

ORIGINAL ARTICLE

Allergen-Specific Immunotherapy and Biologics

Der p 1-specific regulatory T-cell response during house dust mite allergen immunotherapy

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Abstract

Background: Allergen-specific immunotherapy (AIT) is the only available treatment for allergic diseases that can induce specific immune tolerance to allergens. The key mechanisms involved in this process include changes in allergen-specific regulatory T (Treg) cells.

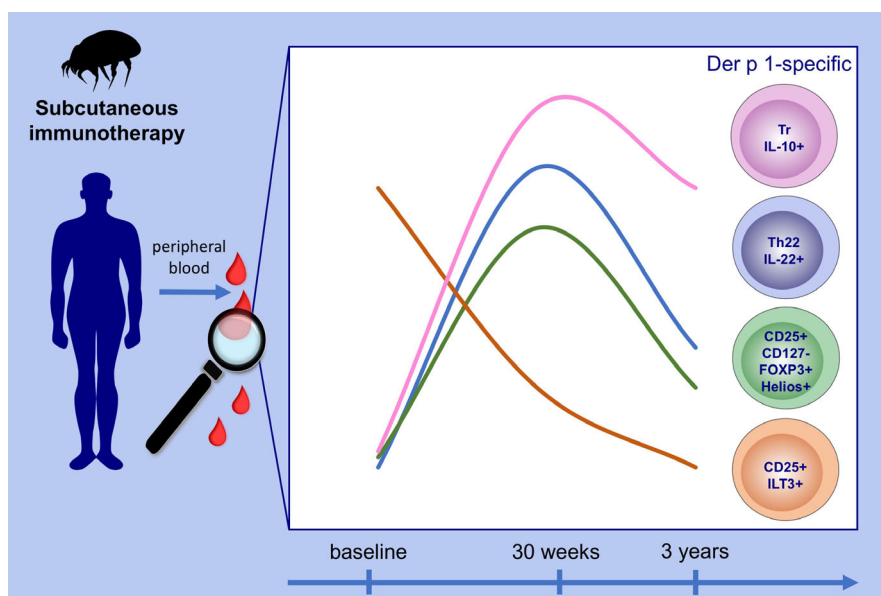
Methods: We studied 25 allergic rhinitis patients undergoing subcutaneous house dust mite-specific immunotherapy. Peripheral blood mononuclear cells were studied before and after 10, 30 weeks, and 3 years of AIT. Der p 1-specific T regulatory cell responses were investigated by characterization of Der p 1-MHC class II tetramer-positive cells and correlated with nasal symptom score.

Results: Twelve of 25 AIT patients matched with their MHC class II expression to the Der p 1 peptide-MHC class II tetramers. A significant increase in the numbers of Der p 1-specific FOXP3⁺Helios⁺CD25⁺CD127⁻ Treg cells after 30 weeks was observed, which slightly decreased after 3 years of AIT. In contrast, Der p 1-specific immunoglobulin-like transcript 3 (ILT3)⁺CD25⁺ Treg cells decreased substantially from baseline after 3 years of AIT. ILT3⁺ Treg cells displayed compromised suppressive function and low FOXP3 expression. In addition, Der p 1-specific IL-10 and IL-22 responses have increased after 30 weeks, but only IL-10⁺ Der p 1-specific Treg cells remained present at high frequency after 3 years of AIT. Increased number of FOXP3⁺Helios⁺ and IL-10⁺ and decreased ILT3⁺ Treg cell responses correlated with improved allergic symptoms.

Conclusion: The results indicate that AIT involves upregulation of the activated allergen-specific Treg cells and downregulation of dysfunctional allergen-specific Treg cell subset. Correction of dysregulated Treg cells responses during AIT is associated with improved clinical response.

KEYWORDS

allergen-specific T cells, antigen-specific immunotherapy, house dust mite allergy, Treg



GRAPHICAL ABSTRACT

Allergen-specific immunotherapy induces an increase in activated Treg cells, IL-10-producing Treg cells, IL-22-producing Th cells, and reduces dysfunctional ILT3⁺ CD25⁺ Treg cells. Increased number of FOXP3⁺Helios⁺, IL-10⁺ and decreased ILT3⁺ Treg cell responses correlated with improved allergic symptoms. Allergen-specific immunotherapy improves clinical symptoms which relates to the correction of dysregulated T cell responses.

1 | INTRODUCTION

Allergies are among the most common diseases worldwide, with highly accelerating prevalence in the last decades.¹ The pathophysiology of allergic disease is complex and influenced by many factors, including genetic susceptibility, route of exposure, antigen/allergen dose, and time of exposure with stimulators of innate immune response, such as infections or commensal bacteria.²⁻⁴ Seasonal and perennial allergens can be inhaled and ingested and can enter the body via the injured skin and induce an IgE-mediated local or systemic inflammatory process.⁵ Currently, allergen-specific immunotherapy (AIT) is the only curative treatment that induces clinical and immunological tolerance to causative allergens in allergic patients.⁶⁻⁹ AIT efficiently decreases symptoms of allergic asthma, rhinitis, and insect sting allergy.¹⁰⁻¹⁴ A primary feature of AIT is to change the course of the disease by changing the underlying pathology on local and systemic immune response.¹⁵ Immune tolerance to allergens is described by an establishment of long-term clinical tolerance.¹⁶

The mechanisms of action of T cells in AIT include induction of allergen-specific T regulatory (Treg) cells, increased secretion of IL-10 and TGF- β , and increased expression of FOXP3, histamine receptor 2, and other suppressive molecules in allergen-specific Treg cells, which lead to the suppression of type 2 immune response in the affected tissues and peripheral blood.¹⁷ Successful AIT augments the key regulator populations of allergen-specific Treg cells that produce IL-10, TGF- β , or both.¹⁸ Naturally occurring FOXP3⁺CD4⁺CD25⁺ Treg cells and inducible type 1 Treg (Tr1) cells contribute to the control of allergen-specific immune responses in several significant ways.¹⁹ IL-10 reduces antigen-specific IgE and increases IgG4 levels.²⁰ Also, the proportion of IL-4-secreting Th 2 and Th17 cells decreases, whereas IFN- γ -secreting Th1 and IL-10-

secreting inducible Treg cells specific for the same allergenic epitope increase in numbers in successful AIT. The same immunological events are also observed in the development of a healthy immune response to high dose of exposure of allergens in nonallergic individuals.^{17,21}

Helios is a marker of T-cell activation and proliferation, and Helios enhances induced Treg (iTreg) cell function in cooperation with FOXP3.^{22,23} Most of the Treg cells in healthy subjects and patients with stable asthma are expressing FOXP3 and Helios, whereas during asthma exacerbations there is a reduction in the number of FOXP3⁺Helios⁺ and an increase in the frequency of FOXP3⁺Helios⁻ Treg cells.²⁴ The co-expression of CD4⁺CD25⁺FOXP3⁺ and immunoglobulin-like transcript 3 (ILT3)⁺ represents the distinct Treg subset.²⁵ As a mechanism, ILT3 regulates the protein kinase CK2 activity and therefore may work as an inactivating factor of the regulatory function of Treg cell, and protein kinase CK2 enables Treg cells to actively suppress excessive Th2 responses.²¹ The frequency of ILT3⁺ cells among human CD4⁺CD25⁺FOXP3⁺ Treg cells of asthmatic patients was found higher than in healthy controls.²⁵ However, Helios and ILT3 expression on Treg cells has not been studied in AIT and we hypothesize that Treg cell subsets might be changed during AIT.

The investigation of allergen-specific immune response by direct analysis of human peripheral blood T cells without any further cell cultures provides an efficient tool for the in-depth investigation of changes in allergen-specific T cells in allergen tolerance during AIT. Peptide (p)-MHC class II tetramer staining is one of the methods to characterize antigen-specific CD4⁺ T cells. Such tetramers classically consist of complexes composed of four MHC class II heterodimers loaded with the peptide of the target allergen binding to a relevant T-cell epitope.

House dust mite (HDM) is the most common indoor aeroallergen. It may also serve as a carrier of bacteria responsible for the

induction of IgE sensitization to microbial antigens.²⁶ Patients allergic to HDM can be successfully treated with HDM AIT.²⁷ HDM-reactive IL-5⁺IL-13⁺CD27⁻CD161⁺CD4⁺ cells and ST2⁺CD45RO⁺CD4⁺ cells were significantly lower in active responders to HDM sublingual immunotherapy.²⁸ In the present study, we treated patients allergic to HDM with subcutaneous AIT, and Der p 1-specific Treg cells were analyzed in peripheral blood samples before and during the course of AIT after 10, 30 weeks, and 3 years. Our results demonstrate that the changes in Der p 1-specific Treg cells during treatment reflect the patient's T-cell response to therapy and are also partially linked to the clinical efficacy of the AIT.

2 | METHODS

2.1 | Subjects

Twenty-five allergic rhinitis (AR) patients who qualified for subcutaneous HDM AIT were recruited from the Allergy Clinic, Department of Medicine, Phramongkutklao Hospital, Bangkok, Thailand. All participants met the following enrollment criteria: a history and objective symptoms of HDM-related and positive skin prick test (wheal diameter ≥ 6 mm² than negative control) with *D. pteronyssinus* extract (Alk-Abello, Round Rock, TX, USA). A 30-week weekly dose escalation protocol was performed with the subcutaneous administration of standardized mite mixes, containing glycerin 50% v/v, phenol 0.4% (preservative), *D. pteronyssinus* 5000 allergy units/mL, and *D. farinae* 5000 allergy units/mL (Alk-Abello). After 30 weeks, patients underwent 0.5 mL (1:10 concentration, 3.5 μ g of Der p and 5 μ g of Der f) every 2–4 weeks until 3 years. Peripheral blood mononuclear cells (PBMCs) were isolated before and after 10, 30 weeks, and 3 years of AIT. PBMCs were kept frozen in liquid nitrogen until analysis. Allergic rhinitis symptoms were monitored with total nasal symptom score (TNSS). The study protocol was approved by the Institutional Review Board of Royal Thai Army's ethics committee (IRBRTA 67/2556) and the Faculty of Medicine, Chulalongkorn University (IRB No. 193/60). All patients were recruited after obtaining written informed consent.

2.2 | HLA-DR typing

For HLA-DR typing, genomic DNA was extracted from PBMCs using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). HLA-DRB1 low-resolution and high-resolution typing were performed using the Aii-Set⁺™ Gold DR Low- and High-Resolution SSP kits (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocols. Twelve out of 25 patients who were compatible with Der p 1 peptide (p)-MHCII tetramers HLA-DRB1*0410, HLA-DRB1*1101, and HLA-DRB1*1501 were enrolled in the study. The patient characteristics are shown in Table S1.

2.3 | Der p 1-specific CD4⁺ T-cell staining

Peripheral blood mononuclear cells were enriched with anti-CD3 magnetic MicroBead (Miltenyi Biotec, Bergisch Gladbach, Germany) and separated by autoMACS separator (Miltenyi Biotec) according to

the manufacturer's protocols. After enrichment, cells were stained with eFluor 780 viability dye (eBioscience, San Diego, CA, USA) for 30 minutes at 4°C and washed in staining buffer (phosphate-buffered saline, pH 7.2, 0.5% bovine serum albumin, and 2 mmol/L EDTA). Cells were incubated with 2 μ g/mL of phycoerythrin (PE)-Der p 1 tetramers in complete RPMI medium at room temperature for 30 minutes. The PE-labeled and empty (without any peptide) HLA-DRB1*0401, 1101, and 1501 tetramers as well as nonMHC-matching tetramers were used as negative control. Epitopes from Der p 1 for HLA-DRB1*0401, DRB1*1101, and DRB1*1501 were identified by tetramer-guided epitope mapping (Table S2).²⁹ Der p 1-specific MHCII tetramer and empty tetramer-labeled with PE were produced by the Benaroya Research Institute (Seattle, WA, USA). The method to measure pMHCII tetramer specificity was described in the previous study.³⁰ Cells were then stained with extracellular antibodies, V500-anti-CD3 (clone SP34-2; BD Biosciences, San Jose, CA, USA), PE/CF594-anti-CD4 (clone RPA-T4; BD Biosciences), APC/Cy7-anti-CD14 (clone HCD14; Biolegend, San Diego, CA, USA), APC/Cy7-anti-CD16 (clone 3G8; Biolegend), APC/Cy7-anti-CD19 (clone HIB19; Biolegend), BV421-anti-CD25 (clone BC6; Biolegend), BV605-anti-CD127 (clone A019D5; Biolegend), and PE/Cy7-anti-ILT3 (clone ZM4.1; Biolegend), at room temperature for 15 minutes. Following this, FOXP3 and Helios intracellular staining buffer (Biolegend) was performed following the manufacturer's protocols. Cells were stained with Alexa Fluor 488-anti-FOXP3 (clone 259D) and Alexa Fluor 647-anti-Helios (clone 22F6, both from Biolegend) at room temperature in the dark for 30 minutes. After washing in staining buffer, cells were resuspended in phosphate-buffered saline with 0.1% paraformaldehyde and 2 mmol/L EDTA. Cells were measured by FACSARIA III (Becton Dickinson, Franklin Lakes, NJ, USA). Data were analyzed by Kaluza software (Beckman Coulter, Indianapolis, IN, USA).

2.4 | Intracellular cytokine staining

The anti-CD3 positively enriched cells were treated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 hours and then with brefeldin A (all from Sigma-Aldrich, Buchs, Switzerland) for 2 hours in cRPMI at 37°C. Then, viability staining was performed first, followed by PE-Der p 1-tetramers and surface antibodies, V500-anti-CD3 (BD Biosciences), PE/CF594-anti-CD4 (BD Biosciences), APC/Cy7-anti-CD14 (Biolegend), APC/Cy7-anti-CD16 (Biolegend), APC/Cy7-anti-CD19 (Biolegend), and PerCP-anti-CD8 (clone SK1; Biolegend). Fixation/permeabilization staining (BD Bioscience) was performed according to the manufacturer's protocol. Cells were then stained with PE/Cy7-anti-IL-10 (clone JES3-9D7) and APC-anti-IL-22 (clone 22URT1, both from Biolegend) for 30 minutes at 4°C. Corresponding isotype-matched antibodies were used as negative controls in all stainings.

2.5 | Treg suppression assay

Peripheral blood mononuclear cells were enriched with CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) and separated by autoMACS separator (Miltenyi Biotec). The enriched CD4⁺ T cells were selected as the final

negative fraction, and the final positive fraction was irradiated and used as cocultured cells. Then, CD4⁺ T cells were stained with viability dye, anti-CD3, anti-CD4, anti-CD25, and anti-ILT3 as described above. ILT3⁺ and ILT3⁻ CD4⁺CD25⁺ Treg cells and CD4⁺CD25⁻ T effector cells were sorted by FACS Aria III (Becton Dickinson). T effector cells were stained with CellTrace Violet Cell Proliferation Kit (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's protocol. Next, Treg, T effector, and irradiated T cells were cultured with anti-CD3-/anti-CD28-coated beads and IL-2 (all from Sigma-Aldrich) in complete RPMI medium in a U-bottom 96-well plate at 37°C and 5% CO₂ for 5 days. Treg cells and T effector cells were cocultured in different Treg:T effector cell ratios. After 5 days, cells were harvested and washed 2 times with phosphate-buffered saline. Cells were stained with 0.5 µL viability dye eFluor 780 (eBioscience) for 30 minutes at 4°C, and then, cells were stained with FITC-anti-CD3 (clone 17A2), AF700-anti-CD4 (clone OKT4), and PE-anti-CD25 (clone BC96, all from Biolegend) for 15 minutes at room temperature. Cells were washed 2 times with staining buffer and analyzed by flow cytometry.

2.6 | Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). The repeated-measures ANOVA with post hoc analysis was used to compare related samples between different time points. Spearman's test was performed for correlation analyses. Statistical analyses and heatmap correlation were performed with GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). *P* value < 0.05 was considered as significant.

3 | RESULTS

3.1 | Der p 1-specific Treg cell responses during AIT

The gating strategy of Der p 1-specific CD4⁺ Th, CD4⁺CD25⁺CD127⁻ Treg, and CD4⁺CD25⁺ILT3⁺ Treg cells is shown in Figure 1A. Before the AIT, the frequency of FOXP3⁺ and Helios⁺ Der p 1-specific Treg cells constituted only 2.73 ± 0.53% of Der p 1-specific CD4⁺ T cells (Figure 1B). We found that the frequency of Der p 1-specific FOXP3⁺Helios⁺ Treg cells was markedly increased peaking after 30 weeks (14.73 ± 1.24%) and slightly decreased after 3 years (6.95 ± 0.95%) of AIT (Figure 1B). The frequency of Der p 1-specific FOXP3⁺Helios⁻ Treg cells did not change significantly during AIT (Figure 1B). Similarly, nonspecific FOXP3⁺Helios⁺ and FOXP3⁺Helios⁻ Treg cells did not change during the therapy (Figure 1C). Thus, AIT induced allergen-specific Treg cell response, but it had no effect on nonspecific Treg cells.

Next, we analyzed the frequency of Treg cell subset, which expresses CD25 and ILT3. The frequency of Der p 1-specific CD4⁺CD25⁺ILT3⁺ Treg cells was significantly lower after 3 years of AIT (Figure 2A). No significant changes in nonspecific CD4⁺CD25⁺ILT3⁺ T cells were observed (Figure 2B). Therefore, we performed in vitro suppressive assay to compare functions of ILT3⁺ and ILT3⁻ Treg cells. Interestingly, CD4⁺CD25⁺ILT3⁺ Treg cells displayed significantly reduced suppressive function as compared to

CD4⁺CD25⁺ILT3⁻ Treg cells which revealed even a higher rate of the proliferation of T effector cells (Figure 2C). In addition, we further examined FOXP3 and Helios expression in ILT3⁺ and ILT3⁻ Treg cells. We found that within ILT3⁺ Treg cells, the frequency of FOXP3⁺Helios⁺ cells was significantly low compared to ILT3⁻ Treg cells. Hence, AIT reduced dysfunctional allergen-specific Treg cell response in addition to increasing FOXP3⁺Helios⁺ Treg cell frequency.

3.2 | Der p 1-specific IL-10- and IL-22-secreting T-cell responses during AIT

To elucidate the cytokine profile of allergen-specific T cells, we determined Der p 1-tetramer⁺CD4⁺ T cells, which are producing IL-10, IL-22, and both IL-10 and IL-22 (Figure 3A). Der p 1-specific IL-10⁺ Treg cells displayed low frequency of Der p 1-specific CD4⁺ T cells before AIT (2.89 ± 0.55%). During AIT, IL-10-producing Der p 1-specific Treg cells expanded already after 10 weeks (5.12 ± 0.67%) and showed the highest frequency after 30 weeks (22.95 ± 3.07%) of AIT (Figure 3B). At 3 years of AIT, the frequency of IL-10-producing Der p 1-specific Treg cells (17.54 ± 1.71%) was slightly decreased compared to the 30-week time point, but was still higher compared to baseline (Figure 3B). Moreover, IL-22-producing Der p 1-specific Th cells increased significantly after 30 weeks of AIT, from 3.78 ± 1.39% to 18.71 ± 3.74% (Figure 3C). In addition, the frequency of Der p 1-specific Treg cells, which secreted both IL-10 and IL-22, significantly increased after 30 weeks of HDM-specific immunotherapy, from 0.89 ± 0.32% to 8.50 ± 1.96% (Figure 3D). For comparison, there was no significant change in IL-10-, IL-22-, and both IL-10- and IL-22-producing nonspecific CD4⁺ T cells during AIT (Figure 3E).

3.3 | Der p 1-specific Treg cell response correlates with allergic symptoms

Allergic patients had significantly decreased total nasal symptom score (TNSS) after 30 weeks (-66.08 ± 6.40%) and 3 years (-86.33 ± 3.35%) of AIT (Figure 4A). To investigate the role of Treg cell changes on clinical improvement in response to AIT, we analyzed the heatmap correlation between allergen-specific Treg subsets, IL-22⁺CD4⁺ T cells, and TNSS (Figure 4B). Improved allergic symptoms by reduced TNSS showed a significant correlation with increased frequency of activated Der p 1-specific FOXP3⁺Helios⁺, IL-10⁺, and IL-10⁺IL-22⁺ Treg cells and IL-22⁺ Th cells (Figure 4B). Moreover, reduced allergic symptoms correlated with decreased frequency of dysfunctional Der p 1-specific ILT3⁺ Treg cells (Figure 4B).

Change in the frequency of effective allergen-specific Treg cells (Der p 1-specific FOXP3⁺Helios⁺CD4⁺CD25⁺CD127⁻) during AIT was parallel with the frequencies of Der p 1-specific IL-10⁺, IL-22⁺, and IL-10⁺IL-22⁺ CD4⁺ T cells (Figure 4C). Meanwhile, the frequency of ineffective allergen-specific Treg cells (Der p 1-specific ILT3⁺ Treg cells) changed opposite with effective, IL-10- and IL-22-producing Treg cells (Figure 4B). The frequency of effective and ineffective Treg cells was approximately equal after 30 weeks of AIT.

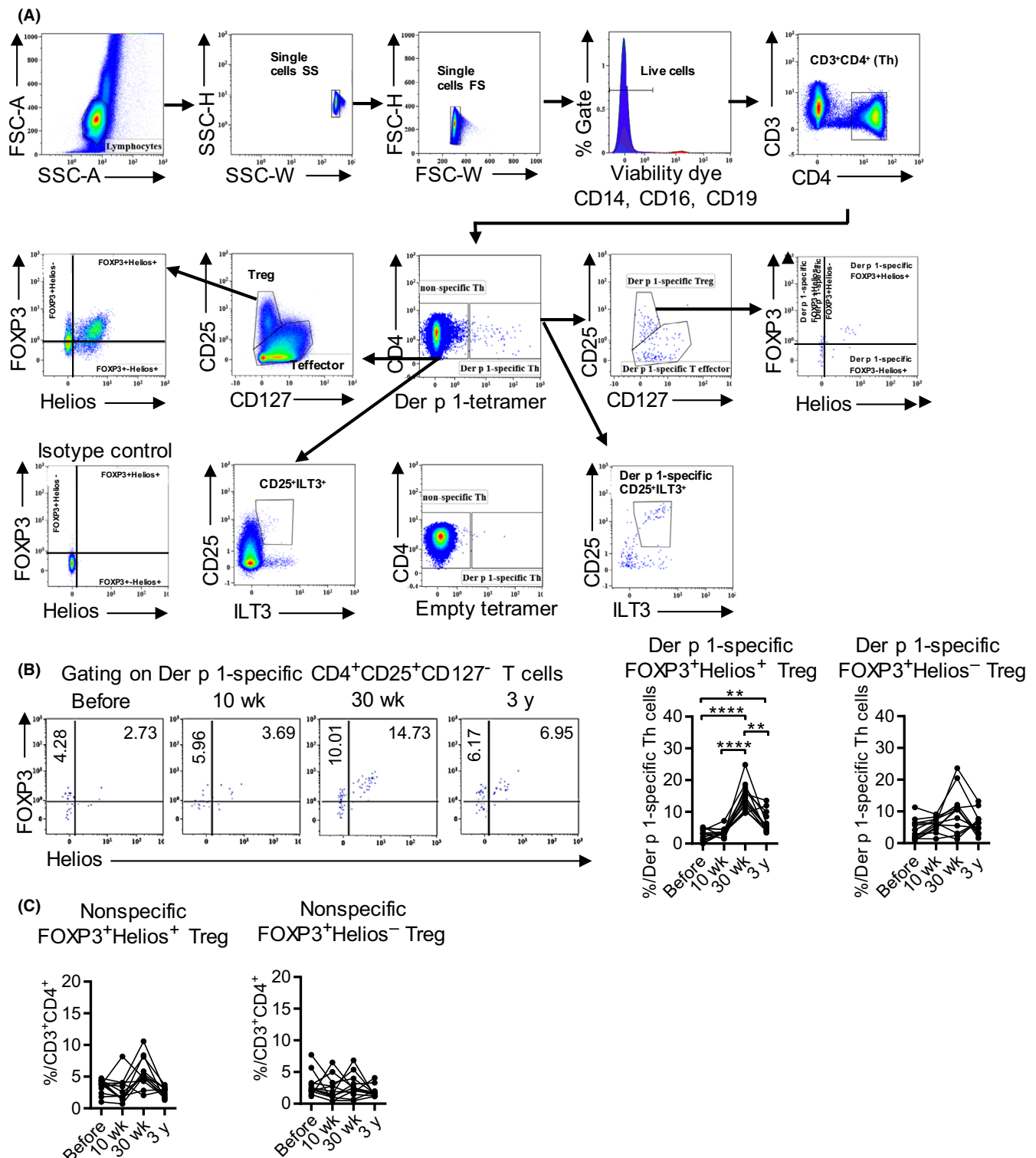
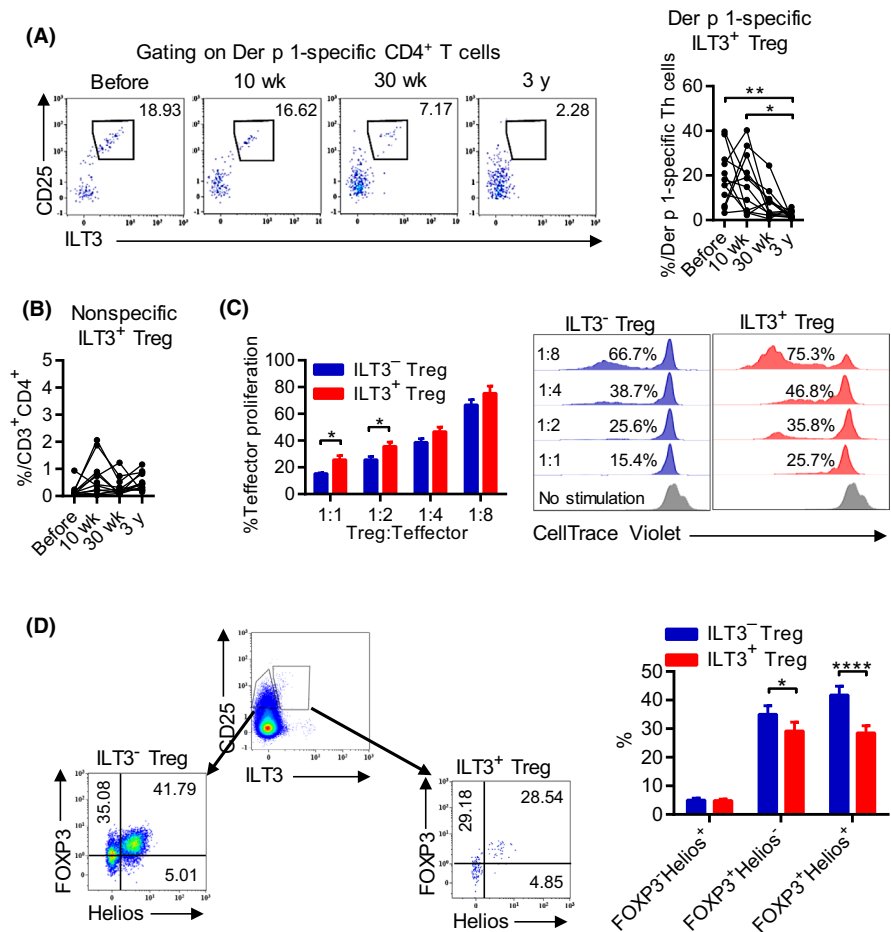


FIGURE 1 Increased frequency of Der p 1-specific FOXP3- and Helios-positive Treg cells during allergen-specific immunotherapy (AIT).

A, Gating strategy of Der p 1-specific Treg cells. Identified cells were first gated on lymphocytes and single cells. Live CD3⁺ cells were gated after gating out dead cell (viability⁺), CD14⁺, CD16⁺, and CD19⁺. Der p 1-specific CD4⁺ T cells were gated on Der p 1 peptide-MHCII tetramer⁺. Empty peptide-MHCII tetramer was used as negative control. Treg cells were identified by the expression of CD4⁺CD25⁺CD127⁻ and then gated on FOXP3 and Helios. Another Treg population was gated on CD3⁺CD4⁺CD25⁺ILT3⁺ cells. Effector T cells were gated on CD4⁺CD25⁻ T cells. B, Representative flow cytometry dot plots (left) and the frequency (right) of Der p 1-specific FOXP3⁺Helios⁺ and FOXP3⁺Helios⁻ Treg cells during AIT (n = 12). C, There was no change in the frequency of nonspecific FOXP3⁺Helios⁺ and FOXP3⁺Helios⁻ Treg cells during AIT. The values are presented as the mean value. **P < 0.01, ****P < 0.0001 [Colour figure can be viewed at wileyonlinelibrary.com.]

FIGURE 2 Decreased frequency of Der p 1-specific ILT3⁺ Treg cells during allergen-specific immunotherapy (AIT). A, Representative flow cytometry dot plots (left) and the frequency (right) of Der p 1-specific ILT3⁺ Treg cells during AIT (n = 12). B, The frequency of nonspecific ILT3⁺ Treg cells during AIT. C, In vitro Treg suppression assay, comparing the suppressive capacity of ILT3⁻ and ILT3⁺ Treg cells. Sorted T effector cells were labeled with CellTrace Violet, stimulated with anti-CD3 and anti-CD28, and cocultured with sorted ILT3⁻ or ILT3⁺ Treg cells for 5 d. The frequency of T effector cell proliferation (left) and the overlay histogram (right) of ILT3⁻ or ILT3⁺ Treg cells at various ratios (n = 6). D, Dot plots (left) and the frequency (right) of FOXP3 and Helios expression on ILT3⁻ and ILT3⁺ Treg cells (n = 48). The values are presented as mean value ± SEM. *P < 0.05, **P < 0.01, ****P < 0.0001 [Colour figure can be viewed at wileyonlinelibrary.com.]



4 | DISCUSSION

The present study demonstrates novel immunological mechanisms linked to the induction of peripheral allergen-specific tolerance by AIT. We demonstrated early increases in functionally efficient Tregs resulting in desensitization compared to delayed suppression of inefficient Tregs as a potential tolerance mechanism after 3 years. Der p 1-MHC class II tetramer staining could be used to monitor peripheral allergy tolerance and clinical response of HDM-specific immunotherapy. It has been previously shown that AIT induces an increase in the percentage of allergen-driven Treg cells.^{31,32} In the present study, we demonstrated that AIT differentially modifies the activation and function of several Treg subsets. AIT in vivo increased subsets of allergen-specific Treg cells with distinct phenotypes, and the mechanisms of action include the CD4⁺CD25⁺CD127⁺FOXP3⁺ nTreg and the CD4⁺IL-10-secreting Tr1 cells.³³⁻³⁸ In addition to changes in allergen-specific Treg cells, we observed that in 9 out of 12 patients, overall IL-10⁺ Tr1 cells also increased during AIT, which is concordant with the previous data reported by others.³⁹

The limitations include relatively small sample size, some samples not being available because of HLA mismatch with tetramers, lack of the placebo control group, lack of robust efficacy outcomes in the clinical trial, and the generalization of the findings obtained from certain

MHC-tetramer match population to the general mite-allergic population. As the availability of recombinant MHC class II molecules is limited, knowledge on T-cell epitopes and recruitment of participants to match with MHC class II tetramers.⁴⁰ Therefore, our study was not powerful enough to examine the differences between responders or nonresponders to the therapy. Interestingly, we found here that TNSS was reduced by 88.5% in matched and by 57.1% in nonmatched HLA-DR patients after 3 years of AIT (Figure S1). The explanation of these results is that our matched HLA-DR patients, who we were able to follow with the tetramers, were relatively good responders to the treatment. Hence, allergen-specific Treg cell subset responses in nonresponders to AIT is still a question and requires further studies. Although the present study focuses on Treg cells, their effect on the control of allergen-specific immune responses goes in parallel to allergen-specific memory B-cell responses and the importance of allergen-specific effector and regulatory B-cell balance should be studied in bigger cohorts.⁴¹

Helios is a specific marker for thymus-derived regulatory T cells.⁴² Combining FOXP3 and Helios intracellular staining is apparently a useful approach to determine activated Treg cells, because FOXP3⁺Helios⁺ Treg cells proliferate in vitro significantly less than FOXP3⁺Helios⁻ Tregs upon TCR stimulation.⁴³ Unlike FOXP3⁺Helios⁻ Tregs, FOXP3⁺Helios⁺ Tregs secrete IL-10, but not other effector cytokines such as IFN- γ or IL-2.⁴³ Moreover, Helios is selectively

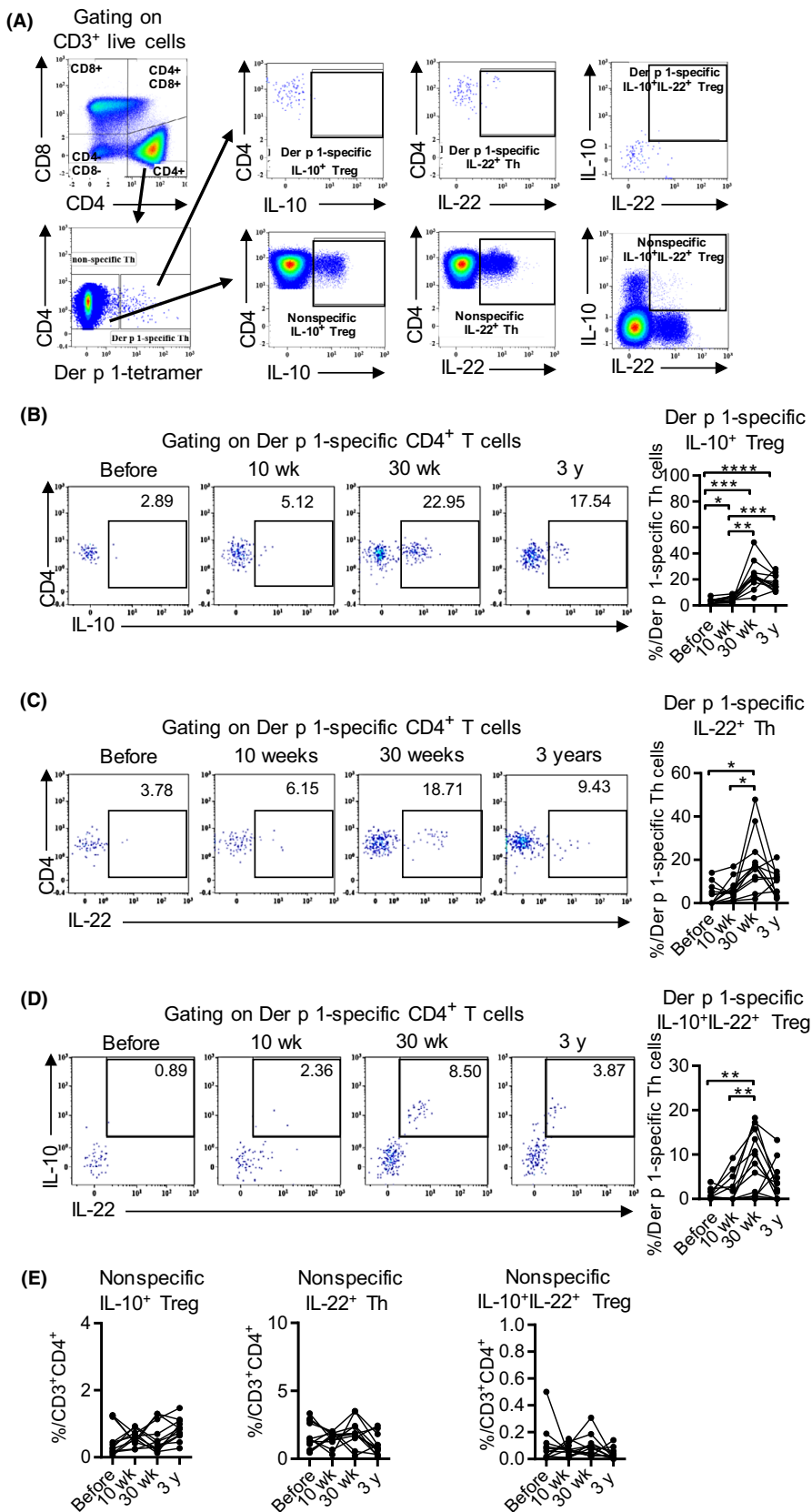


FIGURE 3 Increased IL-10- and IL-22-producing Der p 1-specific CD4⁺ T-cell responses during allergen-specific immunotherapy (AIT). A, Gating strategy of the cytokine profile of nonspecific and Der p 1-specific CD4⁺ T cells. Peripheral blood mononuclear cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 h followed by brefeldin A for 2 h before staining and flow cytometry analysis. B-D, Representative dot plots (left) and the frequencies (right) of Der p 1-specific IL-10⁺ Treg, IL-22⁺ Th, and IL-10⁺IL-22⁺ Treg cells during AIT (n = 12). E, Frequency of nonspecific IL-10-, IL-22-, and both IL-10- and IL-22-producing CD4⁺ T cells during AIT. The values are presented as the mean value. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 [Colour figure can be viewed at wileyonlinelibrary.com.]

upregulated in CD4⁺ T cells during Th2 and T follicular helper cell responses to alum-containing protein vaccines.⁴⁴ The presence of local FOXP3⁺CD25⁺ Treg cells in the nasal mucosa increased number

after immunotherapy, and their association with clinical efficacy and suppression of seasonal allergic inflammation revitalizes the concept of local allergen tolerance depending on Treg cells.⁴⁵

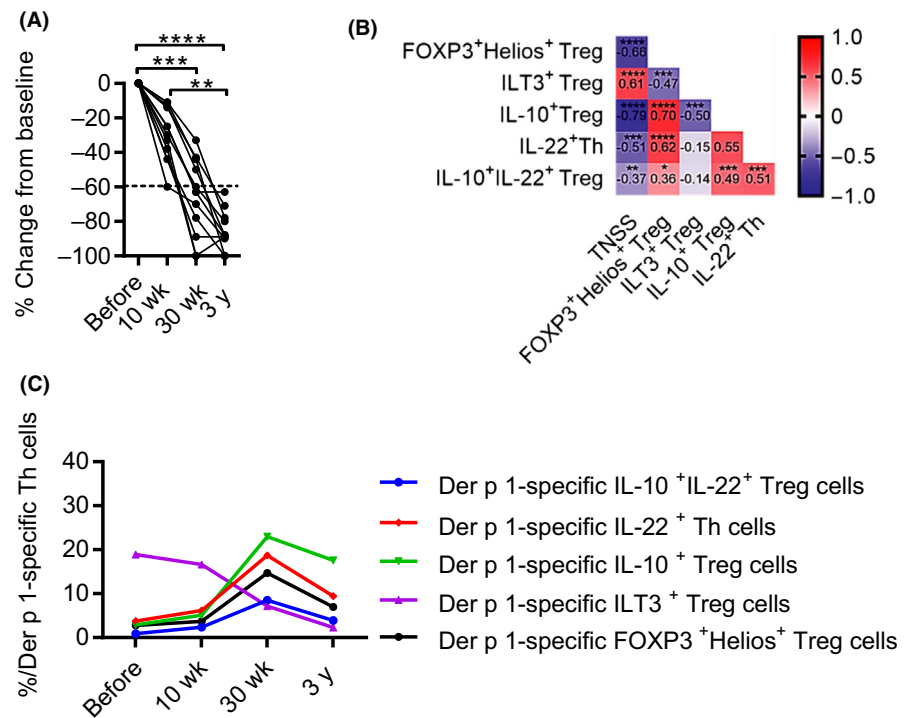


FIGURE 4 Reduced total nasal symptom score (TNSS) during allergen-specific immunotherapy (AIT) was linked to changes in Treg cell responses. A, Percent change of TNSS from baseline of 12 clinical responder patients during AIT. B, Heatmap Spearman correlation between all significantly changed parameters. C, Time course of Der p 1-specific Treg cell subsets changed during AIT. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

The dysfunctional allergen-specific ILT3⁺CD4⁺CD25⁺FOXP3⁺ Treg cells constitute a small subpopulation of allergen-specific Treg cells. We found that the percentage of allergen-specific ILT3⁺ Treg cells was significantly higher before the treatment, which may suggest the inability of Tregs to efficiently suppress allergen-specific Th2 responses. We showed here that human ILT3⁺ Treg cells had reduced suppressive activity and low FOXP3 and Helios expression suggesting a negative role for ILT3⁺ Tregs in allergen tolerance. Supporting our findings, it has been shown in mice that ILT3⁺ Treg cells fail to suppress GATA-3 expression and instead induce expression of IL-5 and IL-13.²⁵ FOXP3 plays an essential role in the maintenance of immunological tolerance to self and innocuous foreign antigens.⁴⁶ FOXP3 homodimers can mediate transcriptional regulation through direct binding to DNA, and FOXP3 has also been shown to interact with several molecular partners forming FOXP3/protein complexes that are required for several regulatory processes.⁴⁶

Recently, new allergen-specific T-cell subsets have been identified in allergic patients during AIT. Special AT-rich sequence-binding protein-1 (SATB1) is repressed in FOXP3⁺ Tregs following grass-specific immunotherapy, and its expression correlates with the clinical response.^{47,48} Allergen-specific CD45RB^{low}CD27⁺CRTH2⁺CD161⁺CD49d⁺ T cells (Th2A) are more frequent in allergic patients compared to nonallergic individuals and suggesting a role for the control of clinical allergic reactions by these cells that are induced by immunotherapy.⁴⁹ Also in long-term grass-specific immunotherapy, a clinical improvement during 2 years of treatment has been associated with the decrease in numbers of the allergen-specific CRTH2⁺CCR4⁺CD27⁺CD4⁺ Th2 cells.⁵⁰ Indeed, there are now many studies confirming the role of decreased Th2 responses in the control of allergic disease and Treg cells play an essential role in the suppression of Th2 cells.

In the present study, IL-10⁺ Treg cells had an early response in low-dose AIT and correlated with the reduction of allergic symptoms. Allergen-specific IL-10-secreting Treg cells are essential components of the induction and maintenance of peripheral allergen tolerance enhanced by AIT.⁵¹ IL-22 is a unique cytokine produced by immune cells, which acts specifically on tissue cells at mucosal sites, since only cells of epithelial origin preferentially express IL-22R1.⁵² IL-22 influences terminal differentiation of keratinocytes by inducing antibacterial protein production, and increases the innate immune defense mechanism against microbes and enhances production of chemokines.⁵³ However, an uncontrolled output of IL-22 may lead to inflammation that has been observed in several diseases including atopic dermatitis, psoriasis, or rheumatoid arthritis.⁵⁴ IL-22 is a member of the IL-10 cytokine family but is usually detected in Th17 cells together with the production of IL-17 and type 3 innate lymphoid cells.⁵⁵ In this study, we found that increased allergen-specific IL-22-secreting CD4⁺ T cells might relate to the increased frequency of allergen-specific IL-10-secreting Treg cells.

It is also important to emphasize that direct analysis of the in vivo situation of circulating allergen-specific T cells is an advantage of the present study compared to cell cultures. Serum specific IgE/total IgE ratio and IgE-FAB, and allergen-specific IgG4 have been considered as potential surrogate candidate biomarkers.⁵⁶ The area is open for further research to discover biomarkers for point-of-care usage because the early prediction of success and to decide when to stop is essential for the patient compliance, cost-effectiveness, and avoidance of side effects. These studies require sophisticated techniques. However, a good biomarker should be studied in easily accessible samples with a rapid method. As similar findings have been reported in healthy immune response to allergens, it may suggest that similar mechanisms can be necessary in the prevention of HDM allergy. Nonetheless, a larger scale study, a randomized

controlled study in particular, comparing HDM-specific antibody-based assays vs Treg-based assays is warranted for identifying a proper biomarkers predicting or monitoring AIT responders.

In conclusion, our results indicate that AIT involves upregulation of the activated allergen-specific Treg cells and downregulation of dysfunctional allergen-specific Treg cell subset. Also, we show that HDM-specific immunotherapy leads to a reduction of allergic symptoms which correlate with Der p 1-specific Treg cell subsets and IL-22-secreting CD4⁺ T-cell responses.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

T.B. carried out the experiment. T.B., M.S., H.M., B.R., J.K., M.W., K.R., and C.A. conceived and planned the experiments. T.B. and M.S. wrote the manuscript with support from K.R., M.A., and C.A. T.B., A.S., P.P., and R.F. recruited the participants. P.T. and S.S. prepared the samples. W.K. provided the peptide-MHCII tetramers. K.R., M.A., and C.A. supervised the study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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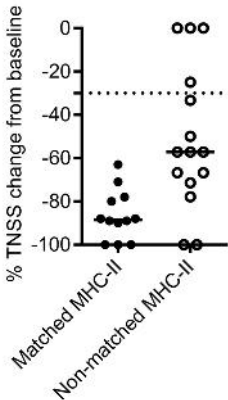


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1 **TABLE S1.** Patient characteristics

Patient no.	Sex	Age	HLA-DRB1	Disease	Total nasal symptom score				SPT Der p (mm)	Total IgE (ng/ml)	Visual analogue scale (mm)	Medication score	
					Before	10 weeks	30 weeks	3 years				Before	3 years
1	Male	33	0401	AR, AD	9.00	5.00	1.00	1.00	7	54.72	8.5	5	0
2	Male	41	1101	AR, AD	9.00	6.00	0.00	1.00	10	81.98	8	6	0
3	Female	22	1501	AR	10.00	4.00	3.00	1.00	9	59.17	9	7	1
4	Female	32	0401	AR	8.00	6.00	3.00	1.00	8	91.11	7.5	6	1
5	Female	27	1501	AR, AD	9.00	8.00	6.00	2.00	6	23.61	9	5	1
6	Male	24	1501	AR	8.00	5.00	0.00	0.00	7	329.69	9.5	6	0
7	Male	35	1101	AR	7.00	6.00	4.00	2.00	6	170.95	6.5	5	2
8	Female	50	1501	AR	9.00	5.00	2.00	0.00	8	98.06	8	6	0
9	Male	25	0401	AR, AA	8.00	6.00	3.00	3.00	6	36.7	7.5	4	1
10	Female	64	1501	AR, AA	10.00	7.00	4.00	2.00	6	46.17	9	6	1
11	Female	52	1101	AR, AA	9.00	8.00	5.00	0.00	6	183.98	7.5	5	0
12	Female	52	0401	AR, AA	8.00	7.00	4.00	1.00	8	112.03	7	4	1

2 AA; Allergic asthma, AD; Atopic dermatitis, AR; Allergic rhinitis, SPT; Skin prick test

3 Total nasal symptom score (TNSS) included rhinorrhea, nasal itching, nasal obstruction and sneezing. The severity is 0 = no symptoms, 1 = mild, 2 = moderate
4 and 3 = severe.

5 Total medication score; 1 point: for beta-2 agonists, antihistamine, pseudoephedrine and montelukast, 2 points: inhaled/intranasal steroids, 3 points: one tablet of
6 corticosteroid

TABLE S2. Der p 1 T-cell epitopes

Tetramer Reagents	Sequence
DR0401/Der p 1 233-252	HSAIAVIIGIKDLDAFRHYD
DR1101/Der p 1 185-204	GVVQESYYRYVAREQSCRRP
DR1501/Der p 1 265-284	PNYHAVNIVGYSNAQGVDYW