

BRIEF COMMUNICATION

IgE-expressing memory B cells and plasmablasts are increased in blood of children with asthma, food allergy, and atopic dermatitis

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Abstract

Despite the critical role of soluble IgE in the pathology of IgE-mediated allergic disease, little is known about abnormalities in the memory B cells and plasma cells that produce IgE in allergic patients. We here applied a flow cytometric approach to cross-sectionally study blood IgE⁺ memory B cells and plasmablasts in 149 children with atopic dermatitis, food allergy, and/or asthma and correlated these to helper T (h)2 cells and eosinophils. Children with allergic disease had increased numbers of IgE⁺CD27⁻ and IgE⁺CD27⁺ memory B cells and IgE⁺ plasmablasts, as well as increased numbers of eosinophils and Th2 cells. IgE⁺ plasmablast numbers correlated positively with Th2 cell numbers. These findings open new possibilities for diagnosis and monitoring of treatment in patients with allergic diseases.

KEYWORDS

asthma, flow cytometry, IgE, memory B cell, plasmablast

1 | INTRODUCTION

IgE is commonly used in the diagnostic workup of patients with allergic disease, but little is known about the cells producing it.^{1,2} All

antibodies, including IgE, are produced by plasma cells.³ IgM⁺, IgG⁺, and IgA-expressing memory B cells and plasma cells can be readily detected in tissue or blood of healthy children and adults. In contrast, IgE-expressing B cells have been enigmatic.⁴ Still, based on the

presence of IgE transcripts isolated from various tissues and blood of healthy controls and allergic subjects,⁵⁻⁷ the presence of IgE-expressing B cells has been anticipated.⁸ We developed a flow cytometric strategy that enabled the detection of IgE-expressing CD27+CD38^{hi} plasmablasts, and CD27+ and CD27- memory B cells.⁹ Although T-cell help is required to generate the former subsets, CD27-IgE+ B cells can be formed independent of cognate T-cell help.^{9,10}

More insights into IgE-expressing B cells and their interactions with other immune cells are needed to better understand the pathogenesis of allergic disease, which could be useful for diagnostic purposes or to uncover targets for treatment. Similar to immunotherapy, anti-IgE antibody treatment can potentially target IgE+ memory B cells and thereby might resolve the underlying allergy.¹¹

We performed a cross-sectional study into IgE-expressing B cells in allergic children and healthy controls. Patients were separated into three groups based on distinct allergic phenotypes of asthma, food allergy, and/or atopic dermatitis. We studied IgE+ memory B-cell and IgE+ plasmablast numbers with flow cytometry, their maturation status with molecular studies, and related these to clinical phenotype and other clinical and immunological parameters.

2 | METHODS

Peripheral blood samples were obtained from children aged 6-18 years who were recruited from the allergy outpatient clinics (KinderHaven and Allergy Department) of the Erasmus University Medical Center Rotterdam. All children were recruited from a tertiary referral center for children with allergic disease and were doctor's diagnosed as moderate-to-severe allergic disease. All children had allergic disease based on clinical examination and confirmed by either atopy skin test (HEP > 0.21), specific IgE serum levels (>0.35 IU/L), immune solid-phase allergy chip (ISAC), or, in the case of food-allergy, double-blind food provocation test. Based on symptoms, clinical findings, and IgE specificity, children with allergic disease were divided into three groups: (1) children with asthma in absence of food allergy (FA) and atopic dermatitis (AD): "Asthma," n = 68; (2) children with asthma in combination with FA and/or AD: "Asthma + FA/AD," n = 48; and (3) children with FA and/or AD in absence of asthma; "FA/AD," n = 33. Age-matched nonallergic controls were recruited among children undergoing surgery in the Sophia Children's Hospital in Rotterdam. Exclusion criteria for the study were as follows: age <6 years, systemic immunosuppressive therapy, and immunological diseases, such as immunodeficiencies and autoimmune diseases.

All peripheral blood samples were collected after written informed consent was obtained and with approval of the Medical Ethical Committee of the Erasmus Medical Center, which complies with the Helsinki Declaration.

Detailed methods on flow cytometry, molecular analysis of IgE transcripts, and statistics can be found in Data S1.

3 | RESULTS

3.1 | Patient characteristics

A total of 149 allergic and 15 nonallergic children were included. All allergic children were sensitized for either aero- or food allergens, as detailed per group in Table S2.

3.2 | Increased numbers of eosinophils, T cells, and B cells in children with allergic disease

Total leukocyte counts were higher in patients with allergic disease than in nonallergic controls (Figure 1A). Similarly, total T- and B-cell numbers were significantly higher in each of the 3 groups with allergic disease than in nonallergic controls (Figure 1B). Total numbers and frequencies of eosinophils were significantly increased in allergic children as compared to nonallergic children (Figures 1C and S2). Leukocyte subsets and total T- and B-cell numbers did not differ between the three allergic groups.

Absolute CD4+ T-cell numbers and subsets within CD4+ T cells were significantly higher in children with allergic disease than in nonallergic children (Figure 1D). Still, frequencies of CD4 T cells within CD3+ T cell were not different from healthy children (Figure 1E). In addition, frequencies of Th1 and Th2 subsets within CD4+ T cells were not different between healthy children and children with allergic disease (Figure 1F). As a result, we did not observe a difference in Th2/Th1 cell ratios (Figure 1G) between patients and controls.

3.3 | Increased numbers of circulating IgE+ memory B cells and IgE-producing plasmablasts

IgE+ memory B cells were defined within the CD19+CD38^{dim} fraction and IgE+ plasmablasts within the CD19+CD38+CD27^{hi} fraction following exclusion of CD19+ B cells expressing IgM, IgD, IgA, or IgG (Figure 2A).⁹

Median cell numbers of both CD27+IgE+ and CD27-IgE+ memory B cells were significantly higher in all three groups of allergic children than in nonallergic controls (Figure 2B,D). In addition, IgE+ plasmablast numbers were significantly increased in allergic children (Figure 2F). IgE+ memory B-cell and IgE+ plasmablast numbers did not significantly differ between patient groups. The increases in IgE+ B-cell numbers in allergic children were not only the result of higher total B-cell counts, because their relative numbers within CD19+ B cells were also significantly higher in all patient groups (Figure 2C,E,G).

To investigate whether the increased numbers constituted normal plasmablasts, we studied the levels of somatic hypermutation (SHM) in IgE transcripts of sorted cells from allergic children (n = 43 unique transcripts) and from nonallergic controls (n = 34 unique transcripts). Transcripts from patients carried fewer mutations in their IGHV genes than controls (Figure 2H). As a measure for antigen-driven selection of the mutations in the IGHV genes, we analyzed the selection for replacement mutations in the complementarity determining regions (CDR) with the BASELINE

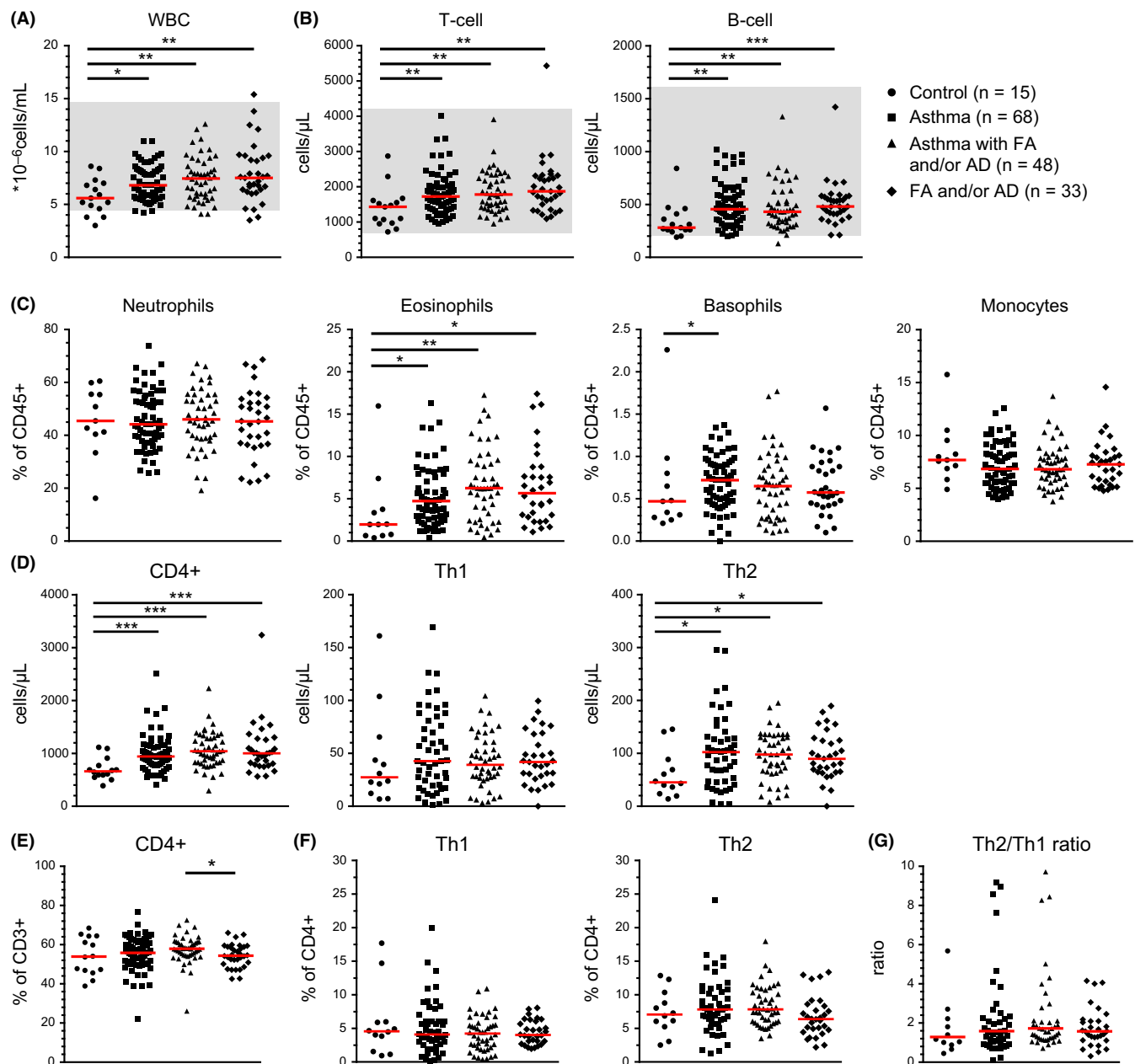


FIGURE 1 Absolute numbers and frequencies of leukocyte subsets and CD4+ helper-T(h) subsets. A, White blood cell count. B, Absolute numbers of T cells and B cells. C, Percentages of granulocyte subsets and monocytes within total CD45+ cells. D, Absolute cell numbers of CD4+ T-cells, Th1 and Th2 cells. E, Percentage of CD4+ T-cells within total CD3+ T-cells. F, Percentage of Th1 and Th2 cells within total CD3+ T-cells. G, ratio of Th2/Th1 cells. Each dot represents one individual and red lines indicate median values. Grey surface represent reference values. Statistical analysis was performed with Mann Whitney U test. * $P < .05$, ** $P < .01$, *** $P < .001$ [Colour figure can be viewed at wileyonlinelibrary.com]

program. Transcripts from healthy controls ($P_{CDR/FWR} -0.48$) nor from children with allergic disease ($P_{CDR/FWR} 0.06$) showed more replacement mutations than expected by random chance in the CDR regions, that is, absence of positive selection (Figure 2I). Thus, the higher numbers of circulating IgE-expressing B cells in children with allergic disease did not show molecular signs of enhanced antigen receptor maturation.

As type 2 cytokines produced by helper T(h) cells are important for IgE class switch recombination (CSR) and children with allergic disease had increased numbers of IgE+ B cells, we investigated the

correlation between Th cells and IgE+ B cells. We observed a correlation between IgE+ plasmablast numbers and Th2 cells ($r = .271$; $P < .01$), whereas there was no correlation between IgE+ memory B-cell subsets and Th2 cells (Figure 2J).

4 | DISCUSSION

In the present study, we extended the immunological phenotype of allergic children. In addition to Th2 cells, eosinophils, basophils, and

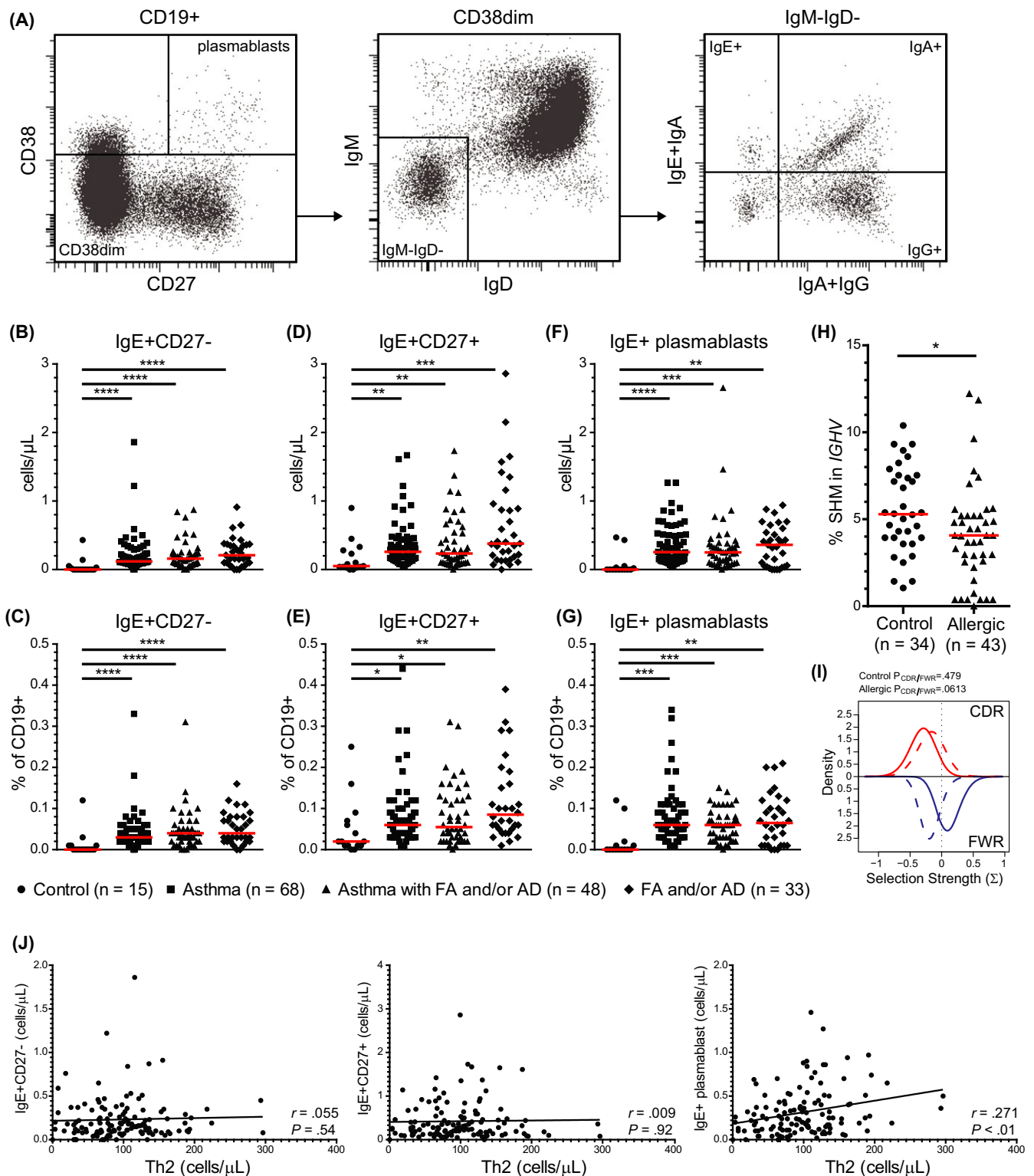


FIGURE 2 IgE+ B-cell subsets. A, Representative flowcytometry image and gating strategy of IgE+ memory B-cells. B-E, Absolute numbers and percentages of IgE+ memory B-cells. F-G, Absolute numbers and percentages of IgE+ plasmablasts. H, Somatic hypermutation (SHM) levels in the *IGHV* gene of IgE+ plasmablasts from healthy children and children with allergic disease (n=5 children each). I, Selection for replacement mutations in the CDR (red) and FWR (blue) regions. J, Correlation of Th2 cell numbers with IgE+CD27-, IgE+CD27+ and IgE+ plasmablasts in children with allergic disease. In panels B-G and J each dot represents one individual; red lines indicate median values; and black lines in panel J indicate linear correlations. Statistical analysis was performed with Mann Whitney *U* test. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$. Correlation was calculated with Spearman *R* [Colour figure can be viewed at wileyonlinelibrary.com]

serum IgE, we found increased numbers of circulating IgE⁺ memory cells and IgE⁺ plasmablasts and a positive correlation between IgE⁺ plasmablasts and Th2 cell numbers.

In our patient group, type 2 sensitization was confirmed by high IgE serum levels, sensitization to various aero- and food allergens, high numbers of eosinophils in blood, and increased Th2 cell numbers.

Our findings of increased numbers of circulating IgE⁺ memory B cells extend previous observations.^{9,12,13} Importantly, our flow cytometric gating strategy enabled exclusion of IgE-binding cells from the true IgE-expressing B cells, which we confirmed by the presence of mature IgE transcripts. We showed that this occurs in children and is independent of the allergic phenotype. A cause for the increased numbers of circulating IgE⁺ memory B cells could be the chronic inflammation. This is specifically thought to occur in patients with atopic dermatitis and to some extent also in asthma. Here, we found increased numbers of both CD27⁺ and CD27[−] IgE-expressing memory B cells in allergic patients. It has been shown that CD27[−] IgE⁺ memory B cells display a limited proliferation history and lower SHM levels in their immunoglobulin genes,⁹ which suggest that they originate from mucosal tissue, analogous to CD27[−] IgA⁺ memory B cells.¹⁴ This is supported by previous detections of IgE transcripts and Sμ-Sε switch circles in nasal tissue of patients with allergic rhinitis and in bronchial tissue of patients with allergic asthma.^{8,15} Several studies have shown that sensitized individuals who are exposed to allergens via nasal tissue or the respiratory tract display a substantial increase in systemic serum IgE levels, thus indicating the direct activation of an established local pool of IgE memory cells with defined specificities as an underlying mechanism.¹⁶

We did not observe a correlation between IgE⁺ memory B-cell and Th2 cell numbers, but we did find a positive correlation between Th2 cells and IgE⁺ plasmablasts. Previous studies show that type 2 innate lymphoid cells (ILC2) might play an important role in the local CSR toward IgE.^{17,18} Furthermore, expansions of ILC2 populations have been observed in allergic disease and are known to produce type 2 cytokines and ILC2 cells facilitate sensitization to local, but not systemic Th2 inducing allergen exposures.¹⁹ Next to that, T follicular helper (Tfh) cells have been linked to IgE class switching through the production of IL-4.^{20,21} Tfh cells express the B-cell follicle homing receptor CXCR5 and mainly reside in germinal centers. Thus, possibly ILC2 cells might be important in local IgE CSR, Tfh cells in germinal center IgE CSR, whereas Th2 cells are important in plasma cell differentiation. In future studies, it would be interesting to study ILC2 cells in mucosal tissue and correlate these to IgE⁺ memory B-cell numbers.

It is uncertain whether circulating IgE⁺ memory B cells sustain the underlying allergy, but in vitro stimulated IgE⁺ memory B cells can differentiate into IgE⁺ plasma cells.⁹ Therefore, it is possible that increased circulating IgE⁺ memory B cells actively contribute to allergic disease.²² To achieve desensitization and possibly even cure allergic disease, it might therefore be important to develop therapies to reduce IgE⁺ memory B cells. Omalizumab treatment in humans has shown to decrease serum IgE in patients with IgE-mediated asthma²³; however, it mainly targets soluble IgE. This is not the case for quilizumab, which only recognizes surface-expressed IgE on B cells,¹¹ and has shown to deplete

IgE-producing B cells in mouse studies.²⁴ Although human clinical trials with quilizumab reduced IgE serum levels for 6 months after treatment cessation, the effects on clinical parameters were variable.^{25,26} Our present findings can possibly contribute to patient selection and treatment monitoring of anti-IgE treatment.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

JJH, JCdJ, JJMvD, and MCvZ designed research. JJH and LR performed research. JJH, LR, and MCvZ analyzed data. NJA, SGP, JCdJ, and GJD evaluated and included patients in the study. JJH and MCvZ wrote the manuscript, and all authors commented on the manuscript and approved the final version.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Supporting information

IgE-expressing memory B cells and plasmablasts are increased in blood of children with asthma, food allergy and atopic dermatitis

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Table of contents

- Data 1. Supplemental Materials and Methods
- Supplemental Table 1. Patient characteristics
- Supplemental Table 2. Antibodies used for flowcytometry
- Supplemental Table 3. Immunophenotypic definitions of T- and B-cell subsets
- Supplemental Figure 1. Representative images from flowcytometry gating strategy for leukocyte subsets and T helper subsets
- Supplemental Figure 2. Absolute counts of neutrophils, eosinophils, basophils and monocytes
- Supplemental References

Data 1. Supplemental Materials and Methods

Flow cytometry of blood samples.

White blood cell (WBC) counts were determined with a Coulter cell counter (Beckman Coulter) within 24 hours of blood sampling. Absolute counts of CD45⁺ leukocytes, CD3⁺ T-cells and CD19⁺ B-cells were obtained with a diagnostic lyse-no-wash protocol using TruCount tubes (BD Biosciences, San Jose, Calif). For detailed 11-color flow cytometry, red blood cells were lysed with NH₄Cl prior to incubation of 2 million nucleated cells for 15 minutes at room temperature in a total volume of 100μL (antibodies listed in Supplemental Table 1). After preparation, cells were measured on a 4-laser LSRFortessa flow cytometer (BD Biosciences) using standardized settings (1). Data were analyzed with FACSDiVa software V8.0 (BD Biosciences). Immunophenotypic definitions of leukocyte and lymphocyte subsets are listed in Supplemental Table 2. Our previously published sequential flowcytometric gating strategy was applied to identify IgE⁺ B-cells (2).

Molecular analysis of IGH gene rearrangements.

RNA was isolated from post-Ficoll mononuclear cells with a GenElute mammalian RNA kit (Sigma-Aldrich, St Louis, Mo) and reverse transcribed to cDNA with random primers (Invitrogen Life technologies). Alternatively, IgE⁺ plasma cells (CD19⁺CD38^{hi}CD27⁺IgE⁺) were single cell sorted on a FACS Aria III (BD Biosciences) into 96-well PCR plates (VWR European, Radnor, PA) containing ice-cold 0.5x PBS, 10mM DTT (Invitrogen, Carlsbad, Calif) and 8 U RNAsin® (Promega, Madison, Wis). Total RNA from these single cells was reversed transcribed into cDNA using 5μM random hexamer primers (Applied Biosystems, Foster City, Calif), 0.5% Triton X (Sigma-Aldrich, St Louis, Mo), 1x first strand buffer

(Invitrogen), 1.25mM dNTPs, 10mM DTT (Invitrogen), 16 U RNAsin® (Promega) and 70 U SuperScript® III reverse transcriptase (Invitrogen) (3).

IGHV gene rearrangements were amplified in a semi-nested multiplex PCR approach using 4 different *IGHV*-family specific leader forward primers (4) in combination with *IGHE*-specific reverse primers (first PCR: 5'-CATCACCGGCTCCGGGAAGTAG-3'; second PCR: 5'-ACGGAGGTGGCATTGGAGGGAAT-3'). PCR products were cloned into a pGEMT easy vector (Promega, Madison WI), amplified by colony PCR, and sequenced on an ABI Prism 3130XL (Applied Biosystems, Foster City, CA). The sequences were analyzed with the IMGT database (www.imgt.org) and BASELINE program (selection.med.yale.edu/baseline) to quantify somatic hypermutations (SHM) and selection for replacement mutations. (5, 6)

Statistical analysis

Frequencies and absolute cell numbers had a non-Gaussian distribution. Therefore, all results were expressed as median (interquartile range) if applicable and were analyzed using the Mann-Whitney *U* test. Linear regression was used to study the strength of association between cell-subsets. Spearman rho was used to define if the correlation was significant. All indicated *P*-values were two-tailed and considered statistically significant if values were lower than 0.05. Statistical analysis was performed using GraphPad Prism software, version 6 (GraphPad Software, La Jolla, Calif).

64 **Supplemental Tables (n=3)**

65

66 **Supplemental Table 1. Patient characteristics**

	Control (n=15)	Asthma (n=68)	Asthma with food allergy and/or atopic dermatitis (n=48)	Food allergy and/or atopic dermatitis (n=33)
Gender – n (%)				
Male	7 (47%)	46 (68%)	31 (65%)	20 (61%)
Female	8 (53%)	22 (32%)	17 (35%)	13 (39%)
Age – mean yr (range)				
	11.1 (6-16)	11.4 (6-17)	10.9 (6-16)	9.0 * (6-15)
Sensitization to aero-allergens – n (%)				
HDM	n.d.	51 (75.0%)	28 (58.3%)	0 (0.0%)
Pollen	n.d.	46 (67.6%)	31 (64.6%)	18 (54.5%)
Dander	n.d.	43 (63.2%)	28 (58.3%)	13 (39.4%)
Sensitization to food allergens – n (%)				
Milk	n.d.	3 (4.4%)	11 (22.9%)	6 (18.2%)
Eggs	n.d.	3 (4.4%)	13 (27.1%)	8 (24.2%)
(shell)fish	n.d.	0 (0.0%)	9 (18.8%)	1 (3.0%)
Fruits	n.d.	1 (1.5%)	4 (8.3%)	0 (0.0%)
Peanut	n.d.	7 (10.3%)	23 (47.9%)	10 (30.3%)
Nuts	n.d.	3 (4.4%)	25 (52.1%)	18 (54.4%)
Wheat	n.d.	0 (0.0%)	6 (12.5%)	2 (6.1%)
Soy	n.d.	1 (1.5%)	5 (10.4%)	2 (6.1%)
Serum immunoglobulins – mean IU/ml (range)				
Total IgE	n.d.	782 (6-9,082)	2561 (13-13,452)	1,346 (252-9,140)
HDM, house dust mite; n.d. not determined				
* significantly lower than Asthma and Asthma with FA and/or AD ($P<0.01$)				

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68 **Supplemental Table 2. Antibody panels used for flowcytometry**

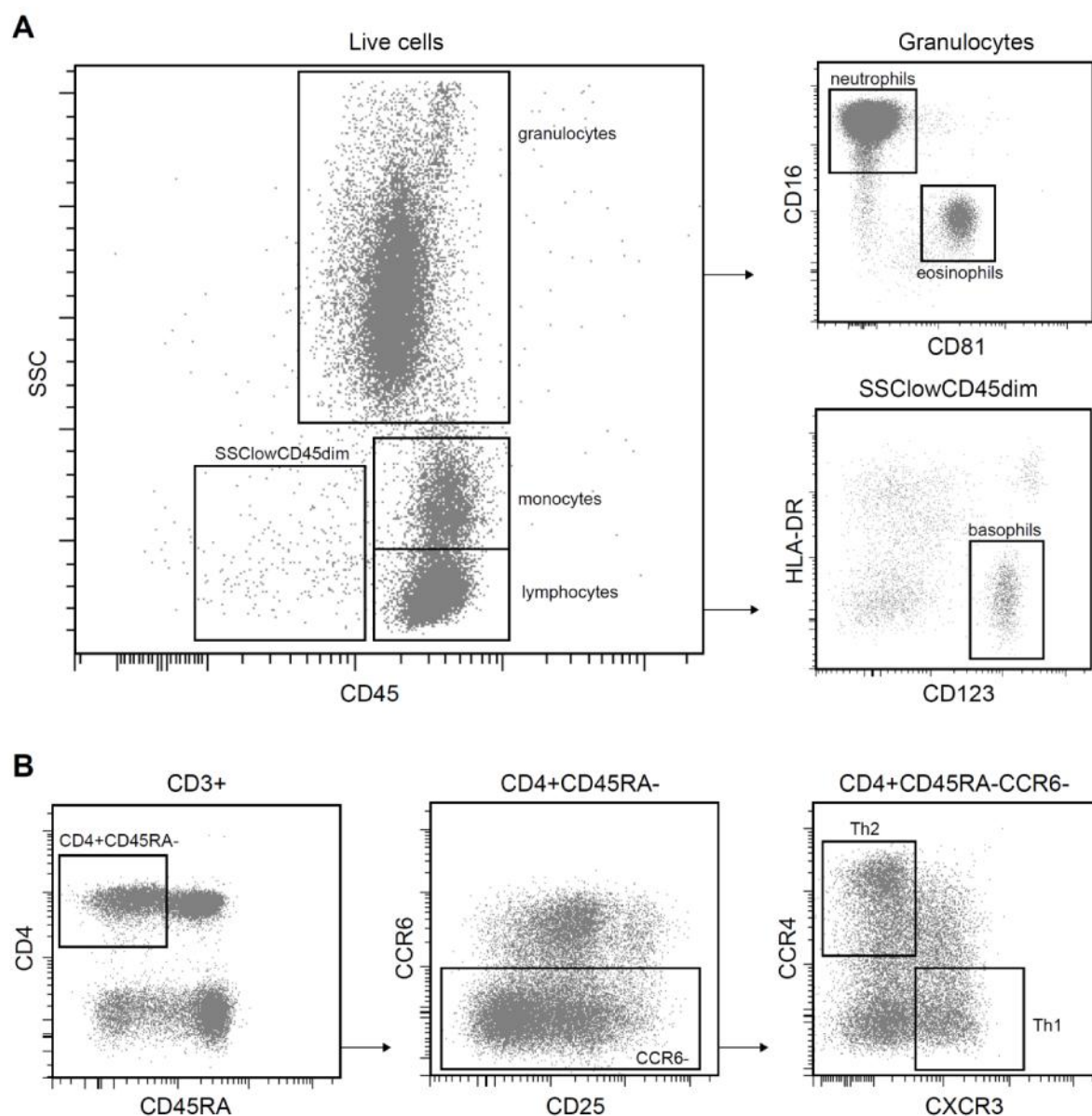
Antibody	Conjugate	Clone	Supplier
Panel 1: T-cell subsets			
CD3	BV711	UCHT1	BD Biosciences, San Jose, CA, USA
CD4	BV510	OKT4	BioLegend, San Diego, CA, USA
CD8	APC-H7	SK1	BD Biosciences
CD25	BV421	BC96	BioLegend
CD45RA	BV605	HI100	BioLegend
CD127	APC	A019D5	BioLegend
CCR4	PE-Cy7	L291H4	BioLegend
CCR6	PerCP-Cy5.5	G034E3	BioLegend
CCR10	PE	314305	R&D systems, Minneapolis, MN, USA
CXCR3	FITC	G025H7	BioLegend
Panel 2: B-cell subsets			
CD19	PE-Cy7	SJ25C1	Beckman Coulter, Indianapolis, IN
CD20	BV605	2H7	BioLegend
CD21	BV711	B-ly4	BD Biosciences
CD23	APC	M-L233	BD Biosciences
CD27	BV421	M-T271	BD Biosciences
CD38	APC-H7	HB7	BD Biosciences
IgA	FITC	IS11-8E10	Miltenyi Biotec
IgA	PE	IS11-8E10	Miltenyi Biotec
IgD	PE-CF594	IA6-2	BD Biosciences
IgE	FITC	Goat polyclonal	Invitrogen, Waltham, MA
IgG	PE	IS11-3B2.2.3	Miltenyi Biotec
IgM	BV510	MHM-88	BioLegend
Panel 3: Leukocyte subsets			
CD15	PE-CF594	W6D6	BD Biosciences
CD16	PerCP	3G8	BD Biosciences
CD45	OC515	GA90	Cytognos
CD62L	BV421	GV421	BioLegend
CD81	APC-H7	JS-81	BD Biosciences
CD117	APC	104D2	BD Biosciences
CD123	PE-Cy7	6H6	eBioscience
CD203c	PE	97A6	Beckman Coulter
HLA-DR	BV605	L243	BioLegend
IgE	FITC	Goat anti-human	Invitrogen
APC, Allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin			

69

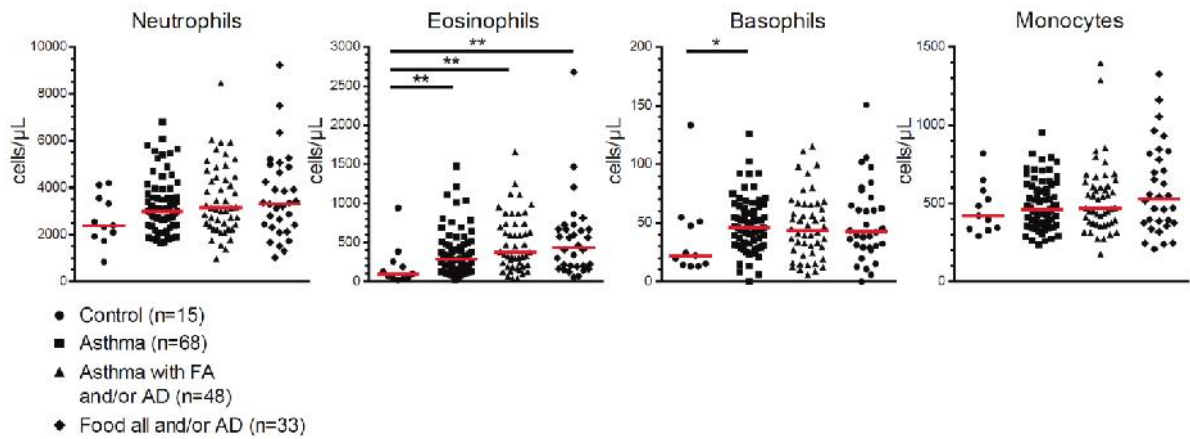
70 **Supplemental Table 3. Immunophenotypic definitions of T- and B-cell subsets**

CD45⁺ Leukocyte subsets	Immunophenotype
Neutrophil	CD45 ⁺ SSC ^{high} CD16 ⁺ CD81 ⁻
Eosinophil	CD45 ⁺ SSC ^{high} CD16 ⁻ CD81 ⁺
Basophil	CD45 ^{dim} SSC ^{low} HLADR ⁻ CD123 ⁺
Monocyte	CD45 ⁺ SSC ^{low}
CD3⁺ T-cell subset	Immunophenotype
Total CD8 ⁺ T cell	CD3 ⁺ CD8 ⁺
Total CD4 ⁺ T cell	CD3 ⁺ CD4 ⁺
T helper 1 cell (Th1)	CD3 ⁺ CD4 ⁺ CD45RA ⁻ CCR6 ⁻ CCR4 ⁻ CXCR3 ⁺
T helper 2 cell (Th2)	CD3 ⁺ CD4 ⁺ CD45RA ⁻ CCR6 ⁻ CCR4 ⁺ CXCR3 ⁻
CD19⁺ B-cell subset	Immunophenotype
IgE ⁺ CD27 ⁻ memory B cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgD ⁻ IgM ⁻ IgE ⁺ CD27 ⁻
IgE ⁺ CD27 ⁺ memory B cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgD ⁻ IgM ⁻ IgE ⁺ CD27 ⁺
Plasmablast	CD19 ⁺ CD38 ^{high} CD27 ⁺
IgE ⁺ plasmablast	CD19 ⁺ CD38 ^{high} CD27 ⁺ IgM ⁻ IgD ⁻ IgE ⁺

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Supplemental Figure 1 Flowcytometry gating strategy for leukocyte subsets and T helper subsets. **A.** Analysis of leukocyte subsets using antibodies listed in Supplemental Table 2 (Leukocyte subsets). **B.** Analysis of T-helper subsets using antibodies listed in Supplemental Table 2 (T-cell subsets)



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79 **Supplemental Figure 2.** Absolute counts of neutrophils, eosinophils, basophils and

80 monocytes per microliter of blood. Each dot represents one individual and red lines indicate

81 median values. Statistical analysis was performed with Mann Whitney *U* test. * $P < 0.05$,82 ** $P < 0.01$

83 Supplemental References

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