

Single B-cell deconvolution of peanut-specific antibody responses in allergic patients

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Background: The frequencies, cellular phenotypes, epitope specificity, and clonal diversity of allergen-specific B cells in patients with food allergy are not fully understood but are of major pathogenic and therapeutic significance.

Objective: We sought to characterize peanut allergen-specific B-cell populations and the sequences and binding activities of their antibodies before and during immunotherapy.

Methods: B cells binding fluorescently labeled Ara h 1 or Ara h 2 were phenotyped and isolated by means of flow cytometric sorting from 18 patients at baseline and 13 patients during therapy. Fifty-seven mAbs derived from allergen-binding single B cells were evaluated by using ELISA, Western blotting, and peptide epitope mapping. Deep sequencing of the B-cell repertoires identified additional members of the allergen-specific B-cell clones.

Results: Median allergen-binding B-cell frequencies were 0.0097% (Ara h 1) or 0.029% (Ara h 2) of B cells in baseline blood from allergic patients and approximately 3-fold higher during immunotherapy. Five of 57 allergen-specific cells belonged to clones containing IgE-expressing members. Almost all allergen-specific antibodies were mutated, and binding to both conformational and linear allergen epitopes was detected. Increasing somatic mutation of IgG₄ members of a clone was

seen in immunotherapy, whereas IgE mutation levels in the clone did not increase.

Conclusion: Most peanut allergen-binding B cells isolated by means of antigen-specific flow sorting express mutated and isotype-switched antibodies. Immunotherapy increases their frequency in the blood, and even narrowly defined allergen epitopes are recognized by numerous distinct B-cell clones in a patient. The results also suggest that oral immunotherapy can stimulate somatic mutation of allergen-specific IgG₄. (*J Allergy Clin Immunol* 2015;■■■:■■■-■■■.)

Key words: Peanut, allergy, B cell, antibody, IgE, IgG₄, immunotherapy, high-throughput DNA sequencing, antigen specific, allergen specific, repertoire, somatic mutation

Antibodies in the blood of allergic patients have been characterized by using serologic methods for decades, and the results of testing for allergen-specific serum IgE are useful in the diagnosis of allergic disease.^{1,2} Changes in allergen-specific antibody populations, particularly increases in amounts of allergen-specific IgG₄, are hallmarks of a successful response to allergen immunotherapy regimens.³⁻⁸ Despite the clear importance of specific antibodies as mediators of allergic disease and as biomarkers or effectors of therapeutic responses, there is little molecular data about the antibody protein sequences found in sera of allergic patients.

Molecular characterization of allergen-specific antibodies in patients could clarify key mechanistic features of allergic disease and immunotherapy responses, including the number of different antibody species and their epitope specificity, somatic mutation levels and affinity maturation, and the clonal identities and antibody isotypes stimulated by using immunotherapy.

Here, we present methods and initial results from measurement of B cells that bind to Ara h 1 or Ara h 2 allergens in 27 patients with peanut allergy at baseline or during immunotherapy, and we report detailed analysis of the antibody sequences expressed by allergen-binding single B cells in 6 of the patients. Allergen-binding B cells are less than 0.03% of total B cells in baseline allergic patients and are approximately 3-fold more frequent in patients during immunotherapy. Almost all allergen-binding B cells expressed somatically mutated antibodies. The allergen-specific antibodies recognize a variety of conformational or linear epitopes. Deep sequencing of patient antibody heavy chain repertoires identified additional members of allergen-specific clones, including IgE-expressing members in 4 clonal lineages. One patient had an allergen-specific clone with IgG₄ members that increased in mutation during oral immunotherapy (OIT), whereas IgE members did not have increased mutation levels, suggesting that mechanisms of OIT might include preferential stimulation of somatic mutation in allergen-specific IgG₄.

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Abbreviations used

CDR: Complementarity determining region
 IGHD: Immunoglobulin heavy chain D
 IGHJ: Immunoglobulin heavy chain J
 IGHV: Immunoglobulin heavy chain V
 OIT: Oral immunotherapy

METHODS

Blood samples were obtained from 27 patients aged 4 to 43 years with double-blind, placebo-controlled food challenge–confirmed peanut allergy undergoing peanut OIT in a phase 1 study (see [Table E1](#) in this article's Online Repository at www.jacionline.org).⁹ Samples were from 14 patients at baseline alone, 9 patients during OIT alone, and 4 patients at baseline and during OIT (9 and 14 months, 9 and 13 months, 16 and 18 months, and 16 and 32 months, respectively). The median age of participants was 8 years for baseline samples and 10 years for OIT samples. Samples from 9 healthy volunteers (median age, 32 years) without peanut allergy or other food allergy and with less than 0.2 kU_A/L of Ara h 1– and Ara h 2–specific serum IgE reactivity were also analyzed (see [Table E1](#)). The Stanford Institutional Review Board committee approved all protocols. OIT samples were collected within 42 months of starting therapy. Specific IgE and IgG₄ levels and component testing were measured according to published techniques (Johns Hopkins University School of Medicine, Baltimore, Md).¹⁰ Clinical reactivity was defined as any sign of allergic reaction (ie, score >1 on Bock criteria).¹¹ Subjects' demographics have been summarized previously.⁹ The protocol was conducted in a hospital setting with trained personnel. Flow cytometric assays, ELISAs, and Western blotting were performed by using commercially available, highly pure full-length Ara h 1 and Ara h 2 from natural sources. Ara h 1– and Ara h 2–specific B cells were isolated by means of flow cytometry from PBMCs, with at least 1 million PBMCs analyzed per sample (median, 9 million; range, 1.2–25 million), and sorted as single cells into 96-well plates. Immunoglobulin heavy and light chains were amplified by using single-cell multiplexed RT-PCR and expressed in 293T cells with linear IgG₁ expression constructs, as previously described,¹² with the following modifications: reverse transcription reactions were primed with random hexamers (Promega, Madison, Wis), 0.3 μ L of AmpliTaq Gold (Invitrogen, Carlsbad, Calif) was used in each PCR reaction, and an IgE constant region primer was added to the multiplex primer mix used for VH PCR (see [Table E2](#) in this article's Online Repository at www.jacionline.org). Supernatants containing expressed mAbs were isolated 3 to 7 days after transfection, and antibodies were quantified by mean of ELISA (eBioscience, San Diego, Calif). Antibody-binding activity was tested with Western blotting, allergen ELISA, and phage display of allergen peptides. Sequencing of immunoglobulin genes was performed with Sanger sequencing for antibodies derived from single-sorted B cells and Illumina Miseq for antibody libraries prepared from bulk PBMC RNA from allergic and healthy subjects.¹³ For high-throughput sequence analysis, parsing of VDJ junctions was carried out with IgBlast software (National Center for Biotechnology Information). Clonal groups were defined as sharing immunoglobulin heavy chain V (IGHV) segment use, equal complementarity determining region (CDR) 3 length, and 80% CDR3 nucleotide identity. Additional detailed descriptions of the materials and methods used in this article are available in the [Methods](#) section in this article's Online Repository.

All DNA sequences used in the study are accessible via the dbGAP database, with study title "Antibody repertoires in peanut allergy" deposited by S. Boyd.

RESULTS**Detection and isolation of peanut allergen-binding B cells**

We used multicolor flow cytometry to quantify, phenotype, and isolate blood B cells that bound fluorescently labeled Ara h 1 or Ara h 2 using a dual-labeling strategy for each antigen to increase cell-staining specificity.^{14–16} The major peanut allergens Ara h 1

and Ara h 2 were selected for these experiments because these proteins are recognized by serum IgE in more than 90% of allergic patients,^{17,18} including all the patients in this study (see [Table E1](#)). Allergens were native proteins purified from natural sources. Only the B cells positive for both dye-labeled versions of an allergen protein were sorted ([Fig 1, A](#), and see [Fig E1](#) in this article's Online Repository at www.jacionline.org). Baseline (n = 18) and OIT (n = 18) samples, as well as samples from healthy donors (n = 9), were analyzed in flow cytometric experiments.

The frequency of allergen-binding B cells as a percentage of total B cells was, on average, higher in OIT samples (Ara h 1 median, 0.035%; Ara h 2 median, 0.084%) compared with baseline samples (Ara h 1 median, 0.0097%; Ara h 2 median, 0.029%) from allergic subjects ([Fig 1, B](#)). Low but detectable frequencies of allergen-binding B cells were also detected in healthy donor peripheral blood samples (Ara h 1 median, 0.0071%; Ara h 2 median, 0.0038%). Frequencies of peanut allergen-binding B cells were somewhat higher than those previously reported for tetanus toxoid–specific cells identified in the blood by using a similar strategy (0.0001% to 0.0011%).¹⁹ As described below, when mAbs generated from allergen-binding B cells were tested for allergen binding in secondary assays (ELISA, Western blotting, or allergen peptide phage display), 67% of the class-switched antibodies and 22% of the non-class-switched antibodies were confirmed for binding activity. To additionally test the specificity of the flow cytometric assay, we attempted to block binding using preincubation of PBMCs with unlabeled allergen (see [Fig E2](#) in this article's Online Repository at www.jacionline.org). Preincubation decreased the frequency of observed double-positive Ara h 1– or Ara h 2–binding B cells by 7.7- and 7.6-fold, respectively, in OIT samples from allergic subjects (see [Fig E2](#) in this article's Online Repository at www.jacionline.org), supporting the specificity of Ara h 1 and Ara h 2 binding. Conversely, preincubation with unlabeled allergen did not significantly decrease double-positive B-cell frequencies in allergic baseline and healthy samples, suggesting that the levels of allergen-binding populations observed in these samples have a higher contribution from nonspecific background binding (see [Fig E2, B](#)).

Peanut antigen-binding B cells in blood show memory and nonmemory phenotypes

In addition to staining with labeled Ara h 1 or Ara h 2 to identify allergen-binding B cells, we analyzed the surface phenotypes for markers of memory (CD27⁺/CD20⁺), plasmablast (CD27^{high}/CD38^{high}/CD20^{low}), and naive and transitional B-cell subsets (see [Fig E1](#)). Roughly half of allergen-binding cells had a CD27⁺ memory phenotype (see [Fig E3, A](#), in this article's Online Repository at www.jacionline.org). Frequencies of class-switched memory (IgD[−]) B cells binding Ara h 1 or Ara h 2 were increased in the OIT samples compared with those in the healthy control samples ($P < .05$ and $P < .01$, respectively), although non-class-switched memory cells binding Ara h 2 were increased in subjects receiving OIT compared with healthy subjects ($P < .001$; [Fig 1, C and D](#)). Plasmablasts made up a smaller fraction of allergen-specific B cells; Ara h 2–binding plasmablasts showed higher levels during OIT compared with baseline and healthy samples ($P < .05$; [Fig 1, E](#), and see [Fig E3, B](#)).

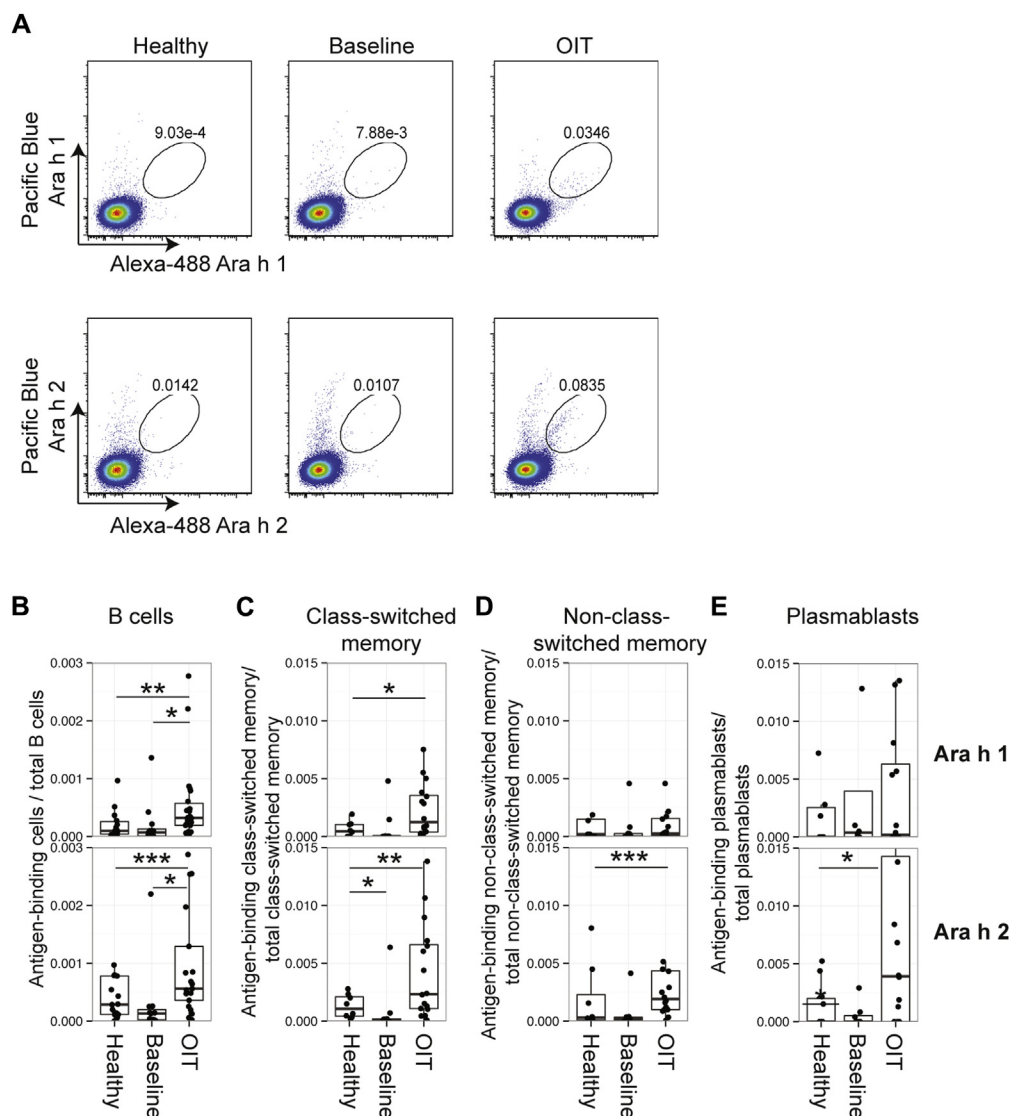


FIG 1. Representative flow cytometric plots of Ara h 1- and Ara h 2-specific B-cell responses in patients with peanut allergy undergoing peanut OIT. Samples are shown from a healthy donor (patient 27, left panels), a patient with peanut allergy at baseline (patient 11, middle panels), and a patient with peanut allergy after 9 months of OIT (patient 5, right panels). **A**, Ara h 1-specific (top panels) and Ara h 2-specific (bottom panels) B cells (CD19⁺/CD3⁺/CD14⁺/CD16⁺/CD235a⁺) were double positive for labeled allergen. **B-E**, Box plots show frequencies of Ara h 1-specific (top panels) and Ara h 2-specific (bottom panels) cells among total B cells (Fig 1, B), class-switched memory B cells (Fig 1, C), non-class-switched memory B cells (Fig 1, D), and plasmablasts (Fig 1, E) for healthy (n = 9), baseline allergic (n = 18), and OIT (n = 18) samples analyzed by means of flow cytometry. Frequencies of allergen-binding B cells within each subset are plotted as the fraction of total B cells within each subset. *P* values were calculated by using Wilcoxon tests: **P* < .05, ***P* < .01, and ****P* < .001.

Peanut-specific mAbs bind conformational and linear Ara h 1 and Ara h 2 epitopes

To further characterize the binding activity of the peanut-specific antibodies present at baseline or during OIT, we amplified heavy and light chain variable regions from single-sorted Ara h 1- or Ara h 2-binding B cells (see Fig E4, A, in this article's Online Repository at www.jacionline.org) from 6 patients (2 at baseline and 4 during OIT) and expressed these as IgG₁ mAbs (Table I). In total, we expressed 55 mAbs from Ara h 1-binding B cells (4 baseline and 51 OIT) and 79 mAbs from Ara h 2-binding B cells (22 baseline and 57 OIT) at concentrations of at least 0.5 μg/mL in supernatants. Antibodies were evaluated by means of ELISA

for native Ara h 1 and Ara h 2 (see Fig E4, B), by using SDS-PAGE and Western blotting with or without reduction by β-mercaptoethanol (see Fig E4, C-E), and by using immunoprecipitation of a phage display library expressing overlapping 24-mer peptides from Ara h 1 and Ara h 2 (see detailed section below). Fifty-seven of the 134 tested antibodies showed binding to allergen proteins in these secondary assays (21 Ara h 1 specific and 36 Ara h 2 specific). Binding in secondary assays was not correlated with expression levels in antibody supernatants (see Fig E4, F). The original isotypes and sequence features of these antibodies are shown in Table E3 in this article's Online Repository at www.jacionline.org. Monoclonal antibodies that did not

TABLE I. Summary of mAbs expressed from allergen-binding single-sorted B cells

Allergen	Time point	mAbs expressed (total)	Isotypes of nonspecific mAbs (total)	Isotypes of validated peanut-specific mAbs (total)
Ara h 1	BL	1 Validated, 3 not validated (4)	2 IgD, 1 not known (3)	1 IgM (1)
Ara h 1	OIT	20 Validated, 31 not validated (51)	18 IgM, 3 IgG ₁ , 1 IgG ₂ , 5 IgA, 4 not known (31)	2 IgM, 2 IgG ₁ , 1 IgG ₂ , 4 IgG ₄ , 11 IgA (20)
Ara h 2	BL	3 Validated, 19 not validated (22)	4 IgD, 12 IgM, 3 not known (19)	1 IgM, 2 IgA (3)
Ara h 2	OIT	33 Validated, 24 not validated (57)	2 IgD, 5 IgM, 4 IgG ₁ , 1 IgG ₂ , 1 IgG ₄ , 6 IgA, 5 not known (24)	1 IgD, 7 IgM, 5 IgG ₁ , 2 IgG ₂ , 2 IgG ₃ , 1 IgG ₄ , 12 IgA, 3 not known (33)

Sequences that did not pass quality thresholds during constant region identification are labeled as "Not known."

BL, Baseline.

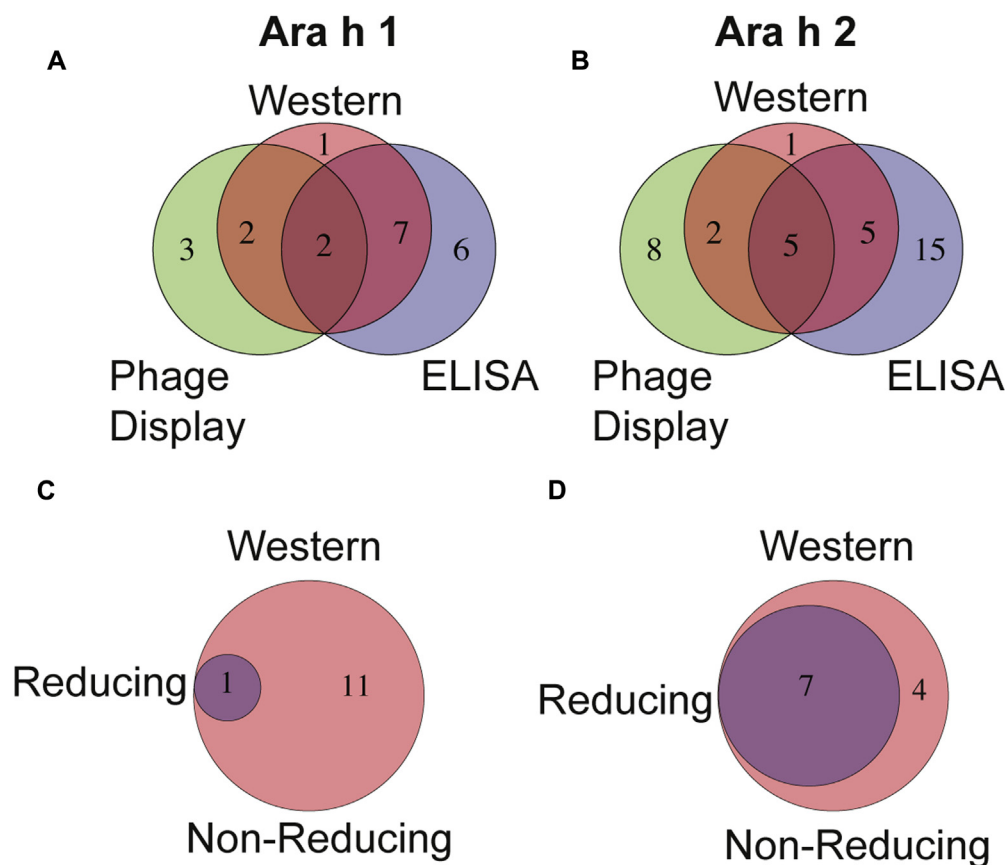


FIG 2. Venn diagrams summarizing the results of Ara h 1 and Ara h 2 binding assays with mAbs generated from B cells of allergic patients isolated by using allergen-specific fluorescence-activated cell sorting. Twenty-one Ara h 1-specific mAbs and 36 Ara h 2-specific mAbs were tested. Shown are results of ELISA, Western blotting, and phage display experiments with Ara h 1 (**A**) and Ara h 2 (**B**) proteins and results of Western blotting experiments with reduced Ara h 1 (**C**) and Ara h 2 (**D**) proteins.

show allergen binding in these secondary assays (hereafter referred to as nonspecific antibodies) were disproportionately derived from IgM- or IgD-expressing cells (see Fig E4, G). These antibodies might represent low-affinity polyreactive species.²⁰ Confirmed allergen-specific antibodies were derived mostly from class-switched B cells expressing IgA (Ara h 1, 52.4%; Ara h 2, 38.9%) or IgG (Ara h 1, 33.3%; Ara h 2, 30.3%; Table I and see Table E3), but a small subset of antibodies with confirmed binding came from non-class-switched B cells (Ara h 1, 13.0%; Ara h 2, 27.3%). Overall, the proportions of allergen-binding B cells in the flow cytometric assay that were confirmed in secondary assays were 15% in baseline samples and 49% in OIT samples, with 22% of non-class-switched antibodies confirmed compared with 67% of class-switched antibodies. IgG₄ antibodies were identified during OIT and not

in baseline samples from allergic patients (Table I), but we note that fewer antibodies were isolated from baseline samples, preventing assessment of the significance of this finding.

Fig 2 summarizes the results of the binding assays performed. Fifteen of 21 Ara h 1 mAbs and 25 of 36 Ara h 2 mAbs were positive in ELISA (Fig 2, A and B). Six of 15 Ara h 1 mAbs and 15 of 25 Ara h 2 mAbs that were positive in ELISA were not positive by using Western blotting, suggesting that a conformational epitope sensitive to denaturation was recognized (Fig 2, A and B). Six Ara h 1 mAbs and 11 Ara h 2 mAbs bound either phage-displayed peptides or in the Western blot assay but not in ELISA (Fig 2, A and B). We hypothesize that these mAbs bind linear or partially unfolded epitopes on Ara h 1 and Ara h 2. We evaluated whether the preparation of fluorescently labeled allergens for the flow sorting protocol might have revealed epitopes not



FIG 3. Peptide epitope mapping of patient-derived Ara h 1- and Ara h 2-specific mAbs. Twenty-two mAbs derived from single cells isolated from 5 patients undergoing OIT bound 24-mer peptides from Ara h 1, Ara h 2, or both expressed in a phage display library. Shown are averaged data from at least 2 independent experiments, and the plotted fold enrichment is relative to a mock control containing no antibody (row 1). Antibodies from each person are presented together. Eleven of these 22 antibodies from Ara h 2-binding B cells from 3 patients bound the same Ara h 2 region (peptides 3-5). Positive control anti-Ara h 1 and anti-Ara h 2 antibodies were polyclonal rabbit antibody preparations.

present in native protein. Supporting this possibility, ELISA experiments with one mAb (AbID 94) showed that it did not bind native Ara h 1 but was able to bind allergen that had first been conjugated with either Alexa Fluor 488 or Pacific Blue dye or had been mock conjugated by means of incubation in labeling buffer (see Fig E4, H). Treatment of Ara h 1 and Ara h 2 by using thiol reduction showed loss of binding in Western blots for 11 of 12 Ara h 1 mAbs but only 4 of 11 Ara h 2 mAbs, indicating that Ara h 1 epitopes are more sensitive to this treatment (Fig 2, C and D, and see Fig E4, C-E). Taken together, the secondary binding assays supported the allergen-binding activity of 57 of the 134 mAbs prepared from single B cells and provide examples of binding to conformational and linear epitopes.

Antibodies from 9 distinct B-cell clones from 3 patients with peanut allergy bind an N-terminal peptide epitope region in Ara h 2

To obtain high-resolution data about the epitopes recognized by patient peanut-specific mAbs, we used phage display of 24-mer peptides of Ara h 1 and Ara h 2 (Fig 3 and see Fig E5

in this article's Online Repository at www.jacionline.org). Consistent with serologic observations detecting reactivity to linear epitopes in Ara h 1 and Ara h 2,²¹⁻²³ a variety of phage-displayed peptides were recognized by patients' mAbs (Fig 3). Eleven mAbs from Ara h 2-binding B cells (AbIDs 131, 388, 408, 413, 415, 424, 425, 429, 433, 434, and 512) from 3 of the 5 subjects bound an Ara h 2 epitope region spanning residues 21 to 64 (peptides 3-5 in the phage library), which was previously reported as a target for serum IgE binding in patients.²² These antibodies included 2 pairs of clonally related mAbs: 413 and 415 and 429 and 433. AbIDs 413 and 415 had very similar binding patterns for the major Ara h 2 epitope region. In contrast, the other clonal pair contained an IgM-derived mAb (AbID 433) that had 2 somatic mutations in its V gene and bound to Ara h 2 peptide 5, as well as a number of Ara h 1 peptides, whereas the other member of the pair (AbID 429) was derived from an IgG₁ antibody with 8 somatic V gene mutations and bound Ara h 2 peptides 3 to 5. This clonal pair might represent an example of increasing binding to the major Ara h 2 epitope region as a result of affinity maturation, as AbID 429 differs by 2 amino acid changes in CDR1 compared with AbID 433. Fig 3 shows other mAbs with cross-reactivity to

Ara h 1 and Ara h 2 epitopes (eg, AbIDs 47, 271, 388, 408, and 424). Polyreactivity has been previously described as a potentially important feature of some antibodies in patients with IgE-mediated allergy.²⁴⁻²⁸ It is possible that these mAbs are examples of the polyreactive antibody species implicated in prior studies.

We grouped the mAbs according to which epitopes they bound and examined them for similarities in V, D, and J gene use; CDR3 length and similarity; or hypermutation levels. No statistically significant patterns of antibody sequences were correlated with epitope binding. These findings lead us to conclude that individual patients generate a range of different paratopes able to bind linear epitope regions, such as residues 21 to 64 of Ara h 2. Therefore even apparently highly focused serologic reactivity can be produced by mixtures of distinct B-cell clones in an individual patient.

Peanut-specific antibodies are not restricted to specific V, D, or J gene segments or CDR3 sequences

We next evaluated *IGHV*, immunoglobulin heavy chain D (*IGHD*), and immunoglobulin heavy chain J (*IGHJ*) gene use by peanut-specific mAbs (Fig 4). Restrictions in V gene use have been reported for antibodies against antigens, including the timothy grass allergen Phl p 2,^{29,30} as well as viral^{31,32} and bacterial³³ antigens. Peanut allergen-specific antibodies in our data did not show significant restriction in *IGHV*, *IGHD*, or *IGHJ* gene use (Fig 4, A-E). No significant differences were observed between validated antibodies and nonspecific antibodies (see Fig E6 in this article's Online Repository at www.jacionline.org). Similarly, the length, hydrophobicity, and charge of CDR3 regions in peanut-specific mAbs did not differ from total repertoires in healthy control subjects.

Most peanut-specific B cells express mutated antibodies

Sequences were grouped by *IGHV* gene mutation level into 4 categories: less than 1% mutation (unmutated), 1% to 5% mutation, 5% to 10% mutation, and greater than 10% mutation frequency. Most peanut-specific antibodies were IgG and IgA isotypes (Fig 4, F), and mutation levels were correlated with isotype, with IgG and IgA being more highly mutated than IgM (Fig 4, G). The most highly mutated antibody isotypes were IgG₄, IgA, and IgG₂, with mean *IGHV* segment mutation levels of 6.9% (SE $\pm$ 1.4%), 7.7% (SE $\pm$ 0.05%), and 9.3% (SE $\pm$ 2.0%), respectively. Of non-class-switched antibodies, 2 of 3 Ara h 1 antibodies and 5 of 11 Ara h 2 antibodies had somatic *IGHV* mutations, suggesting that they were derived from memory B cells expressing IgM, IgD, or both. Median mutation levels for antibodies of a particular isotype were not significantly different between baseline and OIT samples.

Peanut allergen-specific B cells are clonally expanded and enriched for IgE-containing lineages

Peptide epitope mapping experiments have indicated that serum antibodies in allergic patients can be oligoclonal.^{23,34,35} Among the 57 single-cell antibodies we studied, 5 clonally related pairs of sequences were identified from OIT samples (see Table E4 in this article's Online Repository at www.jacionline.org). We sought to identify rare IgE-expressing B cells belonging to the same clonal lineages as the peanut allergen-specific B cells identified

in this study. Deep sequencing of all antibody isotypes from a subset of the sorted baseline and OIT PBMC samples (see Table E1) was performed to a depth of 128,335 to 3,554,943 reads per subject. Searching for antibody heavy chains with the same *IGHV* use, CDR3 length, and 80% or greater nucleotide identity in the CDR3 (Fig 5, A, and see the Methods section in this article's Online Repository), we identified 4 IgE-containing clonal lineages from 3 subjects (patients 1, 7, and 14) that were clonally related to 5 validated peanut allergen-binding mAbs: AbID 14/65, 70, 271, and 705 (Table II). Additionally, 3 IgG₄-containing lineages were identified from patient 7 that were clonally related to peanut-specific mAbs. We assessed whether our set of validated peanut-specific single cells were enriched for lineages that contain IgE compared with all lineages from the overall repertoires (see the Methods section in this article's Online Repository). Table III shows that the peanut-specific clonal lineages detected in patients 1, 7, and 14 were more likely to be members of clones containing IgE-expressing members than randomly selected sequences would be. In 2 of the 3 subjects (patients 1 and 7), this enrichment was statistically significant, as determined by using the Fisher exact test, whereas for patient 14, it had a *P* value of .069.

IgE members of peanut-specific clonal lineages are mutated

We evaluated *IGHV* mutation levels for IgE and other isotype members of the 4 peanut-specific lineages that contained IgE members. IgE members of lineage 271 (originally identified from an IgA₁-expressing B cell) had a mean *IGHV* mutation level of 7.8%. The other 3 lineages contained members from multiple isotypes: lineages 14/65 and 705 contained IgG₁ and IgE members, and lineage 70 contained members from IgG₁, IgG₂, IgG₃, and IgG₄, as well as IgA₁, IgA₂, and IgE (Fig 5, B). The mean *IGHV* percentage mutations of IgE and IgG₁ members of these peanut-specific lineages were 9.5% and 10% (AbID 14), 9.5% and 9.4% (AbID 65), 7.5% and 6.7% (AbID 70), and 9.2% and 9.4% (AbID 705, Table II). No peanut-specific lineages were observed that contained both IgE and IgM members.

IgG₄ immunoglobulins in a peanut-specific lineage become more mutated during OIT

Lineage 70 from patient 7 was striking because it contained multiple examples of IgE and IgG₄ sequences identified over 8 OIT time points from months 9 to 42 of treatment based on 2,948,568 sequences measured from this patient. IgE reads were identified from samples taken at 25, 36, and 42 months of OIT, and IgG₄ reads were found in samples taken at 22, 25, 34, 36, and 42 months of OIT. Linear regression of *IGHV* mutation levels during OIT showed an increasing proportion of more highly mutated sequences over time in IgG₄, but not IgE, members of the lineage (Fig 5, C).

DISCUSSION

By using flow cytometric sorting of allergen-binding B cells, mAb production from single cells, and deep sequencing of antibody heavy chain repertoires, we have characterized peanut allergen-specific human antibody lineages from allergic patients. We measured Ara h 1- and Ara h 2-binding B-cell frequencies in 36 samples from patients with peanut allergy, finding baseline

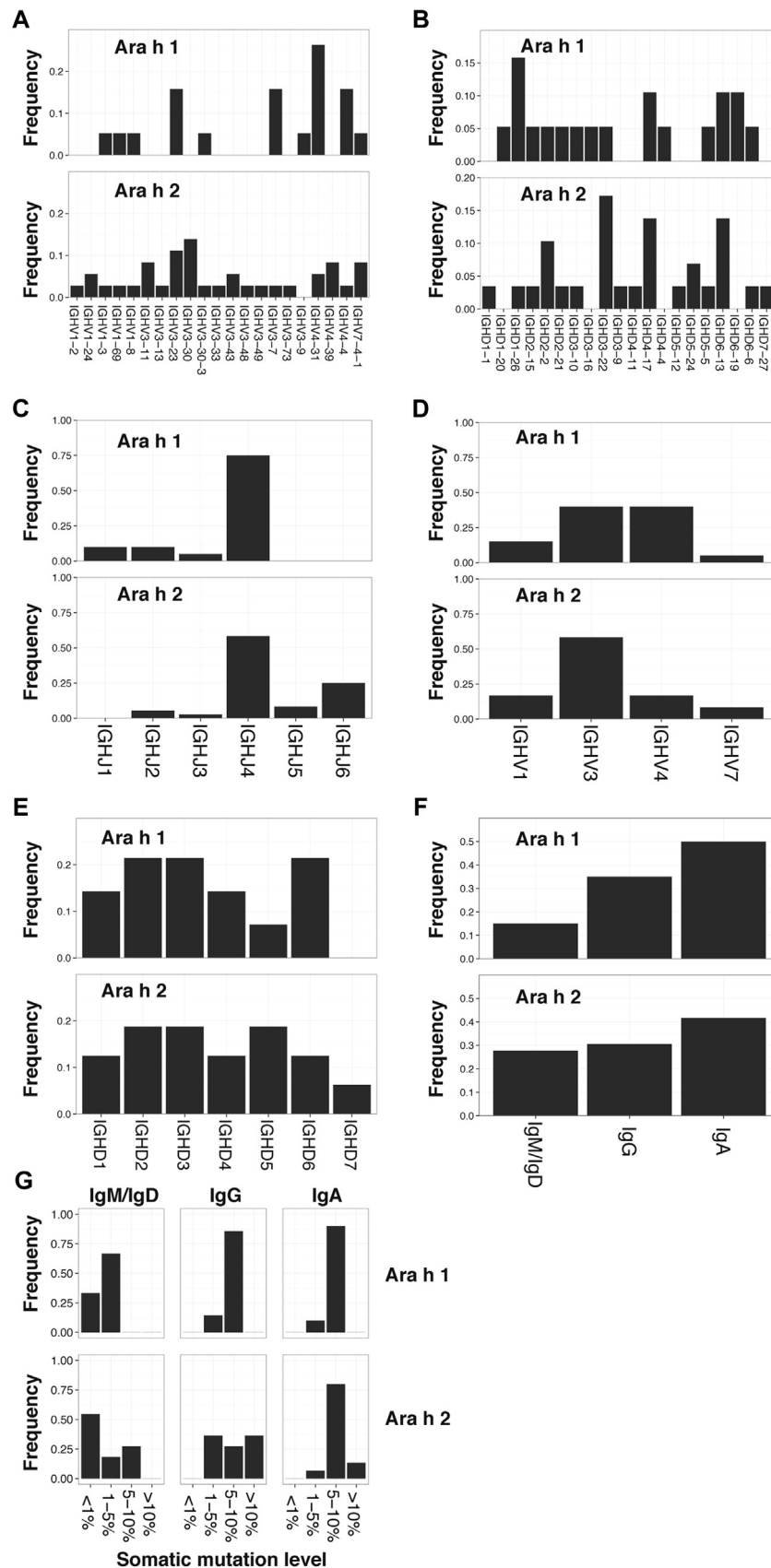


FIG 4. V, D, and J gene use and isotype frequencies of allergen-specific antibody heavy chains. Data were derived from 21 Ara h 1-specific antibodies (*top panels*) and 36 Ara h 2-specific antibodies (*bottom panels*) generated from 6 allergic subjects. V, D, and J gene frequencies (**A-C**) and V and D gene family frequencies (**D-E**) are graphed, as well as isotype frequencies (**F**) and *IGHV* somatic mutation levels for each isotype (**G**).

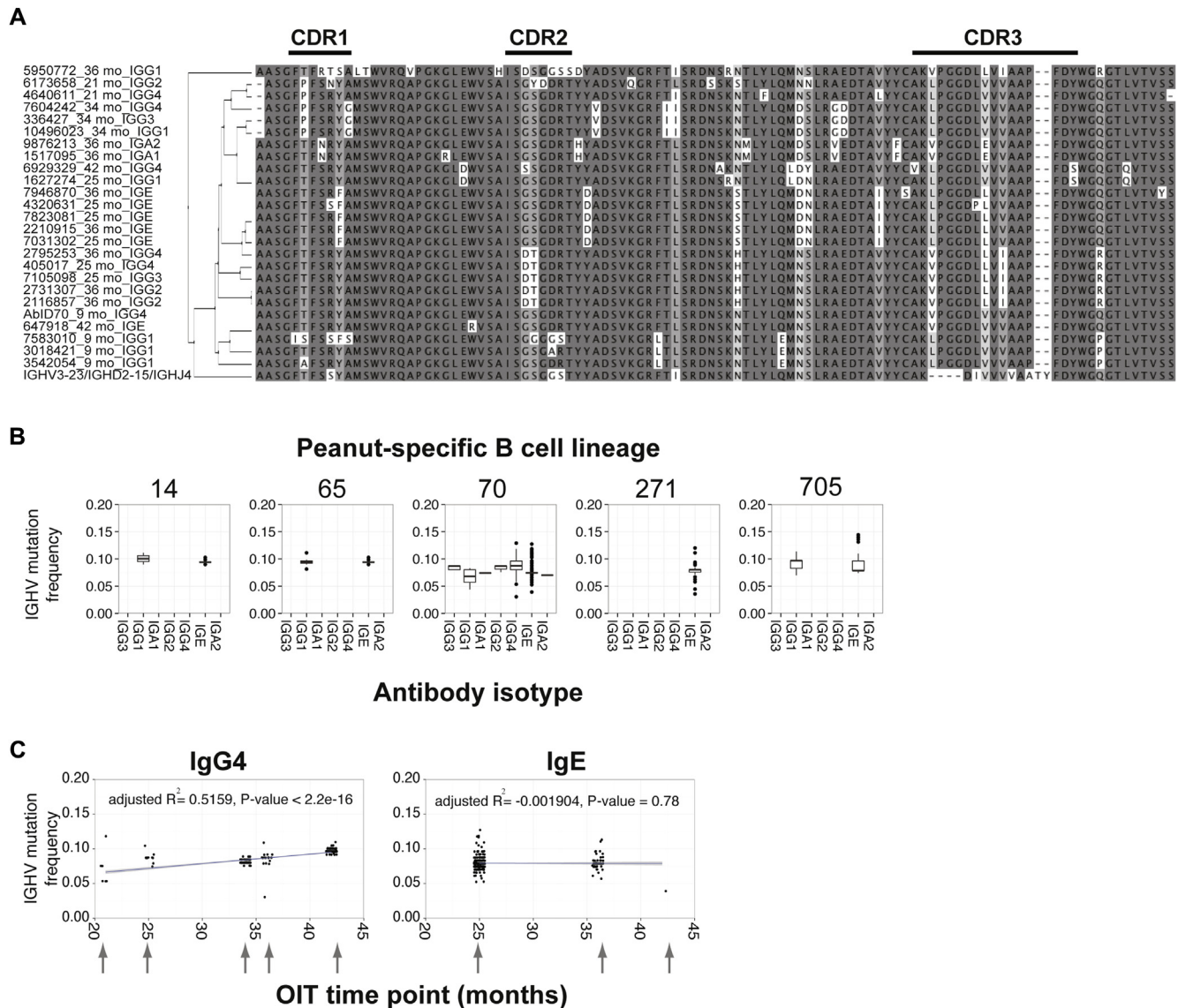


FIG 5. Identification of peanut-specific lineages in deeply sequenced patient antibody heavy chain repertoires. **A**, Representative heavy chain sequences from a lineage clonally related to AbID 70 were obtained from patient 7 at 9, 21, 25, 34, 36, and 42 months during OIT. The sequence derived from the single-cell AbID 70 is also shown. The germline sequences of the gene segments used in this antibody gene rearrangement are shown for reference. Darker shading indicates a higher percentage identity. CDRs are underlined. Average distance trees were computed by using BLOSUM62 on sequences trimmed to the length of the shortest read. **B**, Box plots show IGHV somatic mutation levels for members of IgE-containing lineages clonally related to 5 peanut-specific mAbs. **C**, IGHV mutation levels for IgG₄ (left panel) and IgE (right panel) reads in the AbID 70 lineage are plotted over time. Arrows indicate the time points sampled. Points are plotted with horizontal jitter to prevent superposition. The adjusted R^2 values are indicated in each panel.

levels of 0.0097% for Ara h 1 and 0.029% for Ara h 2 and approximately 3-fold higher levels during OIT (0.035% for Ara h 1 and 0.084% for Ara h 2). Numbers of class-switched memory B cells showed the greatest increase during OIT, but numbers of non-class-switched memory B cells binding Ara h 2 were also increased, suggesting that mobilization or expansion of IgM memory B cells is a feature of the Ara h 2-specific response to OIT.

Of the 57 peanut allergen-specific antibodies we isolated from antigen-specific B cells, 78% were derived from class-switched cells expressing IgG or IgA. Allergen-specific IgG₄ antibodies

were isolated from OIT samples only, a finding that is consistent with the established correlation between specific serum IgG₄ titers and response to allergen immunotherapy.⁸ However, allergen-binding B cells of all types were more rare in baseline allergic samples, and therefore it might be the case that IgG₄-expressing members of allergen-specific clones are present before OIT but are at frequencies too low to be detected in these experiments. Overall, peanut-specific antibodies of switched isotypes showed moderate levels of somatic mutation comparable with those of other antibodies of the same isotype in patient repertoires.

TABLE II. Identification of additional members of allergen-specific B-cell clones with high-throughput DNA sequencing of antibody heavy chain repertoires

Patient ID	Sample time point	Antigen	AbID	Single-cell isotype	mAb V gene	mAb J gene	mAb CDR3 amino acids	Isotypes of related B-cell clones from deeply sequenced repertoires (mean % V gene mutation of all reads for the specified isotype)
7	OIT (9 mo)	Ara h 1	13	IGA1	<i>IGHV3-7</i>	<i>IGHJ4</i>	VRGPYHGASTDYFDF	IgM (7.5), IgD (3.9), IgG ₁ (10.8), IgA ₁ (11.6), IgG ₂ (6.0), IgA ₂ (10.6)
7	OIT (9 mo)	Ara h 1	14	IGG1	<i>IGHV4-31</i>	<i>IGHJ4</i>	ARERYGNTTYLGFDS	IgG ₁ (10.0), IgE (9.5)
7	OIT (9 mo)	Ara h 1	19	IGG4	<i>IGHV1-8</i>	<i>IGHJ3</i>	ARGVDAGL	IgG ₃ (14.9), IgG ₁ (12.7), IgG ₂ (14.1), IgG ₄ (11.6), IgA ₂ (7.0)
7	OIT (9 mo)	Ara h 1	55	IGA1	<i>IGHV3-7</i>	<i>IGHJ4</i>	ARTRCARCSSWYYFDH	IgG ₁ (8.4)
7	OIT (9 mo)	Ara h 1	65	IGG1	<i>IGHV4-31</i>	<i>IGHJ4</i>	ARERYGNTAYLGFDS	IgG ₁ (9.4), IgE (9.5)
7	OIT (9 mo)	Ara h 1	70	IGG4	<i>IGHV3-23</i>	<i>IGHJ4</i>	AKVPGGDLVVVAAPFDY	IgG ₃ (8.4), IgG ₁ (6.7), IgA ₁ (7.4), IgG ₂ (8.3), IgG ₄ (8.8), IgE (7.5), IgA ₂ (7.0)
7	OIT (9 mo)	Ara h 2	123	IGA1	<i>IGHV3-11</i>	<i>IGHJ4</i>	ARTVYYDSSYSFDS	IgA ₁ (9.7)
7	OIT (9 mo)	Ara h 2	130	IGM	<i>IGHV3-30-3</i>	<i>IGHJ6</i>	ARPRDSSSWTSAYYYDMDV	IgM (6.6)
7	OIT (9 mo)	Ara h 2	148	IGA1	<i>IGHV3-43</i>	<i>IGHJ4</i>	VKDNGFRSFGH	IgG ₁ (12.0), IgA ₁ (10.8), IgG ₄ (14.3), IgA ₂ (11.4)
7	OIT (9 mo)	Ara h 2	166	IGG2	<i>IGHV1-3</i>	<i>IGHJ4</i>	ARRGEGYKYDD	IgG ₁ (11.8), IgG ₂ (11.8)
7	OIT (9 mo)	Ara h 2	172	IGG3	<i>IGHV3-13</i>	<i>IGHJ4</i>	AREGYESSGFYD	IgG ₃ (6.6), IgA ₁ (3.6)
14	OIT	Ara h 1	271	IGA1	<i>IGHV1-3</i>	<i>IGHJ4</i>	ARTPIFSRGSSWLSYSDN	IgE (7.8)
14	OIT	Ara h 1	277	IGA1	<i>IGHV4-31</i>	<i>IGHJ4</i>	ARGVLGSSSHNFD	IgD (0.1), IgG ₁ (13.3), IgA ₁ (11.1), IgG ₂ (14.0), IgA ₂ (11.1)
14	OIT	Ara h 1	293	IGG2	<i>IGHV4-4</i>	<i>IGHJ2</i>	VRDRRLPGVTAYWFFDL	IgG ₃ (9.8), IgG ₁ (9.5), IgA ₁ (9.4), IgG ₂ (10.1), IgA ₂ (9.8)
14	OIT	Ara h 1	296	IGA1	<i>IGHV3-30-3</i>	<i>IGHJ4</i>	ARDLAQYCSGDCATFDY	IgM (0), IgG ₁ (11.6), IgA ₁ (6.6), IgG ₂ (10.5)
14	OIT	Ara h 1	305	IGA1	<i>IGHV3-23</i>	<i>IGHJ4</i>	AKDWYSSGWGGLDH	IgG ₃ (13.8), IgG ₁ (14.2), IgA ₁ (12.7), IgG ₂ (14.5), IgA ₂ (11.1)
14	OIT	Ara h 1	314	IGA1	<i>IGHV4-4</i>	<i>IGHJ2</i>	ARDRRLPGTTAYWFFDL	IgG ₃ (9.8), IgG ₁ (9.5), IgA ₁ (9.4), IgG ₂ (10.1), IgA ₂ (9.8)
14	OIT	Ara h 1	320	IGA1	<i>IGHV4-4</i>	<i>IGHJ4</i>	ARDRRVSGYFYFDS	IgG ₃ (8.3), IgG ₁ (8.5), IgA ₁ (9.8), IgG ₂ (8.6), IgA ₂ (10.8)
14	OIT	Ara h 2	344	IGM	<i>IGHV4-39</i>	<i>IGHJ4</i>	AAPYDSSYFDY	IgM (0.9)
14	OIT	Ara h 2	372	IGA2	<i>IGHV7-4-1</i>	<i>IGHJ4</i>	TRGQVVTNVFDY	IgG ₃ (7.5), IgG ₁ (7.7), IgA ₁ (9.6), IgG ₂ (7.5), IgA ₂ (10.0)
14	OIT	Ara h 2	381	IGA1	<i>IGHV3-11</i>	<i>IGHJ6</i>	ARVQLEYSYHGMDI	IgG ₂ (12.8)
14	OIT	Ara h 2	393	IGA1	<i>IGHV3-30</i>	<i>IGHJ4</i>	AKDSGLRALAS	IgG ₁ (11.7)
14	OIT	Ara h 2	403	IGG1	<i>IGHV3-30</i>	<i>IGHJ4</i>	AKDSGLRALDS	IgG ₁ (11.7)
14	OIT	Ara h 2	406	IGD	<i>IGHV3-48</i>	<i>IGHJ4</i>	ARDPPGIEDFDY	IgM (6.8), IgA ₁ (12.1), IgA ₂ (12.2)
14	OIT	Ara h 2	408	IGA1	<i>IGHV4-31</i>	<i>IGHJ4</i>	ARGVLNSSSHNFDY	IgG ₁ (13.3), IgA ₁ (11.1), IgG ₂ (14.0), IgA ₂ (11.1)
14	OIT	Ara h 2	424	IGG3	<i>IGHV7-4-1</i>	<i>IGHJ3</i>	ARDNWSAGNVLDV	IgG ₁ (12.8), IgA ₁ (13.7), IgG ₂ (12.8)
14	OIT	Ara h 2	429	IGG1	<i>IGHV4-39</i>	<i>IGHJ4</i>	ARLDIVVVPAAAFDS	IgD (4.5)
14	OIT	Ara h 2	433	IGM	<i>IGHV4-39</i>	<i>IGHJ4</i>	ARLDIVVVPAAAFDS	IgD (4.5)
14	OIT	Ara h 2	434	IGG2	<i>IGHV3-11</i>	<i>IGHJ2</i>	ARDTHSTNWLYWYFDL	IgG ₁ (8.4), IgA ₁ (10.0), IgA ₂ (10.1)
1	Baseline	Ara h 2	705	IGA1	<i>IGHV3-23</i>	<i>IGHJ4</i>	VKVDRVEMATISFDH	IgG ₁ (9.2), IgE (9.4)

Allergen-specific B cells and B cells that produce IgE in patients have been difficult to study, in part because of the low numbers of these cells in the blood, the presence of FcεRII low-affinity IgE receptors on all B-cell surfaces, and potential low surface immunoglobulin expression in IgE-expressing B cells with a plasmablast phenotype, although recent publications have presented new strategies for studying these cells.³⁶⁻³⁸ We used high-throughput sequencing of allergic patients' B-cell antibody heavy chains to search for additional members of the allergen-specific clones. From 11 million sequences, we identified 4 IgE-containing lineages and 3 IgG₄-containing lineages clonally related to peanut-specific single cells obtained from the same patients. IgE-expressing members of allergen-specific clones showed mutation levels similar to those in IgG members of the same clones (7.5% to 9.5% and 6.7% to 10% for IgE and IgG₁, respectively), suggesting that allergen-specific IgE-expressing B cells can be derived by secondary isotype switching from other

affinity-matured isotype-switched B cells or else undergo somatic mutation themselves.³⁹⁻⁴² We identified an allergen-specific clone with IgG₄ clone members from 5 longitudinal time points during OIT that showed increasing mutation, whereas IgE-expressing clone members showed no significant change in mutation, suggesting that one mechanism for OIT's efficacy might be the generation of more mutated and potentially higher-affinity IgG₄ antibodies that could block IgE binding to allergen.^{8,43} Because this result is based on a single informative clone in our analysis, it will require confirmation from additional clonal lineages in other patients.

Using a panel of immunoassays that tested binding to intact or denatured allergen proteins, as well as linear peptides expressed by means of phage display,⁴⁴ we identified antibodies that bind conformational or linear epitopes in Ara h 1 and Ara h 2. The most prominent epitopes identified in our experiments map to previously reported IgE epitopes in allergic subjects.^{20,21} Our binding data from defined mAbs indicate that even a relatively

TABLE III. Percentage of peanut-specific B cells belonging to IgE-containing clonal lineages compared with percentage of total high-throughput sequencing reads belonging to IgE-containing lineages in antibody heavy chain repertoires of allergic patients

		<u>Peanut-specific cells</u>	<u>Peanut-specific cells in clones containing IgE</u>	<u>HTS reads*</u>	<u>HTS reads in clones containing IgE</u>	<u>Fisher exact test</u>
<u>Lineages</u>		<u>Count</u>	<u>Percent</u>	<u>Count</u>	<u>Percent</u>	<u>P value</u>
Patient 1 (BL)	IgE containing	1	100	310	0.5	5.514E-03†
	Total	1		56,398		
Patient 7 (OIT)	IgE containing	2	20	2,490	0.6	1.828E-03†
	Total	10		384,161		
Patient 14 (OIT)	IgE containing	1	7.1	2,026	0.5	.069
	Total	14		398,040		

BL, Baseline; HTS, high-throughput sequencing.

*Reads sharing 99% IGH nucleotide identity and the same constant region gene were collapsed to a single representative read to avoid overcounting sequence variants arising during PCR and sequencing.

† $P < .01$.

short epitope region of Ara h 2 can be recognized by multiple antibodies with distinct V, D, and J gene use and CDR3 paratope features within a single subject. This ability of numerous distinct B-cell clones to recognize the same epitope might be mechanistically important for responses to OIT because it expands the number of different B-cell clones that could contribute to formation of blocking antibodies *in vivo*.

Taken together, these findings represent an initial evaluation of the antibodies expressed by peanut allergen-specific B cells in human subjects. Extension of these findings to larger numbers of patients and correlative analysis with other immune system features, such as T-cell responses and mast cell and basophil functional status, both before treatment and during OIT should provide additional details and result in an integrated understanding of allergic phenotypes and therapeutic responses in the coming years.

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Key messages

- Peanut allergen-specific B cells in patients express mutated antibody genes, usually of switched isotypes, and bind to both linear and conformational epitopes.
- Even well-defined linear epitopes of allergen proteins can be recognized by multiple independent B-cell clones in a single patient.
- OIT is associated with increased frequencies of allergen-binding B cells in the blood and in one example, progressive somatic mutation of IgG₄, but not IgE, members of an allergen-specific clone.

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Title: Single B Cell Deconvolution of Peanut-specific Antibody Responses in Allergic Patients

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Supplementary Methods

Peanut allergen proteins

Flow cytometry assays, ELISAs, and Western blotting were performed using commercially available, highly pure, full-length Ara h 1 and Ara h 2 from natural sources (Indoor Biotech; NA-AH1-1 and NA-AH2-1). Purity of Ara h 1 and Ara h 2 preparations was validated by immunoblotting using rabbit polyclonal antibodies raised against each antigen.

B cell isolation and cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque-1077 per the manufacturer's instruction (Sigma/Adrich) and cryopreserved prior to use. Cells were stained on ice with PE/Cy5-CD3, PE/Cy5-CD16, APC/Cy7-CD19, PE-IgD, AlexaFluor700-CD38 and APC-CD20 (Biolegend); PE/Cy5-CD14 (US Biological), PE/Cy5-CD235a (BD Pharmingen); PE/Cy7 CD27 (eBioscience); and Aqua amine live/dead stain (Invitrogen). Fluorescently-labeled peanut allergen proteins were prepared with Dylight 488 NHS-Ester (Thermo Scientific) per the manufacturer's instructions. Separate preparations of each peanut allergen protein were labeled with Pacific Blue succinimidyl ester (Invitrogen) per the manufacturer's instructions. Fluorophore-conjugated allergen proteins were purified on Bio-Spin 6 columns (Bio-Rad) to remove unlabelled dye before use. PBMCs were incubated with 0.3 µg of Pacific Blue-Ara h 1 and 0.3 µg of Alexa 488-Ara h 1 per million cells, or 0.3 µg of Pacific Blue-Ara h 2 and 0.3 µg of Alexa 488-Ara h 2 per million cells for identification of allergen-specific B cells, together with a previously described B cell flow cytometry

phenotyping panel¹. Single CD19⁺/CD3⁻/CD14⁻/CD16⁻/CD235a⁻ B cells that exhibited dual positivity for Pacific Blue and Alexa 488 were sorted on a FACSAria II (BD Biosciences, San Jose, CA) into 96-well plates containing reverse-transcription (RT) buffer. Flow cytometry data were analyzed using FlowJo software (TreeStar; Ashland, OR).

PCR of antibody heavy and light chains from single FACS-sorted cells

RT-PCR amplification of single-cell Ig VH and VL chain genes was carried out using described primers per published protocols¹, except: reverse transcription reactions were primed with random hexamers (Promega); 0.3 µL of Amplitaq Gold (Invitrogen) was used in each PCR reaction; and an IgE constant region primer was added to the multiplex primer mix used for VH PCR. Primers designed for this study are listed in Table E2. In total, paired heavy and light chains from sorted cells were successfully amplified and expressed from six patients (2 baseline patient samples, and 4 OIT patient samples, with no antibodies isolated from the same patient at baseline and during OIT).

Deep sequencing of antibody heavy chain repertoires

RNA was isolated from PBMCs (median 8.9 million, range 1.2-25 million) using Qiagen RNeasy Mini kits (Qiagen). cDNA was synthesized according to the manufacturer's instructions using Superscript III RT kits (Invitrogen) with priming by random hexamers. Separate libraries of IGH rearrangements for IgM, IgD, IgG, IgA and IgE were amplified from cDNA templates using Biomed IGHV primers in the framework 1 (FR1) region and isotype specific primers located in the first exon of the constant regions. These primers

also encoded approximately half of the Illumina linker sequences needed for cluster generation and sequencing on the MiSeq instrument (Table E6). Sample identity was encoded by 8-nucleotide multiplex identifier barcodes in each primer. For Illumina cluster recognition, 4 randomized nucleotides were encoded in the primers immediately after the Illumina linker sequence in the constant region primers. Each antibody isotype for each sample was amplified in a separate PCR reaction, to prevent formation of cross-isotype chimeric PCR products. PCR was carried out with AmpliTaq Gold (Roche) following the manufacturer's instructions with the following PCR steps: 94°C 7 min, 35 cycles of (94°C 30 sec, 58°C 45 sec, 72°C 120 sec), and final extension at 72°C for 10 min. A second PCR step was used to add the remaining portion of the Illumina linkers to the amplicons, and was carried out with the Qiagen Multiplex PCR kit (Qiagen) according to the manufacturer's instructions, using 0.4 microliters of the first PCR product as template in a 20 microliter reaction. The PCR program for the second PCR step was 94°C 15 min, 12 cycles of (94°C 30 sec, 60°C 45 sec, 72°C 90 sec), and final extension at 72°C for 10 min. The products of each PCR reaction were quantitated, pooled in equimolar amounts, electrophoresed on agarose gels, and gel extracted with QIAquick gel extraction kits (Qiagen). Sequencing of cDNA libraries was performed on an Illumina MiSeq instrument using 600-cycle sequencing kits.

High-throughput DNA sequence analysis

Paired-end sequence reads were merged using the FLASH algorithm² and were filtered based on exact matches to sample-specific barcodes and IGHV and constant region primers were trimmed prior to identification of gene segments and junctional bases using

the IgBLAST program³. Determination of antibody isotype and subtype was accomplished by selecting the best Smith-Waterman alignment of known constant regions from the IMGT database to the constant region sequence in each amplicon after primer trimming⁴. *IGH*E constant region identity was confirmed by pairwise sequence alignment to non-primer-encoded portions of the observed constant region using BLAST⁵. Reads where the constant region could not be determined unambiguously were discarded from analyses involving isotypes. In total, 11,552,735 sequences were obtained from 18 allergic patients (mean = 608,039 per individual) and 1,821,339 sequences from seven healthy controls (mean = 260,191 per individual).

Identification of clonally-related immunoglobulin sequences and peanut-specific clonal lineages

Immunoglobulin heavy chain sequences from deeply sequenced patient repertoires were clustered into clonal lineages if they met the following criteria: 1) were obtained from the same individual, 2) shared the same *IGHV* gene annotation, 3) had equal CDR3 length, and 4) shared at least 80% nucleotide identity in the CDR3. Peanut-specific clonal lineages were identified based upon passing these four criteria with respect to validated peanut-specific single cell antibodies. Identity of peanut-specific clonal lineages was further evaluated by inspection of nucleotide multiple sequence alignment using ClustalW⁶. Sequences that shared the same randomized N-mer ID, and sequences with 99% identity within a clonal lineage were collapsed to minimize the impact of PCR and sequencing errors on subsequent data analysis.

Expression of monoclonal antibodies derived from single B cells

PCR was used to generate linear expression constructs for paired Ig VH and VL genes as previously described¹. PCR was carried out on three overlapping fragments containing 1) a CMV promoter and an Ig leader sequence, 2) either the VH, V κ or V λ genes amplified from single cells, and 3) either the IgG1 constant region, the Ig κ constant region, or the Ig λ constant region, respectively, upstream of a bovine growth hormone (BGH) poly(A) signal. Primers are listed in Table E2. 293T cells grown in 2% FBS were transfected with VH and VL expression constructs using Effectene transfection reagent (Qiagen), per the manufacturer's protocol. Expressed antibody supernatants were isolated 3-7 days after transfection, and antibodies were quantified by ELISA (eBioscience). To facilitate PCR amplification of the constant regions with a downstream poly(A) signal, which was derived from pDisplay (Life Technologies), each constant region was cloned into a pCR 2.1-TOPO vector (Life Technologies) upstream of a cloned poly(A) signal.

Western Blotting

Whole natural peanut allergens Ara h 1 or Ara h 2 were prepared in 1X XT Sample Buffer (BioRad) and boiled for 10 minutes. For reducing gel electrophoresis, β -mercaptoethanol was added prior to boiling to a final volume of 0.5%. 0.5 μ g of antigen was loaded into each lane of Criterion XT 4-12% Bis-Tris polyacrylamide gels (BioRad). Proteins were transferred onto Immun-Blot PVDF membrane (BioRad) at 100V for 45 minutes. Membranes were blocked in 3% milk in PBST (1X phosphate buffered saline, pH 7.4 plus 0.1% Tween-20) for 1 hour at room temperature, washed three times with PBST, and then incubated overnight at 4C with 293T supernatants diluted 1:100 in 1% milk in PBST. Rabbit polyclonal anti-Ara h 1 (Indoor Biotech, PA-AH1) or anti-Ara h 2 (Indoor Biotech, PA-AH2) antibodies diluted at 1:1000 in 1% milk in PBST were run

alongside 293T antibody supernatants as positive controls in each experiment. Mock transfected 293T supernatants were used as negative control antibodies. Membranes were washed three times with PBST and incubated for 2 hours at room temperature with HRP-conjugated anti-human IgG H+L (Jackson ImmunoResearch Catalog# 709-035-149) or HRP-conjugated anti-rabbit secondary antibodies (GE Healthcare Catalog# NA934V) diluted 1:1000 in 1% milk plus PBST. After washing three times with PBST, bound antibodies were detected by incubation with SuperSignal West Dura Substrate (Thermo Scientific). Blots were imaged on an Alpha-Imager HP (Protein Simple).

ELISA

Maxisorp microtiter plates (Nunc Catalog# 439454) were coated with 250 ng of Ara h 1 or Ara h 2 per well. After blocking overnight, 1:10 diluted monoclonal antibody-containing supernatants or control rabbit polyclonal anti-Ara h 1 (Indoor Biotech PA-AH1, 1:1000) and anti-Ara h 2 (Indoor Biotech PA-AH2, 1:500) or control mouse monoclonal anti-Ara h 1 (Indoor Biotech MA-2C12, 1:1000) and anti-Ara h 2 antibodies (Indoor Biotech MA-1C4, 1:1000) were applied in duplicates to plates and incubated at 4°C overnight. Bound antibodies were detected with HRP-conjugated anti-human IgG H+L (Jackson ImmunoResearch Catalog# 709-035-149), HRP-conjugated anti-rabbit secondary antibodies (GE Healthcare Catalog# NA934V) or HRP-conjugated anti-mouse IgG secondary antibodies (Jackson ImmunoResearch Catalog# 715-035-150) diluted 1:1000 after incubation for 2 hours at room temperature. Plates were then incubated with ABTS substrate (Thermo Scientific) for 10-20 minutes at room temperature before Absorbance 405 nm measurement with a plate reader. Plates were washed three times

with PBST in between incubations. For measurement of monoclonal antibody binding to fluorophore-conjugated Ara h 1, whole native Ara h 1 protein was either conjugated with Dylight-488 or with Pacific Blue according to manufacturer's instructions (see above), and 250 ng of conjugated protein was used to coat each well of 96-well microtiter plates. For measurement of monoclonal antibody binding to allergen that was mock-conjugated with Dylight-488 or Pacific Blue, Ara h 1 was incubated for one hour at room temperature in 0.05M sodium borate buffer pH 8.5 or 0.1M sodium bicarbonate buffer pH 8.3, respectively. Mock-conjugated allergen proteins were buffer exchanged into PBS using Bio-Spin 6 columns before use.

Ara h 1 and Ara h 2 phage-display library construction

Arachis hypogaea Ara h 1 and Ara h 2 protein sequences (Genbank ACF22884.1 and AAN77576.1, respectively) were reverse translated and resulting codons were optimized for protein expression in *Escherichia coli*. 72-nucleotide fragments of these redesigned Ara h 1 and Ara h 2 sequences were picked to tile across the whole sequences and began every 30 nucleotides (Table E5). These fragments were encoded with two half pieces, which were combined to produce the entire fragments using overlap-extension PCR⁷. Ara h 1 was encoded with 61 fragments (Ara h 1 1 to 61) and Ara h 2 was encoded with 17 fragments (Ara h 2, 1 to 17). In producing the fragments with overlap-extension PCR, we introduced restriction sites to flank the fragments (5' EcoRI site and 3' SalI site) and these sites were used to clone the pooled fragments into T7_FLAG, a modified T7Select 10-3b vector (EMD Millipore) that contains a FLAG epitope sequence between NotI and XhoI⁸. Ligation products were packaged into phage, which were then quantitated and plate amplified to produce a high-titer phage library stock that was used in subsequent

experiments, per the manufacturer's instructions. Two independent libraries were used in phage-display experiments and originated from either 8×10^5 or 2.2×10^6 independent plaques.

Ara h 1 and Ara h 2 phage-display binding reactions

Buffers: TBST is Tris-buffered saline with 0.5% Tween 20. Blocking solution is TBST with 3% bovine serum albumin fraction V. Wash solution is 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Nonidet P-40. 96-well untreated plates were blocked overnight at 4 °C in blocking solution on an orbital shaker. Binding reactions were performed overnight at 4 °C on an orbital shaker in 120 µL volumes containing 30 µL of culture supernatant containing a specific monoclonal antibody, 69 µL of TBST, and 21 µL of the phage-display library. Between 3×10^7 and 1.5×10^8 phage, measured in plaque-forming units, were present in each reaction in all experiments. Control reactions without antibody contained 30 µL of culture supernatant from 3T3 cells transfected with an empty construct, while positive control reactions contained either 12 µL of anti-Ara h 1 or 4 µL of anti-Ara h 2 polyclonal antibody (Indoor Biotechnologies; PA-AH1 or PA-AH2). After overnight binding, 1 µL of Protein A and 1 µL of Protein G Dynabeads (Life Technologies) were added to each well and bindings continued at 4 °C for four hours on a platform shaker. Antibody-phage complexes were then captured using a magnet and washed five times with 80 µL wash buffer, after which phage DNA was isolated with 24 µL water and 90 °C treatment for 15 minutes. Phage DNAs were stored at -20 °C until processed.

Ara h 1 and Ara h 2 phage-display high-throughput sequencing.

Prior to high-throughput sequencing on an Illumina Miseq instrument using a Miseq v2 300-cycle kit, phage-insert libraries were constructed using two sequential PCR reactions (Fig E8). Qiagen Multiplex PCR kits were used for all PCR reactions. In the first reaction, a unique pair of 8-mer barcodes and part of an adapter needed for sequencing were added to the 5' and 3' ends of all phage DNA in a given well, allowing for the identification of different samples within the total pooled library that was sequenced. In the second reaction, the remaining necessary part of the sequencing adapter was added. To obtain similar sequencing depth for each sample, the individual second PCR reactions were analyzed by gel electrophoresis and the amount of each PCR reaction added to the final pooled sample was normalized based on the amount of sample visible on the gel. The final pooled sample was purified by gel extraction, quantitated using a Qubit dsDNA HS assay (Life Technologies), denatured, and loaded on a Miseq instrument. Sequence reads from paired-end 300 cycle instrument runs were merged using the FLASH algorithm² sorted by sample of origin by requiring exact matching to the sequence barcodes, and were mapped to the designed phage inserts by exact sequence match to 15-mer nucleotide sequences at the 5' and 3' end of each sequence encoding a peanut allergen peptide. Total counts for each peanut allergen peptide were counted for each pull-down sample and expressed as a fraction of all peptide sequences pulled down in that sample. The fractional representation of each peptide in each pull-down sample was normalized to the fraction of that peptide in a mock pull-down experiment in which all reaction components were present except for the antibody. Data were plotted as fold-enrichment above the mock pull-down condition for each peptide.

References:

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Supplemental Figures and Tables

Fig E1. Gating strategy for isolation and immunophenotypic characterization of antigen-specific B cells. Ara h 1-specific (shown) or Ara h 2-specific (not shown) B cells (CD19⁺/CD3⁻/14⁻/16⁻/235a⁻) were sorted. Allergen-specific B cells were further immunophenotyped as: class-switched memory (CD27⁺/IgD⁻); non-class-switched memory (CD27⁺/IgD⁺); plasmablast (CD38⁺⁺/CD27⁺⁺/IgD⁻/CD20^{lo}); naïve (CD27⁻/CD38⁻/IgD⁺); or transitional (CD27⁻/CD38⁺/IgD⁺).

Fig E2. Representative flow cytometry plots of Ara h 1-specific and Ara h 2-specific B cell populations detected after pre-incubation with unlabeled peanut allergen. Samples are shown from a healthy donor (Patient 27, left panels), a peanut-allergic individual at baseline (Patient 11, middle panels) and a peanut-allergic individual after 9 months of OIT (Patient 5, right panels). A) PBMCs were stained with dual-labeled Ara h 1 or Ara h 2 with (second and fourth rows) or without (first and third rows) pre-incubation with a 10-fold excess of unlabeled allergen. Oval gates show double-positive CD19⁺/CD3⁻/CD14⁻/CD16⁻/CD235a⁻ B cells. B) Boxplots showing quantification of antigen-specific B cells/total B cells for healthy donor samples (n=3; Ara h 1 median = 0.0073%, Ara h 1 blocked median = 0.0016%, Ara h 2 median = 0.0087%, Ara h 2 blocked median = 0.0097%) and allergic patient samples at baseline (n=8; Ara h 1 median = 0.0097%, Ara h 1 blocked median = 0.027%, Ara h 2 median = 0.029%, Ara h 2 blocked median = 0.0056%) and OIT (n=6; Ara h 1 median = 0.035%, Ara h 1 blocked median = 0.0045%,

Ara h 2 median = 0.084%, Ara h 2 blocked median = 0.011%). *P*-values were calculated by Wilcoxon tests ($p^* < 0.05$ and $p^{**} < 0.01$).

Fig E3. The frequency of antigen-binding B cells is increased in the blood during OIT compared to baseline allergic patient blood. Boxplots show the frequency of Ara h 1-binding (top panels) and Ara h 2-binding (bottom panels) cells from A) CD27+ memory and CD27- non-memory compartments or B) memory class-switched, memory non-class-switched, plasmablast, naïve and transitional subsets for all samples from healthy donors ($n = 9$) and allergic individuals at baseline ($n = 18$) and during OIT ($n = 13$) that were analyzed by flow cytometry. *P*-values were calculated by two-tailed Student's *t*-test ($*p < 0.05$, $**p < 0.01$ and $*p < 0.001$).

Fig E4. Validation of monoclonal antibody binding to Ara h 1 and Ara h 2. (A) Amplification of paired Ig heavy and light chain genes from two single cells, 4218 and 4219. (B) Plots of binding of polyclonal anti-Ara h 1 or anti-Ara h 2 control antibodies, isolated mAbs, or control media to Ara h 1 or Ara h 2 by direct ELISA are shown. Points indicate the average of two replicates. mAbs that had an absorbance value three-fold above background (blue line) were considered positive for binding (red line). C-E) Representative Western blots of recombinant human mAbs against Ara h 1 (C) or Ara h 2 (D-E) used as primary antibodies to detect binding to natural purified Ara h 1 and Ara h 2 separated by denaturing gel electrophoresis in the presence or absence of reducing agent. 0.5µg antigen were loaded per lane. Arrow - Ara h 1 monomer (C) or Ara h 2 (D-E); arrowhead- Ara h 1 trimer (C). Lane1- nAra h 1; Lane2- nAra h 1 + 5% βme; Lane3-

nAra h 2; Lane4- nAra h 2 + 5% β me. Molecular weight markers are shown on the left, and values are in kilodaltons. (F) Monoclonal antibody concentrations plotted in $\mu\text{g/mL}$ for validated peanut-specific mAbs and non-specific mAbs that could not be validated by binding to peanut allergens by ELISA, Western or phage-displayed peptides. Statistical comparisons between positive binding and negative binding groups were performed by one-tailed Student's T-test (n.s = not significant). (G) The isotype frequencies for validated (dark bars) and non-validated (light bars) monoclonal antibodies purified by Ara h 1-specific (top panel) and Ara h 2-specific (bottom panel) FACS are plotted. Statistical comparisons between validated antigen binding and non-validated, non-binding groups were performed by Chi-square analysis (** $p < 0.01$ and *** $p < 0.001$) (Ara h 1, Chi-squared = 14.73, dof = 2, $p = 0.0006$; Ara h 2, chi-square = 10.28, dof = 2, $p = 0.006$). (H) Binding of AbID 94 to unconjugated (first panel), Alexa-488 conjugated (second panel), Pacific Blue-conjugated (third panel), Alexa-488 mock-conjugated (fourth panel) or Pacific Blue mock-conjugated (fifth panel) Ara h 1 in direct ELISA are shown. Points indicate the average of two replicates. Binding of commercially available monoclonal antibodies against Ara h 1 and Ara h 2, as well as mock (no antibody) controls, are shown for comparison.

Fig E5. PCR primers and reaction conditions for phage-insert library construction

Fig E6. V, D and J gene usage, and isotype frequencies in antibody heavy chains of non-specific monoclonal antibodies. Data were derived from 34 non-specific antibodies isolated by Ara h 1-binding FACS and 43 non-specific antibodies isolated by Ara h 2-

binding FACS. V, D and J gene frequencies (A-C) and V and D gene family frequencies (D-E) are graphed, as well as isotype frequencies (F) and IGHV somatic mutation levels for each isotype (G).

Table E1. Patient clinical and sample data.

Table E2. Primers designed for single cell PCR and antibody expression for this study.

Table E3. Sequence features of peanut-specific monoclonal antibodies.

Table E4. Expanded B cell clones identified from distinct allergen-specific sorted single cells.

Table E5. Ara h 1 and Ara h 2 DNA and amino acid sequences used in the phage-display system.

Table E6: Sequence of first PCR and second PCR primer sets for generation of templates for human antibody heavy chain sequencing with Illumina Miseq.

Figure E1

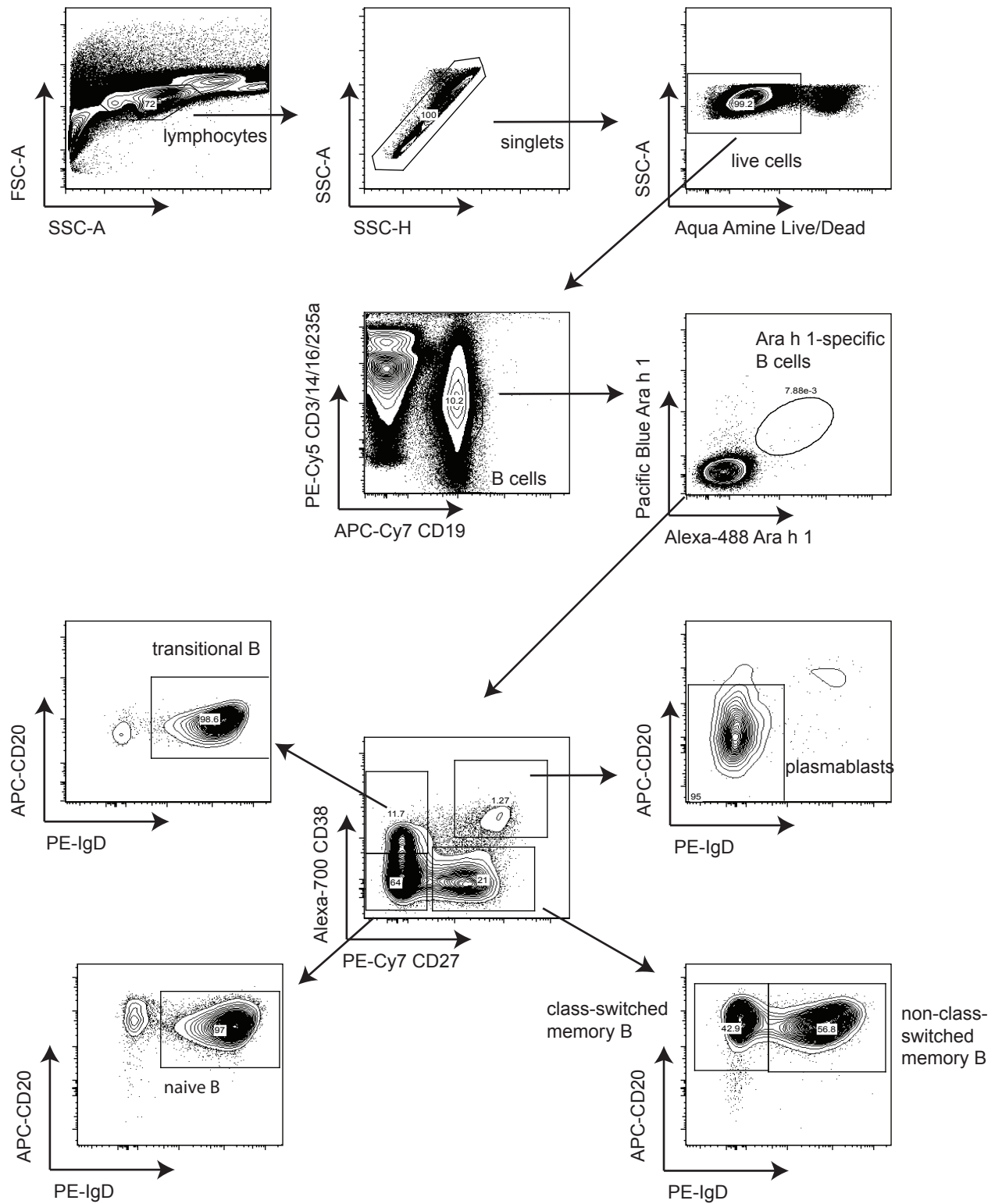


Figure E2

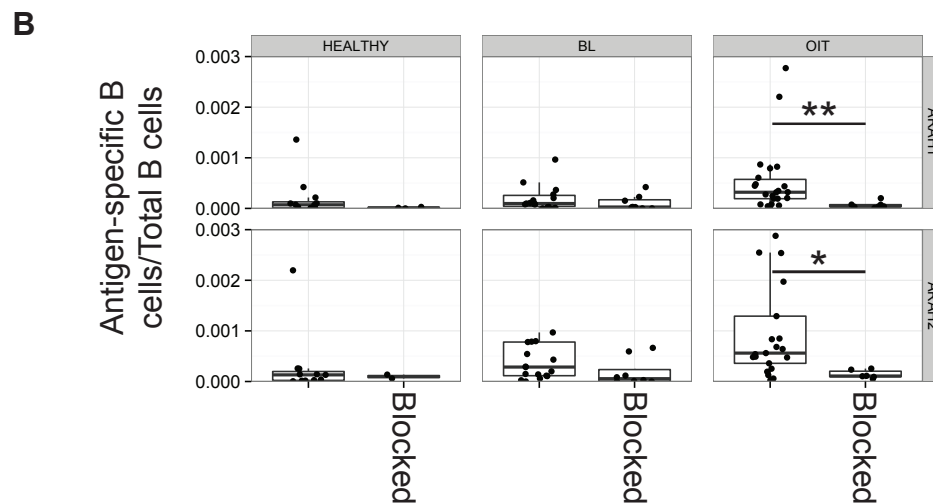
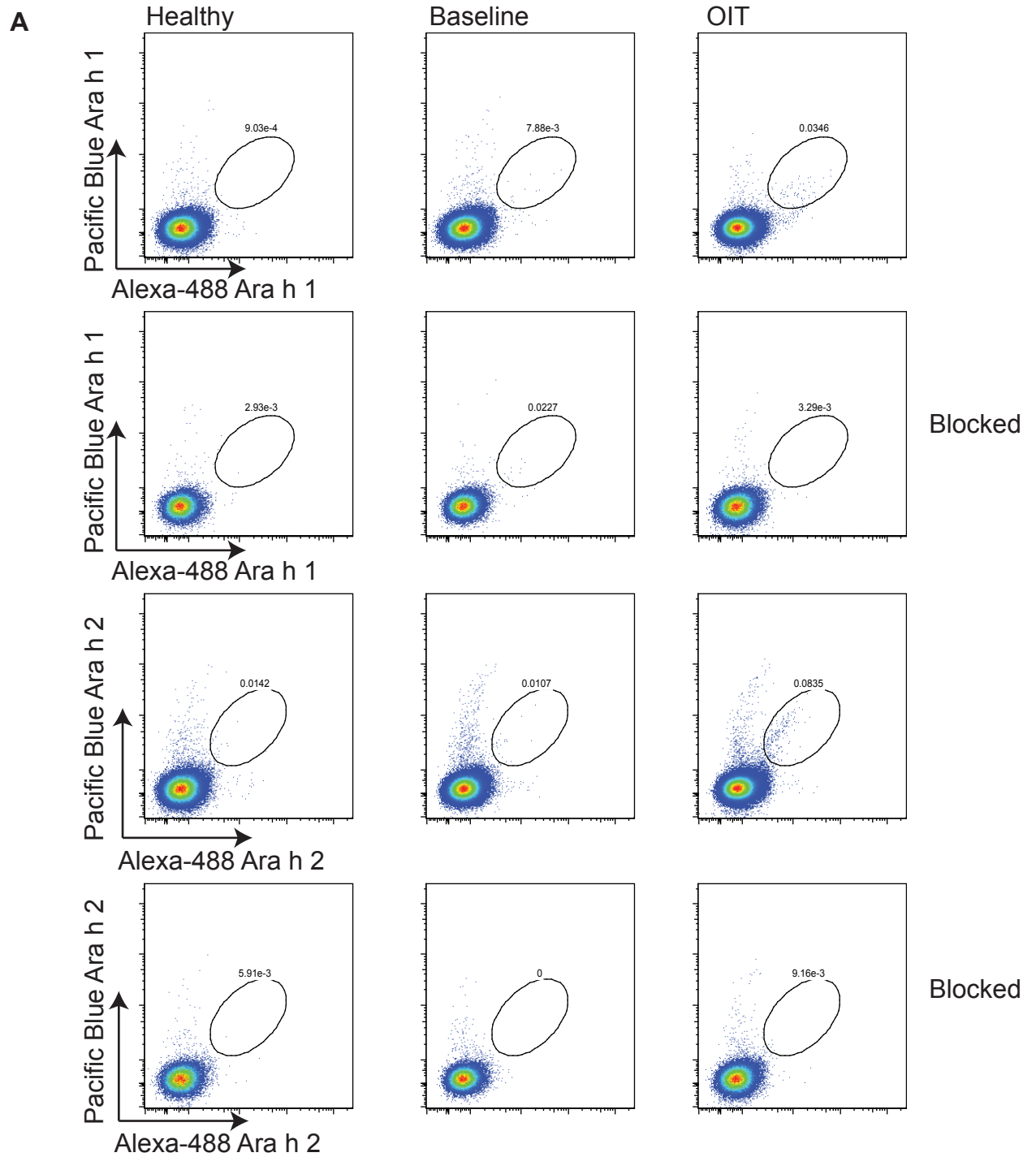
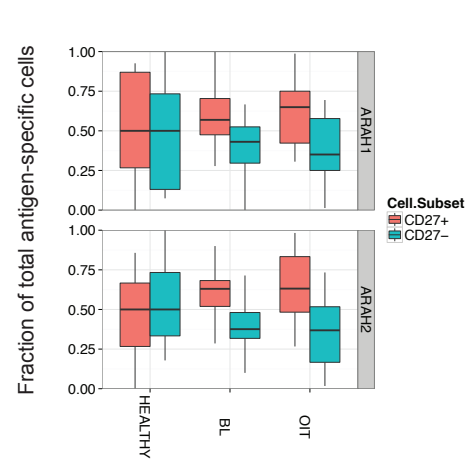


Figure E3

A



B

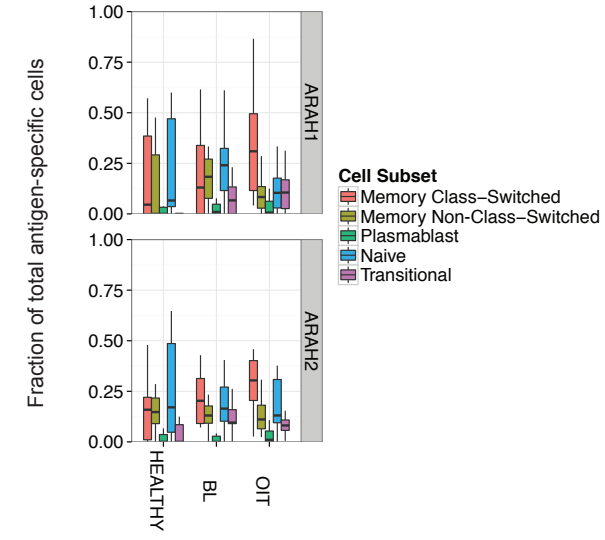


Figure E4

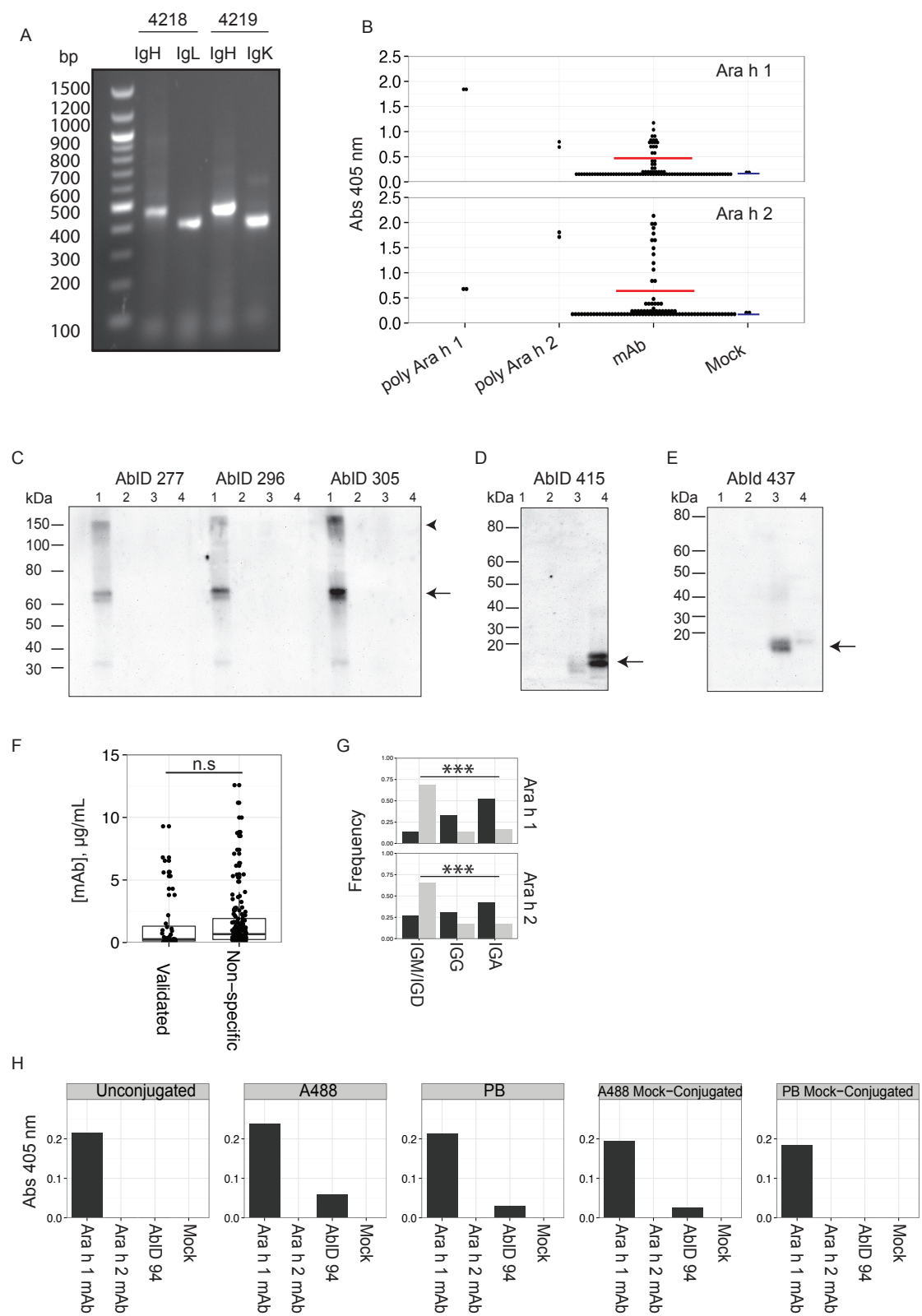


Figure E5. Phage-insert library construction:

Primers used in first PCR reactions:

PCR1_For: 5' ACACTCTTTCCCTACACGACGCTCTTCCGATCG(N₄X₈)GGAGCTGTCGTA
TTCCAGTC 3' (N₄ are random bases, X₈ are barcodes (listed below), and underlined sequence
is from T7Select 10-3b)

PCR1_Rev: 5' GGCATTCCTGCTGAACCGCTCTTCCGATCT(Y₈)AACCCCTCAAGACCCG
TTTA 3' (Y₈ are barcodes (listed below), and underlined sequence is the reverse complement
sequence of T7Select 10-3b)

Primers used in second PCR reactions:

PCR2_For: 5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCT
CTTCCGATC 3'

PCR2_Rev: 5' CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAAC
CGCTC 3'

X₈ barcodes: ACGTAACG; ACGTCGTA; ACGAGACG; AGTGTACG; AGTCCGTA;
CGCATCGT; CGAACTAC; CGAAGCGT; CTCCACGT; CTCCATAC; GTGGTGTA;
GTGTAACG.

Y₈ barcodes: CGTTACGT; TACGACGT; CGTCTCGT; CGTACACT; TACGGACT;
ACGATGCG; GTAGTTCG; ACGCTTCG; ACGTGGAG; GTATGGAG; TACACCAC;
CGTTACAC; TACGAAAC; TACACATC; CGTCCATC; GTACCTTA.

First PCR cycling conditions: 1) 95 °C, 15 min. 2) 25x: 94 °C, 30 sec.; 51 °C, 90 sec.; 72 °C,
60 sec. 3) 60 °C, 30 min.

Second PCR cycling conditions: 1) 95 °C, 15 min. 2) 8x: 95 °C, 30 sec.; 58 °C, 45 sec.; 72 °C,
60 sec. 3) 72 °C, 10 min.

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Figure E6

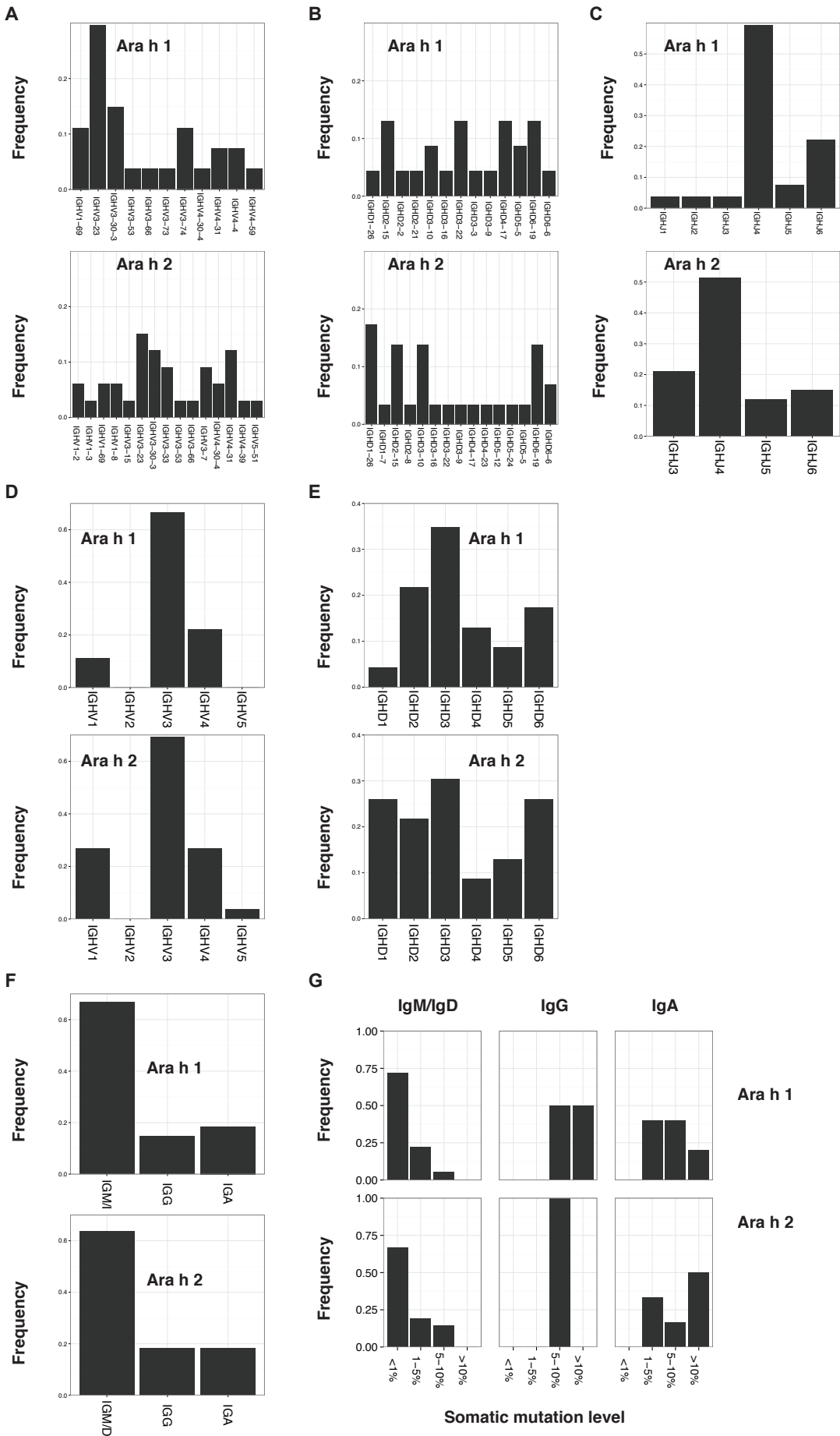


Table E2. Primers designed for single cell PCR and antibody expression for this study.

Primer Name	Primer Sequence (5'-3')	Reaction
IgE-ext	gaggtggcattggaggggaat	Nested single cell PCR for amplification of heavy chains from IgE constant region
IgE-int	tgctgtgccccagaggtttgcagcagcgggtcaaggg	Nested single cell PCR for amplification of heavy chains from IgE constant region
CMVfor	gcgcgcggtgacattgattattgac	Amplification of CMV region for vector generation; amplification of CMV-leader fragments for linear expression constructs
CMVrev	gtcaccagtggaacctggaaccc	Amplification of CMV region for vector generation
PolyA BamHIfor	gccacaggatccagatcagcctcgactgtgccttctag	Amplification of the BGH polyA region for vector generation
PolyA rev	ccccagcatgcctgctattgtcttcc	Amplification of the BGH polyA region for vector generation; amplification of constant region-polyA fragments for linear expression constructs
IgHG1con HindIIIfor	gccacaaagcttgctccaccaagggcccatcg	Amplification of IgG1 heavy chain constant region for vector generation; amplification of constant region fragment for linear expression constructs
IgHG1con BamHIrev	gccacaggatcctcatttaccgggagacaggagag	Amplification of IgG1 heavy chain constant region for vector generation
IgKcon HindIIIfor	gccacaaagcttcgaactgtggctgcaccatctg	Amplification of IgK constant region for vector generation
IgKcon BamHIrev	gccacaggatccctaactctcccctgttgaagc	Amplification of IgK constant region for vector generation; amplification of constant region fragment for linear expression constructs
IgLcon HindIIIfor	gccacaaagcttggtcagcccaaggctgcccc	Amplification of IgL constant region for vector generation
IgLcon BamHIrev	gccacaggatccctatgaacattctgtaggggccac	Amplification of IgL constant region for vector generation;

		amplification of constant region fragment for linear expression constructs
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Table E4. Expanded B cell clones identified from distinct allergen-specific sorted single cells

AbID	Antigen	Participant	Treatment	Isotype	V_gene	D_gene	J_gene	V mutations	D mutations	J mutations	CDR3_amino acids
14	Ara h 1	7	OIT	IGG1	IGHV4-31*03	IGHD2-2*02	IGHJ4*02	23	5	2	ARERYGNTTYLGFDS
65	Ara h 1	7	OIT	IGG1	IGHV4-31*03	IGHD3-22*01	IGHJ4*02	19	2	2	ARERYGNTAYLGFDS
277	Ara h 1	14	OIT	IGA1	IGHV1-3*01	IGHD6-13*01	IGHJ4*02	29	0	1	ARGVLGSSSHNFDS
408	Ara h 1	14	OIT	IGA1	IGHV1-3*01	IGHD6-13*01	IGHJ4*02	24	0	1	ARGVLNSSSHNFY
293	Ara h 1	14	OIT	IGG2	IGHV4-4*07	IGHD1-20*01	IGHJ2*01	19	1	4	VRDRRLPGVTAYWFFDL
314	Ara h 1	14	OIT	IGA1	IGHV4-4*07	IGHD1-26*01	IGHJ2*01	10	0	4	ARDRRLPGTTAYWFFDL
393	Ara h 2	14	OIT	IGA1	IGHV3-30*18	Not identified	IGHJ4*02	25		3	AKDSGLRALAS
403	Ara h 2	14	OIT	IGG1	IGHV3-30*18	Not identified	IGHJ4*02	31		3	AKDSGLRALDS
433	Ara h 2	14	OIT	IGM	IGHV4-39*01	IGHD2-2*03	IGHJ4*02	2	0	1	ARLDIVVVPAASFDS
429	Ara h 2	14	OIT	IGG1	IGHV4-39*01	IGHD2-2*03	IGHJ4*02	8	0	1	ARLDIVVVPAASFDS
415	Ara h 2	14	OIT	IGM	IGHV1-24*01	IGHD4-17*01	IGHJ6*02	0	0	0	ATSRRNGYGDYFFSSYYYYGMDV
413	Ara h 2	14	OIT	IGM	IGHV1-24*01	IGHD4-17*01	IGHJ6*02	0	0	0	ATSRRNGYGDYFFSSYYYYGMDV

Mutation levels in V, D and J gene segments are expressed as nucleotides differing from germline.

Table E5. Ara h 1 and Ara h 2 DNA and amino acid sequences used in the phage-display system.

Peptide	Nucleotide sequence	Amino acid sequence	Amino acid position
ARAH1_1	atgcgcggccgctgagccgctgatctgctgtgggc attctggctgctggcgagcgtgagcgcgaccag	MRGRVSPLMLLLGILVLASVSATQ	1-24
ARAH1_2	ctgctgggcattctgctgctggcgagcgtgagcgcgacc caggcgaaaagcccgtatcgcaaaaccgaaaac	LLGILVLASVSATQAKSPYRK TEN	11-34
ARAH1_3	agcgcgaccagcgcaaaaagcccgtatcgcaaaaccga aaaccctgctgcgcgagcgtgctgagagctgc	SATQAKSPYRK TENPCAQRCLQSC	21-44
ARAH1_4	aaaaccgaaaaccctgctgcgcgagcgtgctgagag ctgcaagcaggaaccggatgatctgaaacagaaa	KTENPCAQRCLQSCQ QEPDDLKQK	31-54
ARAH1_5	ctgcagagctgccagcaggaaccggatgatctgaaacag aaagcgtgcgaaagccgctgcacaaaactggaa	LQSCQ QEPDDLKQKACESRCKLE	41-64
ARAH1_6	ctgaaacagaaagcgtgcgaaagccgctgcacaaaactg gaatatgatccgcgtgctgctgataccggc	LKQKACESRCKLEYDPRCVYDTG	51-74
ARAH1_7	accaaaactggaatatgatccgcgtgctgctgataccg gcgcgaccaaccagcgccatccgcccggcgaa	TKLEYDPRCVYDTGATNQRHPPGE	61-84
ARAH1_8	tatgataccggcgcgaccaaccagcgccatccgcccggg cgaaacaccccgcggccgcaagccggcgattat	YDTGATNQRHPPGERTRGRQPGDY	71-94
ARAH1_9	ccgcccggcgaaacgcaccgcggccgcaagccgggc gattatgatgatgatcgccgagccgcccgcgaa	PPGERTRGRQPGDYDDDRRQPRRE	81-104
ARAH1_10	ccgggcgattatgatgatgatcgccgcaagccgcgcgc gaagaaggcggccgctggggcccgcggaaccg	PGDYDDDRRQPRREEGGRWGPAEP	91-114
ARAH1_11	ccgcgcgcgaaagagcgccgctggggcccgccg gaaccgcgcgaacgcgaacgcgaagagattggcgc	PRREEGGRWGPAEPREEREREEDWR	101-124
ARAH1_12	ccggcggaaccgcgcgaacgcgaacgcgaagagatt ggcgcgagccgcggaagattggcgcgcgcgcgagc	PAEPREEREREEDWRQPREDWRRPS	111-134
ARAH1_13	gaagattggcgccagccgcgcgaagattggcgcgcgc gagccatcagcagccgcgcaaaattcgcccggaa	EDWRQPREDWRRPSHQPRKIRPE	121-144
ARAH1_14	cgccgcccgaagcattcagcagccgcgcaaaattcgccc ggaaggccgcgaaggcgaacagggaatggggcacc	RRPSHQPRKIRPEGREGEQEWGT	131-154
ARAH1_15	attcgcccgaagccgcgaagccgaacagggaatggg gcacccggcgagcgaagtcgcggaagaaaccagc	IRPEGREGEQEWGTPGSEVREETS	141-164
ARAH1_16	gaatggggcaccggcgagcgaagtgcgcgaagaaa ccagccgcaacaaccgctttattttccgagccgc	EWGTPGSEVREETSRNNPFYFPSR	151-174
ARAH1_17	gaagaaccagccgcaacaaccgctttattttcagagcc gcccgtttagcaccgctatggcaaccagaac	EETSRNNPFYFPSRRFSTRYGNQN	161-184
ARAH1_18	tttcgagccgccttttagcaccgctatggcaaccaga acggccgcattcgcgtgctgcagcgctttgat	FPSRRFSTRYGNQNGRIRVLQRFD	171-194
ARAH1_19	ggcaaacagaaacggccgcttcgcgtgctgcagcgcttt gatcagcgcagcaaacagttcagaacctgcag	GNQNGRIRVLQRFDQRSKQFQNLQ	181-204
ARAH1_20	cagcgctttgatcagcgcgaacaacagttcagaacctgc agaaccatcgcttgcagattgaagcgcgc	QRFQDQRSKQFQNLQNHRIVQIEAR	191-214
ARAH1_21	cagaacctgcagaaccatcgcttgcagattgaagcgc gcccgaacaccctggtgctgcggaacatgcg	QNLQNHRIVQIEARPNLTVLPKHA	201-224
ARAH1_22	attgaagcgcgcccgaacaccctggtgctgcggaacat gcggtatcggaataacattctggtgattcagcag	IEARPNTLTVLPKHADADNILVIQQ	211-234
ARAH1_23	ccgaacatcggtatgcggataacattctggtgattcagc agggccagggcgaccgtgaccgtggcgaaaggc	PKHADADNILVIQQGQATVTVANG	221-244
ARAH1_24	gtgattcagcagggccagggcagcgtgaccgtggcgaa cggcaacaaccgcaaaagcttaacctggatgaa	VIQQGQATVTVANGNNRKSFNLDE	231-254
ARAH1_25	gtggcgaacggcaacaaccgcaaaagctttaacctggat gaaggccatgcgctgcgcattccgagcggcttt	VANGNNRKSFNLDEGHALRIPSGF	241-264
ARAH1_26	aacctggatgaaggccatgcgctgcgcattccgagcggc tttattagctattctgaaccgcatgataac	NLDEGHALRIPSGFISYILNRHDN	251-274
ARAH1_27	ccgagcggctttattagctattctgaaccgcatgataac cagaacctgcgcgtggcgaaaattagcatg	PSGFISYILNRHDNQNLRVAKISM	261-284
ARAH1_28	cgccatgataacagaaactgcgcgtggcgaaaattagc atgccggtgaacacccgggagtttgaagat	RHDNQNLRVAKISMPVNTPGQFED	271-294
ARAH1_29	aaaattagcatgccggtgaacacccgggagtttgaag attttttccggcgagcagccgcatcagagc	KISMPVNTPGQFEDFFPASSRDQS	281-304
ARAH1_30	cagtttgaagattttttccggcgagcagccgcgatcagag cagctatctcgagggttttagccgcaaac	QFEDFFPASSRDQSSYLQGF SRNT	291-314
ARAH1_31	cgcgatcagagcagctatctcgagggttttagccgcaaca ccctggaagcggcgtttaacgcggaatttaac	RDQSSYLQGF SRNTLEAAFN AEFN	301-324
ARAH1_32	agccgcaacacctggaagcggcggttaacgcggaattta	SRNTLEAAFN AEFN EIRRVLLEEN	311-334

	acgaaattcgccgctgctgctggaagaaac		
ARAH1_33	gcggaattaacgaaattcgccgctgctggaagaaa acgcgggcgccgaacaggagaacgcggccag	AEFNEIRRVLLEENAGGEQEERGQ	321-344
ARAH1_34	ctggaagaaaacgcggcgccgaacaggagaacgcg gccagcgcccgccagcaccgcagcagcgataac	LEENAGGEQEERGQRRRSTRSSDN	331-354
ARAH1_35	gaacgcggccagcgcgcgcagcaccgcagcagcg ataacgaaggcgtgattgtgaaagtgcgcaagaa	ERGQRRRSTRSSDNNEGIVVKSKE	341-364
ARAH1_36	agcagcgataacgaaggcgtgattgtgaaagtgcgcaaa gaacatgtgcaggaaactgaccaaacatgcgaaa	SSDNEGIVVKSKEHVQELTKHAK	351-374
ARAH1_37	gtgagcaagaacatgtgcaggaaactgaccaaacatgcg aaaagcgtgagcaaaaaaggcagcgaagaa	VSKEHVQELTKHAKSVSKKGSEEE	361-384
ARAH1_38	aaacatgcgaaaagcgtgagcaaaaaaggcagcgaaga agaagatattaccaaccgattaacctgcgcgat	KHAKSVSKKGSEEEEDITNPINLRD	371-394
ARAH1_39	agcgaagaagaagatattaccaaccgattaacctgcgc gatggcgaaccggatctgagcaacaacttggc	SEEEEDITNPINLRDGEPLSNNFG	381-404
ARAH1_40	aacctgcgcgatggcgaaccggatctgagcaacaactttg gccgcctgttgaagtgaacccgataaaaaa	NLRDGEPLSNNFGRLFEVKPDKK	391-414
ARAH1_41	aacaacttggccgcctgttgaagtgaacccgataaaaaa aaaccgcagctgcaggatctggatatgatg	NNFGRLFEVKPDKKNPQLQDLDM	401-424
ARAH1_42	ccggataaaaaaacccgcagctgcaggatctggatatg atgctgacctgcgtggaattaaagaaggcgcg	PDKKNPQLQDLDMMLTCVEIKEGA	411-434
ARAH1_43	ctggatatgatgctgacctgcgtggaattaaagaaggcg cgctgatgctgccgcattttaacagcaaaagcg	LDMLTCVEIKEGALMLPHFNSKA	421-444
ARAH1_44	aaagaaggcgcgctgatgctgcgcattttaacagcaaaag cgatgggtattgtgtgtggaacaaaggcacc	KEGALMLPHFNSKAMVIVVVNKG	431-454
ARAH1_45	aacagcaaaagcagtggtgattgtgtgtggaacaaaggc accggcaacctggaactgtgtggcgtgcgcaaa	NSKAMVIVVVNKG TGNLELVAVRK	441-464
ARAH1_46	aacaaaggcacccggcaacctggaactgtgtggtgcg caaagaacagcagcagcgcggccgcgcaacag	NKG TGNLELVAVRKEQQQRGRREQ	451-474
ARAH1_47	gcggtgcgcaagaacagcagcagcgcggccgcgcg aacaggaatgggaagaagaagaagatgaagaa	AVRKEQQQRGRREQEWEEEEDEE	461-484
ARAH1_48	cgccgcgaacaggaatgggaagaagaagaagatg aagaagaagaaggcagcaaccgcgaagtgcgcgc	RREQEWEEEEDEEEGSNREVRR	471-494
ARAH1_49	gaagatgaagaagaagaaggcagcaaccgcgaagtgc gccgtataccgcgcctgaaagaaggcgatg	EDEEEGSNREVRRYTARLKEGDV	481-504
ARAH1_50	gaagtgcgcgcctataccgcgcctgaaagaaggcgat gtgttattatgccggcgcgcatccgggtgcg	EVRRYTARLKEGDVFIMPAAHVPA	491-514
ARAH1_51	gaaggcgatgtgttattatgccggcgcgcatccgggtg cgattaacgcgagcagcgaactgcactctg	EGDVFIMPAAHVPAINASSELHLL	501-524
ARAH1_52	catccggtggcgattaacgcgagcgaactgcactctg ctgggcttggcattaacgcggaacaaacat	HPVAINASSELHLLGFGINAENNH	511-534
ARAH1_53	ctgcactctggtgcttggcattaacgcggaacaaacac atcgatttttggcggcgataaagataac	LHLLGFGINAENNHRIFLAGDKDN	521-544
ARAH1_54	gaaaacaaccatcgcattttctggcggcgataaagataa cgtgattgatcagattgaaaacaggcgaaa	ENNHRIFLAGDKDNVIDQIEKQAK	531-554
ARAH1_55	gataaagataacgtgattgatcagattgaaaaacaggcga aagatctggcgtttccggcgagcggcgcaacag	DKDNVIDQIEKQAKDLAFPGSGEQ	541-564
ARAH1_56	aaacaggcgaaagatctggcgttccggcgagcggcgga acaggtggaaaaactgattaaaaccagcgcgaa	KQAKDLAFPGSGEQVEKLIKQRE	551-574
ARAH1_57	agcggcgaaacaggtggaaaaactgattaaaaccagcg cgaaagccattttgtgagcgcgcgcgcgcagagc	SGEQVEKLIKQRESHFVSAR PQS	561-584
ARAH1_58	aaccagcgcgaaagccattttgtgagcgcgcgcgcgcag agccagagcccagcagcccggaaaaagaagat	NQRESHFVSAR PQSQSPSSPEKED	571-594
ARAH1_59	cgcccgagagccagagcccagcagcccggaaaaaag aagatcaggaagaagaaccaggcgcgcaaggc	RPQSQSPSSPEKEDQEENQGGKG	581-604
ARAH1_60	agcccgagcagcccggaaaaagaagatcaggaagaag aaaaccaggcgcgcaaggccgcgtgctgagcatt	SPSSPEKEDQEENQGGKGPLLSI	586-609
ARAH1_61	aaagaagatcaggaagaagaaccaggcgcgcaaaag gcccgcgtgctgagcattctgaaagcgtttaac	KEDQEENQGGKGPLLSILKAFN	592-614
ARAH2_1	atggcgaaactgaccatttgggtggcgtggcgtgtttct gtggcgccgcatgagcgcgcgcgcagcag	MAKLTLVALALFLAAHASARQQ	1-24
ARAH2_2	gcgctgtttctgctggcgcatgagcgcgcgcgcag cagtggaactgcaggcgcatgcgcgcgcag	ALFLAAHASARQQWELQEDRRCQ	11-34
ARAH2_3	gcgcgcagcagtggaactgcaggcgcatgcgcgcgtg ccagagccagctggaacgcgcgaacctgcgcgcg	ARQQWELQEDRRCQSQLERANLRP	21-44
ARAH2_4	cgccgctgccagagccagctggaacgcgcgaacctgcg cccgctggaacagcatctgatgcagaaaattcag	RRCQSQLERANLRPCEQHLMQKIQ	31-54

ARAH2_5	aacctgcgcccgtgcgaacagcatctgatgcagaaaattc agcgcgatgaagatagctatggccgcgatccg	NLRPCEQHLMQKIQRDEDSYGRDP	41-64
ARAH2_6	cagaaaattcagcgcgatgaagatagctatggccgcgatc cgtatagcccagcaggatccgtatagcccg	QKIQRDEDSYGRDPYSPSQDPYSP	51-74
ARAH2_7	ggccgcgatccgtatagcccagcaggatccgtatagc ccgagccaggatccgatcgcgcgatccgtat	GRDPYSPSQDPYSPSQDPDRRDY	61-84
ARAH2_8	ccgtatagcccagcaggatccgatcgcgcgatccg tatagcccagcccgtatgatcgcgcggcg	PYSPSQDPDRRDYSPSPYDRRGA	71-94
ARAH2_9	cgcgatccgtatagcccagcccgtatgatcgcgcggc ggggcagcagccagcatcaggaacgctgctgc	RDYSPSPYDRRGAGSSQHQRCC	81-104
ARAH2_10	cgcgcggcgccggcagcagccagcatcaggaacgct gctgcaacgaactgaacgaatttgaaaacaaccag	RRGAGSSQHQRCCNELNEFENNQ	91-114
ARAH2_11	gaacgctgctgcaacgaactgaacgaatttgaaaacaac agcgtgatgtgcgaagcgtgcagcagatt	ERCCNELNEFENNQRCMCEALQOI	101-124
ARAH2_12	gaaaacaaccagcgtgcatgtgcgaagcgtgcagca gattatggaaaaccagagcgtgcctgcagggc	ENNQRCMCEALQOIMENQSDRLQG	111-134
ARAH2_13	ctgcagcagattatggaaaaccagagcgtgcctgcag ggccgcccagcaggaacagcagtttaaacgcgaa	LQOIMENQSDRLQGRQEQQFKRE	121-144
ARAH2_14	cgcctgcaggccgcccagcaggaacagcagtttaaacg cgaactgcgcaactgccgcagcagtgccgcctg	RLQGRQEQQFKRELRLNPQQCGL	131-154
ARAH2_15	tttaaacgcgaactgcgcaactgccgcagcagtgccgc ctgcgcgcgccgcagcgtgcgatctggaagtgc	FKRELRLNPQQCGLRAPQRCDLEV	141-164
ARAH2_16	gaactgcgcaactgccgcagcagtgccgcctgcgcgc gccgcagcgtgcgatctggaagtggaaagcggc	ELRLNPQQCGLRAPQRCDLEVESG	144-167
ARAH2_17	ccgcagcagtgccgcctgcgcgcgccgcagcgtgcga tctggaagtggaaagcggcgccgcgatcgtat	PQQCGLRAPQRCDLEVESGGRDRY	149-172

Table E6: Sequence of first PCR and second PCR primer sets for generation of templates for human antibody heavy chain sequencing with Illumina Miseq.

1 st PCR IgH FR1 V-Segment and Isotype Constant Region Primers, and 2 nd PCR Primers	
1 st -P7-VH1	GGCATTCTGCTGAACCGCTCTTCGATCTXXXXXXXXXGGCCTCAGTGAAGGTCTCCTGCAAG
1 st -P7-VH2	GGCATTCTGCTGAACCGCTCTTCGATCTXXXXXXXXXGTCTGGTCTACGCTGGTGAAACCC
1 st -P7-VH3	GGCATTCTGCTGAACCGCTCTTCGATCTXXXXXXXXXCTGGGGGTCCCTGAGACTCTCCTG
1 st -P7-VH4	GGCATTCTGCTGAACCGCTCTTCGATCTXXXXXXXXXCTTCGGAGACCCGTGTCCTCACCTG
1 st -P7-VH5	GGCATTCTGCTGAACCGCTCTTCGATCTXXXXXXXXXCGGGGAGTCTCTGAAGATCTCCTGT
1 st -P7-VH6	GGCATTCTGCTGAACCGCTCTTCGATCTXXXXXXXXXTCGAGACCCCTCTCACTCACCTGTG
2 nd -P7	CAAGCAGAAGACGGCATAACGAGATCGGTCTCGGCATTCTGCTGAACCGCTC
1 st -P5-IgA	ACACTCTTTCCTACACGACGCTCTTCGATCTNNNNXXXXXXXXXAGCCCTGGACCAGGCA
1 st -P5-IgD	ACACTCTTTCCTACACGACGCTCTTCGATCTNNNNXXXXXXXXXCCTGATATGATGGGAACA
1 st -P5-IgE	ACACTCTTTCCTACACGACGCTCTTCGATCTNNNNXXXXXXXXXGAAGACGGATGGGCTCTGT
1 st -P5-IgG	ACACTCTTTCCTACACGACGCTCTTCGATCTNNNNXXXXXXXXXCAGGCAGCCAGGGC
1 st -P5-IgM	ACACTCTTTCCTACACGACGCTCTTCGATCTNNNNXXXXXXXXXGGGAATTCTCACAGGAGACG
2 nd -P5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCTACACGACGCTCTTCGATC

Base positions marked X encode the barcode sequence used to indicate the sample and replicate library identity. Base positions marked N encode random nucleotides used to ensure base diversity during the first few cycles of the sequencing reaction, which are used for cluster identification.