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Phenotype analyses of IL-10-producing Foxp3$^-$ CD4$^+$ T cells increased by subcutaneous immunotherapy in allergic airway inflammation

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ABSTRACT

Introduction: The mechanisms of allergen immunotherapy are not fully elucidated. Here, we sought to develop a murine model to demonstrate the effectiveness of subcutaneous immunotherapy (SCIT) for allergic responses. As excessive antigen dosages may induce immune tolerance in sensitized mice, the effects of SCIT were assessed by varying the antigen dosage. The mechanisms of SCIT were analyzed by focusing on the induction of Foxp3$^+$ Treg cells and IL-10-producing Foxp3$^-$ CD4$^+$ T cells, as well as on the phenotype of the latter cells.

Methods: Ovalbumin (OVA) + $Al(OH)_3$-sensitized mice received subcutaneous dosages of OVA at 0.01, 0.1 or 1 mg/animal for SCIT, followed by intratracheal challenges with OVA at 5, 50 or 500 μg/animal.

Results: The maximum effects of SCIT were observed with 1 mg/animal of OVA for airway inflammation induced by 5 μg/animal of OVA, in which airway eosinophilia and Th2 cytokine production were markedly suppressed. The increase in the OVA-specific IgE level was significantly suppressed by SCIT. The development of bronchial epithelial thickening and mucus accumulation were also suppressed by SCIT. Concomitantly, IL-10-producing Foxp3$^-$ CD4$^+$ T cells were increased in the lungs by SCIT, but Foxp3$^+$ Treg cells were not. Most of the induced IL-10-producing Foxp3$^-$ CD4$^+$ T cells were negative for either IL-5 or LAG-3, but positive for CD49b.

Conclusion: We successfully developed an airway allergic model for SCIT. It was suggested that most of IL-10-producing Foxp3$^-$ CD4$^+$ regulatory T cells increased by SCIT in the lungs were CD49b$^+$ CD4$^+$ regulatory T cells, but neither Th2 cells nor Tr1 cells.

1. Introduction

Allergic diseases, such as allergic asthma, are induced by repetitive exposure to allergens [1]. The production of allergen-specific IgE and expansion of allergen-specific Th2 cells are characteristic features of the pathogenesis of allergic diseases. Th2 cells produce inflammatory cytokines, such as IL-4, IL-5 and IL-13, leading to eosinophilic airway inflammation [2, 3]. Several drugs, including glucocorticoids and adrenergic β_2 receptor agonistic bronchodilators, have been developed to treat asthma; however, these pharmacotherapies are only symptomatic treatments. Allergen-specific immunotherapy (AIT) is the only treatment that is able to reduce the symptoms of allergic diseases [4–6]. The effectiveness of AITs, such as subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT), has been clinically demonstrated for allergic asthma [7].

SCIT and SLIT treatment induced subsets of regulatory T (Treg) cells in clinical studies. Treg cells are generally divided into two groups, the thymus-derived Treg cells and the peripherally-derived Treg cells, the former of which express the transcription factors, Foxp3 and CD25 [8], whereas the latter include Foxp3$^+$ cells and type 1 regulatory T (Tr1) cells. Tr1 cells highly express CD49b and lymphocyte-activation gene 3 (LAG-3), and highly secrete IL-10, but are negative for Foxp3 [9, 10]. SCIT and SLIT increased not only CD4$^+$ CD25$^+$ Foxp3$^+$ T cells but also IL-10-producing Foxp3$^-$ CD4$^+$ T cells in the peripheral blood of allergic patients [11]. However, it is difficult to analyze the detailed mechanisms of Treg cells using peripheral blood mononuclear cells. Moreover, the phenotype of the increased Treg cells at the local inflammatory site was not fully elucidated. Thus, the use of leukocytes at the local airway inflammatory site is required to elucidate the exact phenotypes of Treg cells. On the other hand, antigen-specific IgG_4 was also induced by SCIT and SLIT treatment in clinical studies [12, 13]. Similar to human IgG_4 [14], murine IgG_1 is unable to activate the complement system by the classical pathway but preferentially binds to inhibitory FcγRIIB [15, 16]. Thus, it has been generally accepted that murine IgG_1 is equivalent to human IgG_4 [17, 18].

In order to clarify the mechanisms of AIT, several murine models

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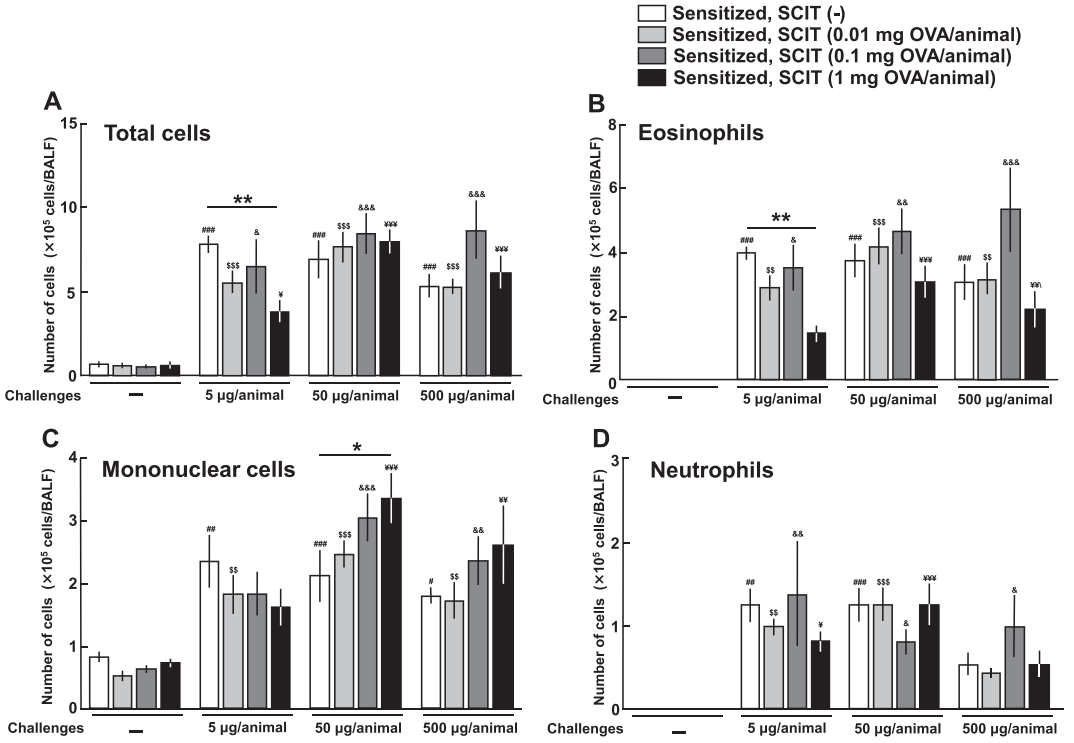

Fig. 1. Effects of subcutaneous immunotherapy (SCIT) on multiple antigen challenge-induced increases in total cells (A), eosinophils (B), mononuclear cells (C) and neutrophils (D) in the BALF in sensitized mice. Mice were i.p. treated with OVA + Al $(OH)_3$ on days 0 and 14. For SCIT treatment, the sensitized mice received subcutaneous injections of OVA solution at 0.01, 0.1 or 1 mg/animal/time/day on days 21, 23 and 25. Mice were challenged with 0.02%, 0.2% or 2% OVA solution at a volume of 25 μl (5, 50 or 500 μg/animal) intratracheally on days 35, 36, 37 and 40 under inhalation anesthesia with isoflurane. BALF was obtained at 4 h after the 4th challenge. Each column represents the mean ± SEM of 4–12 animals. * and **: $P < 0.05$ and 0.01, respectively. #, ## and ###: $P < 0.05$, 0.01 and 0.001 versus non-challenged, non-SCIT-treated mice. $$ and $$$: $P < 0.01$ and 0.001 versus non-challenged, 0.01 mg/animal SCIT-treated mice. &, && and &&&: $P < 0.05$, 0.01 and 0.001 versus non-challenged, 0.1 mg/animal SCIT-treated mice. ¥, ¥¥ and ¥¥¥: $P < 0.05$, 0.01 and 0.001 versus non-challenged, 1 mg/animal SCIT-treated mice.

have been used [18–21]. However, the effectiveness of AIT in mice may vary with the methods for sensitization/challenge and therapeutic treatment with antigens because excessive antigen dosages induce immune tolerance to the relevant antigens [22]. Fox et al. [23] compared 3 models of AIT by intraperitoneal administration of OVA using rapid, intermediate and gradual schedules in OVA + $Al(OH)_3$-sensitized mice, and demonstrated that the effects of AIT on different parameters depended on the duration of AIT treatment. However, intraperitoneal AIT was conducted without antigen challenges [23]; therefore, AIT was assessed without an organ-specific allergic response. A protocol for the development of murine models for SCIT for allergic asthma regarding the clinical effectiveness of SCIT, including effects on IgE production, IgG_1 production, allergic responses and Treg cell induction, has not been reported.

The first goal of the present study was to develop a murine SCIT model demonstrating the effectiveness of SCIT on allergic airway responses, including antigen-specific IgE and IgG_1 production, allergic leukocyte infiltration into the airway tissues, airway remodeling and Treg cell induction. In order to develop the SCIT model, several doses of OVA for intratracheal antigen challenges and SCIT treatment were evaluated in OVA + $Al(OH)_3$-sensitized mice. The second goal of this study was to elucidate the phenotype of a subset of Treg cells induced by the appropriate SCIT model.

2. Materials and methods

2.1. Sensitization/challenge, and subcutaneous immunotherapy (SCIT)

As reported previously [24–30], 5-week-old BALB/c mice (Japan SLC, Hamamatsu) were sensitized by i.p. injections with OVA (Grade V; Sigma Chem., St. Louis, MO, USA) adsorbed to Al $(OH)_3$ at a dose of 50 μg OVA/2 mg Al $(OH)_3$/0.5 ml saline/animal on days 0 and 14. Then, the sensitized mice were challenged with 0.02%, 0.2% or 2% OVA solution at a volume of 25 μl (5, 50 or 500 μg/animal) intratracheally on days 35, 36, 37 and 40 under inhalation anesthesia with isoflurane. For SCIT treatment, the sensitized mice received subcutaneous injections of OVA solution at 0.01, 0.1 or 1 mg/animal/time/day on days 21, 23 and 25.

This animal study was approved by the Experimental Animal Research Committee at Setsunan University.

2.2. Analyses of cells recovered by bronchoalveolar lavage (BAL)

As we previously reported [24–27, 30], mice were sacrificed 4 h after the fourth airway antigen challenge by lethal i.p injection of pentobarbital and xylazine. Peripheral blood was drawn from the abdominal aorta, and bronchoalveolar lavage fluid (BALF) was obtained from the right lung lobe for measurement of cytokines and antibodies in the BALF and serum, respectively. To determine differential cell counts, cells were centrifuged onto a glass slide using a cell settling chamber followed by staining with Diff-Quik solution (Sysmex, Kobe, Japan) after treatment with ACK lysis buffer. The left lung lobe was isolated for histological studies.

2.3. Measurement of OVA-specific IgE and OVA-specific IgG_1 in the serum

Amounts of OVA-specific IgE were measured by ELISA using anti-IgE (clone R35-72, BD Biosciences) as a capture antibody, horseradish peroxidase (HRP)-labeled OVA (Abcam, Cambridge, UK) as a detection reagent and OVA-specific IgE (clone E-C1, Chondrex, Redmond, WA,