

CDR3 apex and the peptide. The available data suggest that an increased cysteine index is a specific biomarker of defective cortical tolerance mechanisms. The hydrophobic index appears more sensitive for detection of a self-tolerance defect but is not specific for either cortical or medullary tolerance mechanisms. Thus the cysteine and hydrophobic indices provide complementary information in the diagnosis and classification of T-cell self-tolerance defects.

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Supported by the Monash Biomedicine Discovery Institute, the National Health and Medical Research Council (grant 1107464), and the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

Disclosure of potential conflict of interest: L. D. Notarangelo is supported by the National Institute of Allergy and Infectious Diseases; has board memberships with the *Journal of Clinical Immunology*, *Clinical Immunology*, and *Frontiers in Immunology*; and has received royalties from UpToDate. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online April 30, 2019.
<https://doi.org/10.1016/j.jaci.2019.03.022>

Human BCR analysis of single-sorted, putative IgE⁺ memory B cells in food allergy



To the Editor:

The biology of IgE⁺ memory B cells (MBCs) remains enigmatic. The body of data from murine models demonstrates that IgE⁺ MBCs are extremely rare, at best, therefore suggesting that IgE-mediated recall responses are derived from non-IgE-expressing MBCs, particularly IgG₁.¹ In stark contrast, several human studies have claimed that a population of IgE⁺ MBCs can be identified in PBMCs of healthy donors, atopic donors, and donors with food allergy,²⁻⁴ as well as in the sputum of asthmatic patients.^{5,6} Moreover, the presence of these cells in the circulation has been proposed as a prognostic marker of allergy.²

Divergent findings on the existence of IgE-expressing MBCs between mice and human subjects could be partly due to the precision of techniques used in their quantification. While the identification of IgE-expressing MBCs in mice has been comprehensive, identification of these cells in human subjects has relied on flow cytometry.¹ In light of this, we sought to establish a method to validate the flow cytometric identification of IgE⁺ MBCs through genetic analysis of the BCR isotypes from single-sorted cells (see the [Methods](#) section in this article's Online Repository at www.jacionline.org).

We modified a single-cell nested PCR amplification method⁷ to amplify the variable region of IgE transcripts with primers shown specific to the 5' end of IgE heavy chain constant region (see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org).³ Then amplicons underwent Sanger sequencing, and resulting sequences were aligned to human heavy chain constant region alleles of all antibody isotypes to assess homology. To evaluate that the PCR technique amplifies IgE transcripts with high specificity and sensitivity, we single-sorted a human IgE-expressing cell line and performed IgE RT-PCR according to the same methodology (see [Fig E1, B](#)).

Our protocol demonstrated high sensitivity, amplifying, on average, more than 90% of the cells tested, sequences of which all

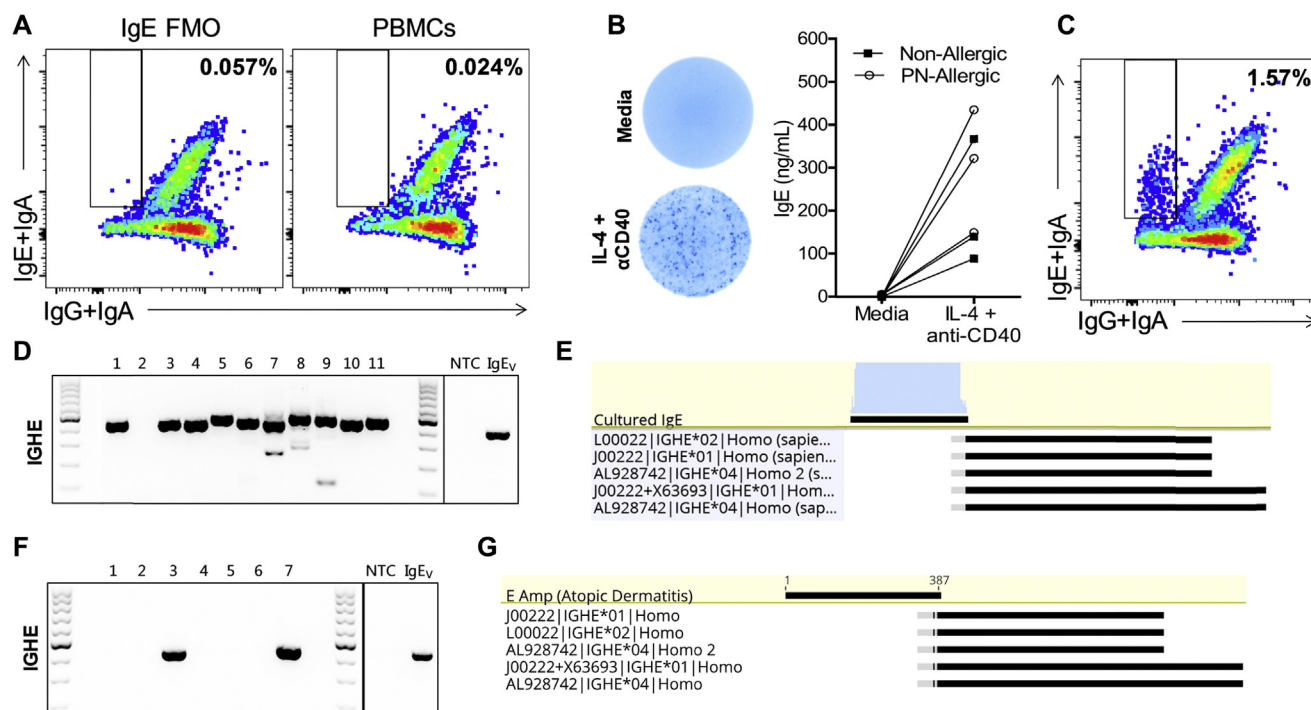


FIG 1. Enhanced flow cytometric method for detection of IgE⁺ MBCs. **A**, Cytometric detection of IgE⁺ MBCs. FMO, Fluorescence minus one control. **B** and **C**, PBMCs cultured with IL-4 plus anti-CD40 were analyzed by means of ELISpot and ELISA (Fig 1, B) and single-sorted (Fig 1, C). PN, Peanut. **D-G**, Amplification of IgE transcripts of single-sorted cells from positive control (Fig 1, D and E) or patients with atopic dermatitis (Fig 1, F and G) and alignment to the constant region of IGHE. Data are representative of 2 independent experiments (1-2 donors per experiment and 12-24 single-sorted cells per donor).

aligned to IgE constant region (IGHE) alleles (see Fig E1, C). Additionally, we generated a DNA vector containing a human IgE backbone (IGE_v) for use as a positive control. To determine that our technique specifically amplifies IgE transcripts, we single-sorted peripheral blood B cells (CD20⁺) that did not stain for IgE (see Fig E1, D). Using the same RT-PCR strategy as in Fig E1, A, none of the IgE⁻ B cells were amplified (see Fig E1, E), indicating that the technique is both sensitive and specific. Together, these data demonstrate that this system accurately amplifies rearranged IgE heavy chain variable sequences, specifically in single-sorted IgE-expressing cells.

Reported frequencies of IgE-expressing MBCs in peripheral blood vary depending on the flow cytometric identification strategies.^{2,3,6,8} A basic approach to identify these cells involves intracellular staining of IgE without preventing staining of cytotropic IgE.^{5,6} A more stringent detection method involves the stepwise exclusion of each BCR isotype through extracellular staining.³ With our single-cell IgE amplification protocol, we sought to validate the frequency of peripheral blood IgE-expressing MBCs in donors with peanut allergy using these previously reported flow cytometric approaches.

MBCs were identified as live singlet CD20⁺CD38^{lo-med}CD27⁺ and IgD⁻IgM⁻ cells, and IgE⁺ MBCs were further identified through basic IgE staining or the stepwise exclusion approach (see Fig E2, B). Twelve putative IgE-expressing MBCs were single-sorted from each staining technique for subsequent single-cell nested PCR. Basic IgE staining revealed a 3.4% frequency of IgE⁺ MBCs from B cells. However, no cells were amplified with IgE-specific RT-PCR (see Fig E2, C). The stepwise exclusion approach reported a frequency of putative

IgE-expressing MBCs 20 times lower (0.17%), but likewise, none of the cells were amplified with IgE-specific RT-PCR (see Fig E2, D).

To delineate the BCR identity of the spurious cells that fell into the IgE gate, we generated a cocktail of primers specific to IgG, IgA, and IgM heavy chain regions (GAM). By using our single-cell nested PCR strategy, more than 90% of the sorted cells were amplified with GAM primers, and the rest did not amplify (<10%). Amplicons were confirmed to align predominantly with IGHG and to a minor extent with IGHA or IGHM through Sanger sequencing. These data demonstrate that previously reported IgE⁺ MBC flow cytometry detection protocols result in a high rate of false-positive events.

The demonstration that previously reported flow cytometric methods for IgE⁺ MBC identification are faulty prompted us to ascertain the true frequency of these cells. Because the stepwise exclusion method generated a substantially lower number of spurious events than the basic IgE staining method, we sought to modify the former to remove contamination from non-IgE-expressing cells, which largely originated from IgG⁺ MBCs carrying cytotropic IgE (data not shown). This enhanced protocol consisted of purifying B cells from PBMCs and a stepwise exclusion of IgD⁺, IgM⁺, IgA⁺, and IgG⁺ cells (Fig 1, A). Furthermore, use of a polyclonal anti-IgG antibody markedly contributed to resolving the double-negative population of MBCs (IgD⁻IgM⁻IgG⁻IgA⁻IgE⁻) compared with the previous stepwise exclusion method (3.8% vs 58.6%). Because B cells canonically express at least 1 BCR isotype, we reasoned that the population arose from B cells with a low BCR surface density, in which use of a polyclonal (rather than monoclonal)

TABLE I. Quantification of IgE⁺ MBCs in healthy and allergic donors

Tissue	Donor ID	Allergic status	Mononuclear cells	Purified B cells	Events in CD20 ⁺ CD38 ^{lo-med} gate	Events in IgE gate	Sorted cells	IGHE amplification
Blood	P001	—	250,000,000	9,540,000	600,304	21	6	0
	P003	—	123,000,000	8,640,000	169,267	29	12	0
	P007	—	125,000,000	2,685,000	645,055	5	3	0
	P009	—	98,600,000	4,140,000	325,734	12	12	0
	P014	—	109,000,000	2,820,000	605,535	8	8	0
	P021	—	173,000,000	26,000,000	440,963	2	1	0
	P025	—	86,600,000	1,401,000	246,090	20	20	0
	P026	—	94,200,000	1,494,000	250,018	14	10	0
	P030	—	78,800,000	1,128,000	132,157	4	4	0
	P031	—	85,400,000	945,000	172,260	4	4	0
	P008	Peanut	125,000,000	8,160,000	335,641	20	20	0
	P011	Peanut	250,000,000	7,150,000	520,021	6	3	0
	P013	Peanut	210,000,000	5,450,000	690,015	13	12	0
	P016	Peanut	125,000,000	1,068,000	143,759	5	5	0
	P017	Peanut	125,000,000	1,467,000	213,345	11	10	0
	P020	Peanut	124,000,000	3,900,000	537,856	4	1	0
	P024	Peanut	71,800,000	1,026,000	166,313	3	1	0
	P028	Peanut	94,800,000	1,440,000	157,340	1	1	0
	P029	Peanut	54,000,000	1,467,000	277,359	2	2	0
Tonsils	TP-9	—	10,000,000	—	204,125	10	7	0
	TP-10	—	10,000,000	—	68,461	15	12	0
	TP-11	—	10,000,000	—	56,127	6	6	0

antibody to stain for surface IgG was superior. Notably, the improved staining resulted in a significantly lower frequency of cells in the IgE gate, at 0.006% of total B cells, which was considered background as it was comparable to the frequency observed in the fluorescence minus one control (0.01% of total B cells; Fig 1, A).

To validate that our enhanced staining technique was capable of detecting IgE⁺ MBCs, we stimulated PBMCs in culture with IL-4 plus anti-CD40. As expected, culturing under these conditions resulted in the robust emergence of IgE-secreting cells and IgE, as detected by using total IgE ELISpot and ELISA, respectively (Fig 1, B). A population of putative IgE⁺ MBCs was observed through the enhanced stepwise exclusion method (Fig 1, C), and their IgE identity was confirmed through single-cell nested RT-PCR and Sanger sequencing (Fig 1, D and E). Further validation was carried out in PBMCs from 4 patients with atopic dermatitis and serum IgE levels of between 2370 and 6350 kIU/L. *Bona fide* IgE⁺ MBCs, confirmed with Sanger sequencing, were identified in 2 of these 4 patients at a frequency of 0.0015% from total B cells (Fig 1, F and G).

With our enhanced detection method, we conducted analyses on PBMCs of 20 donors, which included patients with peanut allergy (n = 9; mean serum total IgE level, 196 kIU/L [11-890 kIU/L]) and nonallergic subjects (n = 10, see Table E1 in this article's Online Repository at www.jacionline.org). We detected similar frequencies of putative IgE⁺ MBCs (percentage from total B cells: 0.0019 for patients with peanut allergy and 0.0046 for nonallergic subjects). However, in all instances there was no IgE amplification (Table I).

To ensure that IgE⁺ MBCs were not being missed through our exclusion of CD27[−] cells, we sorted CD27[−] IgE⁺ MBCs because it has been speculated that MBCs arising

extrafollicularly³ might not gain CD27, the canonical MBC marker. Similarly, no PCR amplification occurred with IgE primers (data not shown).

Furthermore, we investigated the possibility that the polyclonal anti-IgG antibody could bind nonspecifically to IgE⁺ MBCs because of serum IgG or IgA bound to MBCs, thus masking IgE⁺ MBCs cells in the IgG⁺ or IgA⁺ populations. Here we stained for IgA and IgG on the same fluorophore and flow-sorted class-switched MBCs that were positive for IgE (see Fig E3, A and B, in this article's Online Repository at www.jacionline.org). The frequency of this population was 0.074%, and genetic analysis demonstrated that these MBCs were of a non-IgE identity that presumably bound secreted IgE. Additionally, we single-sorted class-switched MBCs from the IgG, IgA, and (residual) double-negative gate. There was no PCR amplification with IgE primers but with GAM (see Fig E3, C and D), thus supporting that the methodology used did not underestimate the frequency of IgE⁺ MBCs.

The value of scientific knowledge relies to a large extent on the fidelity of the tools used to generate such knowledge. In this context, we provide a validated method to identify *bona fide* IgE⁺ MBCs. Our data demonstrate the extreme rarity of these cells in the circulation of allergic patients, at least orders of magnitude lower than previously reported,^{2,3} and are in agreement with human genetic studies that reported few IgE transcripts in the circulation but without unambiguously defining the B-cell phenotype (eg, MBCs and plasmablasts).⁹ This finding strengthens the concept that the reservoir of IgE-secreting cells resides in MBCs of a non-IgE isotype and, as such, informs future research directions. Of note, it is possible that tissues from allergic subjects could harbor IgE⁺ MBCs; this remains both a challenge and a prime venue for future efforts. Nevertheless, it is evident that the proposal that circulating

IgE⁺ MBCs could be a clinical marker for allergic disease is unproved.

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This study was supported by grants from Food Allergy Canada, the Delaney family, the Zych family, the Walter and Maria Schroeder Foundation and AllerGen NCE (grant 16CanFAST5 to Drs Wasserman and Jordana, International Visit Award to Dr Jiménez-Saiz and Summer studentship to Yosef Ellenbogen).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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Available online April 5, 2019.

<https://doi.org/10.1016/j.jaci.2019.04.001>

Allogeneic hematopoietic stem cell transplant outcomes for patients with dominant negative *IKZF1*/IKAROS mutations



To the Editor:

The transcription factor IKAROS is encoded by the *IKZF1* gene and plays a crucial role in lymphopoiesis.^{1,2} Somatic and, more recently, germline mutations of *IKZF1* are associated with

a number of hematologic malignancies, most notably B-cell precursor acute lymphoblastic leukemia.^{3,4} A germline mutation in *IKZF1* was first reported as a monogenic cause of human disease in a single neonate in 2012 after similarities were noted between the patient's phenotype and an IKAROS knockout mouse model.⁵ Subsequently, autosomal dominant mutations leading to haploinsufficiency were discovered to underlie a proportion of patients with common variable immunodeficiency and low B-cell numbers.⁶

Most recently, patients with a more profound combined immunodeficiency (CID) phenotype were discovered to have unique mutations affecting the amino acid N159, which result in a dominant negative effect, unlike the other mutations.⁷ These patients all exhibited infections characteristic of T-cell deficiency, including *Pneumocystis jirovecii* pneumonia. Because of the early-onset, severe, and progressive nature of the disease, which also includes leukemia susceptibility, some patients were treated with allogeneic hematopoietic stem cell transplantation (HSCT).

At this time, there is very little known regarding allogeneic HSCT outcomes for patients with dominant negative *IKZF1* mutations. Allogeneic HSCT is a high-risk procedure associated with potential for serious complications, including graft-versus-host disease (GVHD). Previous mouse models of hematopoietic cell transplantation have convincingly demonstrated a greater severity of acute GVHD in IKAROS-knockout and dominant negative mice,⁸ suggesting that patients who undergo allogeneic HSCT might be at increased risk of more severe acute GVHD. Survival was decreased in both of the IKAROS-deficient mouse models and was lowest for those with a dominant negative mutation, suggesting that patients with dominant negative *IKZF1* mutations might also be at an increased risk of mortality after allogeneic HSCT. To determine whether patients with *IKZF1* dominant negative mutations are at increased risk of adverse events after allogeneic HSCT, we collected transplantation data from 4 patients who underwent allogeneic HSCT at transplantation centers around the world.

Clinical presentations of this cohort were similar before diagnosis, including opportunistic infections, T-cell abnormalities, hypogammaglobulinemia with absent to nearly absent B cells before the age of 1 year, and progressive myeloid involvement. Additionally, all patients had *Pneumocystis jirovecii* pneumonia early in life or even repeated episodes. Other notable infectious complications included respiratory syncytial virus in patients 1 and 2, *Cryptosporidium* species cholangitis in patient 3, and pneumonia in patients 3 and 4. Patient 4 initially presented at 9 months of age with acute respiratory distress syndrome and was found to have a CID for which she received an unconditioned CD34⁺ haploidentical transplant with subsequent graft failure. This patient then continued to have infections and had T-cell acute lymphoblastic leukemia at the age of 13 years (which prompted their second HSCT, which is described here).⁹ Only patient 1 had a genetic diagnosis before transplantation.

Patients underwent a variety of conditioning preparative regimens before transplantation (Table I). Conditioning regimens were selected by the primary treating physician based on their standard-of-care practices. Neutrophil engraftment occurred between days +12 and +51 after transplantation. Platelet engraftment occurred between days +8 and +167. Patient 3 did not

METHODS

Flow cytometry

Antibodies were obtained from BioLegend (San Diego, Calif), Columbia Biosciences (Frederick, Md), BD Biosciences (San Jose, Calif), Miltenyi Biotec (Bergisch Gladbach, Cologne, Germany), eBioscience (Carlsbad, Calif), or Thermo Fisher Scientific (Waltham, Mass): CD38–phycoerythrin (PE)-Cy7 (clone HIT2); IgE-allophycocyanin (Columbia Biosciences SKU: D3-110-E); IgG-PE (clones G18-145 and HP6017 and Thermo Fisher Scientific Catalog #12-4998-82); IgA-PE (clone IS11-8E10); IgA-allophycocyanin (clone IS11-8E10); IgM–Brilliant Violet 510 (clone MHM-88); IgD–Brilliant Violet 421 (clone IA6-2); IgG-biotin (Thermo Fisher Scientific Catalog #A18815); CD20–Alexa Fluor 700 (clone 2H7); and CD27–fluorescein isothiocyanate (clone O323). In all assays 1×10^6 cells were first incubated with Human TruStain FcX (Fc Receptor Blocking Solution, BioLegend) or anti-human CD32 (FcγRII Blocker; STEMCELL Technologies, Vancouver, British Columbia, Canada) for 15 minutes on ice to block nonspecific staining and then incubated with fluorochrome-conjugated antibodies for 30 minutes on ice and protected from light. When IgG-biotin was used to label IgG⁺ cells, cells were incubated for an additional 30 minutes with streptavidin-PE (BioLegend) on ice and protected from light. Dead cells were excluded by using the fixable viability dye eFluor780 (eBioscience) and by gating on singlets. Fluorescence minus one was used for gating. Data were acquired on a Fortessa (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, Ore), and single cells were sorted on a MoFlo XDP Cell Sorter (Beckman Coulter, Fullerton, Calif).

PCR amplification

Single cells were sorted into 96-well PCR plates (Thermo Fisher) containing 20 U of RNasin Ribonuclease Inhibitors (Promega, Madison, Wis), 2 μL of First Strand buffer (Thermo Fisher), and nuclease-free water to a volume of 10 μL per well. The cells were centrifuged at 4°C and 550g for 1 minute and immediately placed in a –80°C freezer. Next, heat lysis was performed by adding 3 μL of Nonidet P-40 Substitute (G-Biosciences, St Louis, Mo) and 150 ng of random hexamers (Thermo Fisher). The reaction was performed at 65°C for 10 minutes and then 25°C for 3 minutes. All thermocycler reactions were done with the Mastercycler Pro S (Eppendorf). cDNA was synthesized, as follows: 2 μL of 5× First Strand buffer (Thermo Fisher), 2 μL of 0.1 mol/L dithiothreitol (Qiagen, Hilden, Germany), 1 μL of 10 mmol/L of each dNTP, and 0.5 μL of SuperScript III (Thermo Fisher) were added to the plate containing the heat lysis contents (final volume, 19.5 μL). Reverse transcription was performed at 37°C for 1 hour and then 70°C for 10 minutes.

IgH amplification was accomplished by using a 2-step nested PCR, as previously described. Briefly, a mix of 6 forward primers^{E1} and either a reverse primer specific to *IGHF*^{E2} (first 5'-CATCACCGGCTCCGGGAAGTAG-3' and second 5'-GTTTTCGACGACGGGTCAAG-3') or a pool of reverse primers specific to *IGHM*, *IGHA*, and *IGHG* were used (*IGHM*: first 5'-CAGGA GACGAGGGGAAAAG-3' and second 5'-GAAAAGGGTTGGGGCG GATGC-3'; *IGHA*: first 5'-GCTCAGCGGGAAGACCTT-3' and second 5'-GACCTTGGGGCTGGTCGGGGA-3'; and *IGHG*: first 5'-GCCAGGGGGA GACSGATG-3' and second 5'-GACSGATGGGCCCTTGGTGA-3').^{E3} The first PCR reaction contained 8 μL of cDNA mixture, 1 U of HotStar Plus Taq (Qiagen), 200 nmol/L of each primer, 400 μmol/L dNTP (Thermo Fisher), 5 μL of 10× PCR buffer (Qiagen), and nuclease-free water to a final volume of 50 μL. The reaction was performed starting with 3 cycles of preamplification of 95°C for 45 seconds, 45°C for 45 seconds, and 72°C for 45 seconds, followed by 30 cycles of 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 1 minute and 45 seconds and a final extension of 72°C for 10 minutes. The second PCR reaction contained 4 μL of PCR 1 product, 5 μL of 10× Pfu buffer (Agilent, Santa Clara, Calif), 1.25 μL of 10 mmol/L dNTP (Thermo Fisher), 400 nmol/L of each primer, 1.25 U of Pfu polymerase (Agilent), and nuclease-free water to a final concentration of 50 μL. The reaction was performed for 30 cycles at 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 1 minute and 45 seconds, followed by a final extension of 72°C for 10 minutes.

The second PCR product was visualized on a 1.5% agarose gel, and the expected band size was approximately 400 bp. Amplified IgH sequences were enzymatically purified by using ExoSAP-IT PCR Product Cleanup Reagent

(Thermo Fisher) and subsequently Sanger sequenced by using the forward and reverse primers (GENEWIZ). The sequences were analyzed by using IMG/HighV-QUEST (<http://imgt.org/HighV-QUEST>) for V, D, and J sequences with the highest identity, as well as nucleotide and amino acid mutations from their germline sequences.

Generation of a DNA vector containing a human IgE backbone

To generate a human IgE, we started with a heavy chain IgG₁ vector (gifted by Michel C. Nussenzweig) previously modified to include an Ara h 2 variable chain.^{E4} The human ε constant region was amplified from an anti-OVA human IgE vector^{E5} with primers (5' TTTT GTCGAC GGCGACCA 3' and 5' TTTT AAGCTT CTCAATGGTGGTGATGTTA 3') to add flanking *Sall* and *HindIII* restriction enzyme sites. The human ε constant region then replaced the γ1 constant region under the CMV promoter by using the *Sall* and *HindIII* restriction enzyme sites. Sanger sequencing confirmed successful insertion of the ε constant region.

Study population

A cohort of 10 blood donors with peanut allergy and 10 nonallergic blood donors were recruited from McMaster University (Hamilton, Ontario, Canada). Allergy to peanut was ascertained by using a peanut-specific IgE ImmunoCAP performed at LRC Hamilton (McMaster Children's Hospital) and by using skin prick testing. Patients with peanut allergy were considered for inclusion with peanut-specific serum IgE levels of greater than 0.35 kU/L and skin prick test responses of 3 mm or more larger than those elicited by the saline control. Total serum IgE levels were quantified by using the IMAGE 800 (Beckman Coulter) performed at LRC Hamilton for a general measure of atopy. We recruited an additional 4 participants with total serum IgE levels of greater than 2300 kIU/L and received 3 tonsil discards from subjects undergoing routine tonsillectomies. Exclusion criteria for all recruited donors included the following: allergen immunotherapy, previous or current omalizumab (Xolair) treatment, other systemic immunomodulatory treatments (ie, rituximab), or autoimmune/immunodeficiency diseases. Patients' demographics and allergic indicators are summarized in Table E1. All donors were recruited with written consent and ethical approval from Hamilton Integrated Research Ethics Board.

Mononuclear cell isolation and B-cell enrichment

Up to 80 mL of peripheral blood was collected into heparinized tubes (BD), and tonsils were crushed into a single-cell suspension. PBMCs were isolated through Ficoll-Paque (GE Healthcare, Chicago, Ill) density gradient centrifugation. Immediately afterward, B cells were isolated from PBMCs by using a negative selection magnetic separation kit (19054; STEMCELL Technologies) with at least 70% purity.

PBMC culture

PBMCs were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% human AB serum (Corning, Corning, NY), 10 mmol/L HEPES, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 55 μmol/L 2-mercaptoethanol, 1% L-glutamine, and 1% penicillin-streptomycin. Cells were plated at a density of 1.5×10^6 cells/mL in 24-well plates. Stimulated cells were treated with 68.7 ng/mL (8000 IU) IL-4 (Sigma-Aldrich, St Louis, Mo) and 5 μg/mL anti-CD40 (Bio X Cell, West Lebanon, NH) on day 1. Cells were incubated at 37°C and 5% CO₂ for the duration of culture. On days 4 and 8 of culture, 1 mL of cell-free supernatant was withdrawn and replaced with fresh media. Supernatants were also withdrawn on day 11 of culture and stored at –80°C for later analysis of total IgE by means of ELISA. After 11 days in culture, cells were harvested, and IgE-secreting cells were quantified by using ELISpot.

ELISA and ELISpot

For total IgE ELISA, MaxiSorb plates (Thermo Fisher Scientific) were coated with 0.5 μg/mL anti-human IgE (555894; BD PharMingen, San Jose,

Calif) in carbonate-bicarbonate buffer overnight at 4°C. Coated wells were blocked with 5% skim milk in PBS for 2 hours at room temperature, followed by 3 washes (1× PBS and 0.05% Tween 20). Cell-free supernatant samples and a serial dilution of purified human IgE (401152; Calbiochem, San Diego, Calif) were incubated overnight at 4°C. Wells were washed 3 times and incubated with 1 µg/mL biotinylated anti-human IgE (A18803; Invitrogen, Carlsbad, Calif) in 1% skim milk for 2 hours at room temperature. Subsequently, wells were washed 3 times and incubated with alkaline phosphatase streptavidin for 1 hour at room temperature. Plates were developed with *p*-nitrophenyl phosphate, and the reaction was stopped with 2N NaOH. ODs were measured at 405 nm (Multiskan FC; Thermo Scientific).

A commercially available ELISpot kit (3810-2H; Mabtech, Stockholm, Sweden) was used for detection of IgE-secreting cells. On day 11 of culture, samples were plated in duplicates at 4×10^6 cells/mL. Plates were imaged with the ImmunoSpot S6 Analyzer, and spots were counted independently by 2 blinded investigators.

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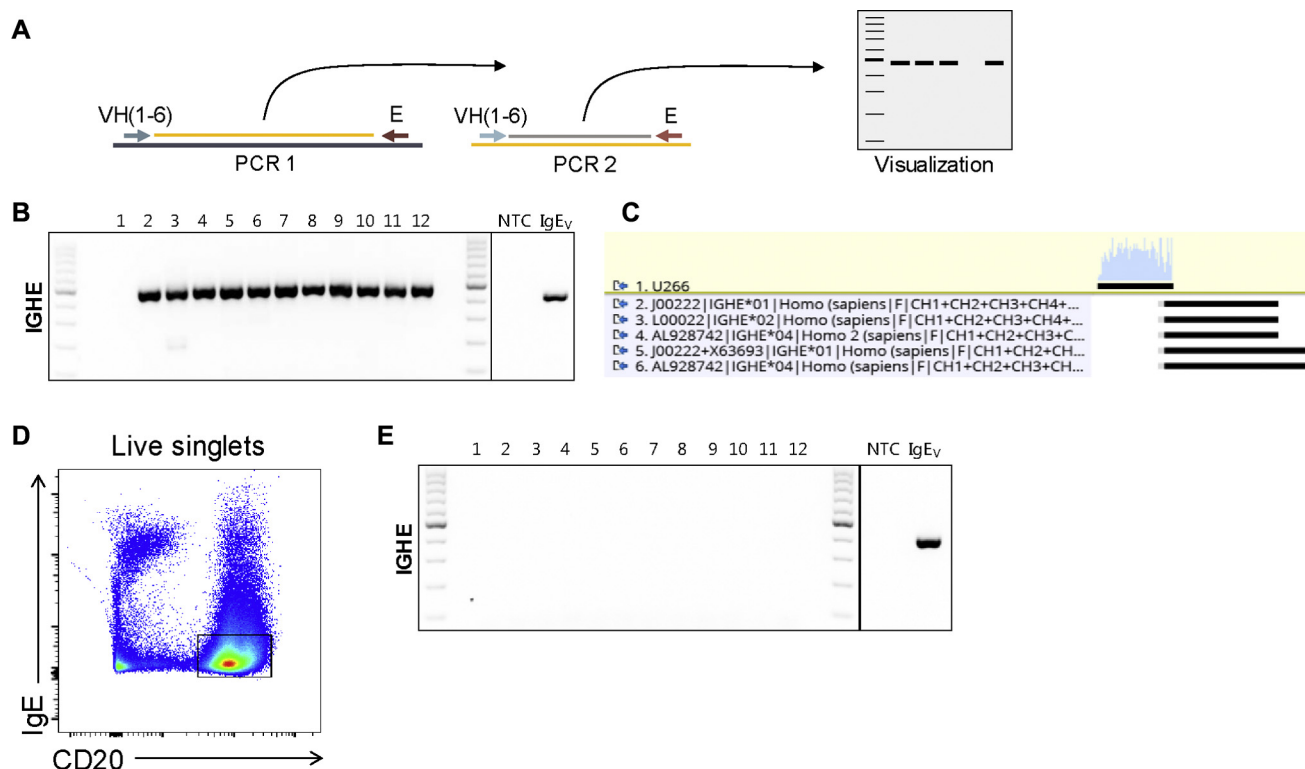


FIG E1. Methodology to genetically validate the IgE identity of single-sorted cells. **A**, Schematic of the method. **B**, Single-sorted human IgE-expressing myeloma cells were assayed by using nested PCR. **C**, IgE transcripts were shown to align with *IGHE* alleles. **D**, Peripheral blood CD20⁺ B cells that stained negative for IgE were single-sorted. **E**, IgE transcripts did not amplify, as evidenced by using DNA gel electrophoresis.

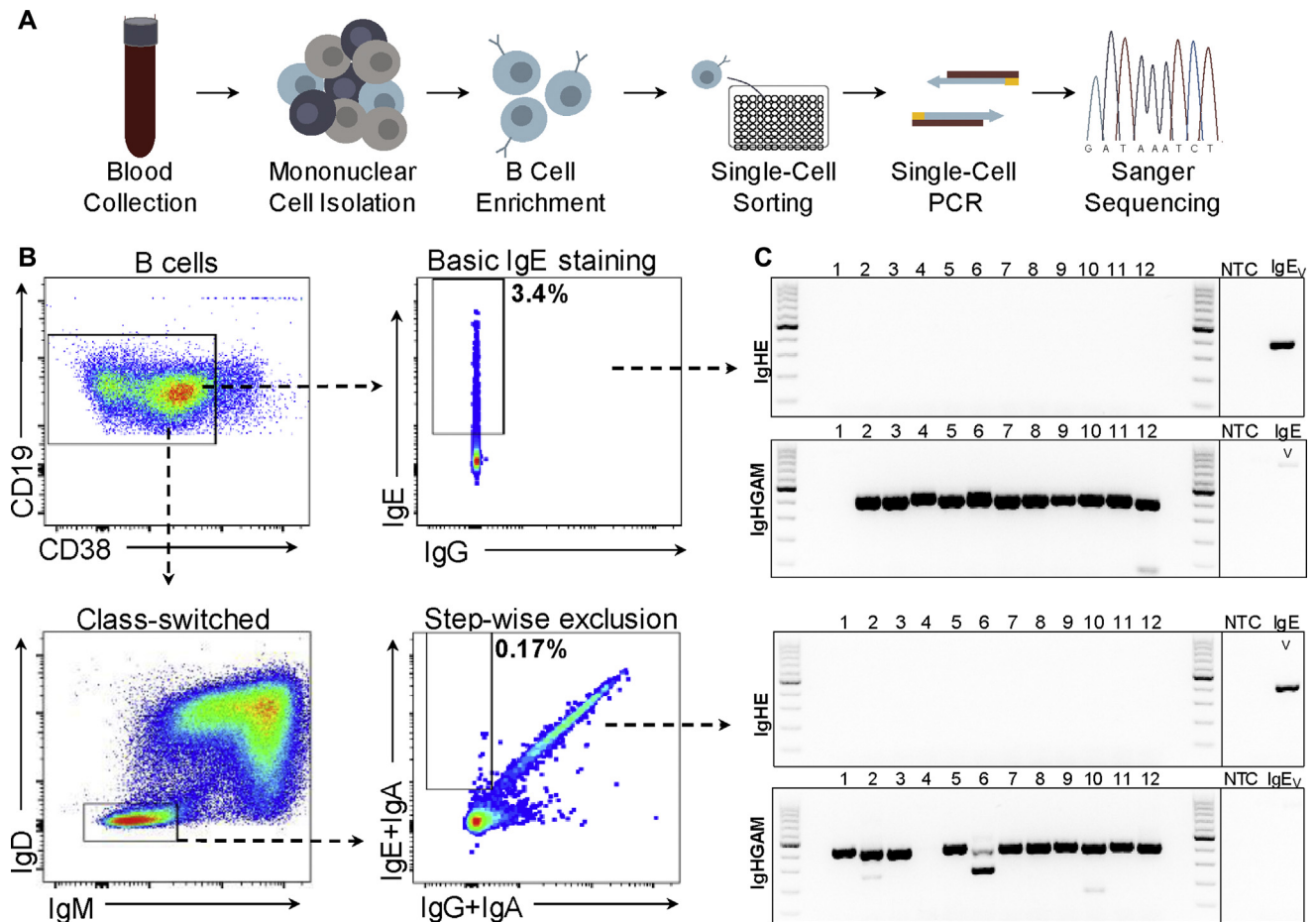


FIG E2. BCR analysis of single-sorted, putative IgE⁺ MBCs stained with commonly used methods demonstrates a non-IgE identity. **A**, Schematic of the experimental design. **B**, Gating strategy used with a basic IgE staining (*upper*) or stepwise exclusion (*lower*) method. **C**, BCR amplification with primers specific for *IGH*E or a mix (*IGH*GAM). Data are representative of 5 independent experiments (1-2 donors per experiment and 12-24 single-sorted cells per donor).

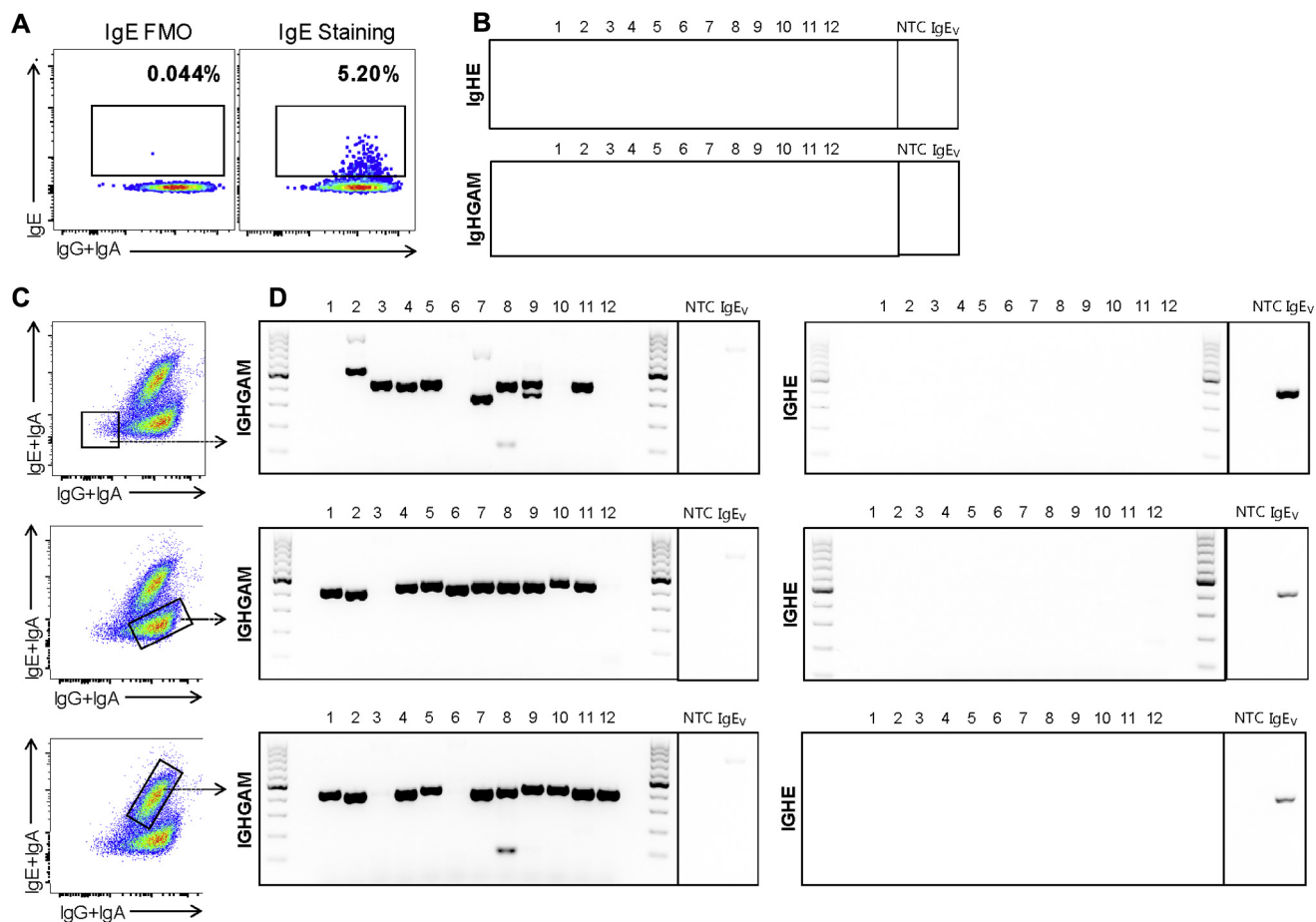


FIG E3. Assessment of cytotropic (IgG and/or IgA) and negative staining of IgE⁺ MBCs. **A** and **C**, Cytometric detection and sorting of class-switched MBCs from different gates. *FMO*, Fluorescence minus one control. **B** and **D**, BCR amplification with primers specific for IgE (*IGH*E) or a mix specific of IgG, IgA, and IgM (*IGH*GAM) of single-sorted cells. Data are representative of 2 independent experiments (2 donors per experiment and 12-18 single-sorted cells per donor).

TABLE E1. Patients' profiles

	Donor ID	Sex	Age	Serum IgE (kIU/L)	Atopic dermatitis	Clinical reactivity to peanut	Peanut IgE (kU/L)	Skin prick test (S/H/P)
Nonallergic subjects	P001	M	20	<5	No	No	<0.1	2/5/2
	P003	M	23	96	No	No	<0.1	1/3/1
	P007	M	23	<5	No	No	<0.1	1/4/1
	P009	F	24	<5	No	No	<0.1	1/4/1
	P014	M	22	230	No	No	0.34	2/5/2
	P021	M	21	45	No	No	<0.1	1/4/2
	P025	F	34	40	No	No	<0.1	1/5/1
	P026	M	19	37	No	No	0.11	1/6/1
	P030	M	24	9	No	No	<0.1	1/4/1
	P031	M	23	12	No	No	<0.1	1/5/1
	TP09	M	2	NA	No	No	NA	NA
	TP10	M	30	NA	No	No	NA	NA
	TP11	M	5	NA	No	No	NA	NA
Patients with atopic dermatitis	P006	F	23	5430	Yes	Yes	13.48	1/4/5
	P034	F	39	3760	Yes	No	10.29	NA
	P035	M	53	2370	Yes	No	NA	NA
	P036	F	20	6350	Yes	No	NA	NA
Patients with peanut allergy	P008	M	32	130	No	Yes	15.87	1/4/7
	P011	M	19	210	No	Yes	0.66	1/5/7
	P013	M	20	890	No	Yes	28.09	1/5/4
	P016	M	21	91	No	Yes	1.66	1/4/8
	P017	M	22	120	No	Yes	43.28	1/4/10
	P020	M	26	256	No	Yes	7.56	1/4/12
	P024	M	60	37	No	Yes	5.54	1/4/10
	P028	M	25	27	No	Yes	1.11	1/4/7
	P029	F	23	11	No	Yes	3.79	1/4/6

F, Female; M, male; NA, not available; S/H/P, saline/histamine/peanut.