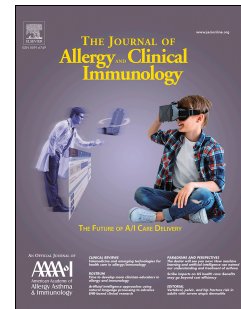


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IL-10–producing innate lymphoid cells increased in house-dust-mite allergic rhinitis patients following immunotherapy

Tadech Boonpiyathad, MD, PhD, Pongsakokorn Tantilipikorn, MD, PhD, Kiat Ruxrungtham, MD, Panitan Pradubpongsa, MD, Wat Mitthamsiri, MD, Aurélie Piedvache, MSc, Pattarawat Thantiworasit, MSc, Sunee Sirivichayakul, PhD, Alain Jacquet, PhD, Narissara Suratannon, MD, Pantipa Chatchatee, MD, Naho Morisaki, MD, PhD, Hirohisa Saito, MD, PhD, Atik Sangasapaviriya, MD, Kenji Matsumoto, MD, PhD, Hideaki Morita, MD, PhD



PII: S0091-6749(20)31554-2

DOI: <https://doi.org/10.1016/j.jaci.2020.10.029>

Reference: YMAI 14819

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 30 March 2020

Revised Date: 18 September 2020

Accepted Date: 16 October 2020

Please cite this article as: Boonpiyathad T, Tantilipikorn P, Ruxrungtham K, Pradubpongsa P, Mitthamsiri W, Piedvache A, Thantiworasit P, Sirivichayakul S, Jacquet A, Suratannon N, Chatchatee P, Morisaki N, Saito H, Sangasapaviriya A, Matsumoto K, Morita H, IL-10–producing innate lymphoid cells increased in house-dust-mite allergic rhinitis patients following immunotherapy, *Journal of Allergy and Clinical Immunology* (2020), doi: <https://doi.org/10.1016/j.jaci.2020.10.029>.

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IL-10–producing innate lymphoid cells increased in house-dust-mite allergic rhinitis patients following immunotherapy

Tadech Boonpiyathad, MD, PhD^a, Pongsakokorn Tantilipikorn, MD, PhD^b, Kiat Ruxrungtham, MD^{c,d}, Panitan Pradubpongsa, MD^a, Wat Mitthamsiri, MD^a, Aurélie Piedvache, MSc^e, Pattarawat Thantiworasit, MSc^c Sunee Sirivichayakul, PhD^{c,d}, Alain Jacquet, PhD^d, Narissara Suratannon, MD^f, Pantipa Chatchatee, MD^f, Naho Morisaki, MD, PhD^e, Hirohisa Saito, MD, PhD^g, Atik Sangasapaviriya, MD^a, Kenji Matsumoto, MD, PhD^{g,*} and Hideaki Morita, MD, PhD^{g,*}

^aDepartment of Medicine, Phramongkutklao Hospital, Bangkok, Thailand

^bDepartment of Otorhinolaryngology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

^cDepartment of medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

^dCenter of Excellence in Vaccine Research and Development, Chulalongkorn University, Bangkok, Thailand

^fPediatric Allergy and Clinical Immunology Research Unit, Division of Allergy and Immunology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, The Thai Red Cross society, Bangkok, Thailand

^eDepartment of Social Medicine and ^gDepartment of Allergy and Clinical Immunology, National Research Institute for Child Health and Development, Tokyo, Japan

* To whom correspondence should be addressed:

Hideaki Morita

2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan

Tel.: +81 (3) 3416 0181

Fax: +81 (3) 5494 7173

E-mail: morita-hi@ncchd.go.jp

and

Kenji Matsumoto

E-mail: matsumoto-k@ncchd.go.jp

Conflicts of interest

H. Saito reports personal fees from Torii Pharmaceutical Co. Ltd, and Shionogi & Co., Ltd., outside of this work. K. Matsumoto reports payment for lectures including service on speakers bureaus from Kyorin Pharmaceutical Co., Ltd and Torii Pharmaceutical Co. Ltd, AstraZeneca K.K. and Alcon Pharma, outside of this work. The rest of the authors have no conflicts of interest to declare in relation to this work.

Capsule summary: IL-10⁺CTLA-4⁺ innate lymphoid cells were induced during allergen-specific immunotherapy (AIT) in responders and may be associated with improvement in the symptoms after AIT.

Key words: Allergen-specific immunotherapy; innate lymphoid cell; IL-10; regulatory ILC; IL13⁺IL4⁺ ILC2; house dust mites; allergic rhinitis; exhausted-like ILC2

To the Editor:

Allergen-specific immunotherapy (AIT) is a well-recognized and effective treatment for allergic rhinitis (AR) and asthma. Its efficacy is based on induction of allergen-specific regulatory immune cells, such as regulatory T cells (Tregs) and regulatory B cells (Bregs).^{1, 2} Those cells suppress effector cell subsets of type-2 immune responses through production of suppressive cytokines (such as IL-10) and inhibitory molecules, leading to decreased inflammation at local sites in the tissues.³ Innate lymphoid cells (ILCs) are recently-identified innate-type immune cells that include three effector subsets resembling T helper subsets, but they have no antigen receptors.⁴ We recently found a new subset of ILCs, IL-10-producing regulatory ILCs (ILCregs), which resemble Tregs and suppress proliferation of Th2 cells and ILC2s through IL-10 production.⁵ ILCregs can be converted from ILC2s by retinoic acid (RA) and express Treg-associated molecules such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and CD25.⁵ However, the roles of IL-10-producing ILCs in AIT remain unclear, and this study aimed to clarify those roles.

This study is a part of the MITAR study conducted in Thailand.^{1,2} Detailed information regarding the subjects and methods is described in Fig E1, Table E1 and in the Methods section in this article's Online Repository at www.jacionline.org. In brief, peripheral blood mononuclear cells (PBMCs) obtained from 20 patients (10 AIT treatment responders, 5 treatment non-responders and 5 placebo-treated patients) at four different time points were cultured in the presence of antigen and cytokines.

We first determined the percentage of each ILC subset in the PBMCs using canonical surface markers as well as those of CTLA-4⁺ ILCs (Fig E2). The percentages of Lin⁻CD127⁺CD161⁺CRTH2⁺ ILC2s decreased in the AIT responders: -10.1% [-21.1%, 0.9%] from the baseline to after 2 years of treatment as shown in an earlier report.⁶ The percentages of Lin⁻CD127⁺CD161⁺CRTH2⁻c-kit⁺ ILC3s were comparable in all three groups. In contrast, the percentages of Lin⁻CD127⁺CD161⁺CTLA-4⁺ ILCs had increased progressively by 2.4% [-0.9%, 5.7%] at 6 months, 3.1% [-0.2%, 6.4%] at 12 months and 5.0% [1.7%, 8.3%] at 24 months in the responders, but not in the non-responders and placebo-treated patients (Fig 1, A and Fig E3, A).

Next, to clarify the modulation of ILC functions during AIT, we determined the percentages of cytokine-producing ILCs by intracellular staining of IL-4 and IL-10. The percentages of IL-4-producing ILC2s were slightly reduced after 2 years of AIT (-6.6% [-15.3%, 2.1%]) in the responders, but not in the non-responders or placebo-treated patients (Fig 1, B, Fig E3, B and Fig E4, A). In line with these findings, we previously reported that expression of CD69, an activation marker of leukocytes, on ILC2s was significantly decreased after 2 years of AIT in responders, but not in non-responders.⁷ Taken together, AIT may suppress activated ILC2s in responders, but not in non-responders. In sharp contrast, the percentages of IL-10⁺CTLA4⁺ ILCs in the responders after 2 years of AIT were increased by 3.2% [0.7%, 5.7%] and were higher than in the non-responders (-0.9% [-4.5%, 2.7%]) and placebo-treated patients (1.2% [-2.4%, 4.8%]) (Fig 1, B, Fig E3, B and Fig E4, B). These findings suggest that IL-10⁺CTLA4⁺ ILCs were induced in the responders, but not in the non-responders or placebo-treated patients after 2 years of AIT. However, the levels of IL-10 in the culture supernatant were below the detection limit (data not shown), probably because of the limited number of cells. When we analyzed the IL-4⁺CRTH2⁺/IL-10⁺CTLA4⁺ ILC ratio, we found that it decreased gradually in the responders, but did not change in the non-responders or placebo-treated patients (Fig 1, C and Fig E3, C). These findings imply that an increase in IL-10⁺CTLA4⁺ ILCs is associated with a decrease in IL-4⁺CRTH2⁺ ILC2s.

We also investigated for correlations between the symptom-medication score (SMS) (Fig E5) and the percentage of cytokine-producing ILCs at 0, 6, 12 and 24 months after AIT, for responders, non-responders and placebo-treated patients. We found that for each unit increase in the SMS, the percentage of IL-10⁺CTLA4⁺ ILCs decreased by -0.30% [-0.51%, -0.10%] (p=0.005) in responders and by -0.48% [-0.87%, -0.10%] (p=0.014) in placebo-treated patients. Conversely, the IL-4⁺CRTH2⁺/IL-10⁺CTLA4⁺ ILC ratio increased by 0.79%

[0.43%, 1.15%] ($p < 0.001$) in responders. The percentage of IL-4⁺CRTH2⁺ILC2s increased by 0.73% [0.01%, 1.45%] for each unit increase in SMS, only among responders (Fig 2, A and C, and Fig E6, A and C). For non-responders, we found no significant relationship for any of the percentages, partly because of the limited range of SMS scores (Fig 2, B and Fig E6, B). These findings suggest that induction of IL-10⁺CTLA4⁺ ILCs may be associated with the improvement in symptoms during AIT in responders.

We previously showed that RA is a critical factor that converts ILC2s into IL-10⁺ ILCregs, and this is the only currently known pathway. Since RA-synthesizing DCs were reported to be present in the dermis and cutaneous lymph nodes, like in the gastrointestinal tract, those DCs might be the source of RA in the skin during subcutaneous AIT.⁸ However, it is still not clear how RA and IL-10⁺ ILCs, innate-type immune cells, are induced in an antigen-specific immune response in AIT. Recent findings in mice suggested that chronic antigen exposure might induce a subtype of ILC2s called “exhausted-like ILC2s” that express IL-10 and inhibitory molecules via yet-unknown mechanisms.⁹ Therefore, IL-10⁺CTLA-4⁺ ILCs found in AR patients might be “exhausted-like ILC2s”, rather than ILCregs. However, the characteristics of IL-10-producing ILCregs and exhausted-like ILC2s are similar, as a result of which we are unable to settle this issue at this time.

In conclusion, we demonstrated for the first time that IL-10⁺CTLA-4⁺ ILCs were induced during AIT in responders and may be associated with the improvement in symptoms after AIT. However, the mechanisms of IL-10⁺CTLA-4⁺ ILCs induction during AIT and the fine characteristics of those cells remain unclear. This study has limitations regarding the sample size, variation of the data and the number of the cells analyzed because of the technical reason. Further studies to elucidate those issues are needed, and the findings may lead to the development of new therapeutic targets for allergic diseases.

Acknowledgments

We thank Mrs. Jitta Tubkate for help in contacting the patients. We thank the members of the Department of Allergy and Clinical Immunology, National Research Institute for Child Health and Development (Ms. Masako Fujiwara, Ms. Michiko Yamada, Ms. Ayako Yamada and Dr. Keisuke Orimo) for their technical assistance. The MITAR study is supported by the Health System Research Institute (HSRI) in Thailand. We acknowledge the MITAR study team for their support of this clinical study. This research was supported by a Grants-in-Aid for Young Scientists (B) (to H.M.), a grant from Nipponham Foundation for the Future of Food (to H.M.) and a grant of National Center for Child Health and

Development (#29-2; to H.M.) and Chulalongkorn Academic Advancement (2nd Century Project-CUAASC, to A.J.).

Tadech Boonpiyathad, MD, PhD^a

Pongsakokorn Tantilipikorn, MD, PhD^b

Kiat Ruxrungtham, MD^{c,d}

Panitan Pradubpongsa, MD^a

Wat Mitthamsiri, MD^a

Aur lie Piedvache, MSc^e

Pattarawat Thantiworasit, MSc^c

Sunee Sirivichayakul, PhD^{c,d}

Alain Jacquet, PhD^d

Narissara Suratannon, MD^f

Pantipa Chatchatee, MD^f

Naho Morisaki, MD, PhD^e

Hirohisa Saito, MD, PhD^g

Atik Sangasapaviriya, MD^a

Kenji Matsumoto, MD, PhD^{g,*}

Hideaki Morita, MD, PhD^{g,*}

^aDepartment of Medicine, Phramongkutklao Hospital, Bangkok, Thailand

^bDepartment of Otorhinolaryngology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

^cDepartment of medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

^dCenter of Excellence in Vaccine Research and Development, Chulalongkorn University, Bangkok, Thailand

^fPediatric Allergy and Clinical Immunology Research Unit, Division of Allergy and Immunology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, The Thai Red Cross society, Bangkok, Thailand

^eDepartment of Social Medicine and ^gDepartment of Allergy and Clinical Immunology, National Research Institute for Child Health and Development, Tokyo, Japan

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Figure legends

FIG 1. Frequencies of each ILC subset during AIT.

A, Differences from baseline in the percentages of Lin⁻CD127⁺CD161⁺CRTH2⁻c-kit⁻ ILC1s, Lin⁻CD127⁺CD161⁺CRTH2⁺ ILC2s, Lin⁻CD127⁺CD161⁺CRTH2⁻c-kit⁺ ILC3s and Lin⁻CD127⁺CD161⁺CTLA4⁺ ILCs per total Lin⁻CD127⁺CD161⁺ ILCs in PBMCs at different time points of AIT (6, 12 and 24 months after starting AIT). **B**, Differences from baseline in the percentages of Lin⁻CD127⁺CD161⁺IL-4⁺CRTH2⁺ ILC2s and Lin⁻CD127⁺CD161⁺IL-10⁺CTLA4⁺ ILCs. **C**, Differences from baseline in the ratio of IL-4⁺CRTH2⁺/IL-10⁺CTLA4⁺ ILCs. Data represent the mean and confidence interval. Bonferroni's method was used to calculate differences between groups. **P*<.05, ***P*<.01, and ****P*<.001.

FIG 2. Correlations between the percentages of ILCs and symptom-medication score (SMS) during AIT.

A – C, Correlations between the SMS and the percentages of IL-4⁺CRTH2⁺ ILC2s and IL-10⁺CTLA4⁺ ILCs per total ILCs, and the ratio of IL-4⁺CRTH2⁺/IL-10⁺CTLA4⁺ ILCs in

243 PBMCs from AIT responders (A, n = 10), non-responders (B, n = 5) and placebo-treated
244 patients (C, n = 5). Each dot represents the data at one time point for each individual subject.
245 (Baseline: red triangles; 6 months: orange rhombuses; 12 months: blue squares; and 24
246 months: green circles).

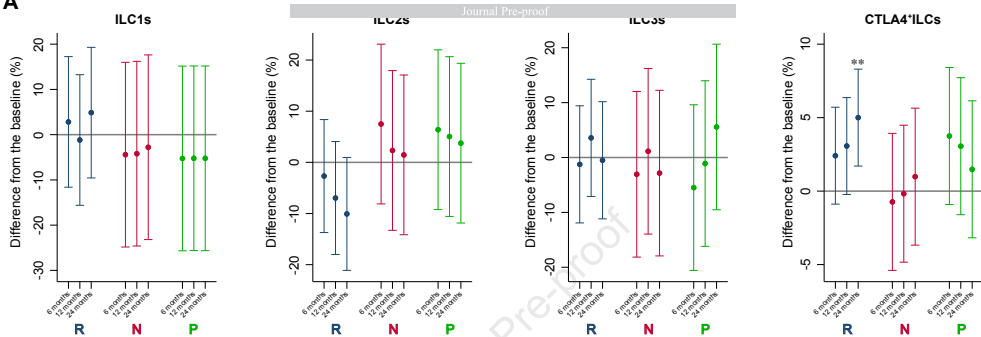
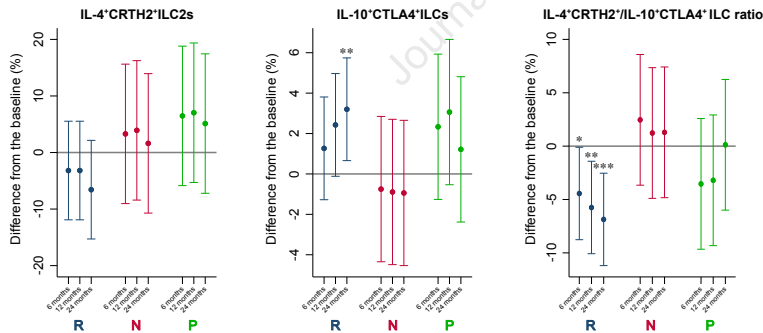
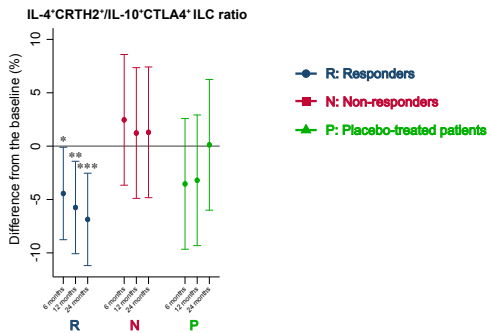
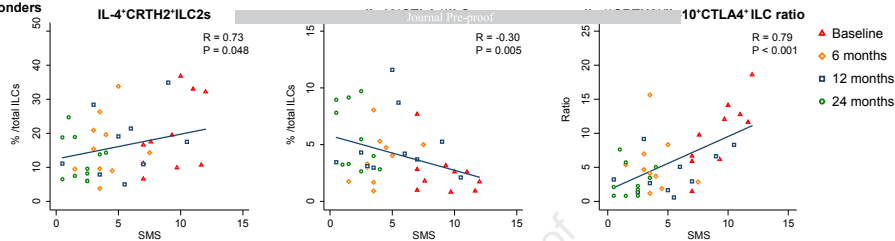
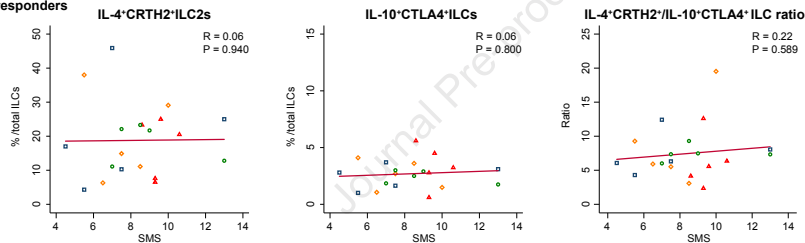
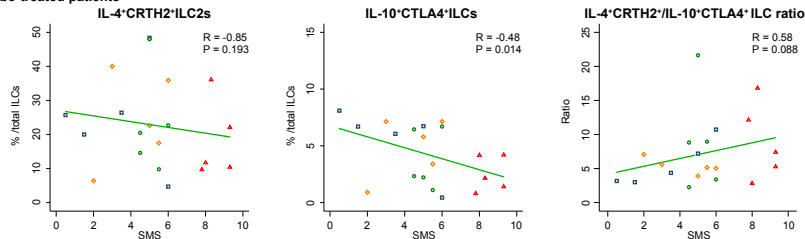
A**B****C**

Figure 1 Boonpiyathad et al

A Responders**B Non-responders****C Placebo-treated patients**

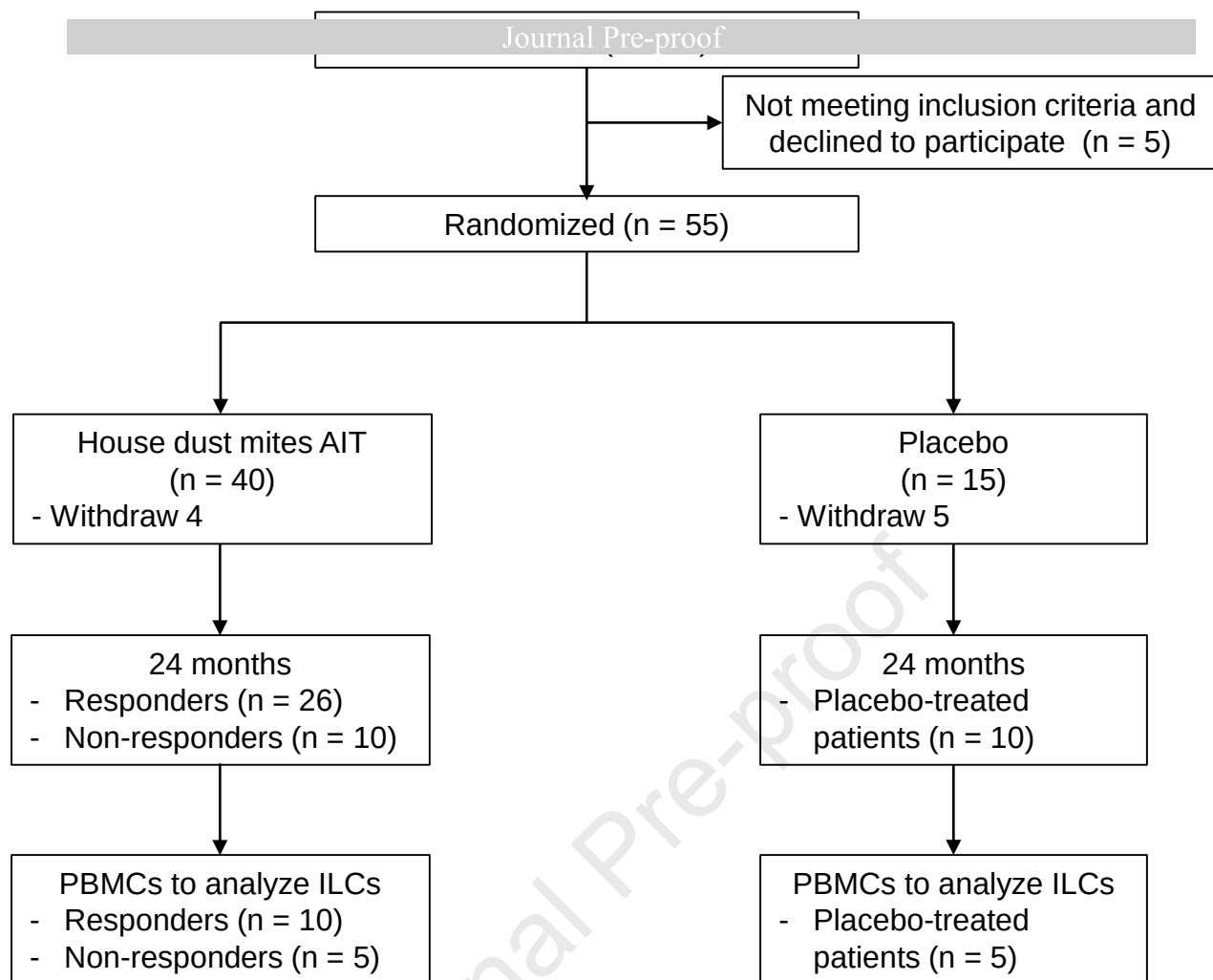


Figure E1. Boonpiyathad et al.

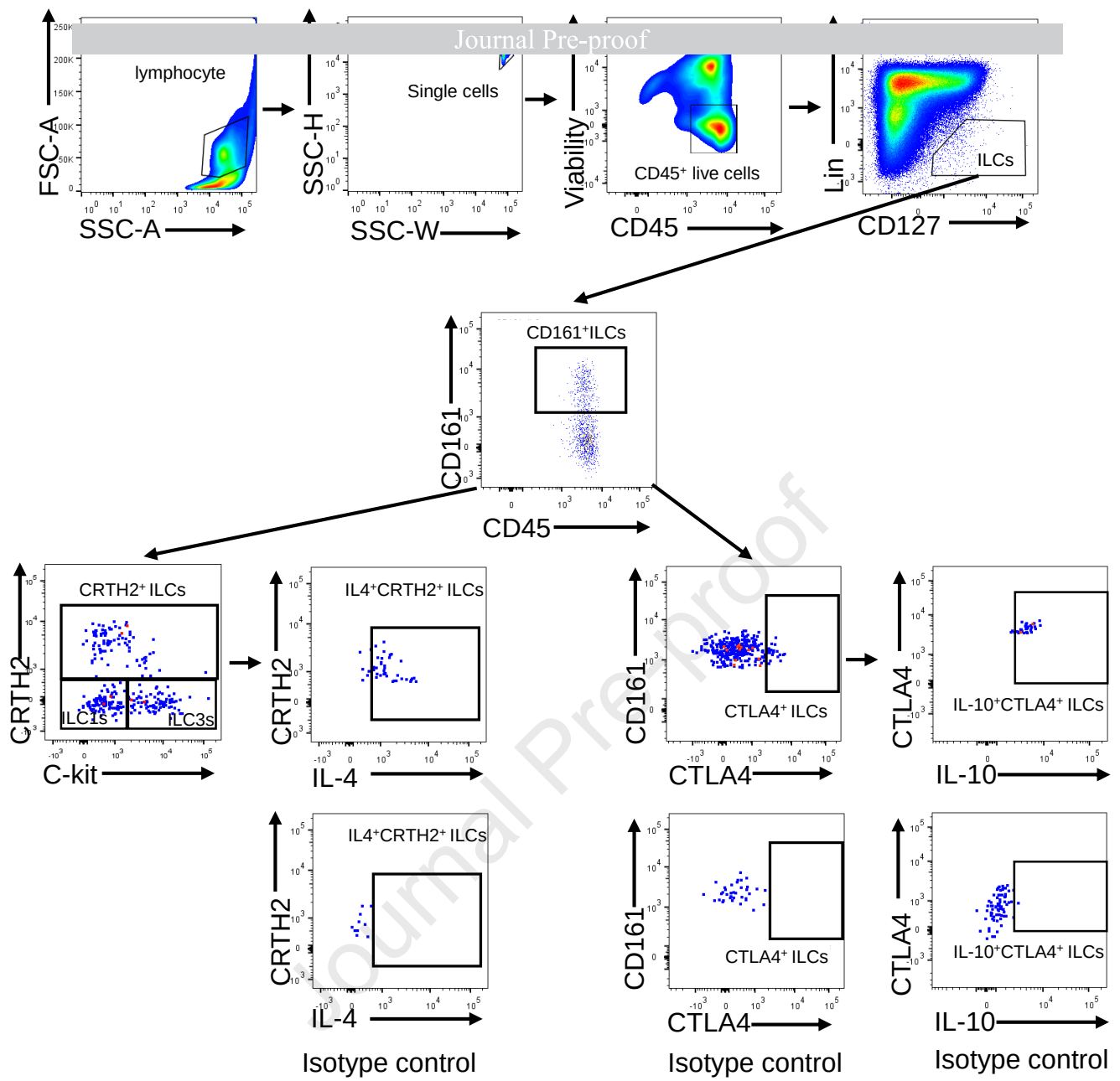
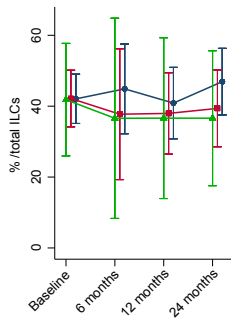
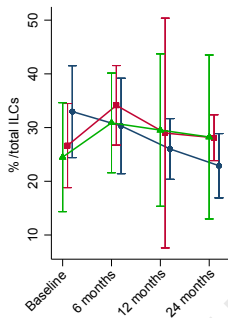
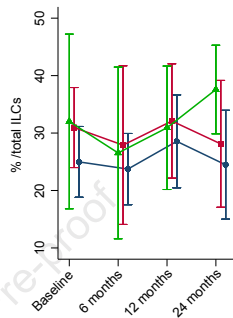
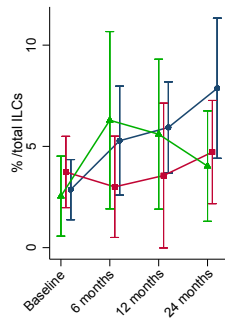
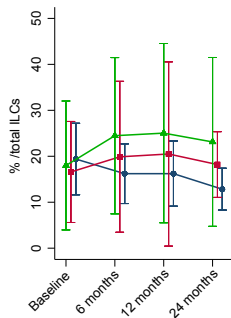
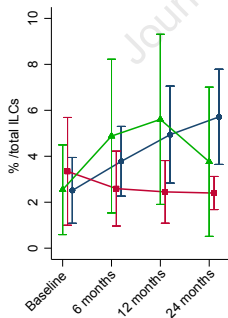
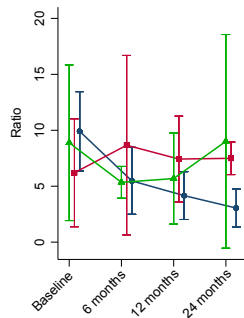
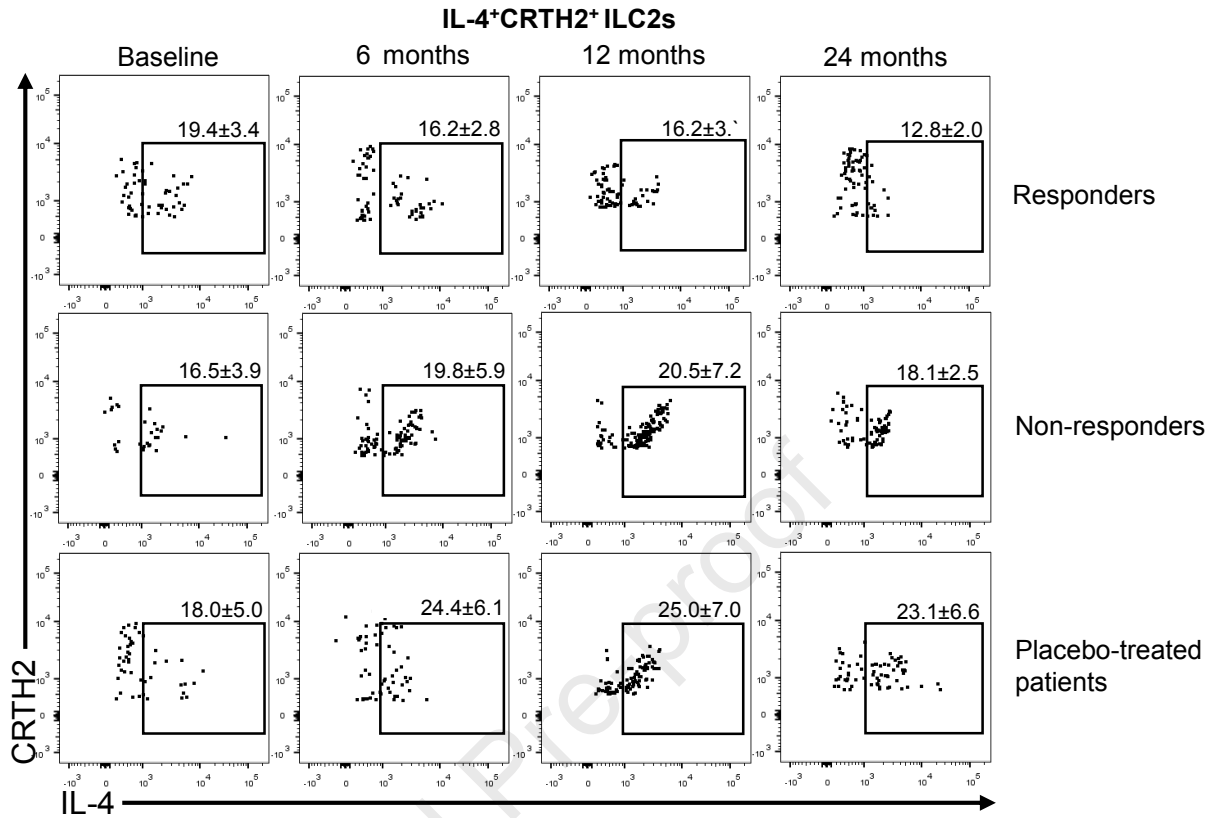


Figure E2. Boonpiyathad et al.

A**ILC1s****ILC2s****ILC3s****CTLA4^{hi} ILCs****B****IL-4^{hi}CRTH2^{hi} ILC2s****IL-10^{hi}CTLA4^{hi} ILCs****C****IL-4^{hi}CRTH2^{hi} / IL-10^{hi}CTLA4^{hi} ILC ratio**

● Responders
 ■ Non-responders
 ▲ Placebo-treated patients

A



B

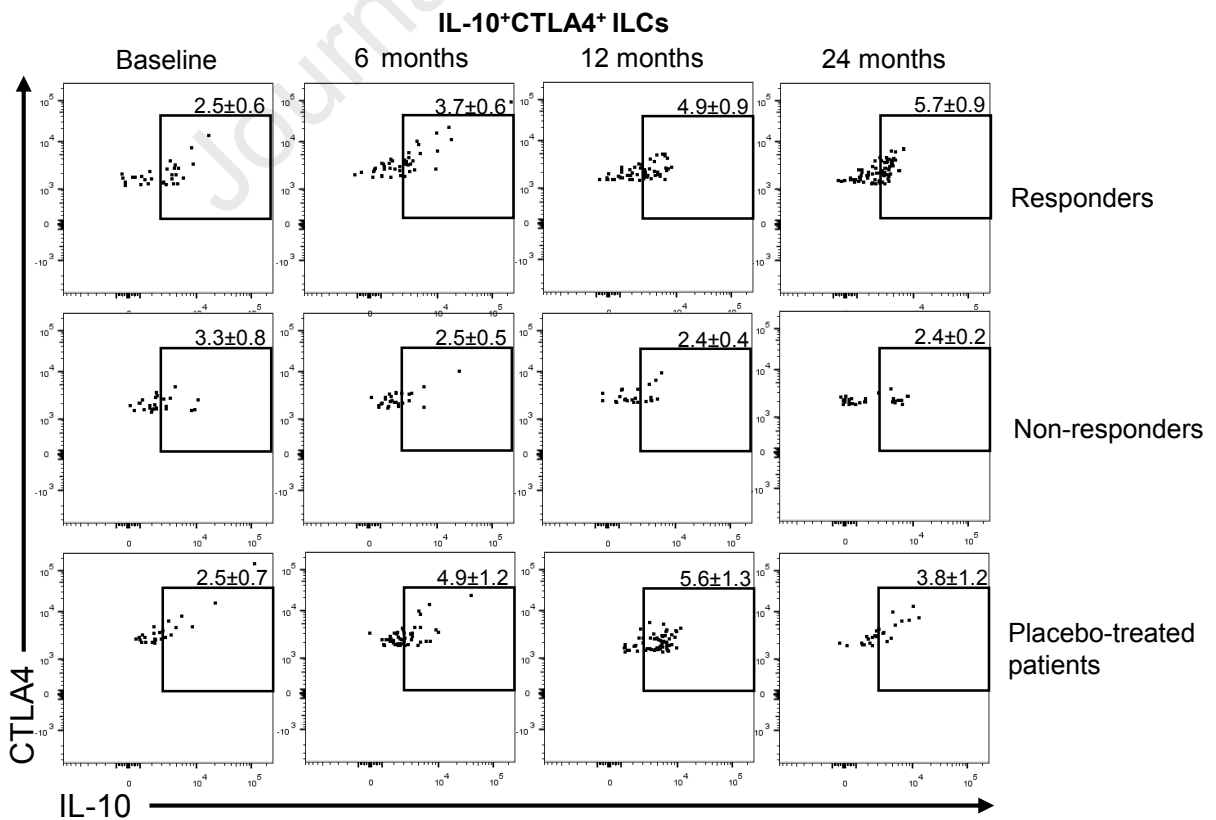


Figure E4. Boonpiyathad et al.

Symptom-medication score

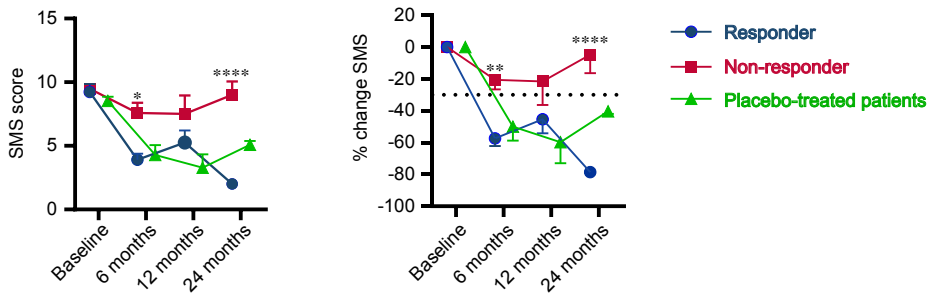
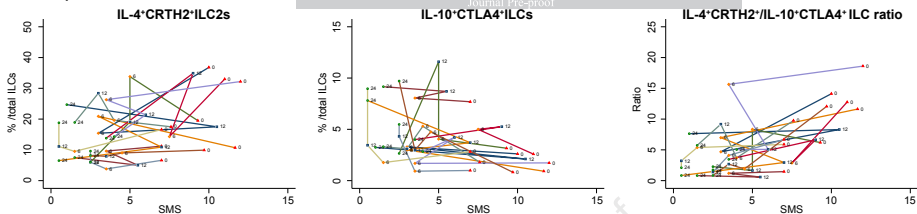


Figure E5. Boonpiyathad et al.

A Responders

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1 **Table E1.** Patient baseline characteristics

	Responders n = 10	Non-responders n = 5	Placebo- treated patients n = 5
Sex, Female (%)	5 (50)	4 (80)	4 (80)
Age (years±SD)	29.5±17.1	38.2±15.6	29.2±11.4
Allergic disease, n (%)			
Allergic rhinitis	10 (100)	5 (100)	5 (100)
Asthma	0	0	0
Atopic dermatitis	0	0	0
Type of allergen sensitization, n (%)			
Der p monosensitization	8 (80)	5 (100)	5 (100)
Der p dominant polysensitization	2 (20)	0	0
Allergic rhinitis status, n (%)			
Mild	0	0	0
Moderate to severe	10 (100)	5 (100)	5 (100)
Allergic rhinitis symptom status, n (%)			
Intermittent	0	0	0
Persistent	10 (100)	5 (100)	5 (100)
Duration of allergic symptoms, n (%)			
<5 years	1 (10)	0	0
5–10 years	1 (10)	1 (20)	1 (20)
>10 years	8 (80)	4 (80)	4 (80)

IL-10-producing innate lymphoid cells increased in house-dust-mite allergic rhinitis patients following immunotherapy

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^aDepartment of Medicine, Phramongkutklao Hospital, Bangkok, Thailand

^bDepartment of Otorhinolaryngology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

^cDepartment of medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

^dCenter of Excellence in Vaccine Research and Development, Chulalongkorn University, Bangkok, Thailand

^fPediatric Allergy and Clinical Immunology Research Unit, Division of Allergy and Immunology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, The Thai Red Cross society, Bangkok, Thailand

^eDepartment of Social Medicine and ^gDepartment of Allergy and Clinical Immunology, National Research Institute for Child Health and Development, Tokyo, Japan

† To whom correspondence should be addressed:

Hideaki Morita

2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan

Tel.: +81 (3) 3416 0181

Fax: +81 (3) 5494 7173

E-mail: _morita-hi@ncchd.go.jp

and

Kenji Matsumoto

E-mail: matsumoto-k@ncchd.go.jp

34

35 **Methods**

36 **Study population**

37 This study was a part of the MITAR study (A multicenter, randomized, placebo-controlled
38 house dust mite (HDM) subcutaneous allergen immunotherapy study of HDM persistent
39 allergic rhinitis), which was initiated in Thailand. The primary objective of the MITAR study
40 was to investigate the efficacy of allergen-specific immunotherapy in allergic rhinitis
41 patients, while the secondary objective was to elucidate the novel mechanisms and
42 biomarkers of immune tolerance. The study was registered at www.clinicaltrials.in.th, and its
43 study ID is TCTR20140127001. The study was conducted in accordance with the Declaration
44 of Helsinki and in compliance with the Good Clinical Practice guidelines. The study protocol
45 was approved by the Royal Thai Army Medical Department Ethics Committee, and all
46 subjects gave written informed consent before the start of study activity. Twenty patients (10
47 AIT treatment responders, 5 treatment non-responders and 5 placebo-treated patients) were
48 recruited in Phramongkutklao Hospital, Bangkok, Thailand since 2017 (Fig E1), and Table
49 E1 shows the characteristics of the participants in each group. All participants met the
50 inclusion criteria, including a history and objective symptoms of HDM-related allergic
51 rhinitis, a positive skin-prick test (a wheal diameter ≥ 6 mm larger than the negative control)
52 to *D. pteronyssinus* extract (Alk-Abello, TX, USA), and absence of allergic asthma and
53 atopic dermatitis. The symptom-mediation score (SMS) combines the total nasal symptom
54 score (TNSS) and the medication score (which represents the treatment efficacy) and was
55 used to assess the severity of allergic symptoms.^{1,2} The cut-off point for AIT responders has
56 been defined as a decrease in the SMS of more than 30% after 2 years of AIT compared with
57 before starting AIT (Fig E4).³⁻⁵ A rush protocol was used, consisting of subcutaneous
58 administration of a standardized mite mix that contained glycerin 50% v/v, phenol 0.4%
59 (preservative), sodium chloride, sodium bicarbonate, *D. pteronyssinus* 5,000 Allergy
60 Units/ml and *D. farinae* 5,000 Allergy Units/ml (Alk-Abello). Peripheral blood mononuclear
61 cells (PBMCs) were isolated at baseline, and at 6 months, 12 months and 2 years after
62 starting AIT. The PBMCs and plasma samples were cryopreserved in liquid nitrogen until
63 analysis.

64

65 **Cell culture and flow cytometry analysis**

Cryopreserved PBMCs were thawed and incubated in 10 ml of warm cRPMI (Nacalai Tesque; Kyoto, Japan) supplemented with 100 U DNase I recombinant (Roche; Mannheim, Germany) at 37°C and 5% CO₂ for 1 hour. The cells were then washed and cultured in Yssel's medium supplemented with 5% Human AB serum (both from Gemini Bio Products; West Sacramento, CA, USA) in the presence of 70 U/ml IL-2 (Sigma-Aldrich, St. Louis, MO, USA), 50 ng/ml IL-33 (PeproTech; Rocky Hill, NJ, USA) and 10 ug/ml recombinant Der p 2 (Alain J. Laboratory, Chulalongkorn University, Bangkok, Thailand) at 37°C and 5% CO₂ for 3 days. Subsequently, the cells were stimulated for 4 hours with 48 ng/mL PMA (Sigma-Aldrich) plus 1 mg/mL ionomycin (Sigma-Aldrich) and 1 µM Monensin (Sigma-Aldrich) and stained using eF780 viability dye (eBioscience; San Diego, CA, USA) at 4°C for 30 min. The lineage markers were stained with the following antibodies: Alexa Fluor (AF) 700-streptavidin-biotin-lineage marker (anti-CD1a (HI149), anti-CD3 (OKT3), anti-CD11c (3.9), anti-CD14 (HCD14), anti-CD16 (3G8), anti-CD19 (HIB19), anti-CD34 (581), anti-CD94 (DX22), anti-CD123 (6H6), anti-CD303 (201A) and anti-FcεRIα (AER-37) (all from BioLegend; San Diego, CA, USA). Other surface molecules were also stained with phycoerythrin (PE)/Cy7-conjugated anti-CD127 (A019D5), peridinin-chlorophyll-protein complex (PerCP)/Cy5.5-conjugated anti-CD161 (HP-3G10), Brilliant Violet 421-conjugated anti-CD117 (104D2) and Brilliant Violet 510-conjugated anti-CD45 (HI30) (all from BioLegend); and AF 647-conjugated anti-CD294 (BM16, BD Biosciences; San Jose, CA, USA). Since the markers of NK cells differ depending on the cells' developmental stage, various cell markers, such as CD34, CD117, CD94 and CD16, were included as lineage markers to exclude NK cells in all developmental stages. For intracellular cytokine staining, cells were fixed and stained with the following antibodies using a Cytofix/Cytoperm Kit (BD Biosciences): AF-488-conjugated anti-IL-4 (8D4-8, BioLegend), PE-CF594-conjugated anti-IL-10 (JEF3-19F1, BD Biosciences) and PE-conjugated anti-CTLA4 (BNI-3, BD Biosciences). All samples were analyzed using a BD LSRFortessa (BD Biosciences). Data were analyzed using FlowJo software (FlowJo LLC; Ashland, OR, USA). The levels of IL-10 in the culture supernatant after 3 days of culture were determined by multiplex assay (Merck Millipore; Burlington, MA, USA).

Statistical analysis

A mixed-effects linear regression model—adjusted for treatment with Kroger's method,⁶ which takes into account repeated measures, small sample size, and unbalanced data—was used to assessed for correlations between the three biomarkers, i.e., IL-10⁺CTLA4⁺ILCs, IL-

4⁺CRTH2⁺ILC2s and the IL-4⁺CRTH2⁺ILC2s/IL-10⁺CTLA4⁺ILCs ratio, and the symptom-medication score. Then, we assessed the time-changes in these biomarkers plus 4 others (ILC1s, ILC2s, ILC3s and CTLA4⁺ILCs) in each group: responders, non-responders and placebo-treated patients. We computed the mean differences and the 95% the confidence intervals using a mixed model that adjusted for group, time, and interaction groupXtime. Multiple comparison was corrected by the Bonferroni method. Statistical analysis was performed using Stata 15.1.

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Legends to Supplementary Figures

FIG E1. Study design. A CONSORT diagram showing how the subjects progressed through the study and how the final study samples were obtained.

FIG E2. Gating strategy for detecting ILCs. Lymphocytes were first gated from the forward-scatter (FSC) and side-scatter (SSC) plots. After doublet discrimination, live CD45⁺ cells were gated. ILCs were defined as CD45⁺Viable Lin⁻CD127⁺CD161⁺ cells.

FIG E3. Frequencies of each ILC subset during AIT. **A,** The percentages of Lin⁻CD127⁺CD161⁺CRTH2⁻c-kit⁻ ILC1s, Lin⁻CD127⁺CD161⁺CRTH2⁺ ILC2s, Lin⁻CD127⁺CD161⁺CRTH2⁻c-kit⁺ ILC3s and Lin⁻CD127⁺CD161⁺CTLA4⁺ ILCs per total Lin⁻CD127⁺CD161⁺ ILCs in PBMCs at different time points of AIT (baseline, 6 months, 12 months and 24 months after starting AIT). **B,** The percentages of Lin⁻CD127⁺CD161⁺IL-4⁺CRTH2⁺ ILC2s and Lin⁻CD127⁺CD161⁺IL-10⁺CTLA4⁺ ILCs. **C,** The ratios of IL-4⁺CRTH2⁺/IL-10⁺CTLA4⁺ ILCs with time. Data represent the mean and confidence interval.

FIG E4. Representative dot-plots of IL-4⁺CRTH2⁺ILC2s (**A**) and IL-10⁺CTLA4⁺ILCs (**B**) at baseline, 6 months, 12 months and 24 months of AIT in responders, non-responders and placebo-treated patients. Data are shown as means $\pm$ SEM.

FIG E5. The clinical response was assessed based on the symptom-medication score (SMS) and the percentage change in SMS during 2 years of AIT (responder patients, n = 10; non-responder patients, n = 5; placebo, n = 5). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

FIG E6. Plots of the relationship between the percentage of each ILC and the symptom-medication score (SMS) during AIT. **A – C,** Relationships between the SMS and the percentages of IL-4⁺CRTH2⁺ ILC2s and IL-10⁺CTLA4⁺ ILCs per total ILCs, and the ratio of IL-4⁺CRTH2⁺/IL-10⁺CTLA4⁺ ILCs in PBMCs from AIT responders (**A**, n = 10), non-responders (**B**, n = 5) and placebo-treated patients (**C**, n = 5). Each plot represents one subject, and the numbers (0, 6, 12, 24) indicate the 4 different time-points of AIT (baseline, 6, 12 and 24 months after starting AIT).