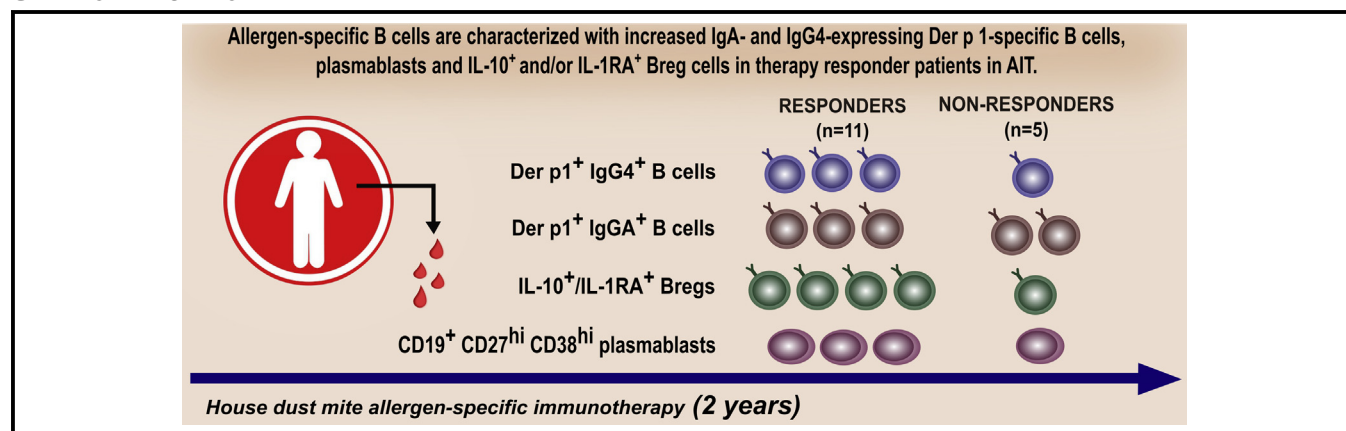


Role of Der p 1-specific B cells in immune tolerance during 2 years of house dust mite-specific immunotherapy



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GRAPHICAL ABSTRACT



Background: Long-term follow-up of allergen-specific B cells in terms of immunoglobulin isotype expression, plasmablast differentiation, and regulatory B (Breg) cell development during allergen-specific immunotherapy (AIT) has not been reported. **Objective:** Allergen-specific B-cell responses during 2 years of house dust mite AIT were compared between responder and nonresponder patients. **Methods:** B cells specific for Der p 1 were detected by using the fluorochrome-labeled allergen method. The frequency of IgA⁺, IgG1⁺

and IgG4-switched Der p 1-specific B cells, plasmablasts, and IL-10- and IL-1 receptor antagonist (IL-1RA)-producing Breg cells were investigated and correlated to clinical response to AIT.

Results: Sixteen of 25 patients completed the 2-year study. Eleven responder patients showed a successful response to AIT, as measured by a decrease in symptom-medication scores from 13.23 ± 0.28 to 2.45 ± 0.24 ($P = .001$) and a decrease in skin prick test reactivity to house dust mite from 7.0 ± 1.3 to 2.7 ± 0.5 mm ($P = .001$). IgG4⁺ and IgA⁺ Der p 1-specific B cells showed a significant increase after AIT, with a significantly greater frequency in responders compared with nonresponders in the IgG4⁺ but not the IgA⁺ fraction. The frequency of plasmablasts and IL-10- and/or IL-1RA-producing Breg cells was greater among responders compared with nonresponders after 2 years. The increased frequency of Der p 1-specific IgG4⁺ B cells, plasmablasts, and IL-10⁺ and dual-positive IL-10⁺IL-1RA⁺ Breg cells significantly correlated with improved clinical symptoms over the course of AIT.

Conclusion: Allergen-specific B cells in patients responding to AIT are characterized by increased numbers of IgA- and IgG4-expressing Der p 1-specific B cells, plasmablasts, and IL-10⁺ and/or IL-1RA⁺ Breg cells. (J Allergy Clin Immunol 2019;143:1077-86.)

Key words: Allergen-specific immunotherapy, house dust mite, B cells, plasmablasts, immunoglobulin isotype, IgG4, IgA, regulatory B cells, IL-10, IL-1 receptor antagonist, immune tolerance

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Allergen-specific immunotherapy (AIT) is the only curative treatment inducing clinical and immunologic tolerance to causative allergens in allergic patients.¹⁻³ Investigation of B-cell

Abbreviations used

AIT:	Allergen-specific immunotherapy
BCR:	B-cell receptor
Breg:	Regulatory B
DC-SIGN:	Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
HDM:	House dust mite
IL-1RA:	IL-1 receptor antagonist
PE:	Phycoerythrin
SMS:	Symptom-medication score
TMS:	Total medication score
Treg:	Regulatory T
VAS:	Visual analog scale

responses among patients receiving venom immunotherapy and naturally exposed beekeepers suggested a similar functional immunoregulatory role for B cells, enhanced IL-10-secreting regulatory B (B_R1) cells, and IgG₄-secreting phospholipase A₂-specific B cells in allergen tolerance.⁴ AIT also induces IL-10-producing regulatory B (Breg) cells and allergen-specific IgG₄.⁴⁻⁶

IL-10 is produced by T_H1 and B_R1 cells and also produced by T_H2 cells in combination with T_H2 cytokines. IL-10 has multiple functions in the regulation of immune responses by suppressing antigen-presenting cells, eosinophils, and basophils and allergen-specific CD4⁺ T cells through direct and indirect mechanisms and induction of IgG₄ isotype antibodies by B cells.⁷⁻¹⁰ Allergen-specific IgG₄ can function as a blocking antibody by preventing allergen-IgE binding, thereby inhibiting mast cell and basophil degranulation.^{11,12} Its production is selectively regulated by T_H1 and B_R1 cells.⁵ In addition, AIT can induce somatic mutation of IgG₄-switched allergen-specific B cells, leading to generation of high-affinity antibodies.¹³ Furthermore, AIT results in reduced expression of antigen-specific IgE and increased expression of allergen-specific IgA as a delayed response after months to years. Increased allergen-specific IgA₂ responses correlating with increased local TGF-β expression and increased monocyte IL-10 expression have also been reported.¹⁰

IL-1 is a potential mediator of inflammation and tissue damage in multiple organs. The IL-1 family is a group of 11 cytokines,¹⁴ with IL-1α and IL-1β as the most studied members. IL-1 receptor antagonist (IL-1RA) binds to IL-1 receptor and thereby prevents binding of IL-1α and IL-1β and reduces the inflammation related to IL-1 in patients with various diseases.¹⁴ IL-1RA deficiency causes a very rare autoinflammatory disease with an inappropriate lack of control of inflammation.¹⁵ IL-1RA is produced by monocytes, macrophages, neutrophils, epithelial cells, fibroblasts, and mesenchymal stem cells.¹⁶

House dust mite (HDM) is a common cause of perennial allergy. Dust mites can survive throughout the year in warm and humid indoor conditions. Der p 1 is the major HDM allergen inducing IgG and specific IgE antibody synthesis.¹⁷ Its cysteine protease activity cleaves CD40 on dendritic cells, plays a role in directing dendritic cells to induce T_H2 differentiation, and stimulates the epithelial-mesenchymal transition in airway epithelial cells, leading to airway remodeling.^{18,19} Intranasal administration of proteolytic active Der p 1 to sensitized mice leads to enhanced inflammatory cellular infiltration in the lung and systemic production of IgE compared with inactive Der p 1, which has no effect.²⁰ Der p 1 can activate protease-activated receptor 2 signaling pathway

through Akt and pp65 to increase IL-6 and IL-8 expression.²¹ Moreover, *Dermatophagoides pteronyssinus* can be endocytosed into dendritic cells through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and reduces DC-SIGN expression to change the polarization of dendritic cells, leading to the T_H2 cytokine immune response.²²

In the present study we investigated the role of allergen-specific B cells in induction of clinical and immune tolerance to HDM. Allergen-specific B cells are present in circulation at very low frequencies.²³ Small fractions (<0.05%) of B cells express a B-cell receptor (BCR) that binds fluorescent dyes, such as phycoerythrin (PE).²⁴ Therefore accurate detection of allergen-specific B cells requires staining with antigens labeled with 2 structurally unrelated fluorescent dyes.

We present here a reproducible technique to identify Der p 1-specific B cells using dual-color allergen-labeled fluorescence staining. Multicolor flow cytometry was used to determine and follow-up the immunoglobulin isotype expressed by Der p 1-specific B cells during 2 years of HDM AIT. The purpose of this article was to identify a biomarker for immune response to AIT. Although our data did not show a clinically useful biomarker, we identified AIT responders and nonresponders after 2 years and demonstrated that especially IgA⁺ and IgG₄⁺ Der p 1-specific B cells, as well as plasmablasts, expanded in patients who responded to therapy. In parallel, CD73⁺CD25⁺CD71⁺ expressing Breg cells, which also produce IL-10 and IL-1RA, increased *in vivo* during AIT. Numbers of allergen-specific IgG₄⁺ B cells, plasmablasts, and IL-10⁺ and/or IL-1RA⁺ Breg cells positively correlated with improved clinical response during AIT.

METHODS

Subjects

In a prospective observational study between April 2013 and September 2016, 25 patients with HDM allergy were recruited in the Phramongkutklao Hospital (Bangkok, Thailand). All participants met the criteria, including a history and objective findings of HDM-related rhinitis, asthma, or atopic dermatitis. The allergic patients had positive skin prick test responses (wheal diameter ≥6 mm larger than that elicited by the negative control) to *D pteronyssinus* extract (ALK-Abelló, Hørsholm, Denmark). Allergic symptoms were monitored with a visual analog scale (VAS), which is a continuous scale 100 mm in length that is validated to use among patients with allergic rhinitis and those with allergic asthma.²⁵⁻²⁷ The patients marked on a line the point they believed represented their perceptions of their current state.¹⁷ Patients had to record on the same questionnaire when they used medication (1 point = use of β₂-agonists, antihistamine, pseudoephedrine, and montelukast; 2 points = use of inhaled/intranasal corticosteroid; and 3 points = use of 1 tablet of corticosteroid) and were calculated as the total medication score (TMS).²⁸ The symptom-medication score (SMS) combines clinical symptom (VAS) scores and TMSs, which represent treatment efficacy and assess the different outcome measures to discriminate between effective treatment and placebo.^{29,30} Baseline patients' characteristics, VAS scores, mean wheal diameters of positive responses to *D pteronyssinus*, and TMSs are shown in Table E1 in this article's Online Repository at www.jacionline.org. The cutoff points for the responders have been defined as an SMS decrease of greater than 50% in comparison between before and after 2 years of AIT.

PBMCs were isolated at baseline and after 10 weeks, 30 weeks, and 2 years of AIT. During the AIT period, PBMCs and plasma samples were kept frozen in liquid nitrogen until analysis. A 30-week weekly dose-escalation protocol was used with subcutaneous administration of standardized mite mix and contained 50% vol/vol glycerin, 0.4% phenol (preservative), sodium chloride, sodium bicarbonate, 5000 allergy units/mL *D pteronyssinus*, and 5000 allergy units/mL *Dermatophagoides farinae* (ALK-Abelló).

Sixteen of 25 patients completed the study, and 9 patients discontinued AIT, 3 for economic reasons, 5 for logistic challenges, and 1 because of local reactions. The protocol of HDM-specific immunotherapy is shown in Table E2 in this article's Online Repository at www.jacionline.org. The study protocol was approved by the Royal Thai Army Medical Department Ethics Committee, IRBRTA 67/2556, and conducted with the written consent of all participants.

Flow cytometry

PBMCs (10^7) were stained with eF780 viability dye (eBioscience, San Diego, Calif) to define the frequency of Der p 1-specific B cells. Then cells were washed in staining buffer (PBS, pH 7.2, 0.5% BSA, and 2 mmol/L EDTA) and resuspended in 100 μ L staining buffer. Fc receptor blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to cells, according to the manufacturer's instructions. Afterward, cells were stained with antibodies against surface markers (see Table E3 in this article's Online Repository at www.jacionline.org) at 4°C for 15 minutes. Alexa Fluor 488-labeled IgA (Jackson Laboratory, Bar Harbor, Me) and Dy405-labeled IgG₁ (Sanquin, Amsterdam, The Netherlands) were stained to detect IgA and IgG₁, respectively. For IgG₄ staining, anti-IgG₄ antibody (Alta Bioscience, Birmingham, United Kingdom) labeled with biotin (Innova Biosciences, Cambridge, United Kingdom) and Streptavidin-PE/Dazzle 594 (BioLegend, San Diego, Calif) was used. For a detailed description of the procedure used to identify Der p 1-specific B cells, see the Methods section and Fig E1 in this article's Online Repository at www.jacionline.org.

For intracellular cytokine staining, 5×10^6 PBMCs were stimulated for 48 hours with 1 μ mol/L of the Toll-like receptor 9 ligand CpG 2006 (Mycosynth, Balgach, Switzerland) in 2 mL of complete RPMI 1640 medium (Sigma-Aldrich, St Louis, Mo). After 48 hours, PBMCs were stimulated with 20 ng/mL phorbol 12-myristate 13-acetate and 1 μ g/mL ionomycin for 2 hours, followed by 2 hours in the presence of 10 μ g/mL brefeldin A (all from Sigma-Aldrich). After surface staining, cells were washed with staining buffer and fixed and permeabilized with Cytofix/Cytoperm Kit (BD Biosciences, San Jose, Calif) and stained with anti-IL-10-peridinin-chlorophyll-protein complex/Cy5.5 (clone JES3-9D7; BioLegend) and anti-IL-1RA-fluorescein isothiocyanate (clone CRM17; eBioscience, San Diego, Calif), incubated for 30 minutes in the dark at 4°C, and washed with staining buffer. All samples were analyzed and sorted with a FACSria III (Beckton Dickinson, Franklin Lakes, NJ). Data were analyzed with Kaluza Software (Beckman Coulter, Indianapolis, Ind).

Isolation of Der p 1-specific B cells

Purified rDer p 1 (Allergopharma AG, Therwil, Switzerland) was labeled with the Lightning-Link Dy633 Rapid Conjugate System and the Lightning-Link PE/Cy7 Tandem Conjugate Kit (both from Innova Biosciences). Conjugated Der p 1 constructs were titrated for optimal staining concentrations. Der p 1-specific class-switched B cells were gated as CD19⁺IgM⁺ and double positivity for Der p 1-Dy633 and Der p 1-PE/Cy7. As a negative control, cells were incubated with purified unlabeled Der p 1 for 15 minutes in the dark at 4°C to block Der p 1-specific BCRs before adding the conjugated Der p 1.

Der p 1-specific antibody detection

Der p 1 (5 μ g/mL) was coated on a Nunc Maxisorb microtiter plate (Thermo Scientific, Waltham, Mass) at 4°C overnight and then blocked with blocking buffer (PBS [pH 7.4], 1% BSA, and 0.05% Tween 20) to measure Der p 1-specific immunoglobulin levels. Diluted plasma samples (in the blocking buffer) were added and incubated for 2 hours at room temperature. For specific IgG₄ detection, mouse anti-human IgG₄ (Bionostics, Devens, Mass) was used, followed by incubation with peroxidase-conjugated goat anti-mouse IgG (Thermo Scientific). For specific IgA detection, goat anti-human IgA-horseradish peroxidase (Bethyl Laboratories, Montgomery, Tex) and tetramethylbenzidine substrate were used. The reaction was stopped by 2 mol/L sulfuric acid, and OD450 values were measured with a Mithras LB 940

spectrophotometer (Berthold Technologies, Bad Wildbad, Germany). Results were expressed as arbitrary units based on the positive in-house pool of plasma from patients with HDM allergy.

Statistical analysis

Data were expressed as means $\pm$ SEMs. ANOVA and Dunn multiple comparison tests were used, comparing related samples at different time points. The Mann-Whitney *U* test was used to compare differences between responder and nonresponder groups. The Spearman test was performed for correlation analyses. The correlation network was performed with R software (version 3.4.2; R Foundation for Statistical Computing, Vienna, Austria). Statistical analysis was performed with GraphPad Prism 7.0 software (GraphPad Software, La Jolla, Calif). A *P* value of less than .05 was considered statistically significant.

RESULTS

Characterization of responder and nonresponder patients after 2 years of AIT

Sixteen patients who completed 2 years of AIT were classified as responders or nonresponders by using SMSs measured at baseline and after 2 years of AIT. Eleven patients in the responder group improved clinically based on decreases in SMSs from 13.23 ± 0.28 to 2.45 ± 0.24 ($P = .001$), whereas SMSs decreased from 13.1 ± 1.1 to 10 ± 0.9 ($P = .12$) in 5 clinical nonresponder patients before and after 2 years of AIT, respectively (Fig 1, A). Also, the percentage change in SMSs decreased significantly in responders more than in nonresponders (Fig 1, A). In the responder group mean wheal diameter of skin prick test reactivity to HDM decreased from 7.0 ± 1.3 to 2.7 ± 0.5 mm ($P = .001$), whereas it decreased from 7.6 ± 1.1 and 5.6 ± 0.5 mm ($P = .06$) in nonresponder patients (Fig 1, B). Moreover, the relative percentage difference in mean wheal diameter was reduced significantly in responders compared with nonresponders (Fig 1, B). In addition, SMSs and mean wheal diameters of positive skin prick test responses showed positive correlation in responders ($r = 0.77$, $P = .0002$) and nonresponders ($r = 0.18$, $P = .43$) after 2 years (see Fig E2, A, in this article's Online Repository at www.jacionline.org). VAS scores and TMSs also decreased significantly in responders more than in nonresponders (see Fig E2, B and C).

Increased frequency of Der p 1-specific B cells during the 2 years of AIT

Der p 1-specific class-switched B (CD19⁺IgM⁺Der p 1⁺) cells were analyzed by using flow cytometry to study allergen-specific B-cell responses during AIT. The gating strategy and control are shown in Fig E3 in this article's Online Repository at www.jacionline.org. The frequency of Der p 1-specific B cells was investigated before the start, at 10 and 30 weeks of AIT, and after 2 years of AIT. In responder patients AIT significantly increased the frequency of Der p 1-specific class-switched B cells after 30 weeks and 2 years of AIT compared with baseline. Mean changes were from $0.049\% \pm 0.012\%$ at baseline to $0.073\% \pm 0.019\%$ at 10 weeks, $0.097\% \pm 0.021\%$ at 30 weeks, and $0.165\% \pm 0.029\%$ after 2 years (Fig 2, A and B).

Next, we analyzed a biomarker for AIT treatment efficacy. Increased numbers of Der p 1-specific B cells from baseline to 2 years were observed in responders (3.60 ± 1.20 -fold increase

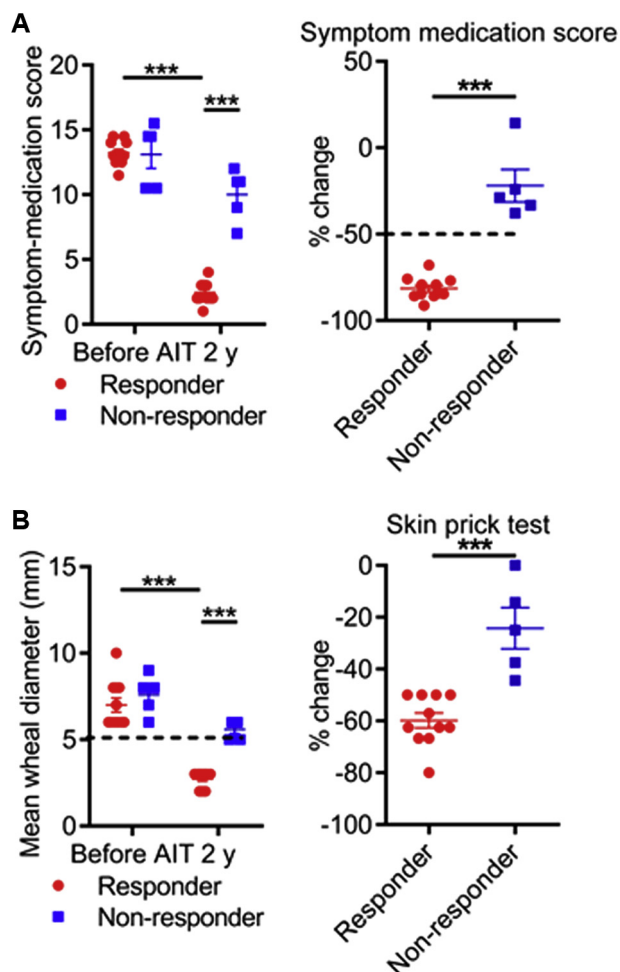


FIG 1. Decreased clinical symptoms and HDM sensitization after 2 years of AIT in responder patients. **A**, Clinical response was assessed based on SMSs in patients with HDM allergy undergoing subcutaneous injection AIT (responder patients, $n = 11$; nonresponder patients, $n = 5$) and percentage change in SMS between before and after 2 years of AIT. **B**, Mean wheal diameter and percentage change in positive *D pteronyssinus* skin prick test responses compared before and after 2 years of AIT. *** $P < .001$.

after 2 years) compared with nonresponder patients (0.59 ± 0.41 -fold increase; Fig 2, C).

Increased IgA and IgG₄ isotypes of Der p 1-specific B cells and antibodies during AIT

We measured expression of IgA, IgG₁, and IgG₄ immunoglobulin isotypes on Der p 1-specific class-switched B cells. The representative gating strategies used to investigate the expression of IgA, IgG₁, and IgG₄ of Der p 1-specific (Der p 1⁺) class-switched B cells are shown in Fig E3. The frequency of IgA⁺- and IgG₄⁺-secreting Der p 1⁺ B cells as a percentage of total B cells increased significantly after 2 years (Fig 3, A-E). IgA⁺ Der p 1-specific B cells showed expansion after 10 weeks and continued to increase during AIT in responder patients (Fig 3, B), without showing any significant difference between responder and nonresponder patients (Fig 3, C). IgG₄⁺ Der p 1-specific B cells were detected at very low frequencies in allergic patients before the start of AIT

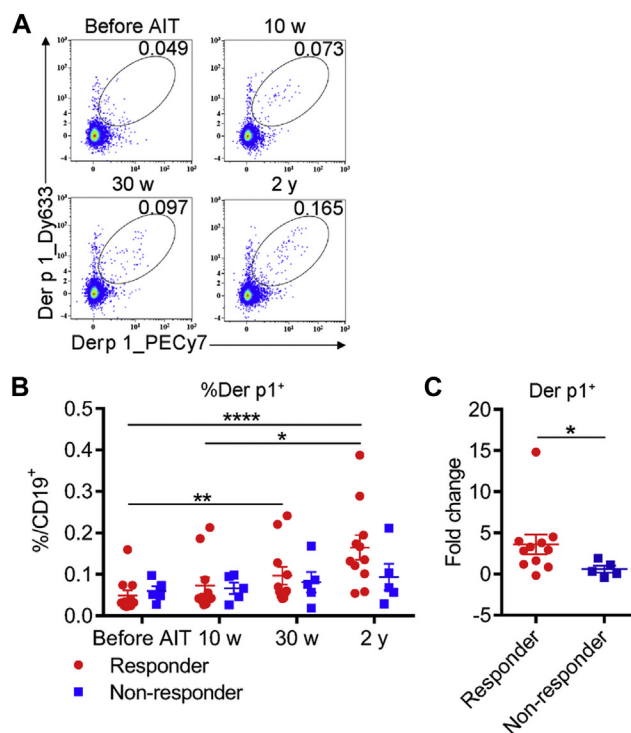


FIG 2. Increased Der p 1-specific class-switched B-cell frequencies in responder patients during AIT. **A**, Representative dot plot of a representative responder patient. Cells were gated as CD19⁺IgM⁻, and Der p 1-specific cells were identified as dual stained for Der p 1-Dy633⁺ and Der p 1-PECy7⁺. **B**, Frequency of Der p 1-specific class-switched B cells before and after 10 weeks, 30 weeks, and 2 years of AIT. **C**, Fold change of Der p 1-specific class-switched B cells between before and after 2 years of AIT among responder patients ($n = 11$) and nonresponder patients ($n = 5$). * $P < .05$, ** $P < .01$, and **** $P < .0001$.

and showed a significant increase after 30 weeks and continued to increase after 2 years of AIT (Fig 3, D). AIT responder patients showed a significantly greater frequency of IgG₄⁺ Der p 1-specific class-switched B cells ($0.27\% \pm 0.006\%$) compared with nonresponder patients ($0.008\% \pm 0.002\%$) after 2 years of AIT (Fig 3, D). Moreover, the degree of change (between before and after 2 years) of IgG₄⁺ Der p 1-specific B cells was significantly greater in responders (20.52 ± 5.34 -fold difference) compared with nonresponders (6.97 ± 1.91 -fold difference) after 2 years (Fig 3, E). The number of IgG₁⁺ Der p 1-specific B cells did not show any difference between responders and nonresponders at baseline or during the course of AIT (Fig 3, F and G).

Plasma levels of Der p 1-specific IgA and IgG₄ were also determined during the course of AIT. At 2 years, we observed that plasma levels of Der p 1-specific IgA were increased compared with baseline levels in responders, which were greater compared with those in nonresponders (Fig 3, H). Plasma levels of Der p 1-specific IgG₄ continued to increase only in responder patients until 2 years and was significantly higher compared with those in nonresponder patients (Fig 3, H). Collectively, our data demonstrate that plasma levels of Der p 1-specific IgA and IgG₄ increase during the course of AIT in responder patients.

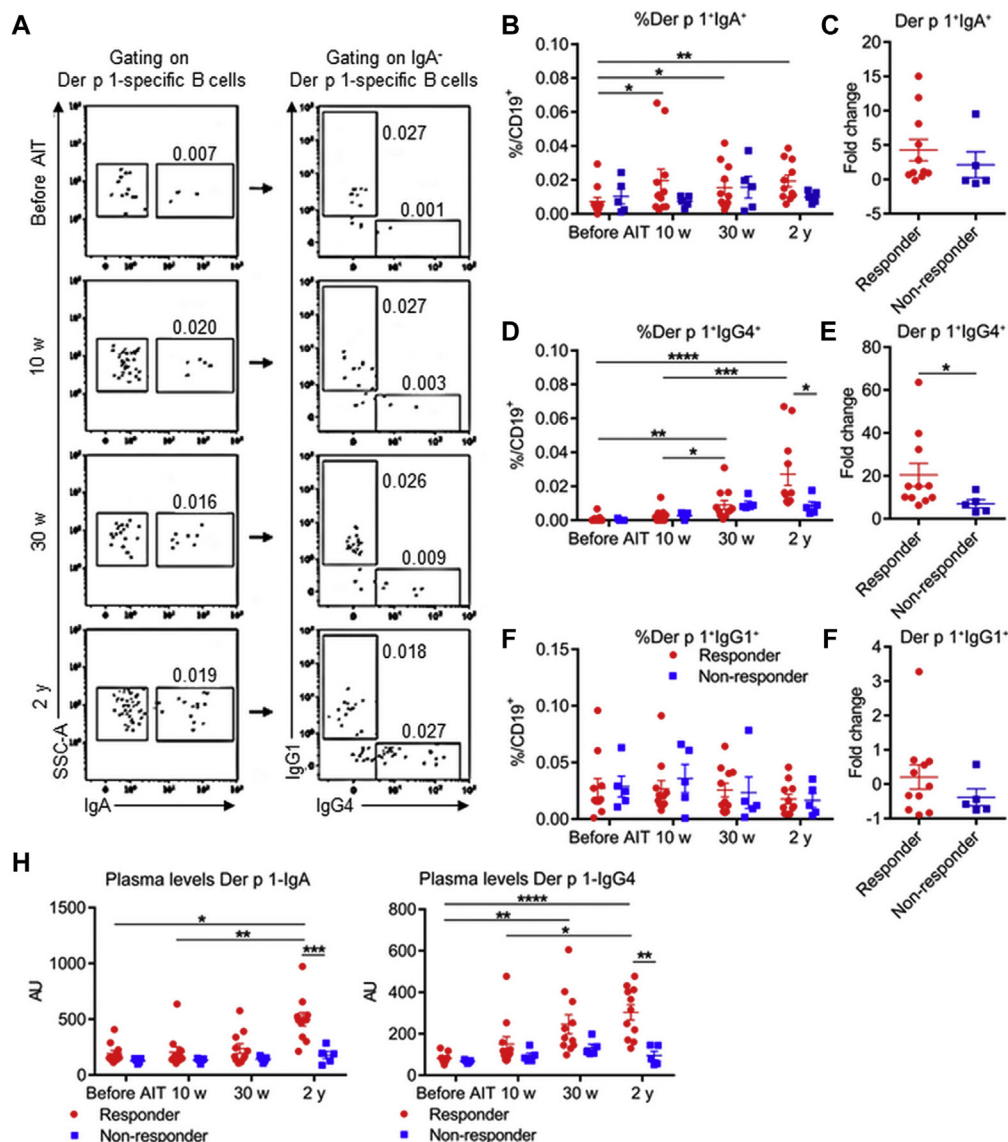


FIG 3. Increased Der p 1-specific IgA- and IgG₄-switched B-cell frequencies in responder patients during AIT. **A**, Representative dot plots of Der p 1-specific IgA⁺, IgG₄⁺, and IgG₁⁺ B cells of responder patients. SSC, Side scatter. **B–G**, Frequencies of Der p 1-specific IgA⁺, IgG₄⁺, and IgG₁⁺ B cells before and after 10 weeks, 30 weeks and 2 years of AIT and fold change between before and after 2 years of AIT compared between responder (n = 11) and nonresponder (n = 5) patients. **H**, Plasma levels of Der p 1-specific IgA and IgG₄ during AIT. Values are presented in arbitrary units (AU). **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001.

Increased circulating plasmablast response during AIT

Plasmablasts were identified as CD19⁺CD27^{hi}CD38^{hi} cells in the circulation. Their gating strategy is shown in Fig E3. The percentage of circulating plasmablasts increased in both groups, with their frequencies significantly increased from 0.20% ± 0.01% to 0.30% ± 0.02% after 30 weeks and to 0.43% ± 0.03% after 2 years in responder patients during the course of AIT, and the increase in plasmablast frequency in nonresponders was not significant (Fig 4, A and B). The mean frequency of plasmablasts in responder patients was also significantly greater compared with that in nonresponder patients after 2 years (Fig 4, B). Moreover, plasmablasts increased 1.21 ± 0.2-fold from baseline after

2 years and were 0.52 ± 0.16-fold greater than in nonresponders (Fig 4, C).

IL-10 and IL-1RA single- or double-positive Breg cells expand in responders during AIT

To identify Breg cells, PBMCs were stimulated with the Toll-like receptor 9 ligand CpG 2006 for 48 hours before flow cytometric analysis, and we investigated IL-10- and IL-1RA-producing Breg cells.⁴

In this study we demonstrate that 3 subsets of Breg cells are detectable in peripheral blood. The subsets are defined by staining positively for IL-10, IL-1RA, or both. The gating strategy for

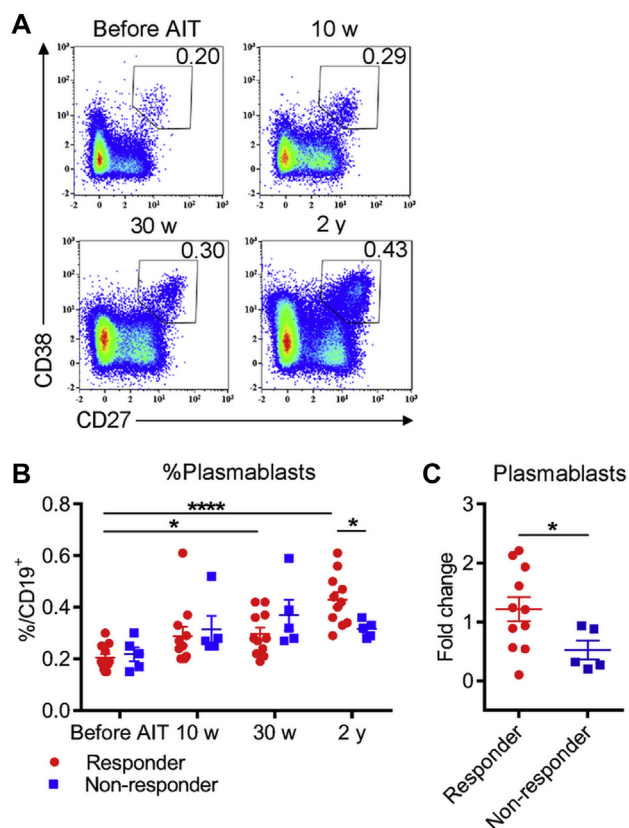


FIG 4. Increased frequency of plasmablasts in responder patients during AIT. **A**, Representative dot plot of a representative responder patient. Cells were first gated for CD19⁺ viable cells, and plasmablasts were defined as CD27^{hi}CD38^{hi} cells. **B** and **C**, Frequencies of plasmablasts before and after 10 weeks, 30 weeks, and 2 years of AIT and fold change between before and after 2 years of AIT in responder patients (n = 11) and nonresponder patients (n = 5). **P* < .05 and *****P* < .001.

Breg cells is shown in Fig E4 in this article's Online Repository at www.jacionline.org, and the frequency of CD73⁺CD25⁺CD71⁺ Breg cells during AIT is shown in Fig E5 in this article's Online Repository at www.jacionline.org. Within the CD73⁺CD25⁺CD71⁺ population, the frequency of IL-10⁺ Breg cells showed a significant difference between responder and nonresponder patients. At 30 weeks of AIT, the frequency of total IL-10⁺ Breg cells was significantly greater in responders (2.27% ± 0.30%) compared with that in nonresponders (1.24% ± 0.13%; Fig 5, A and B). Furthermore, the frequency of total IL-10⁺ Breg cells was significantly increased in AIT responders (2.60% ± 0.21%) compared with that in nonresponders (1.75% ± 0.21%) after 2 years (Fig 5, B). The frequency of total IL-1RA⁺ Breg cells also increased during AIT and showed a significant difference between responders (2.29% ± 0.18%) and nonresponders (1.47% ± 0.41%) after 2 years of AIT (Fig 5, C). In addition, the frequency of dual-positive IL-10⁺IL-1RA⁺ Breg cells significantly increased during AIT (Fig 5, D). After 2 years, responders almost doubled the frequency of IL-10⁺IL-1RA⁺ Breg cells (1.26% ± 0.09%) compared with nonresponder patients (0.65% ± 0.12%; Fig 5, D). As shown in Fig 5, B and D, either single or double IL-10⁺ and IL-1RA⁺ Breg cells showed a tendency to be already greater at the beginning of the study in responders.

Clinical symptoms and B-cell responses during AIT

A correlation analysis of all investigated markers of the study between responder and nonresponder patients before and 2 years after AIT are shown in Fig 6, A. It appeared that a strong immune response that includes multiple parameters of antigen-specific B cells and Breg cells during the course of AIT defines those who respond to therapy. An extensive correlation between B-cell responses was observed in responders compared with nonresponders (Fig 6, A). In addition, the correlation between the clinical symptoms observed with SMSs and the frequency of B-cell subsets was analyzed to investigate the relationship between clinical tolerance and Breg cell-related immune tolerance (Fig 6, B). The IgG₄-secreting Der p 1-specific B cells showed a negative correlation with SMSs in both responders (*r* = −0.75, *P* < .0001) and nonresponders (*r* = −0.89, *P* = .0001). Also, the frequency of plasmablasts negatively correlated with SMSs in both responder (*r* = −0.69, *P* = .0002) and nonresponder (*r* = −0.62, *P* = .04) patients. Moreover, increased numbers of IL-10⁺ Breg cells negatively correlated with clinical symptoms during AIT in both responder (*r* = −0.67, *P* = .0005) and nonresponder (*r* = −0.67, *P* = .03) patients. Also, SMSs negatively correlated with dual-positive IL-10⁺IL-1RA⁺ Breg cells among responder (*r* = −0.42, *P* = .04) and nonresponder (*r* = −0.67, *P* = .03) patients. The frequency of IgG₄-secreting Der p 1-specific B cells also correlated positively with the frequency of plasmablasts and IL-10⁺ Breg cells in both groups (Fig 6, B).

In all these correlation analyses responders showed stronger correlation compared with therapy in nonresponders. The *r* values of correlation and significance of all data of responder and nonresponder patients are shown in Fig E6 in this article's Online Repository at www.jacionline.org.

DISCUSSION

Nonallergic subjects typically have immunologic tolerance to allergens with active immunoregulatory mechanisms or do not show any immune response at all, whereas allergic subjects have an allergic immune response. Molecular mechanisms that drive these processes are better understood; however, there are still many remaining open questions. One of the strengths of this study was direct analyses of human material without any cultures representing the direct *in vivo* situation. *In vivo* human models of natural high-dose allergen exposure and AIT demonstrate that the generation of functional allergen-specific regulatory T (Treg) and Breg cells is an essential component of allergen tolerance induction. However, studies providing detailed analyses of allergen-specific B-cell responses during induction of allergen tolerance remain sparse. One major handicap has been the extremely low frequencies of allergen-specific T and B cells. The present study focuses on HDM allergen, particularly Der p 1-specific immune responses during 2 years of AIT in peripheral blood samples taken before and 10 weeks, 30 weeks, and 2 years after HDM extract subcutaneous AIT. The data presented here highlight important aspects of peripheral allergen-specific B-cell tolerance and link some of the findings to clinical tolerance. As a key finding, the frequency of certain circulating allergen-specific B cells, Breg cell subsets, and plasma cells changed during the course of AIT with differences between responder and nonresponder patients.

In this study a dual-labeling method was used to identify Der p 1-specific B cells. A similar approach has been used for identification of Ara h 1- and Ara h 2-specific B cells in patients

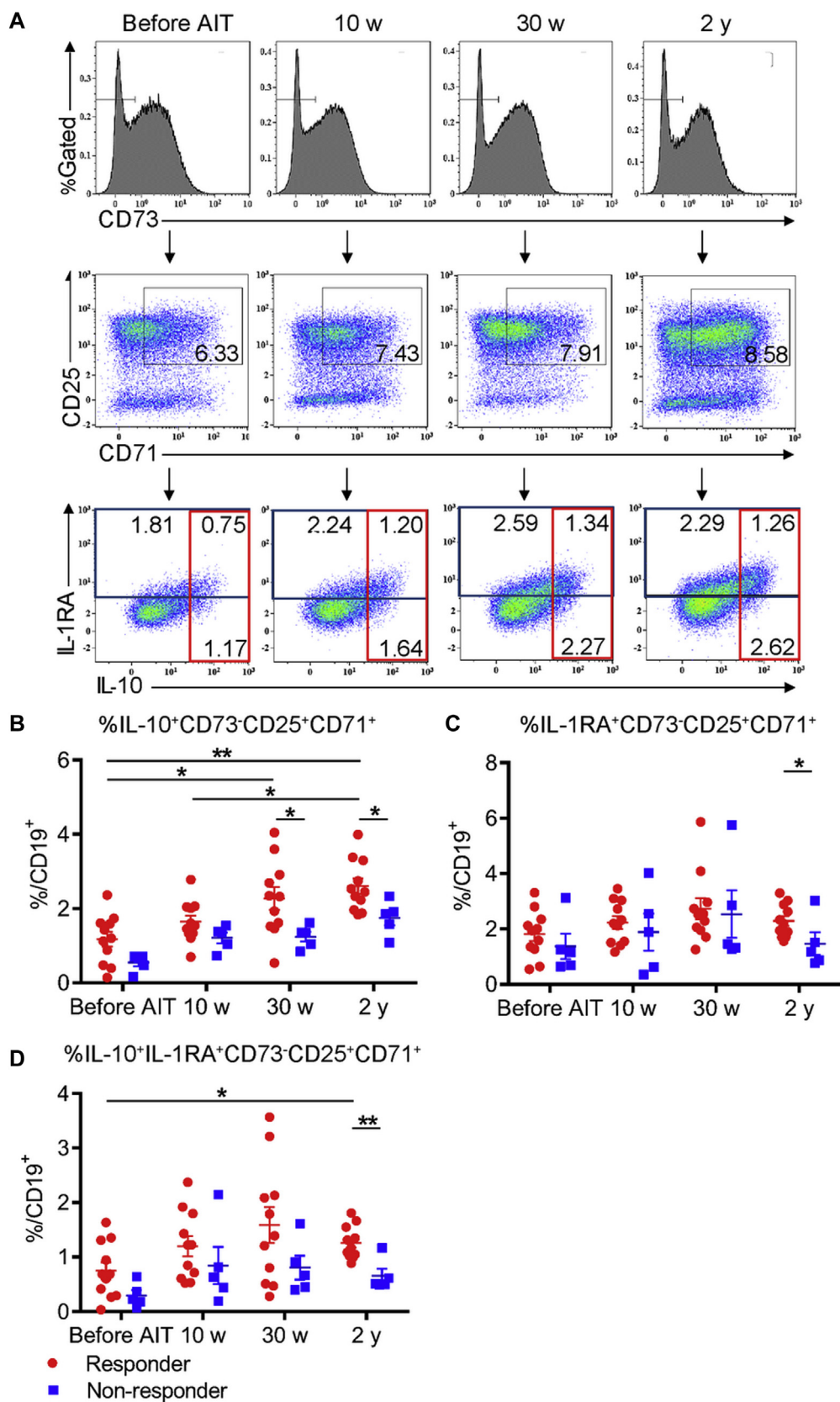


FIG 5. Frequencies of IL-10- and IL-1RA-producing Breg cells increased in responder patients during 2 years of AIT. **A**, Representative dot plots of responder patients. Cells were first gated from CD19⁺ viable cells, and cells were gated as Breg cells. IL-10 and IL-1RA were gated on Breg cells and described as total IL-10⁺ (red), total IL-1RA⁺ (blue), and dual-positive IL-10⁺IL-1RA⁺ Breg cells. **B-D**, Frequencies of total IL-10⁺, total IL-1RA⁺, and dual-positive IL-10⁺IL-1RA⁺ Breg cells before and after 10 weeks, 30 weeks, and 2 years of AIT among responder patients (n = 11) and nonresponder patients (n = 5). **P* < .05 and ***P* < .01.

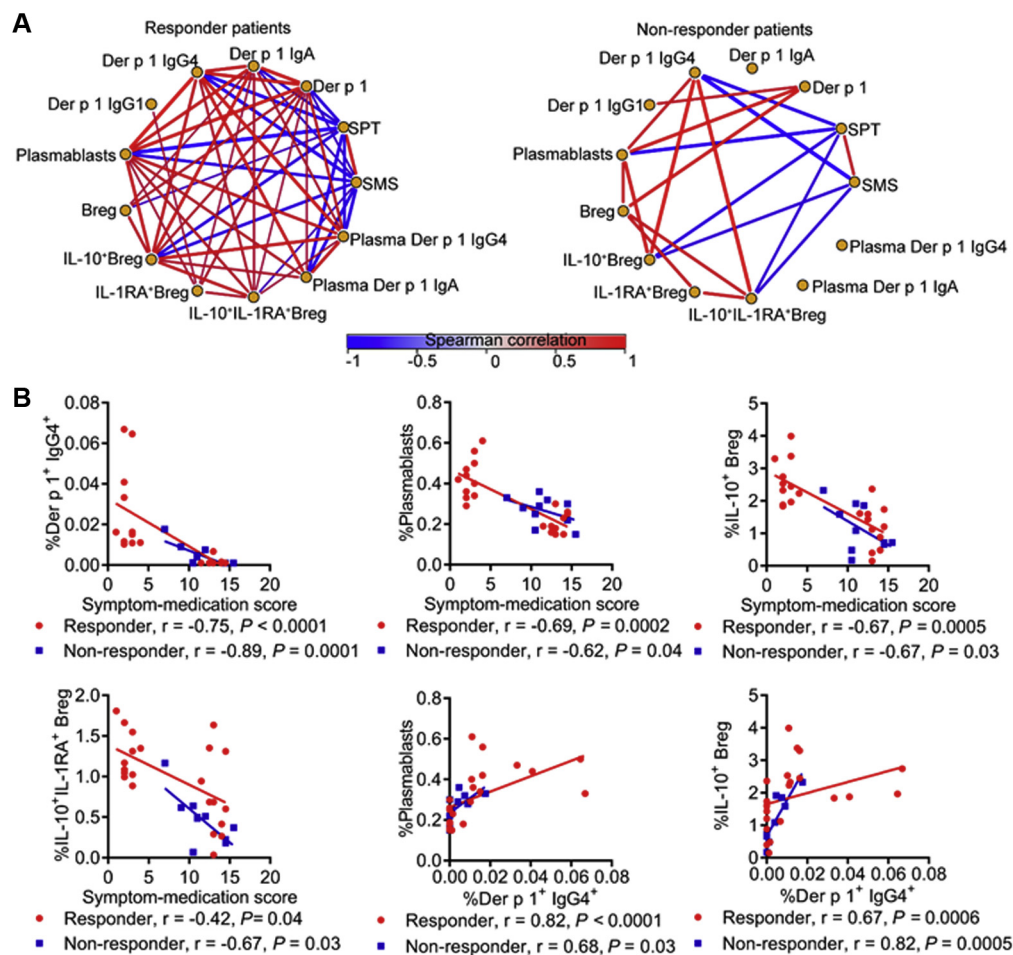


FIG 6. Correlation data of responder and nonresponder patients. **A**, Correlation network displaying Spearman correlation between all comparisons in responder and nonresponder patients. **B**, Correlation between SMSs and Der p 1-specific IgG4⁺ B-cell, plasmablast, total IL-10⁺ cell, and dual-positive IL-10⁺IL-1RA⁺ Breg cell frequencies during AIT between before and after 2 years of AIT.

with peanut allergy and phospholipase A-specific B cells in patients with venom allergy.^{5,9,20} One of the first findings in the present study is the significant increase in Der p 1-specific peripheral blood B-cell frequencies during 2 years of HDM AIT. Interestingly, this increase in total numbers of Der p 1-specific B cells was up to 3-fold in 2 years in average responder patients. These findings suggest a continuous and relatively slow increase in responder patients and show a difference compared with infectious disease vaccine-specific B-cell responses and responses after booster injections of vaccines, which show a stronger and rapid increase after the booster doses.³¹ The difference in the dose and time of intervals between subcutaneous immunotherapy and vaccines for infectious diseases likely results in different immune responses.

In this study we observed an increase in frequencies of allergen-specific IgA⁺ and IgG4⁺ B cells during AIT, which were greater in responder compared with nonresponder patients. Because of their very low frequency, IgE⁺ B cells could not be detected with the same method. Increases in serum allergen-specific IgA and IgG4 levels were also found in responder patients with egg allergy during oral immunotherapy.^{32,33} In grass pollen immunotherapy serum Phl p 5-specific IgA₁ and IgA₂ concentrations also increased after 2 years of treatment.¹⁰ The increase in

Der p 1-specific IgG4⁺ B-cell frequencies showed an initial response after low-dose allergen-specific subcutaneous injection. Bee venom immunotherapy and high levels of environmental allergen exposure in beekeepers promote the generation of functional IL-10-producing Treg cells and high levels of allergen-specific IgG4.³⁴⁻³⁷ The same phenomenon occurs in nonallergic cat owners. The increase in numbers of functional Treg cells and levels of allergen-specific IgG4 is associated with tolerance induction without new sensitizations or asthma development.

IgG4 has multiple features that might suggest a role in immune tolerance. IgG4 does not function in antibody-dependent cellular cytotoxicity because it does not have a specific Fc receptor.³⁸ Moreover, IgG4 might restrict inflammation because of an inability to fix complement and mitigate allergy as a result of competing with IgE as a blocking antibody for allergen binding to IgE Fc receptor-expressing cells.^{11,39,40}

We demonstrate here that numbers of circulating plasmablasts are increased during AIT and even highly expressed at 2 years in responder compared with nonresponder patients. It was an interesting finding to see a non-allergen-specific population change in an antigen-specific treatment, which might be due to a general vaccine effect. This observation needs further research. The frequency of plasmablasts in patients with HDM allergy

treated with AIT seems to be much greater than that in patients with bee venom allergy. In a previous study we observed an increased frequency of plasmablasts during venom immunotherapy, but phospholipase A₂-specific plasmablasts were rarely detected.⁴ Numbers of Ara h 2-specific plasmablasts also increased during peanut oral immunotherapy.¹³ Intralymphatic immunotherapy induces grass pollen antigen-specific plasmablasts and increases tolerance to skin prick testing in a pilot study.⁴¹

In the present study we could not detect Der p 1-specific plasmablasts because they were less than the detection limit of flow cytometry. There are several possible explanations for the fact that most of the remaining plasmablasts did not bind Der p 1. First, there are other allergens present in the HDM extract, and a fraction of the plasmablasts might be specific for some of these allergens. Second, B cells downregulate the expression of their BCR on differentiation to plasmablasts and plasma cells. When BCR is not present on the cell surface, Der p 1 cannot be bound to the BCR. Therefore we cannot exclude the possibility that some of these cells make Der p 1-specific antibodies, but we do not detect this because the BCR is downregulated from the surface. Assessment of immunoglobulin isotypes in allergen-specific plasmablasts by using multicolor flow cytometry before immunotherapy is challenging because the number of antigen-specific plasmablasts is very low. Antigen-specific plasmablasts expressing specific BCR increase intensely after vaccination or natural infection.²⁷⁻²⁹

Here we demonstrated that B_R1 cells not only produce IL-10 but also produce IL-1RA. Numbers of IL-10⁺IL-1RA⁺ Breg cells expanded significantly during AIT and were greater among responder patients. We previously reported that IL-10-secreting CD73⁺CD25⁺CD71⁺ Breg cells expanded in response to high-dose antigen exposure, both among patients receiving venom immunotherapy and among beekeepers during the beekeeping season.⁴ In addition, IL-10⁺ Breg cells suppress T_H cell proliferation and upregulate IgG₄ secretion.⁵

The present study introduces IL-1RA as a Breg cell cytokine in human subjects. The link of IL-1RA to IL-10 in B cells was first shown with increased IL-1RA in only IL-10-transfected and overexpressing human B cells, which showed that B cells overexpressing IL-10 gained a suppressive and anti-inflammatory function with increased IL-1RA and decreased proinflammatory cytokine levels.⁴² Various anti-inflammatory functions of IL-1RA have been reported. IL-1 triggers a cascade of inflammatory mediators, chemokines, and other cytokines. IL-1RA is an IL-1 inhibitor effective in reducing inflammation by blocking proinflammatory signals through the IL-1 receptor.⁴³ Administration of IL-1RA to mice reduced inflammation in various diseases.⁴⁴ In human subjects IL-1RA reduces the inflammatory effects of IL-1 and preserves cell function in diabetes and subarachnoid hemorrhage.^{43,45} IL-1RA reduces numbers of IL-17-producing T cells and induces Treg cell proliferation.^{30,46} IL-1RA might support IL-10 to induce immune tolerance by and in B cells. In a murine model IL-1RA produced by mesenchymal stem cells decreased the differentiation of B cells toward plasmablasts and induced the differentiation of macrophages toward an M2-like phenotype.¹⁹ However, the role of IL-1RA directly on class-switched B cells in human subjects requires further study.

In correlation analyses between multiple parameters, we demonstrated here a distinct profile between responders and nonresponders. We observed a strongly correlating overall immune response with multiple parameters of B-cell responses in therapy responders. B-cell immune response parameters also

showed a negative correlation with SMSs and SPT responses, suggesting a good link to clinical improvement. It is not fully possible to identify which of these changes is fully linked to treatment success. However, increases in numbers of IgG₄⁺ allergen-specific B cells, plasmablasts, and IL-10- and/or IL-1RA-producing Breg cell responses during AIT are particular markers associated with successful treatment. As previously described, increases in allergen-specific IgA and IgG₄ levels and decreases in allergen-specific IgE levels are markers of successful immune response during AIT.^{13,47-51} We aimed to demonstrate a biomarker to predict patient responsiveness to AIT. Numbers of IL-10-producing Der p 1-specific B cells showed a significant difference after 30 weeks of AIT from baseline. Changes in the frequency of IgG₄ class-switched B cells differed between responder and nonresponder patients. The responder patients also showed expansion of plasmablasts. In addition, increased numbers of IL-10- and IL-1RA-producing Breg cells in responder patients were observed. However, all of these might represent important targets that can predict clinical therapy response, and high numbers are needed.

It was not possible to demonstrate a single factor that identifies nonresponder patients in the present study. Many factors, such as sex, age, disease duration, socioeconomic factors, occupational factors, medical adherence, house environment, psychology, and body mass index might play a role. In addition, additional allergen exposure and sensitization or degree of dust mite exposure might explain the variability in clinical response. One major handicap of using one of these immunologic observations as a biomarker is that these analyses require a sophisticated laboratory approach and cannot be done at the point of care.

In conclusion, the present study demonstrates Der p 1-specific B-cell and Breg cell responses over a 2-year period during AIT and suggests novel mechanisms of allergen tolerance, such as an increase in numbers of circulating allergen-specific memory B cells and IL-1RA production by Breg cells. However, no clinically useful biomarker for response to HDM AIT was identified. Extension of the study to a larger number of patients and extending the study period should provide additional information for identifying biomarkers of long-term tolerance and successful AIT response.

We thank Mrs Jitta Tubkate for help in contacting the patients.

Key messages

- AIT induces IgA- and IgG₄-producing antigen-specific B cells, and these can be involved in development of mucosal tolerance.
- Numbers of plasmablasts and IL-10- and IL-1RA-producing Breg cells expanded during AIT and were greater in responders compared with nonresponders.
- Frequencies of allergen-specific IgG₄⁺ B cells, plasmablasts, and IL-10⁺ and/or IL-1RA⁺ Breg cells positively correlated with improved clinical response during AIT.

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METHODS

Identification of Der p 1–specific B cells

We selected BV510-CD19 (violet laser; maximum excitation, 415 nm) and labeled Der p 1 with Dy633 (red laser; maximum excitation, 633 nm) and PE-Cy7 (blue laser; maximum excitation, 496 nm) to detect Der p 1–specific B cells using flow cytometry. After labeling of Der p 1 with the Lightning-Link Dy633 Rapid Conjugate System and the Lightning-Link PE-Cy7 Tandem Conjugate Kit (both from Innova Biosciences), conjugated Der p 1 constructs were titrated for optimal concentrations for staining. After viability dye staining, Fc receptor blocking reagent (Miltenyi Biotec) was added to cells for blocking unspecific binding. We prepared the mixture of labeled Der p 1 allergens containing Der p 1–Dy633 and Der p 1–PE-Cy7, and added all surface staining. We added the mixture into the sample to avoid blocking of the Der p 1–specific BCR that can occur in the case of adding Der p 1 conjugates sequentially.

We purified double-positive Der p 1–Dy633⁺/Der p 1–PE-Cy7⁺, single-positive Der p 1–Dy633⁺, and single-positive Der p 1–PE-Cy7⁺

class-switched memory B cells (Fig E1, A). After 7 days of culturing with CD40 ligand and rIL-21, cells were immortalized by means of transduction with BCL6 and BCL-xl, according to the method of Kwakkenbos et al.^{E1}

After *in vitro* expansion of immortalized B cells, we found that more than 95% of the double-positive Der p 1–Dy633⁺/Der p 1–PE-Cy7⁺ B cells retained their double-color positive staining, whereas single-positive B cells did not (Fig E1, B). More importantly, we also measured Der p 1–specific IgG levels in the culture supernatant after 7 days using ELISA. Der p 1–Dy633⁺/Der p 1–PE-Cy7⁺ B cells showed high Der p 1–specific IgG levels, whereas single-color Der p 1⁺ B cells did not produce Der p 1–specific IgG (Fig E1, C).

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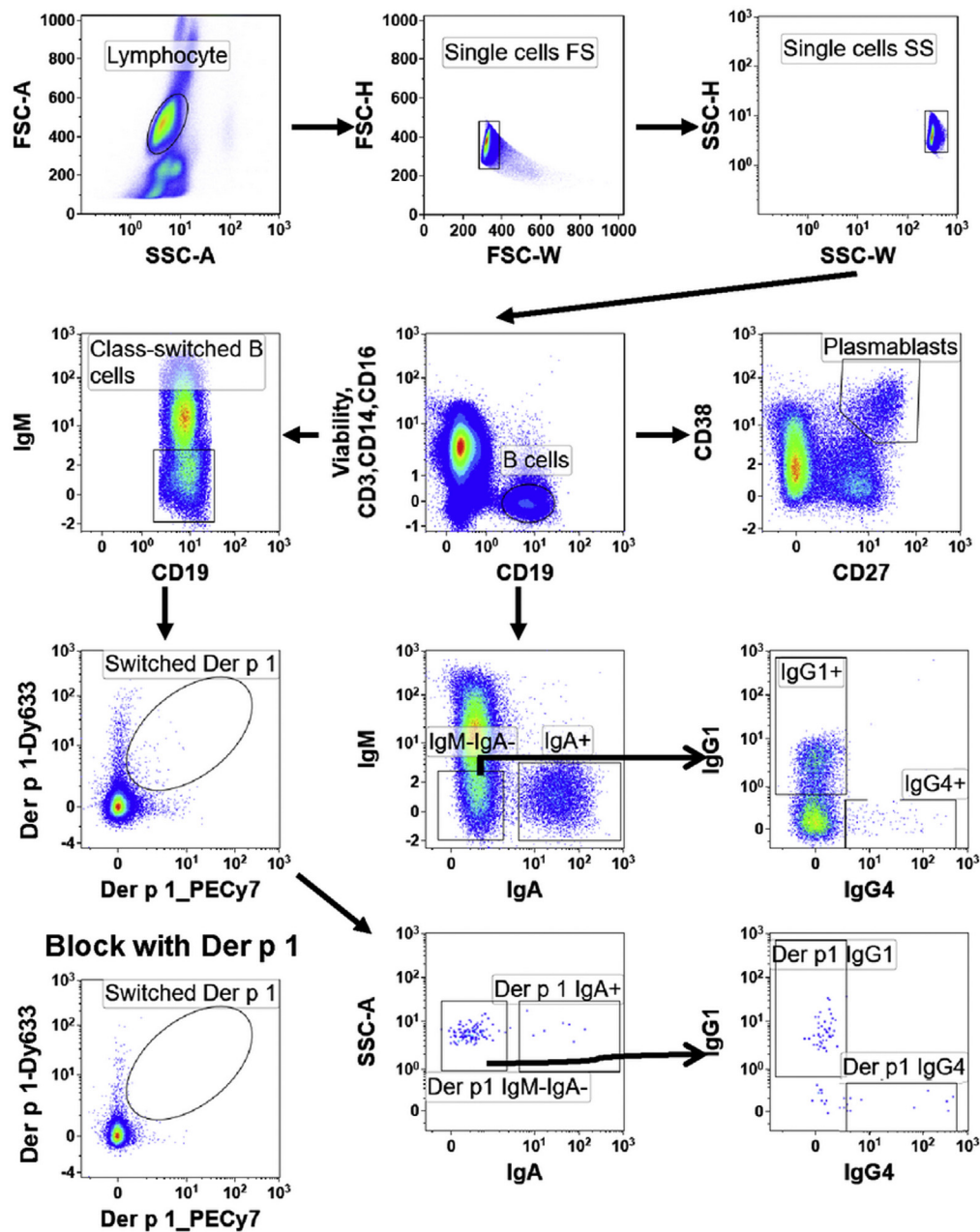


FIG E1. Gating strategy for Der p 1-specific cells in PBMCs. Lymphocytes were first gated from the forward scatter (FSC) and side scatter (SSC) plot. After doublet discrimination, live CD19⁺CD3⁺CD14⁺CD16⁺ cells were gated. Then IgM⁺ (class-switched B) cells were gated. Der p 1-Dy633⁺ and Der p 1-PE-Cy7⁺ cells were identified as Der p 1-specific B cells. Negative control cells were incubated with an excess of nonlabeled Der p 1 to block subsequent binding of labeled Der p 1 to the B cells receptor. Der p 1-specific IgA⁺ B cells were gated on Der p 1-specific B cells, and Der p 1-specific IgG1⁺ and IgG4⁺ B cells were gated on Der p 1-specific IgA⁺ B cells.

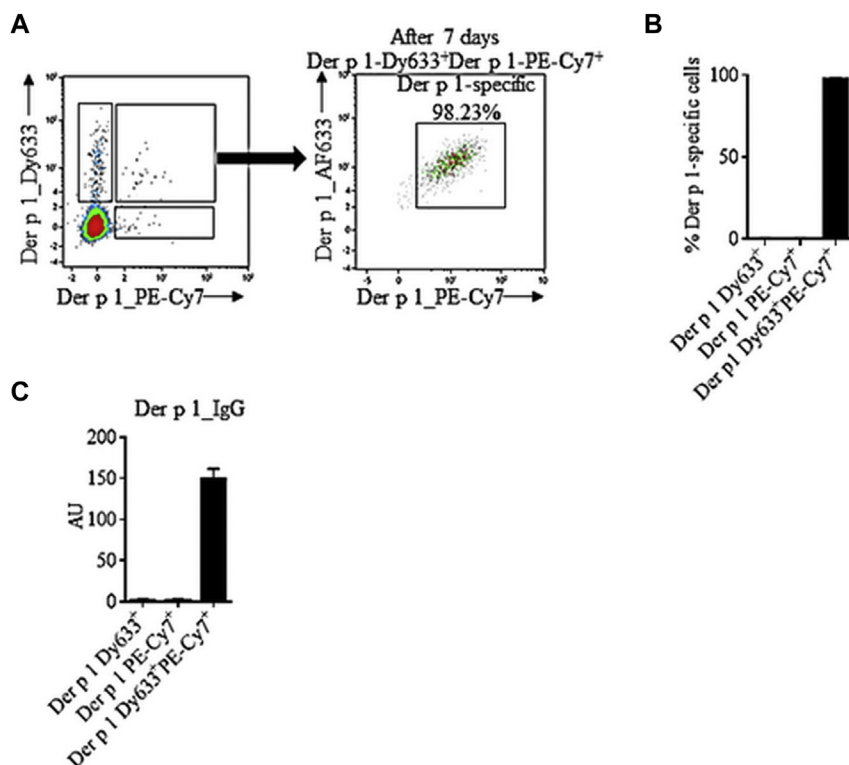


FIG E2. **A**, We purified double-positive Der p 1-Dy633⁺/Der p 1-PE-Cy7⁺, single-positive Der p 1-Dy633⁺, and single-positive Der p 1-PE-Cy7⁺ class-switched memory B cells. Cells were cultured with CD40 ligand and rIL-21 cells and immortalized by means of transduction with BCL6 and BCL-xL. After 7 days, the cells were stained with Der p 1-Dy633 and Der p 1-PE-Cy7 to identify Der p 1-specific B cells. **B**, Frequency of Der p 1-specific B cells after 7 days of *in vitro* expansion of immortalized B cells (n = 5). **C**, Der p 1-specific IgG levels in culture supernatants after 7 days, as determined by means of ELISA.

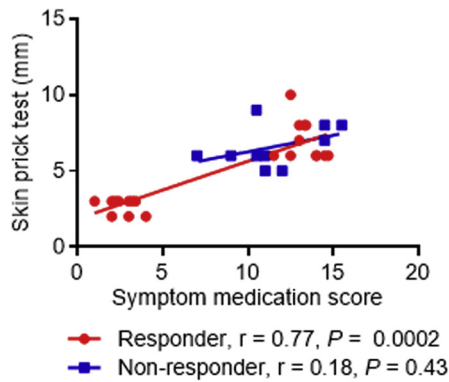
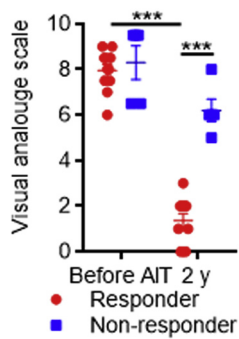
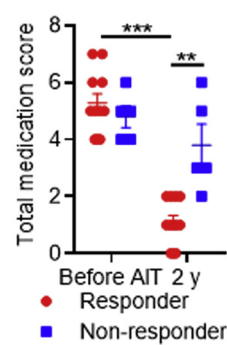
A Symptom medication score and skin prick test**B** Visual analogue scale**C** Total medication score

FIG E3. **A**, Correlation between SMSs and positive *D pteronyssinus* skin prick test responses in responder and nonresponder patients. **B** and **C**, Clinical response was assessed by using VAS scores and TMSs in patients with HDM allergy undergoing subcutaneous injection AIT compared before and after 2 years of AIT: responder patients, $n = 11$; nonresponder patients, $n = 5$. ** $P < .01$ and *** $P < .001$.

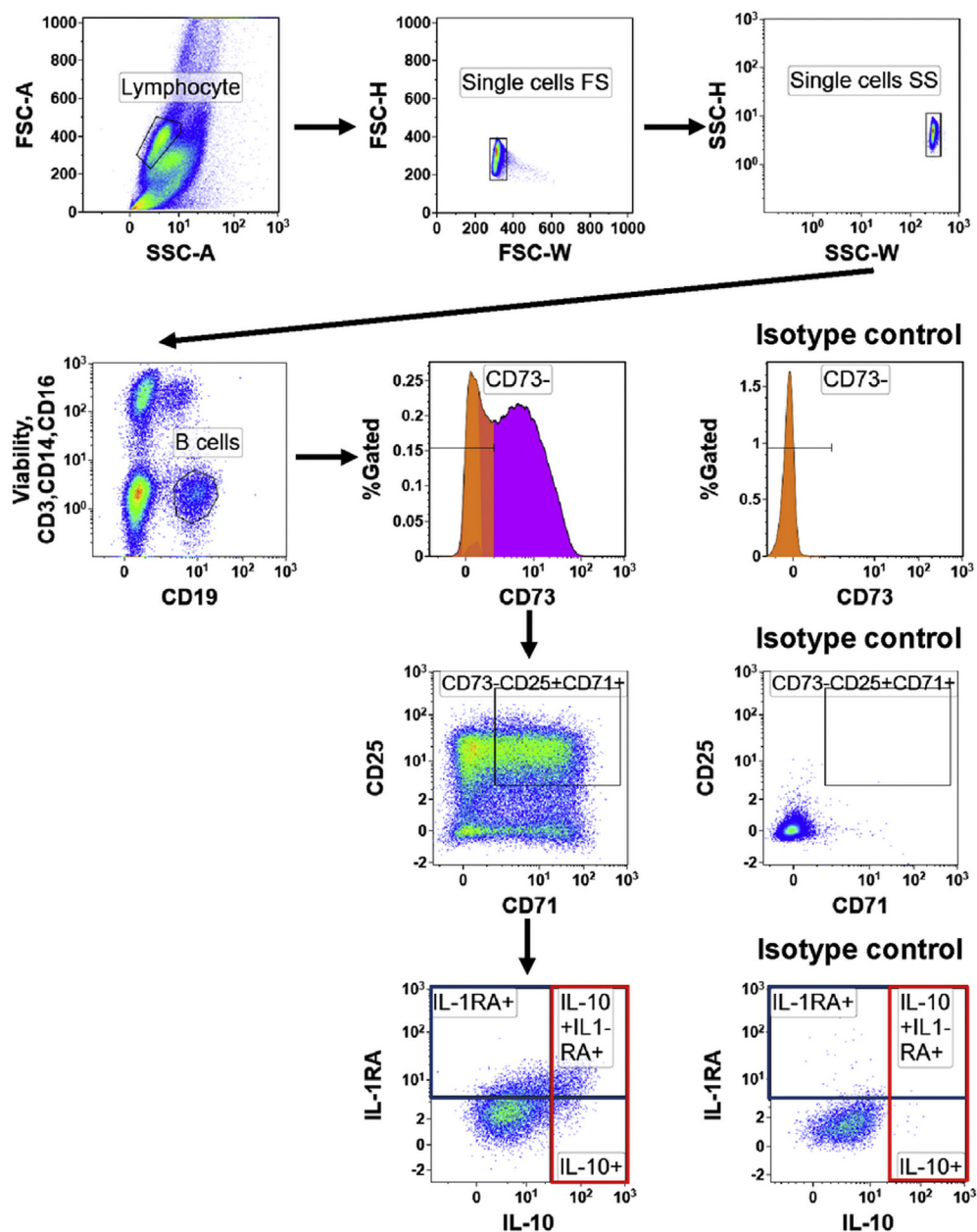


FIG E4. Gating strategy for Breg cells in PBMCs. Lymphocytes were first gated from the forward scatter (FSC) and side scatter (SSC) plot. After doublet discrimination, live $CD19^+CD3^-CD14^-CD16^-$ cells were gated. Then $CD73^-$ and $CD71^+CD25^+$ cells were gated. Afterward, IL-10 and IL-1RA were gated to show total IL-10 (red box) and total IL-1RA (blue box) and dual IL-10- and IL-1RA-producing Breg cells.

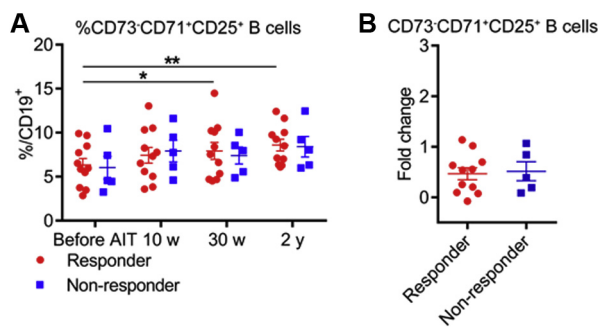
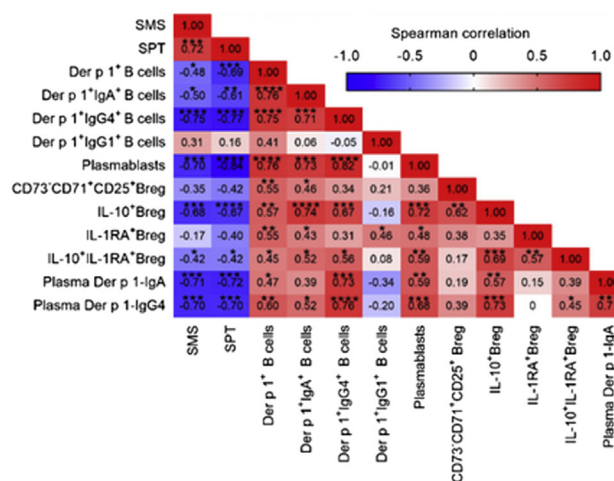


FIG E5. Frequency of CD73⁻CD71⁺CD25⁺ B cells before and after 10 weeks, 30 weeks, and 2 years of AIT and fold change between before and after 2 years of AIT among responder (n = 11) and nonresponder (n = 5) patients. **P* < .05 and ***P* < .01.

Data correlation of responders



Data correlation of non-responders

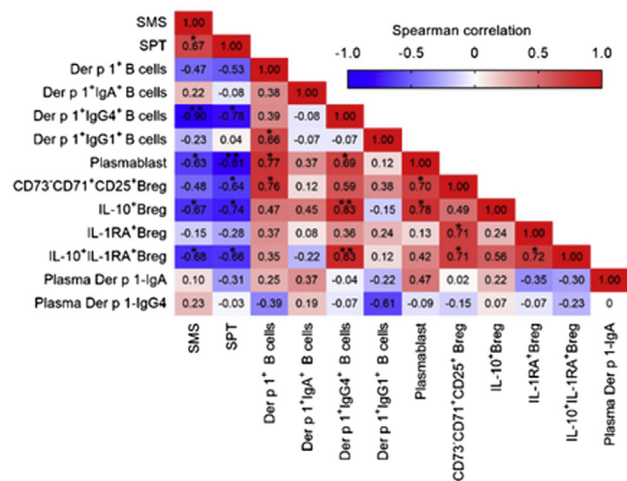


FIG E6. Heat map displaying Spearman correlation between all comparisons in responder and non-responder patients.

TABLE E1. Patients' characteristics

Patient code	Sex	Age (y)	Diagnosis	<i>D pteronyssinus</i> SPT response (mm)		VAS score				TMS		Total nasal score			
				Before	2 y	Before	10 wk	30 wk	2 y	Before	2 y	Before	10 wk	30 wk	2 y
Responder_AIT1	Male	35	AR	7	3	8	4	1	2	5	1	8	4	1	2
Responder_AIT2	Female	50	AR	6	3	8.5	5	0	2	6	1	8.5	5	0	2
Responder_AIT3	Male	25	AR, AA	8	3	7.5	3	2	1	7	2	7.5	3	2	1
Responder_AIT4	Male	41	AR	10	2	8	5	3	2	5	0	8	5	3	2
Responder_AIT5	Female	26	AR	6	2	8.5	7	2	2	4	1	8.5	7	2	2
Responder_AIT6	Male	60	AA	8	3	9	8.5	1	0	5	2	7	4.5	0	0
Responder_AIT7	Female	52	AR, AA	6	3	7	4.5	0	0	6	2	7.5	5	2	1
Responder_AIT8	Male	24	AR	8	3	7.5	5	2	1	4	0	6	4	1	0
Responder_AIT9	Female	52	AR, AA	6	2	6	4	1	0	7	2	8.5	7	2	3
Responder_AIT10	Female	27	AR, AD	6	2	8.5	7	2	3	4	1	9	7.5	3	2
Responder_AIT11	Male	20	AR	6	3	9	7.5	3	2	5	0	8	6	2	2
Nonresponder_AIT1	Male	24	AR	8	6	9.5	9	4	6	5	3	9.5	9	4	6
Nonresponder_AIT2	Female	64	AR, AA	8	5	9.5	7	2.5	6	6	5	9.5	7	2.5	6
Nonresponder_AIT3	Female	18	AR	6	6	6.5	6.5	3	5	4	2	6.5	6.5	3	5
Nonresponder_AIT4	Female	15	AR, AD	7	6	9.5	8	4	8	5	3	9.5	8	4	8
Nonresponder_AIT5	Male	22	AA	9	5	6.5	4	4	6	4	6	6.5	4	4	6

For TMSs, 1 point is defined as use of β_2 -agonists, antihistamine, pseudoephedrine, and montelukast; 2 points is defined as use of inhaled/intranasal steroids; and 3 points is defined as use of 1 tablet of corticosteroid.

AA, Allergic asthma; AD, atopic dermatitis; AR, allergic rhinitis.

TABLE E2. Schedule of injections for mite AIT

Visit	Antigenic dilution concentration	Volume (mL)	Antigen (AU)	Accumulate antigen (AU)
W1	1:1000 (5 AU/mL)	0.05	0.25	0.25
W2	1:1000 (5 AU/mL)	0.1	0.5	0.75
W3	1:1000 (5 AU/mL)	0.15	0.75	1.5
W4	1:1000 (5 AU/mL)	0.2	1	2.5
W5	1:1000 (5 AU/mL)	0.25	1.25	3.75
W6	1:1000 (5 AU/mL)	0.3	1.5	5.25
W7	1:1000 (5 AU/mL)	0.35	1.75	7
W8	1:1000 (5 AU/mL)	0.4	2	9
W9	1:1000 (5 AU/mL)	0.45	2.25	11.25
W10	1:1000 (5 AU/mL)	0.5	2.5	13.75
W11	1:100 (50 AU/mL)	0.05	2.5	16.25
W12	1:100 (50 AU/mL)	0.1	5	21.25
W13	1:100 (50 AU/mL)	0.15	7.5	28.75
W14	1:100 (50 AU/mL)	0.2	10	38.75
W15	1:100 (50 AU/mL)	0.25	12.5	51.25
W16	1:100 (50 AU/mL)	0.3	15	66.25
W17	1:100 (50 AU/mL)	0.35	17.5	83.75
W18	1:100 (50 AU/mL)	0.4	20	103.75
W19	1:100 (50 AU/mL)	0.45	22.5	126.25
W20	1:100 (50 AU/mL)	0.5	25	151.25
W21	1:10 (500 AU/mL)	0.05	25	176.25
W22	1:10 (500 AU/mL)	0.1	50	226.25
W23	1:10 (500 AU/mL)	0.15	75	301.25
W24	1:10 (500 AU/mL)	0.2	100	401.25
W25	1:10 (500 AU/mL)	0.25	125	526.25
W26	1:10 (500 AU/mL)	0.3	150	676.25
W27	1:10 (500 AU/mL)	0.35	175	851.25
W28	1:10 (500 AU/mL)	0.4	200	1051.25
W29	1:10 (500 AU/mL)	0.45	225	1276.25
W30	1:10 (500 AU/mL)	0.5	250	1526.25

After 30 weeks, patients undergo 0.5 mL (1:10 concentration) every 2 to 4 weeks until 2 to 3 years.

AU, Arbitrary units.

TABLE E3. Antibodies used in flow cytometry

Antigen	Parameter	Clone	Manufacturer
CD19	BV510	HIB19	BioLegend, San Diego, Calif
CD27	AF700	M-T271	BioLegend
IgM	BV605	MHM-88	BioLegend
IgM	PerCP/Cy5.5	MHM-88	BioLegend
CD38	BV786	HIT2	BD Biosciences, San Jose, Calif
CD3	eFlour780	UCHT1	eBioscience, San Diego, Calif
CD14	APC/Cy7	HCD14	BioLegend
CD16	APC/Cy7	3G8	BioLegend
CD73	PE	AD2	BioLegend
CD25	PE/Dazzle594	M-A251	BioLegend
CD71	PE/Cy5	M-A712	BD Biosciences
CD24	BV421	ML5	BioLegend
Streptavidin	PE/Dazzle594		BioLegend
IgA	AF488		Jackson Laboratory, Bar Harbor, Me
IgG ₁	Dy405		Sanquin, Amsterdam, Netherland
IgG ₄	Biotin		Alta Bioscience, Birmingham, United Kingdom

AF, Alexa Fluor; APC, allophycocyanin; BV, Brilliant Violet; PerCP, peridinin-chlorophyll-protein complex.