis without decrease in specific IgE levels to JCP.[49] We also found that significant elevation in JCP-specific IgG4 titers after RIT. There was no changes in JCP-induced histamine release from purified basophils[50] after RIT (Fig. 4). In passive sensitization experiments, the patients' sera obtained both before and after RIT showed essentially similar sensitizing capacity for basophils, corroborating the fact that specific IgE did not change. In contrast, basophil degranulation in response to the pollen extract was effectively suppressed by addition of post-RIT serum samples, which correspond with the elevation of specific IgG4 in the serum.[51] These results suggest that the CD203c test can detect blocking activity of IgG antibodies and other factors induced by immunotherapy. We also extend application of the assay to diagnosis of food allergy, especially of tolerance. Although specific IgE levels roughly predict sensitivity to food allergens,[52] markers that represent tolerance levels during outgrow phase of food allergy in childhood are not well-known. We found that the CD203c test effectively predicts sensitivity as well as tolerance to egg, milk (manuscript in preparation), and wheat[53] in children with food allergy.

CONCLUSIONS

Allergen immunotherapy is a promising disease-modifying therapy for allergic diseases including Japanese cedar pollinosis. To successfully initiate, maintain, and complete immunotherapy, predictive biomarkers have to be developed. Some prospects of biomarkers in the mechanisms of immunotherapy were reviewed in this article. Measurement of "blocking" activity of IgG such as IgE-facillitated allergen binding assay may efficiently monitor treatment effect of immunotherapy. Quantification of enhanced expression of CD203c on basophils employing whole blood during reaction with allergen may represent not only sensitization status but also tolerance levels in immunotherapy-treated patients. Larger scale studies are needed to standardize the CD203c assay for general laboratory use.

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