

LETTER TO THE EDITOR

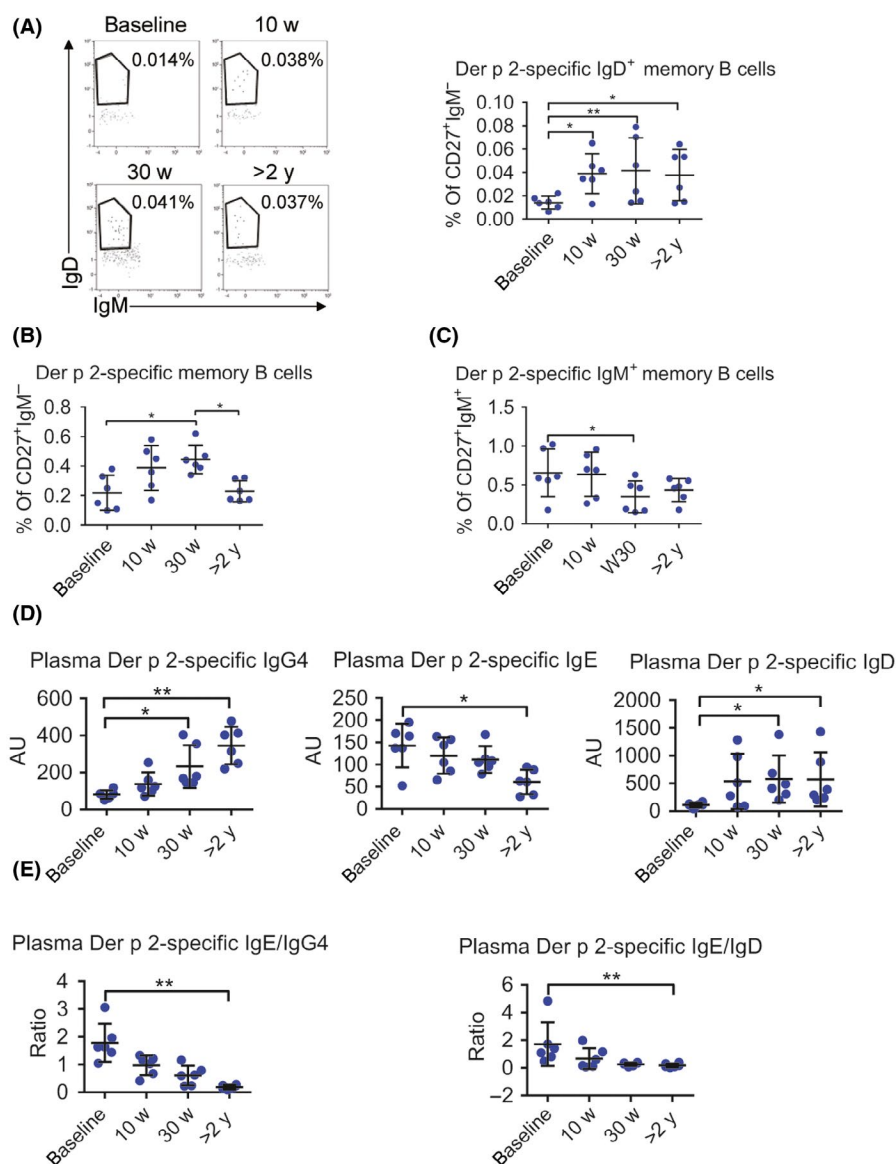
Allergen-specific immunotherapy boosts allergen-specific IgD production in HDM-sensitized asthmatic patients

To the Editor,

Whereas the biology of IgG, IgA, and IgE has been extensively studied in allergic diseases, the regulation and function of IgD remain poorly investigated. IgG4 is a blocking antibody, which prevents the cross-linking of receptor-bound IgE. IgD is unable to recruit complement protein which is similar to mucosal IgA.¹ IgD was shown to

increase Th2 cell polarization in germinal centers, boosting Th2 cytokine production.² IgD-activated basophils also enhance IgG1 and IgE production by allergen-specific B cells. Interestingly, IgD could interfere with IgE-mediated basophil degranulation supporting its role in the development of allergen immune tolerance.² Of note, beekeepers with tolerance to bee venom developed higher serum

FIGURE 1 Increased Der p 2-specific IgD⁺ class-switched memory B cells and plasma Der p 2-specific IgD levels during AIT in HDM asthmatic patients. A, Representative dot plot (left) and frequency (right) of Der p 2-specific IgD⁺ class-switched memory B cells. B and C, The frequencies of Der p 2-specific class-switched and nonclass-switched memory B cells. D, Plasma Der p 2-specific IgG4, IgE, and IgD levels during AIT. E, Der p 2-specific IgE/IgG4 and gE/IgD ratios during AIT (n = 6). *P < .05, **P < .01



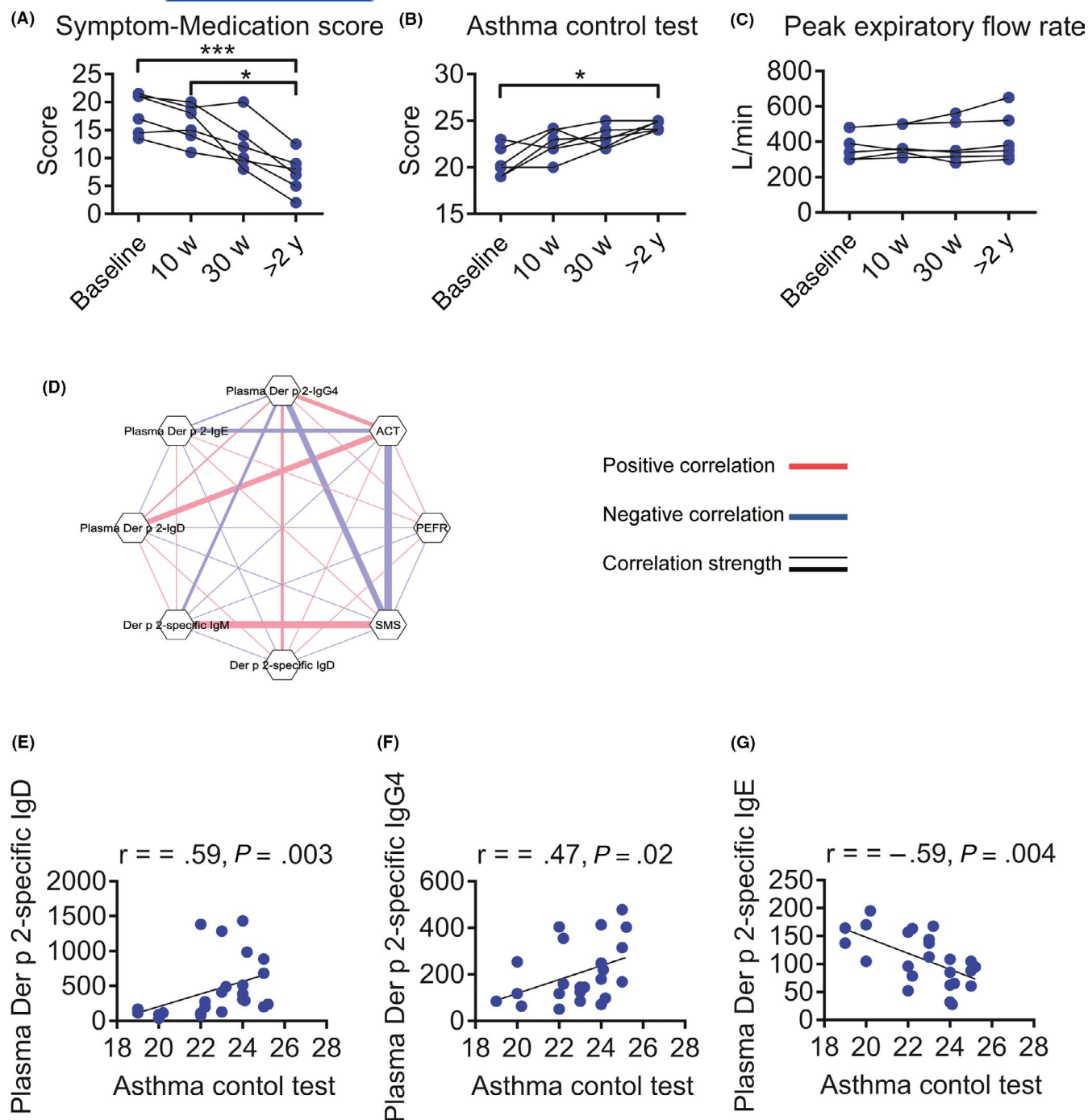


FIGURE 2 Improved asthma control correlated with plasma Der p 2-specific IgD levels. A-C, Clinical response was assessed using symptom-medication score (SMS), asthma control test (ACT) score, and peak expiratory flow rate (PEFR) of patients with allergic asthma during AIT ($n = 6$). D, Correlation network displaying Spearman's correlation between all significant comparisons. E-G, Correlation graph between ACT score and plasma IgD, IgG4, and IgGE levels. * $P < .05$, *** $P < .001$

IgD-specific phospholipase A2 (PLA2) levels and lower total IgD levels compared with healthy controls.² Moreover, milk- and egg-allergic children treated with oral immunotherapy showed increased serum allergen-specific IgD responses compared with placebo controls.²

Allergen-specific immunotherapy (AIT) is the only treatment which induces long-term clinical tolerance.³ AIT enhanced allergen-specific IgG4-, IgG2-, and IgA-producing B cells, plasmablasts, IL-10⁺IL-1RA⁺Breg cells and reduced allergen-specific

IgE-producing B cells.⁴⁻⁶ In addition, an increase in allergen-specific-IgG4⁺ B cells was shown to correlate with a reduction in symptom score in successful AIT.⁴ Whereas AIT is an effective treatment in the patients with house dust mites (HDM) allergy, the role of HDM allergen-specific IgD remains to be fully evaluated.^{4,7} The major HDM allergens are represented by Der p/f 1, Der p/f 2, and Der p/f 23.⁸ In this report, we have shown that Der p 2-specific IgD-producing B cells increased during the course

of AIT that might be correlated to the successful development of HDM tolerance.

From a cohort study of 75 HDM allergic patients undergoing SCIT with HDM allergen extracts, we selected 6 treated asthmatic patients to investigate Der p 2-specific B cells.^{4,7} The patient characteristics are shown in the Table S1. Peripheral blood mononuclear cells (PBMCs) and plasma samples were collected at four time points: before AIT, 10 weeks, 30 weeks, and >2 years of AIT. The study protocol was approved by the Institutional Review Board of the Royal Thai Army's Ethics Committee, IRBRTA 67/2556. All patients were recruited after obtaining written informed consent.

For a detailed description of reagents and procedures, see the method section in this article's Supplementary data. In brief, to detect Der p 2-specific B cells, recombinant Der p 2 (rDer p 2) was fluorescently labeled with Lightning-Link Dy633 Rapid Conjugate System and Lightning-Link PE-Cy7 Tandem Conjugate Kit (both from Innova Biosciences, Cambridge, UK). Therefore, PBMCs were stained using eF780 viability dye (eBioscience, San Diego, CA, USA), a mixture of Der p 2- Dy633 and Der p 2-PE-Cy7, anti-IgD-FITC, anti-CD19-PE, anti-CD27- PE/Dazzle 594, anti-IgM- PerCP/CY5.5, and anti-CD3- APC/Cy7 (all from BioLegend).

The gating strategy of Der p 2-specific B cells is shown in Figure S1. We found that the number of Der p 2-specific IgD memory B cells (IgD⁺IgM⁻ CD27⁺Der p 2⁺) was very low at baseline and was significantly upregulated during the course of AIT (Figure 1A). The mean changes were from $0.014 \pm 0.005\%$ at the baseline to $0.038 \pm 0.017\%$ ($P = .04$) at 10 weeks, $0.041 \pm 0.028\%$ ($P = .005$) at 30 weeks and $0.037 \pm 0.02\%$ ($P = .04$) after 2 years. The frequency of Der p 2-specific class-switched memory B cells also increased significantly after 30 weeks of AIT compared with baseline ($0.22 \pm 0.11\%$ to $0.44 \pm 0.09\%$, $P = .01$) (Figure 1B and Figure S2A). Meanwhile, Der p 2-specific nonclass-switched memory B cells (CD19⁺IgM⁺CD27⁺Der p 2⁺) gradually decreased during the course of AIT and this reduction was significant at 30 weeks compared with baseline ($0.66 \pm 0.3\%$ to $0.34 \pm 0.2\%$, $P = .04$) (Figure 1C and Figure S2B).

Next, we analyzed plasma Der p 2-specific IgD, IgM, IgE, and IgG4 antibody levels by ELISA. After 2 years of treatment, plasma Der p 2-specific IgG4 and IgE levels significantly increased and decreased from baseline, $P = .003$ and $P = .04$, respectively (Figure 1D). Interestingly, plasma Der p 2-specific IgD levels increased fivefold after 30 weeks ($P = .02$) and 2 years ($P = .04$) of AIT compared with baseline (Figure 1D). No statistically significant difference was found for plasma Der p 2-specific IgM levels. We also analyzed the Der p 2-specific antibody ratio as an interesting biomarker to monitor the AIT response.^{9,10} Similar to Der p 2-specific IgE/IgG4 ratio, Der p 2-specific IgE/IgD ratio decreased substantially after 2 years of AIT (Figure 1E).

Asthma symptoms were monitored using the symptom-medication score (SMS), the asthma control test score (ACT), and the peak expiratory flow rate (PEFR). Following 2 years of AIT, asthmatic patients improved both SMS and asthma control (Figure 2A and 2). PEFR was increased in the majority of patients but without statistical significance (Figure 2C). The clinical improvements were correlated with B-cell immune responses (Figure 2D). The proportion of Der p 2-specific IgD memory B cells correlated with Der p 2-specific IgM

memory B cells ($r = -.42$, $P = .04$) and plasma Der p 2-specific IgG4 levels ($r = .45$, $P = .02$). Plasma Der p 2-specific IgD levels showed a significant correlation with asthma control ($r = .59$, $P = .003$) (Figure 2E). Moreover, the ACT score correlated with plasma Der p 2-specific IgG4 and IgE levels ($r = .47$, $P = .02$ and $r = -.59$, $P = .004$) (Figure 2F and G).

Our study evidenced the expansion of allergen-specific IgD-producing B cells during AIT. Moreover, increasing secreted allergen-specific IgD correlated with the ability to control asthma. Our data support the hypothesis that allergen-specific IgD might play a role in allergen immune tolerance and allergen-specific IgD levels might represent a new biomarker to monitor successful AIT. The limitation of the study is the low numbers of the HDM allergic patients and that all the selected subjects in this study were responders to AIT. Further study with larger cohort of HDM AIT-treated patients should explore more in details the exact contribution of specific IgD in immune tolerance to HDM.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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REFERENCES

1. Gutzeit C, Chen K, Cerutti A. The enigmatic function of IgD: some answers at last. *Eur J Immunol*. 2018;48(7):1101-1113.
2. Shan M, Carrillo J, Yeste A, et al. Secreted IgD amplifies humoral T helper 2 cell responses by binding basophils via galectin-9 and CD44. *Immunity*. 2018;49(4): 709-724.
3. Sokolowska M, Boonpiyathad T, Escribese MM, Barber D. Allergen-specific immunotherapy: power of adjuvants and novel predictive biomarkers. *Allergy*. 2019;74(11):2061-2063.
4. Boonpiyathad T, van de Veen W, Wirz O, et al. Role of Der p 1-specific B cells in immune tolerance during 2 years of house dust mite-specific immunotherapy. *J Allergy Clin Immunol*. 2019;143(3):1077-1086.
5. Sugimoto M, Kamemura N, Nagao M, et al. Differential response in allergen-specific IgE, IgGs, and IgA levels for predicting outcome of oral immunotherapy. *Pediatr Allergy Immunol*. 2016;27(3):276-282.
6. Couroux P, Ipsen H, Stage BS, et al. A birch sublingual allergy immunotherapy tablet reduces rhinoconjunctivitis symptoms when exposed to birch and oak and induces IgG4 to allergens from all trees in the birch homologous group. *Allergy*. 2019;74(2):361-369.
7. Boonpiyathad T, Sokolowska M, Morita H, et al. Der p 1-specific regulatory T-cell response during house dust mite allergen immunotherapy. *Allergy*. 2019;74(5):976-985.
8. Thomas WR. Hierarchy and molecular properties of house dust mite allergens. *Allergol Int*. 2015;64(4):304-311.
9. van de Veen W, Akdis M. Role of IgG4 in IgE-mediated allergic responses. *J Allergy Clin Immunol*. 2016;138(5):1434-1435.
10. Caubet JC, Lin J, Ahrens B, et al. Natural tolerance development in cow's milk allergic children: IgE and IgG4 epitope binding. *Allergy*. 2017;72(11):1677-1685.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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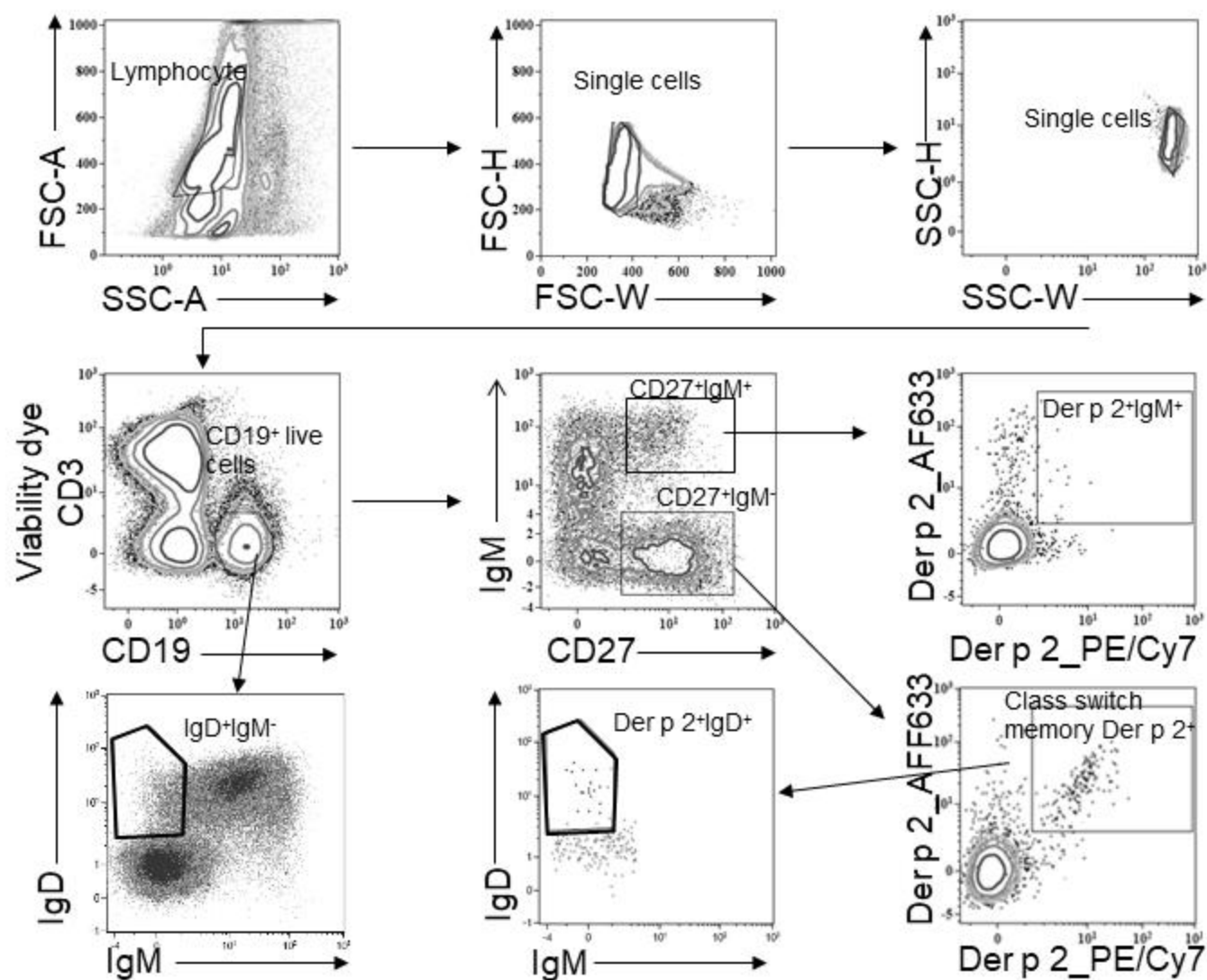


Figure S1. Boonpiyathad et al.

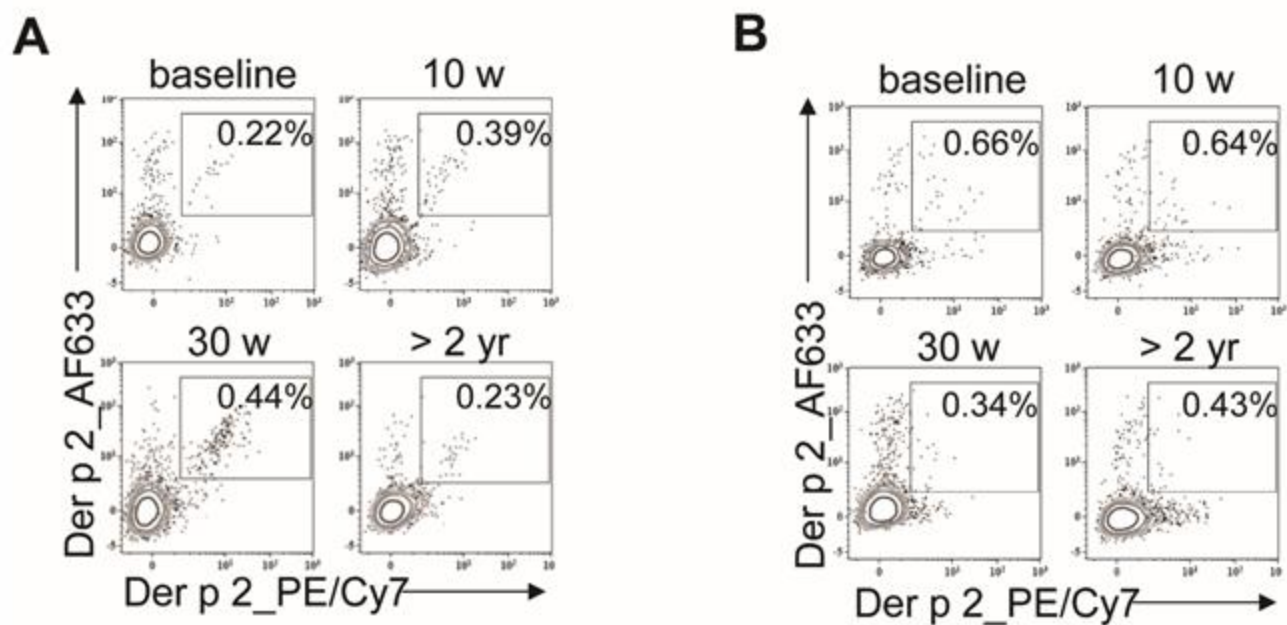


Figure S2. Boonpiyathad et al.

**Allergen-specific immunotherapy boosts allergen-specific IgD production in
HDM-sensitized asthmatic patients**

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MATERIALS and METHODS

Subjects

Seventy-five house dust mite (HDM) allergic patients were recruited from the Allergy Clinic, Department of Medicine, Phramongkutklao Hospital, Bangkok, Thailand since 2013 for a prospective observational study of SCIT with HDM extracts, receiving treatment for subcutaneous HDM AIT. The majority of the patients suffered from allergic rhinitis. Ten HDM asthmatic patients were enrolled in the study, but only 6 patients achieved the 2-year observation study. All participants had objective symptoms of HDM-related and positive skin prick test (wheal diameter ≥ 6 mm than negative control) with *D. pteronyssinus* extract (Alk-Abello, Round Rock, TX, USA). A 30-week weekly dose escalation protocol was used with the subcutaneous administration of standardized mite mixes, which contain glycerin 50% v/v, phenol 0.4% (preservative), sodium chloride, sodium bicarbonate, *D. pteronyssinus* 5,000 Allergy Units/ml and *D. farinae* 5,000 Allergy Units/ml (Alk-Abello) which has been described elsewhere (1). Peripheral blood mononuclear cells (PBMCs) and plasma were collected before, after 10 weeks, 30 weeks and >2 years of AIT. PBMCs were kept frozen in liquid nitrogen until analysis. Allergic asthma symptoms were monitored using an asthma control test score (ACT), peak expiratory flow rate (FEFR) and symptom-medication score (SMS) that combines the visual analogue scale (VAS) and the medication score (1 point for beta-2 agonists, antihistamine, pseudoephedrine and montelukast, 2 points for inhaled/intranasal steroids with long-acting beta-2 agonists and 3 points for one tablet of corticosteroid) (1). The Asthma control test (ACT) is a patient self-questionnaire for identifying the level of controlled asthma. The scores range from 5 (poor control of asthma) to 25 (complete control of asthma), with higher scores representing a greater asthma control. An ACT score > 19 indicates well-controlled asthma (2). The study protocol was approved by the Institutional Review Board of the Royal Thai Army's Ethics Committee, IRBRTA 67/2556. All patients were recruited after obtaining written informed consent.

Flow cytometry to detect Der p 2-specific B cells

Purified recombinant Der p 2 was labeled with Lightning-Link Dy633 Rapid Conjugate System and Lightning-Link PE-Cy7 Tandem Conjugate Kit (both from Innova Biosciences, Cambridge, UK) according to the manufacturer instructions. The detection of Der p 2-specific B cells have been described in detail elsewhere (1). The fluorescently labeled Der p 2 was titrated for optimal concentration before staining the in patient samples. A dual-labeling method was used to identify Der p 2-specific B cells in this study (1, 3, 4).

To detect Der p 2-specific B cells, cryopreserved 10^7 PBMCs, following thawing, were treated with 100 U recombinant DNase I (Sigma-Aldrich, St Louis, MO, USA) and stained using eF780 viability dye (eBioscience, San Diego, CA, USA) at 4°C for 30 min and then cells were washed in staining buffer (PBS, pH 7.2, 0.5% bovine serum albumin and 2 mM EDTA). Afterward, cells were stained with a mixture of labeled Der p 2

allergens containing Der p 2- Dy633 and Der p 2-PE-Cy7 and followed by antibodies against surface markers anti-IgD-FITC (clone IA6-2, Biolegend, San Diego, CA, USA), anti-CD19-PE (clone HIB19, Biolegend), anti-CD27-PE/Dazzle 594 (clone M-T271, Biolegend), anti-IgM-PerCP/CY5.5 (clone MHM-88, Biolegend) and anti-CD3-APC/Cy7 (clone HIT3a, Biolegend) at room temperature for 30 min. Finally, cells were washed twice in staining buffer and resuspended in 500 μ l. All samples were analyzed with a Gallios flow cytometer (Beckman Coulter, Indianapolis, IN, USA). The acquired cell number during flow cytometry analysis was 1.5 million. If the cell number was more than 2 million, cell samples were subdivided into 2 – 4 polypropylene tubes. The FACs files were merged and analyzed using FlowJo Software (FlowJo LLC, Ashland, OR, USA).

ELISA to detect Der p 2-specific antibody

To measure Der p 2-specific antibody, ELISA plates (Nunc plate Thermo Scientific, Waltham, MA, USA) were coated with 500 ng/well rDer p 2 at 4°C overnight and then blocked with blocking buffer (PBS pH7.4, 1% BSA, 0.05% Tween 20). Serial plasma samples (in the blocking buffer) were added and incubated at room temperature for 2 hours. Anti-human IgD, IgM, IgE (all from Bethyl Laboratories, Montgomery, TX, USA) and IgG4 (Cusabio, Houston, TX, USA) were used to detect Der p 2-specific antibody levels measured by ELISA as described (1). The detection limit to measure Der p 2-specific IgD, IgM, IgE and IgG4 by ELISA was performed without calibration standard. The results were expressed as arbitrary units (AU) based on the positive inhouse pool plasma of patients with HDM allergic asthma.

Statistical analysis

Data were expressed as a mean $\pm$ standard deviation (SD). Shapiro-Wilk normality test and by Kolmogorov-Smirnov test were used to assess the distribution of data. ANOVA and Dunn's multiple comparison tests were used to compare related samples between different time points. The Mann-Whitney U test was used to compare between 2 time points and correlation data were analyzed using Spearman's test. The correlation network was performed with Cytoscape 3.7.1 (National Institute of General Medical Sciences of the National Institute of Health, USA). Statistical analysis was performed with GraphPad Prism 7.0 Software (GraphPad Software, La Jolla, CA, USA). A *p*-value of less than 0.05 was considered statistically significant.

References

1. Boonpiyathad T, van de Veen W, Wirz O, Sokolowska M, Ruckert B, Tan G, et al. Role of Der p 1-specific B cells in immune tolerance during 2 years of house dust mite-specific immunotherapy. *J Allergy Clin Immunol.* 2019;143(3):1077-86 e10.
2. Schatz M, Kosinski M, Yaras AS, Hanlon J, Watson ME, Jhingran P. The minimally important difference of the Asthma Control Test. *J Allergy Clin Immunol.* 2009;124(4):719-23 e1.
3. Boonpiyathad T, Meyer N, Moniuszko M, Sokolowska M, Eljaszewicz A, Wirz OF, et al. High-dose bee venom exposure induces similar tolerogenic B-cell responses in allergic patients and healthy beekeepers. *Allergy.* 2017;72(3):407-15.
4. Hoh RA, Joshi SA, Liu Y, Wang C, Roskin KM, Lee JY, et al. Single B-cell deconvolution of peanut-specific antibody responses in allergic patients. *J Allergy Clin Immunol.* 2016;137(1):157-67.

Table S1. Baseline patient characteristics

Patient donor	Age (yr)	Sex	BMI	FEV1/	PEFR	ACT	VAS	Medication	Der p	other	Co-morbid	Total IgE (kU/L)
				FVC	(L/min)		(mm)	score	Skin prick test (mm)	sensitization	disease	
BC_1	68	F	26.3	74.3	480	23	75	7	6x6	Der f, Bla g	HT, DLP	157
VS_2	61	M	21.1	68.3	300	22	95	12	6x6	Der f,	AR, HT	143
MM_3	60	F	29.9	72.6	390	20	60	11	8x8	Der f, Bla g	AR	125
CS_4	63	M	26.7	75.3	340	20	65	7	6x6	Der f,	AD	231
LN_5	61	F	24.0	78.2	480	19	90	12	8x8	Der f,	AR, HT	175
TC_6	62	F	25.7	71.4	300	19	70	8	6x6	Der f, Bla g	AR	112

BMI, body mass index; FEV1, forced expiratory volume in the first second; FVC, forced volume vital capacity; PEFR, peak expiratory flow rate; ACT, asthma control test; VAS, visual analogue scale; Der p, *Dermatophagoides pteronyssinus*; Der f, *Dermatophagoides farina*; Bla g, *Blattella germanica*; HT, hypertension; DLP, dyslipidemia; AR, allergic rhinitis, AD, atopic dermatitis

Figure legend

Figure S1. Gating strategy used to identify circulating of Der p 2-specific B cell subsets. Lymphocytes were first gated from the forward and side scatter plot. After doublet discrimination, live CD19⁺CD3⁻ cells were gated. Then CD27⁺IgM⁻ (class-switched memory B cells) and CD27⁺IgM⁺ (non-class-switched memory B cells) were gated. Gated on CD27⁺IgM⁺, Der p 2-AF633⁺ and Der p 2-PECy7⁺ were identified as Der p 2-specific non-class-switched memory B cells. Der p 2-specific IgD⁺ memory B cells, were gated on Der p 2-specific memory B cells and then gated on IgD⁺IgM⁻ B cells.

Figure S2. Representative dot plot Der p 2-specific class-switched (**A**) and Der p 2-specific IgM⁺ non-class-switched (**B**) memory B cells during AIT.