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Original Article

Suppression of Immunotherapy on Group 2 Innate Lymphoid Cells in Allergic Rhinitis

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Abstract

Background: Group 2 innate lymphoid cells (ILC2s) are regarded as a novel population of lineage-negative cells that induce innate Type 2 responses by producing the critical Th2-type cytokines interleukin (IL)-5 and IL-13. ILC2s as key players in the development of allergic rhinitis (AR) have been proved, however, the effect of subcutaneous immunotherapy (SCIT) with dermatophagoides pteronyssinus extract (Der p-SCIT) on ILC2s in AR patients is not clear. This study aimed to investigate the response of ILC2s of peripheral blood in house dust mites (HDM)-sensitized Chinese patients with AR who received SCIT with Der P extract.
Methods: Seven healthy controls without symptoms of AR who had negative reactions to any of the allergens from skin-prick testing, nine patients diagnosed with persistent AR according to the Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines, and 24 AR patients who received Der p-SCIT for 1.0–3.5 years were recruited for the study. ILC2s in the peripheral blood were evaluated using flow cytometry. The severity of their symptoms of all participants was rated based on the Total 5 symptom score.
Results: Among 40 participants, 9 AR patients were assigned to the untreated group, 24 AR patients receiving Der p-SCIT were assigned to the immunotherapy group, and 7 healthy controls without symptoms of AR were assigned to healthy control group. The mean Total 5 symptom score of immunotherapy group was significantly lower than that of untreated group (4.3 ± 1.4 vs. 10.1 ± 2.5, $P < 0.001$). Similarly, the levels of ILC2s in the peripheral blood of immunotherapy group were significantly reduced compared with that in untreated group ($P < 0.001$), but were not significantly different from healthy controls ($P = 0.775$). Further subgroup analysis based on the duration of SCIT therapy (1.0–2.0 years [$SCIT_{1-2}$], 2.0–3.0 years [$SCIT_{2-3}$], and 3.0–3.5 years [$SCIT_{3-3.5}$]) showed that the percentage of ILC2s was not significantly different between $SCIT_{1-2}$, $SCIT_{2-3}$, and $SCIT_{3-3.5}$ groups ($SCIT_{1-2}$ vs. $SCIT_{2-3}$: $P = 0.268$; $SCIT_{1-2}$ vs. $SCIT_{3-3.5}$: $P = 0.635$; and $SCIT_{2-3}$ vs. $SCIT_{3-3.5}$: $P = 0.787$).
Conclusions: The present study highlighted the suppression of Der p-SCIT on ILC2s in HDM-AR patients. ILC2s identified in peripheral blood can be used as an effective biomarker for Der p-SCIT.

Key words: Allergic Rhinitis; Group 2 Innate Lymphoid Cell; House Dust Mite; Immunotherapy

Introduction

Allergic rhinitis (AR) is a chronic inflammatory disorder of the nasal mucosa, characterized by an immunoglobulin E (IgE)-mediated Type 2 immune response.[1] A review of the studies investigating the prevalence, incidence of comorbid allergic diseases, and trends and patterns of sensitizing allergens of AR in adults and children in China has indicated that the prevalence of AR with sensitivity to house dust mites (HDM) has increased dramatically in China.[2] Furthermore, evidence suggested that HDM sensitization in patients with AR was associated with an increased risk for the development of allergic asthma, independent of other

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allergens.[3,4] HDM allergen has been shown to be the most prevalent indoor allergen in China,[3] and therefore avoidance to this allergen is often difficult and rarely achievable. Similarly, the benefits of pharmacotherapy are not sustained after discontinuation of the medication. In this respect, subcutaneous immunotherapy (SCIT) is thought to be an effective and safe approach leading to long-term symptom remission for AR patients, as SCIT induces T-cell tolerance by the generation of Type 1 T-regulatory (Tr1) cells and blocks IgE-mediated responses in favor of serum-specific IgG4 antibodies.[5-7] Thus, Tr1 cells and IgG4 antibodies may be recognized as potential biomarkers to monitor responses to SCIT.

Group 2 innate lymphoid cells (ILC2s) in peripheral blood have recently been described in the initiation and maintenance of human allergic diseases, such as AR, atopic dermatitis, and allergic asthma.[8-11] ILC2s not expressing cell lineage markers associated with T-cells, B-cells, dendritic cells, macrophages, and granulocytes have been shown to respond to epithelial cell-derived interleukin (IL)- 25, IL-33, or thymic stromal lymphopoietin by producing high levels of Th2 cytokines (IL-5 and IL-13).[12-14] Similarly, we have reported that the percentage of ILC2s was significantly increased in HDM-AR patients compared with healthy controls, and ILC2s mediated major Type 2 innate immunity in stimulation with IL-33 and/or IL-25 combined with IL-2 *in vitro*. This suggested that ILC2s may have parallel functions to Th2 cells and mast cells in promoting the development of AR. Lao-Araya *et al*.[9] have demonstrated that the proportion of ILC2s was elevated in AR patients sensitized to *Phleum pratense,* and SCIT for 8–36 months can induce a reduction of ILC2s in AR patients.[9] Unlike the effect of grass pollen SCIT-induced suppression of ILC2s noted in the peripheral blood of AR patients, the effect of SCIT using dermatophagoides pteronyssinus extract (Der p-SCIT) on ILC2s in AR patients is not clear. The aim of the present study was, therefore, to determine whether Der p-SCIT can result in the suppression of ILC2s in HDM-sensitized Chinese patients with AR.

Methods

Participants

The study was approved by the Ethics Committee of Beijing Institute of Otolaryngology, and informed consent was obtained from all participants before the commencement of the study. Nine patients diagnosed with persistent AR according to Allergic Rhinitis and its Impact on Asthma guidelines[1] and with an equal or greater than Class 2 serum-specific IgE response against *Dermatophagoides pteronyssinus/Dermatophagoides farina* (EUROBlotMaster 44, Lübeck, Schleswig-Holstein, Germany) and 24 AR patients sensitized to HDM who had received Der p-SCIT (Alutard SQ, ALK-Abelló A/S; Hørsholm, Denmark) for 1.0–3.5 years were enrolled from the AR Clinic at Beijing Tongren Hospital between June and August 2014. Patients received Der p-SCIT were allocated to receive a cluster protocol, followed by a dose maintenance phase.[15] In addition, seven healthy controls without symptoms of AR and with negative skin prick test reactions to any of a panel of common allergens (including *D. pteronyssinus/D. farina*, *Chenopodium album*, animal hair, tree mix, grass mix, cereal mix, dandelion, giant ragweed, *Humulus* species, locust bean, *Blattella germanica*, mugwort, pine, plantain, *Cochliobolus lunatus*, *Candida albicans*, *Penicillium notatum*, *Alternaria tenuis*, and *Aspergillus fumigatus*) were also enrolled in the study.

The severity of symptoms of sneezing, rhinorrhea, nasal obstruction, nasal pruritus, and ocular pruritus were rated on a scale of 0–3 (0 = symptoms not present; 1 = mild symptoms present, but not bothersome; 2 = moderate symptoms bothersome, but easily tolerated; and 3 = severe symptoms difficult to tolerate), which were added and expressed as the Total 5 symptom score. Exclusion criteria were as follows: (1) antihistamines, steroids, or leukotriene receptor antagonist therapy within 4 weeks; (2) allergic reaction to any drug during the past 2 weeks; (3) acute infection within the past 4 weeks; (4) smokers within the past 12 months; or (5) pregnancy.

Analysis of Group 2 innate lymphoid cells by flow cytometry

ILC2s were analyzed according to the method reported by Mjösberg *et al*.[16] Briefly, peripheral blood cells were stained simultaneously using an antihuman fluorescein isothiocyanate-conjugated lineage cocktail, phycoerythrin (PE)-conjugated CRTH2, and PE-CY7-conjugated CD127, or appropriate isotype controls (all from BD Pharmingen, San Diego, CA, USA). The cell lineage cocktail comprised antibodies to CD3, CD16, CD14, CD19, CD34, CD123, CD11c, T-cell receptor (TCR) αβ, and TCRγδ expressed on T-cells, B-cells, monocytes, macrophages, mast cells, dendritic cells, and hematopoietic progenitor cells. Lymphocytes lacking any of these lineage markers, as well as expressing CRTH2 and CD127, were considered to be the ILC2 population [Figure 1]. Cell counts were performed using the FACSAria II flow cytometry device (BD Biosciences, San Diego, CA, USA), and all data were analyzed using the FACSDiva software (BD Biosciences). The proportion of ILC2s was expressed as a percentage of total lymphocytes.

Statistical analysis

All data were shown as mean ± standard deviation (SD) and analyzed using the SPSS software version 19.0 (IBM, Armonk, NY, USA), and graphs were generated using Prism software version 4 (GraphPad, La Jolla, CA, USA). Qualitative data were compared between groups using Chi-square test, Total 5 symptom scores of patients before and after Der p-SCIT, and nonparametric data were analyzed using Mann-Whitney *U*-test. All tests were two-tailed, and a $P < 0.05$ was considered statistically significant.

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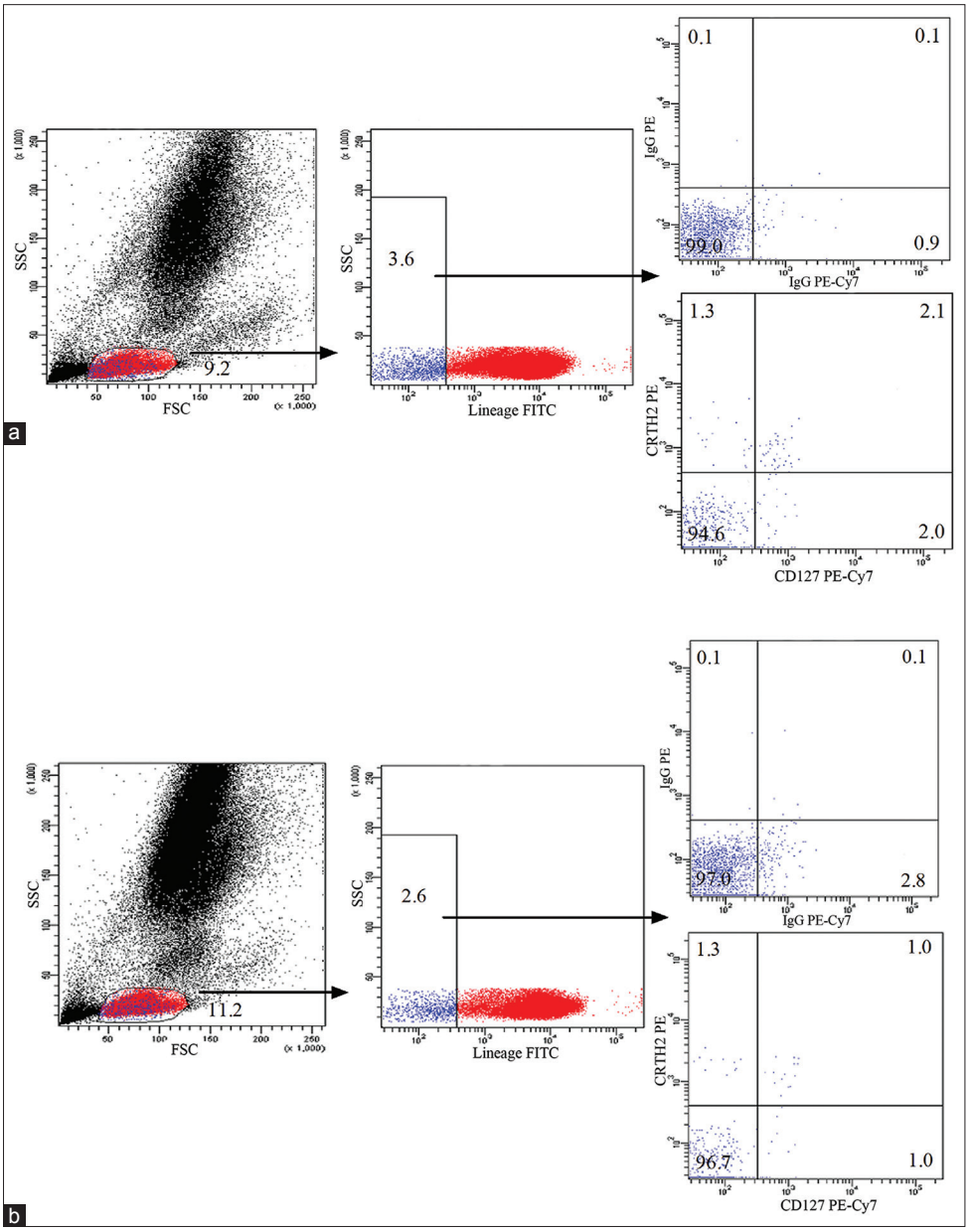

Figure 1: Gating strategy to identify peripheral blood ILC2s. Lymphocytes were detected from peripheral blood mononuclear cells and lineage-negative cells. Lineage-negative cells were further assessed for expression of CD127 and CRTH2 or isotype control staining, and ILC2s were identified as lineage-CRTH2$^+$ CD127$^+$ lymphocytes. The cell lineage cocktail consisted of antibodies to CD3, CD16, CD14, CD19, CD34, CD123, CD11c, TCRαβ, and TCRγδ. Representative flow plots are shown for untreated group (a) and immunotherapy group (b). FSC: Forward scatter; SSC: Side scatter; FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; ILC2s: Group 2 innate lymphoid cells; TCR: T-cell receptor.

Results

Among 40 participants, 9 AR patients were assigned to the untreated group, 24 AR patients receiving Der p-SCIT were assigned to the immunotherapy group, and 7 healthy controls without symptoms of AR were assigned to healthy control group. The mean ages of patients in untreated, immunotherapy, and healthy control groups were 29.0 ± 9.4 years, 28.9 ± 13.8 years, and 30.0 ± 9.3 years, respectively. Similarly, the proportion of males in the untreated, immunotherapy, and healthy control groups was 22.2%, 54.2%, and 28.6%, respectively. The mean period of Der p-SCIT in immunotherapy group was 2.2 ± 0.9 years. The differences with respect to age, gender, or diseases among the three groups were not statistically significant (all $P > 0.05$). The mean Total 5 symptom score of immunotherapy group was significantly lower than that of untreated group (4.3 ± 1.4 vs. 10.1 ± 2.5, $Z = -4.367$, $P < 0.01$).

To determine the effect of immunotherapy on ILC2s, we assessed the levels of ILC2s in the peripheral blood of untreated group, immunotherapy group, and healthy controls using flow cytometry. The level of ILC2s was significantly lower in the peripheral blood of immunotherapy group compared with that in untreated

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group [Figure 2, $Z = -4.320$, $P < 0.001$], but there was no statistically significant difference between immunotherapy group and healthy controls [Figure 2, $Z = -0.286$, $P = 0.775$]. In addition, the level of ILC2s in the untreated group was significantly higher compared with that in healthy controls [Figure 2, $Z = -3.342$, $P = 0.001$). Moreover, further subgroup analysis based on the duration of SCIT therapy (1.0–2.0 years [$SCIT_{1-2}$], 2.0–3.0 years [$SCIT_{2-3}$], and 3.0–3.5 years [$SCIT_{3-3.5}$]) showed that the percentage of ILC2s was not significantly different between $SCIT_{1-2}$, $SCIT_{2-3}$, and $SCIT_{3-3.5}$ groups [$SCIT_{1-2}$ vs. $SCIT_{2-3}$: $Z = -1.108$, $P = 0.268$; $SCIT_{1-2}$ vs. $SCIT_{3-3.5}$: $Z = -0.475$, $P = 0.635$; and $SCIT_{2-3}$ vs. $SCIT_{3-3.5}$: $Z = -0.270$, $P = 0.787$; Figure 2].

Discussion

This study demonstrated that the proportion of ILC2s was significantly decreased in the peripheral blood of immunotherapy group compared with untreated group, but was not significantly different compared with healthy controls. Moreover, the levels of ILC2s appeared to be decreased in the 1st year of Der p-SCIT to the normal level and were not significantly altered for at least the next 2 years. These findings suggested that the therapeutic strategy of SCIT to target ILC2s was likely to be effective in modifying the course of allergic inflammation in individuals with HDM-AR.

Although the precise molecular mechanisms by which ILC2s are downregulated in allergic inflammatory disorders by SCIT are presently not clear, Salimi *et al*.[17] have reported that E-cadherin, an adhesion protein pivotal for maintaining the integrity of airway epithelia, might play a significant role, as E-cadherin ligation on cultured ILC2s isolated from skin of patients with atopic dermatitis significantly downregulated the expression of GATA3 and transcription of IL-5 and IL-13, as well as reduced the proliferation of the skin ILC2s *in vitro*.[10] In the absence of E-cadherin, ILC2 cytokine production was unhindered. Other studies have suggested that inhibition of ILC2s may be influenced by E-cadherin-mediated signaling pathway through inhibitory killer cell lectin-like receptor G1 (KLRG1),[18] which was expressed as high levels in activated ILC2s of allergic individuals.[10] As cleavage or loss of E-cadherin in the nasal resulting from eosinophil infiltration has been suggested to trigger the initial step of subsequent epithelial destruction in allergic states[19,20] and administration of exogenous IL-10 has been shown to attenuate the decline in the expression of several intestinal epithelial junctions, including E-cadherin, zonula occludens-1, and occluding, as well as a loss of intestinal barrier function,[21] it was tempting to speculate that a mechanism involving IL-10 and E-cadherin may be operative in AR patients receiving SCIT, particularly as IL-10 has been shown to be elevated in AR patients who received SCIT.[5,7,22] Thus, in HDM-sensitized AR patients, SCIT induced IL-10, which resulted in the production of E-cadherin and subsequent E-cadherin-KLG1-mediated inhibition of ILC2s. Collectively, these findings suggested that ILC2s were a critical target to treat allergic diseases and that the IL-10-E-cadherin-KLRG1 axis may represent a mechanism for suppression of ILC2s in AR patients who received SCIT. However, further research on the inhibitory signals is needed to elucidate the relative contribution of ILC2s to AR as well as SCIT.

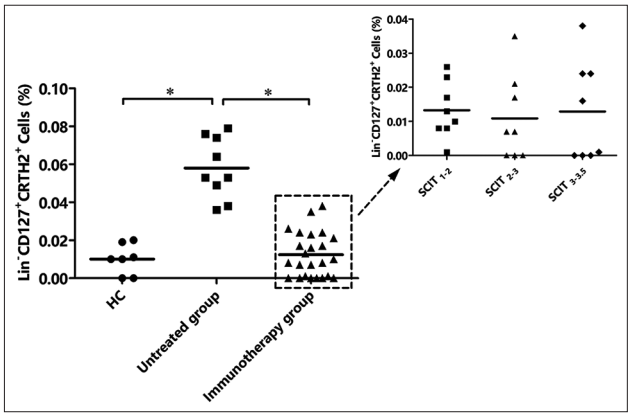

Figure 2: Flow cytometric analysis of ILC2s levels in HCs ($n = 7$), untreated group ($n = 9$), immunotherapy group ($n = 24$), and the three subgroups of immunotherapy group based on duration of Der p-SCIT ($SCIT_{1-2}$: 1.0–2.0 years; $SCIT_{2-3}$: 2.0–3.0 years; $SCIT_{3-3.5}$: 3.0–3.5 years). Each point represents individual patient samples, and the horizontal bar represents the mean level. The number of ILC2s was expressed as a percentage of all lymphocytes. The differences was analyzed by Mann-Whitney *U*-test (*$P < 0.01$). ILC2s: Group 2 innate lymphoid cells; Der p-SCIT: Subcutaneous immunotherapy using dermatophagoides pteronyssinus extract; HC: Healthy control.

The Total 5 symptom scores in patients receiving Der p-SCIT were significantly lower than that in those before SCIT treatment, which indicated that SCIT was an effective treatment for AR patients. A limitation of this study, however, was that baseline data for ILC2s prior to SCIT treatment in immunotherapy group were not available. Despite this limitation, the current study has demonstrated that the levels of ILC2s in AR patients who had received SCIT for 1.0–3.5 years were similar to those in healthy controls, whereas both the Total 5 symptom scores and ILC2s levels in the AR patients who had not received SCIT were significantly higher. The findings of this study reflected the inhibited effect of SCIT therapy on ILC2s in HDM-sensitized AR patients accurately, which was consistent with the study of Lao-Araya *et al*.,[9] but these finding need to be further confirmed in much larger cohort of patients in the future. Furthermore, targeting ILC2s as a potential therapeutic strategy for AR patients in well-controlled trials may provide a valuable insight into the role of these cells in allergic diseases.

In conclusion, this experimental study has suggested that the relatively high level of ILC2s in AR patients sensitized to HDM may be treated by Der p-SCIT, and a reduction of ILC2 levels might contribute to symptom remission and immunologic tolerance in AR. Furthermore, ILC2s identified in peripheral blood might be used as an effective biomarker for therapeutic response to Der p-SCIT in AR patients.

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Conflicts of interest

There are no conflicts of interest.

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