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ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

Distinction between peanut allergy and tolerance by characterization of B cell receptor repertoires

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Abstract

Background: Specific IgE against a peanut 2S albumin (Ara h 2 or 6) is the best predictor of clinically relevant peanut sensitization. However, sIgE levels of peanut allergic and those of peanut sensitized but tolerant patients partly overlap, highlighting the need for improved diagnostics to prevent incorrect diagnosis and consequently unnecessary food restrictions. Thus, we sought to explore differences in V(D)J gene transcripts coding for peanut 2S albumin-specific monoclonal antibodies (mAbs) from allergic and sensitized but tolerant donors.

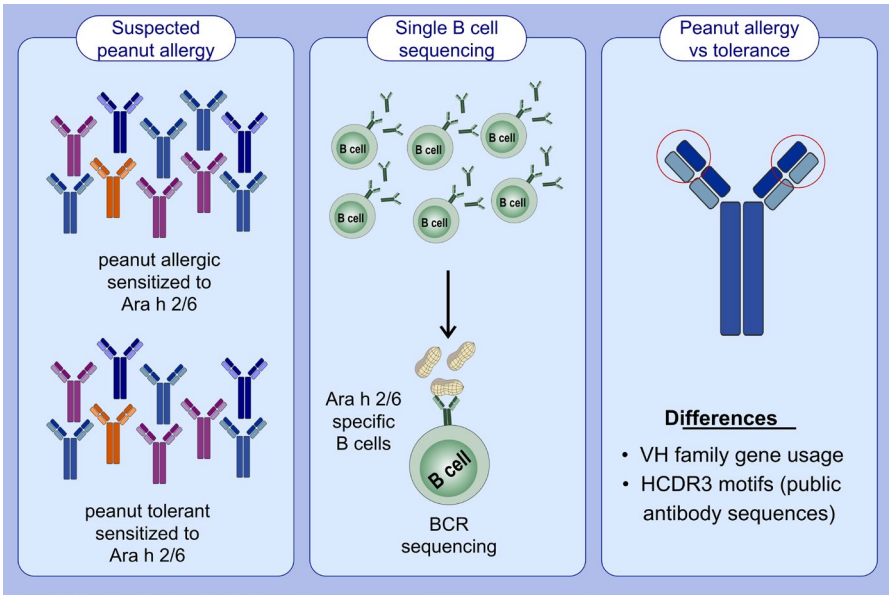
Methods: 2S albumin-binding B-cells were single-cell sorted from peripheral blood of peanut allergic (n=6) and tolerant (n=6) donors sensitized to Ara h2 and/or 6 (≥ 0.1 kU/l) and non-atopic controls (n=5). h 2 and/or 6 (≥ 0.1 kU/l). Corresponding h heavy and light chain gene transcripts were heterologously expressed as mAbs and tested for specificity to native Ara h2 and 6. HCDR3 sequence motifs were identified by Levenshtein distances and hierarchically clustering.

Results: The frequency of 2S albumin-binding B cells was increased in allergic (median: 0.01%) compared to tolerant (median: 0.006%) and non-atopic donors (median: 0.0015%, $p = 0.008$). The majority of mAbs (74%, 29/39) bound specifically to Ara h 2 and/or 6. Non-specific mAbs (9/10) were mainly derived from non-atopic controls. In allergic donors, 89% of heavy chain gene transcripts consisted of VH3 family genes, compared with only 54% in sensitized but tolerant and 63% of non-atopic donors. Additionally, certain HCDR3 sequence motifs were associated with allergy ($n = 4$) or tolerance ($n = 3$) upon hierarchical clustering of their Levenshtein distances.

Conclusions: Peanut allergy is associated with dominant VH3 family gene usage and certain public antibody sequences (HCDR3 motifs).

KEYWORDS
clinically irrelevant sensitization, food allergy diagnostics, monoclonal antibodies, peanut allergy, VH family gene usage

Abbreviations: BAT, Basophil activation test; DBPCFC, double-blind placebo-controlled food challenge; HCDR3, complementary-determining region 3 of the heavy chain; mAbs, monoclonal antibodies; OD, optical density; PBMCs, peripheral blood mononuclear cells; sIgE, specific IgE; V(D)J, rearranged variable (V), diversity (D) and joining (J) gene segments; VH, V (variable) gene of the heavy chain.



GRAPHICAL ABSTRACT

2S albumin-specific IgM+B cells from peanut allergic patients show partly a high number of non-silent mutations. VH3-family genes are predominantly used in 2S albumin-specific B-cells of peanut allergic patients. Certain public antibody sequences (HCDR3 sequence motifs) are associated with peanut allergy or tolerance in patients sensitized to Ara h 2 and/or 6.

Abbreviations: BCR, B cell receptor; HCDR3, heavy chain complementarity-determining region 3; VH, variable (V) gene of the heavy chain

1 | INTRODUCTION

Current food allergy diagnostics comprise careful history, skin prick tests, measuring specific IgE (sIgE) and double-blind placebo-controlled food challenges (DBPCFC) as the gold standard. However, DBPCFCs are burdensome for the patient, costly and require dedicated hospital facilities.[1,2] On the other hand, current technologies to measure sIgE detect both clinically relevant and irrelevant sensitization, potentially leading to incorrect diagnosis and consequently unnecessary food restrictions.[3,4] Clinically relevant peanut sensitization is associated with sIgE against the major peanut allergens belonging to the 2S albumin family, Ara h 2 and 6.[5] In previous studies with adults, 100% positive predictive values for sIgE against Ara h 2 and 6 were found using sIgE positivity thresholds of, respectively, 1.75 kU/L and 1.8 kU/L. Specific IgE levels below these thresholds, however, overlapped between allergic and tolerant subjects, preventing precise diagnosis.[6,7]

The occurrence of clinically irrelevant sensitization to Ara h 2 and 6 might be explained by differences in peanut (Ara h 2 and 6) specific antibody repertoires comprising IgE and non-IgE antibodies. These differences may include the presence of non-IgE antibodies blocking sIgE binding to clinically relevant epitopes,[8,9] whose recognition induces degranulation and leads to the presentation of allergic symptoms. Moreover, differences may also be based on sIgE antibody affinity and recognition patterns of clinically relevant and irrelevant epitopes.[10] However, no clear differences between allergic and tolerant subjects were observed by current epitope mapping approaches.[11] We hypothesize that these evaluations may have been hampered by the use of patient sera consisting of polyclonal specific IgE and non-IgE antibodies. Sera of allergic subjects may usually contain mixtures of antibodies recognizing both clinically relevant and irrelevant epitopes. On the other hand, sera from tolerant subjects may contain sIgE antibodies recognizing clinically relevant epitopes with insufficient affinity for successful FcεRI receptor crosslinking, sIgE antibodies recognizing clinically irrelevant epitopes and non-IgE blocking antibodies.[12] Hence, deep analysis of monoclonal antibodies (mAbs) from specific B cells may provide more insights into differences in specific IgE and non-IgE antibody repertoires between allergic and tolerant subjects.

To this end, we analyzed gene sequences encoding the variable region of peanut 2S albumin-specific mAbs from 6 allergic and 6 sensitized but tolerant adults. In particular, sequence motifs of the HCDR3 region, the most important region for recognizing antigens,[13] were hierarchically clustered. Clustering of HCDR3 regions resulted in four motifs exclusively present in allergic donors and three motifs associated with tolerance.

2 | METHODS

Blood samples (100 ml) were drawn from allergic (n = 6) and tolerant (n = 6) adults (≥18 years) sensitized to Ara h 2 and/or 6 (≥0.1 kU/L[14]). Classification was achieved by DBPCFC or convincing history (n = 2) of tolerated peanut ingestion. Non-atopic donors (n = 5) served as reference group. The present study was approved by the ethical committee of the University Medical Center Utrecht (No. 17-945), and informed consent was given by all participants.

2S albumins isolated from roasted peanuts were used for tetramer formation with Streptavidin-APC or -PE.[15,16] B cells double positive for tetramer-PE/APC staining were single cell sorted and the V(D)J gene transcript of the heavy and corresponding light chain were amplified as described previously.[15,17,18] Quality checked sequences of successfully amplified gene transcripts were evaluated with the IgBlast web

TABLE 1 Patient characteristics

	Sex	Age	Last reaction	Müller score[a]	Subj. ED [mg]	Obj. ED [mg]	Ara h2 [kU/L]	Ara h 6 [kU/L]	Total IgE [kU/L]
Allergic group									
Pt-05	f	34	3 years	3	10	3000	1.0	1.4	625
Pt-09	f	29	1 year	3	4	4000	2.4	5.3	807
Pt-01	f	39	1.5 years	1	100	3000	3.3	0	14.5
Pt-04	m	27	7 years	1	10	-	8.2	1.7	417
Pt-10	f	27	3 years	4	100	100	23	7.9	252
Pt-06	m	41	2 years	3	100	100	72	13.1	83.3

	Sex	Age	Last consumption	Amount	Processing/ Manufacturing	Ara h2 [kU/L]	Ara h 6 [kU/L]	Total IgE [kU/L]
Tolerant group								
Pt-02	m	45	<2 weeks	<1 g	contained in products	0	0.9	14.2
Pt-07	f	29	<2 weeks	<1 g	contained in products	0.2	0	250
Pt-08	m	36	<2 weeks	>20 g	roasted	0.6	0.2	98.3
Pt-11	m	27	1–3 days	>20 g	roasted, peanut butter	1.3	0.8	>1000
Pt-12	f	31	<2 weeks	<1 g	contained in products	2.3	0.3	>1000
Pt-03	m	63	2 days	>20 g	peanut butter	1.7	0	208

[a]Müller score 0: oral cavity, 1: skin and mucous membranes, 2: gastrointestinal, 3: respiratory, 4: cardiovascular

interface.[19] pFUSEss vectors (Invivogen), containing the IgE or IgG1 backbone, were used for antibody cloning and expression in HEK293 cells. Generated mAbs were tested for their specificity to nAra h 2 and/or 6[20] in comparison with their binding to transferrin. The ability of generated mAbs to induce degranulation was determined by CD63 upregulation of re-sensitized and stimulated human basophils.

Descriptive gene lineage analysis consisted of isotype distribution, mutational status and VH family gene usage.[21,22] Sequence motifs were identified by calculating Levenshtein distances of HCDR3 regions combined with hierarchically clustering. HCDR3 sequences with five or less differences were defined as one motif. Detailed descriptions are shown in Supporting Information. These sequence data have been submitted to the GenBank database under submission number 2395667 (MW2971045-MW271525).

3 | RESULTS

3.1 | Specific IgE levels overlapped between allergic and tolerant donors

In order to study differences in antibody repertoires between adult peanut allergic and peanut sensitized (IgE) but tolerant patients, blood was drawn from peanut allergic (n = 6, age: 27–41) and tolerant (n = 6, age: 27–63) donors sensitized to Ara h 2 and/or 6 (≥0.1 kU/L). Specific IgE levels to Ara h 2 ranged from 1.0–72 kU/L in the allergic and from 0 to 1.7 kU/L in the tolerant group. Specific IgE levels to Ara h 6 were lower compared with sIgE levels to Ara h 2 and ranged from 0 to 13 kU/L in the allergic and from 0 to 0.9 kU/L in the tolerant group. Mono-sensitization to either Ara h 2 or 6 was detected in both groups, although it occurred more often within the tolerant group (allergic: Pt-01, tolerant: Pt-02, 03, 07) as shown in Table 1.

3.2 | Frequency of peanut 2S albumin-binding B cells was significantly higher in allergic donors

As a first step, the frequencies of 2S albumin-binding B cells were compared between peanut allergic patients, peanut sensitized but tolerant patients and non-atopic controls. 2S albumin-binding B cells, double-positive for allergen-tetramer staining, were defined as putatively specific, and their frequency was expressed as percentage of total B cells acquired from the respective sample. While the frequency in allergic patients (median: 0.01%, 95% CI: 0.005–0.164) was only slightly increased compared to the tolerant patients (median: 0.006%, 95% CI: 0.0016–0.014), the frequency was significantly elevated compared with non-atopic controls (median: 0.002%, 95% CI: 0.0004–0.004, p = 0.008). Those 2S albumin-binding B cells were single-cell sorted and served as source for heavy and corresponding light chain gene transcript amplification. No correlation was found between the frequency of 2S albumin-binding B cells and the number of 2S albumin-binding B cells from which the heavy and the corresponding light chain gene transcripts were successfully amplified and sequenced (R^2 = 0.1825, p = 0.0872). A high number of V(D)J gene transcripts were successfully amplified from the B cells of patients 4 (n = 63) and 6 (n = 61)—both allergic—while none and only one V(D)J gene transcript was successfully amplified from B cells of patient 2 (tolerant) and 9 (allergic), respectively. For both of these patients, only a small number of 2S albumin-binding B cells were sorted (Pt-2: 8, Pt-9: 12). Frequencies, number of sorted 2S albumin-binding B cells and successfully amplified corresponding

V(D)J gene transcripts are shown in Figure 1[1]. Taken together, those successfully sequenced gene transcripts—heavy chain: 280, light chain: 221—provide a good basis for further analyses.

3.3 | 2S albumin-binding B cells show specific binding to Ara h 2 and 6

In order to verify the specificity of identified 2S albumin-binding B cells, 42 different sets of heavy and corresponding light chain gene transcripts were randomly heterologously expressed as IgE or IgG1 mAbs to enable the examination of specificity (IgE and IgG1), ability to induce degranulation (IgE) and inhibitory potencies (IgG1, Figures S3 and S5). These mAbs derived from allergic (except Pt 9), tolerant (except Pt 2 and 8) and non-atopic donors (except NA-3). The majority of these mAbs (81%, 34/42), mostly derived from allergic and tolerant patients, showed specific binding to a mixture of Ara h 2 and 6 at 10 µg/ml and lower in a concentration-dependent manner (1–10 µg/ml, Figure 2A). These heterologously expressed mAbs originated from 2S albumin-binding IgM+ (41%, 14/34), IgG1+ (3%, 1/34), IgG2+ (23%, 8/34), IgE+ (3%, 1/34), IgA1+ (18%, 6/34) and IgA2+ (9%, 3/34) B cells. While the two mAbs with relatively high optical density (OD) values originated from IgG1+ and IgA1+ B cells, mAbs with relatively low OD values originated predominately from IgM+ B cells. About two-third of the specific mAbs (70%, 19/27) tested for separate binding to Ara h 2 and 62 or 6 recognized both allergens with comparable OD values. Eight mAbs exclusively recognized either Ara h 2 or 6 (15%, 4/27 Ara h 2 and 15%, 4/27 Ara h 6) (Figure 2B). Notably, the OD values measured for separate binding for either Ara h 2 or 6 tended to be higher compared with the OD values measured for binding to the mixture of Ara h 2 and 6. The remaining antibodies without specific binding to Ara h 2 and 6 at 10 µg/ml originated predominately from IgM+ B cells of non-atopic controls (88%, 7/8). Additionally, specific binding to the peanut allergens Ara h 1 and 3 was assessed as it has been described that sIgE to Ara h 2 and 6 can cross-react with those peanut allergens.[23-25] Of our mAbs, 67% (22/33) bound additionally to Ara h 3 while only 9% (3/33) of them bound additionally to Ara h 1 (Figure 2D). These data confirm the specificity of most selected 2S albumin-binding B cells, which supports the relevance of the gene analyses described in the following paragraphs.

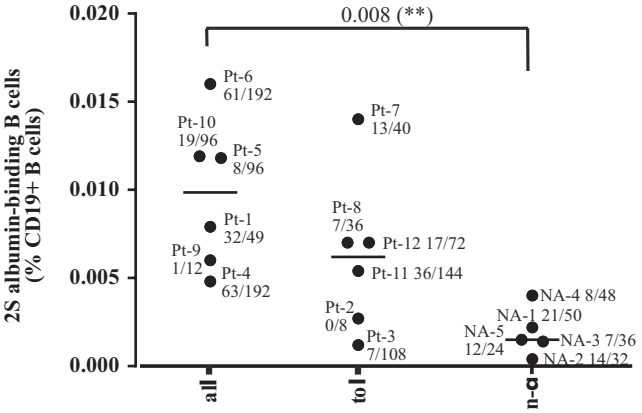
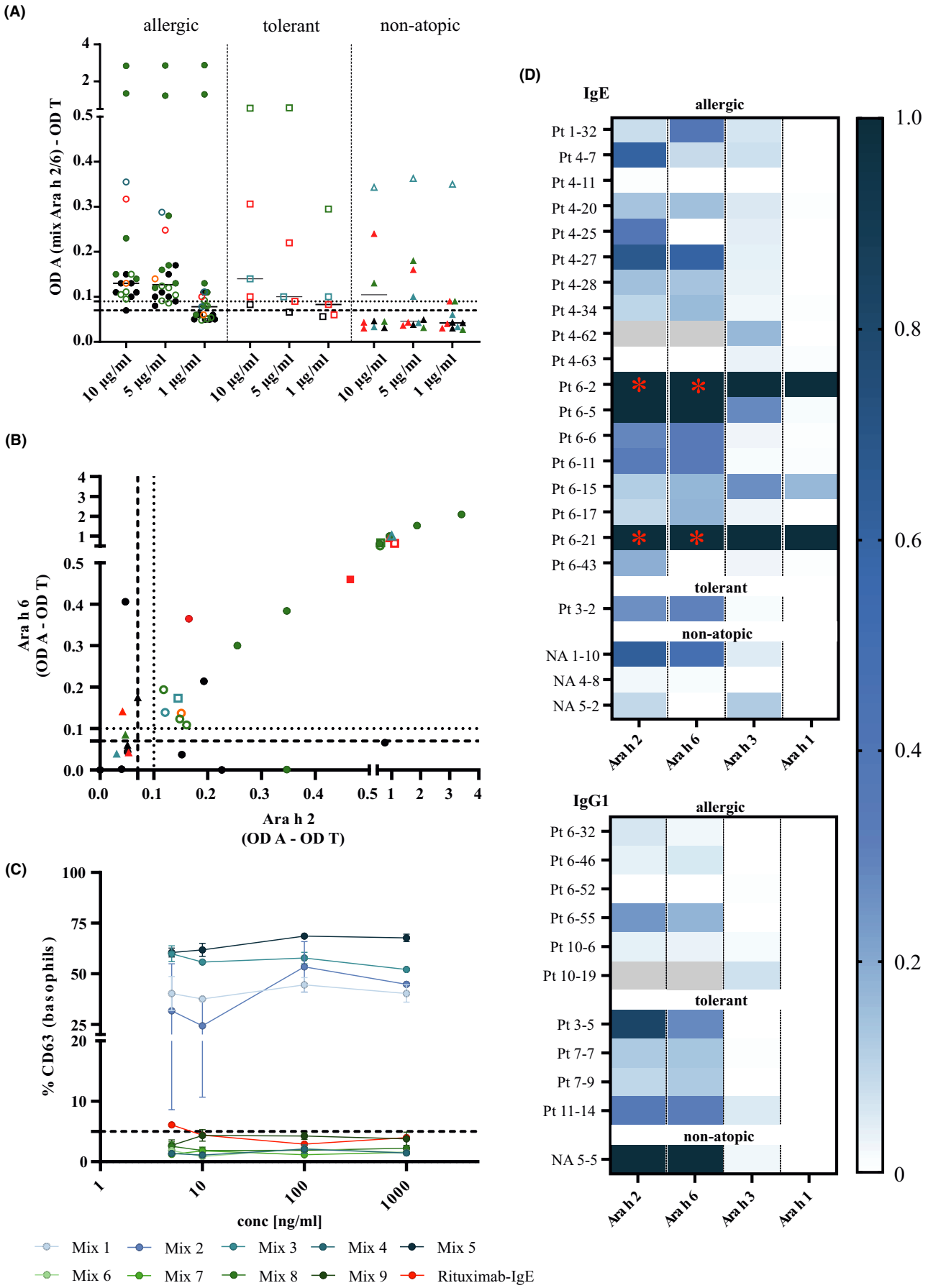


FIGURE 1 Frequency of 2S albumin-binding B cells. Frequency of 2S albumin-binding B cells, double-positive for allergen-tetramer (PE and APC) staining, expressed as percentage of the total B-cell number acquired from the respective sample, indicated with the median. Each frequency is labelled with the respective patient number and the number of successfully amplified corresponding heavy and light V(D)J gene transcripts in relation to sorted B cells. Statistical significance was proven with the Kruskal-Wallis test and corrected for multiple testing using Dunn's multiple comparison test (adjusted *p*-value); all, allergic, tol, tolerant, *n*-a, non-atopic

3.4 | Selected 2S albumin-specific mAbs can induce degranulation

As IgE B-cell memory is potentially stored in IgG1+ memory B cells and to a lesser extent in B cells belonging to another isotype, the functionality of mAbs originated from non-IgE 2S albumin-binding B cells was examined.[26-28] To determine mAb functionality, stripped human basophils were loaded with mixtures of randomly selected mAbs (IgE) and subsequently stimulated with Ara h 2 and 6 (Figure 2C and Figure S4). Stimulated basophils showed individual

FIGURE 2 Specificity and functionality of heterologously expressed mAbs. (A) Concentration-dependent (1 to 10 µg/ml) binding of heterologously expressed mAbs (*n* = 42) to a mixture of Ara h 2 and 6 defined as OD values corrected for their binding to transferrin; filled dots indicate mAbs expressed as IgE and open dots mAbs expressed as IgG1; bold dash line is the cut-off for IgE mAbs and non-bold dash line the cut-off for IgG1 antibodies; dot = allergic (Pt 1 = red, Pt 4 = black, Pt 5 = orange, Pt 6 = green, Pt 10 = blue, square = tolerant (Pt 3 = red, Pt 7 = black, Pt 11 = green, Pt 12 = blue), triangle = non-atopic (NA 1 = red, NA 2 = black, NA 4 = green, NA 5 = blue); A = allergen, T = transferrin. (B) Binding of heterologously expressed mAbs to Ara h2 and 6 (*n* = 33, 10 µg/ml) defined as OD values corrected for their binding to transferrin; filled dots indicate mAbs expressed as IgE and open dots mAbs expressed as IgG1, bold dash line is the cut-off for IgE mAbs and non-bold dash line the cut-off for IgG1 mAbs; dot = allergic (Pt 1 = red, Pt 4 = black, Pt 5 = orange, Pt 6 = green, Pt 10 = blue, square = tolerant (Pt 3 = red, Pt 7 = black, Pt 11 = green, Pt 12 = blue), triangle = non-atopic (NA 1 = red, NA 2 = black, NA 4 = green, NA 5 = blue); A = allergen, T = transferrin. (C) Upregulation of CD63 upon loading of human basophils with mAb mixture and subsequent stimulation with Ara h 2 and 6 in a concentration-dependent manner (5 to 1000 ng/ml); the dots indicate the mean of duplicate measurements and their range; Mix 1 = Pt-6-2, Mix 2 = Pt-6-2 + Pt-6-17, Mix 3 = Pt-6-2 + Pt-3-2 + Pt-6-11, Mix 4 = Pt-6-17, Pt-3-2, Pt-6-11, Mix 5 = Pt-1-32, Pt-6-11, Pt-6-6, Pt-6-8, Mix 6 = NA-1-10 + Pt-4-28 + Pt-4-34 + Pt-6-2, Mix 7 = Pt NA-1-10 + Pt-4-28 + Pt-4-34, Mix 8 = Pt-1-32 + Pt-6-15 + Pt-6-21, stripped basophils before loading, Rituximab expressed as IgE; Figure S2: comparison to stimulation with cow's milk extract, dash line: threshold level set to 5%. (D) Binding of heterologously expressed mAbs to Ara h 1, 2, 3 and 6 (*n* = 33, 5 µg/ml) defined as OD values corrected for their binding to transferrin and the respective blank value (HEK cell supernatant); heat map at the top: binding of mAbs expressed as IgE, heat map at the bottom: binding of mAbs expressed as IgG1, red star =corrected OD value >1.0


(A)
allergic
tolerant
non-atopic
OD A (mix Ara h 2/6) - OD T
10 µg/ml
5 µg/ml
1 µg/ml
(B)
Ara h 6
(OD A - OD T)
Ara h 2
(OD A - OD T)
(C)
% CD63 (basophils)
conc [ng/ml]
Mix 1
Mix 2
Mix 3
Mix 4
Mix 5
Mix 6
Mix 7
Mix 8
Mix 9
Rituximab-IgE
(D)
IgE
allergic
Pt 1-32
Pt 4-7
Pt 4-11
Pt 4-20
Pt 4-25
Pt 4-27
Pt 4-28
Pt 4-34
Pt 4-62
Pt 4-63
Pt 6-2
Pt 6-5
Pt 6-6
Pt 6-11
Pt 6-15
Pt 6-17
Pt 6-21
Pt 6-43
tolerant
Pt 3-2
non-atopic
NA 1-10
NA 4-8
NA 5-2
Ara h 2
Ara h 6
Ara h 3
Ara h 1
IgG1
allergic
Pt 6-32
Pt 6-46
Pt 6-52
Pt 6-55
Pt 10-6
Pt 10-19
tolerant
Pt 3-5
Pt 7-7
Pt 7-9
Pt 11-14
non-atopic
NA 5-5

CD63 upregulation upon stimulation when loaded with mixtures containing the mAb Pt-6-2 (Mix-1, 2, 3 and 6). Such responses were already achieved at low concentrations (5 ng/ml Ara h 2/6). Moreover, the overall CD63 upregulation induced by Mix 3 and 6 was even higher than the CD63 upregulation induced by the anti-IgE control at all allergen concentrations, pointing to an additive effect by using mAb Pt-6-2 (Mix-1) in combination with additional mAbs. The functionality of mAb Pt-6-2 and the observed additive effect confirm the selection of specific and functional B cells.

3.5 | Allergic and tolerant donors possess more class-switched 2S albumin-binding B cells than non-atopic controls

All successfully sequenced heavy chain gene transcripts were initially analyzed for isotype distribution, as class-switching is mostly accompanied by somatic hypermutation maturation upon antigen challenge. Overall, IgM was the most dominant isotype across all groups. Notably, its prevalence was much lower in allergic (47%) and tolerant (53%) donors than in the non-atopic reference group (94%), indicating more antigen-challenged and matured specific B cells originated from allergic and tolerant patients. Specific binding assessment in Figure 2A and B showed that IgM+B cells derived from the non-atopic control group reflected a certain degree of non-specific selection of IgM+ B cells. However, IgM+ B cells from allergic and tolerant donors were nearly exclusively specific to Ara h 2 and 6 (93%, 14/15). Moreover, the prevalence of IgA2-expressing B cells was increased in the tolerant group (20%), while a comparable distribution was observed in allergic and non-atopic donors (2%), as shown in Figure 3A. This finding suggests potential protection by specific IgA in tolerant patients, as IgA is generally able to prevent mucosal antigen crossing.[29] Regarding IgE, only a small number of V(D)J gene transcripts derived from IgE+ B cells were successfully amplified (allergic: 1; tolerant: 2), possibly due to the extreme low abundance (0.002–1%) of IgE+B cells within the circulation.[30] Even though slightly higher proportions are known for IgG3+ (~1%) and IgG4+ B cells (~0.75%),[31,32] no V(D)J gene transcripts derived from IgG3+ and IgG4+ B cells were amplified. Overall, the individual isotype distribution varied for each patient, for example 2S albumin-binding B cells of the allergic patient 4 were predominantly IgM+ B cells, while those of patient 1 were predominantly IgG+ B cells with a shift to class-switched B cells in peanut allergic and tolerant patients. This indicates that the 2S albumin-binding B cells from allergic and tolerant patients were more often matured and antigen-challenged B cells compared with the non-atopic control group.

3.6 | IgM+ B cells from allergic donors partly show strong maturation by somatic hypermutations

The introduction of non-silent (mutations on amino acid level) somatic hypermutations can increase target specificity and affinity during B-cell maturation. As shown in Figure 3B, the median number of non-silent mutations was increased in VDJ heavy chain and VJ light chain gene transcripts of class-switched B cells and was the highest for IgA1+ and IgA2+ B cells (heavy chain IgA1: 0–24 mutations, median: 11; heavy chain IgA2: 0–16 mutations, median: 16). The number of non-silent mutations in VDJ heavy chain gene transcripts of IgM expressing B cells, on the other hand, ranged from 0 to more than 10 in all study groups. Of note, a small number of IgM+ B cells, mostly originating from the allergic group, consisted of highly mutated heavy chain gene transcripts with mutation numbers comparable to the other isotypes, pointing to potential maturation of 2S albumin-binding IgM+ B cells by antigen challenge.

3.7 | VH3 family genes are predominately used in gene transcripts of allergic donors

The variable region of the heavy chain gene transcript consists of recombined V, D and J genes. The V gene accounts for most nucleotides of the variable region and contributes, besides D and J genes, to the CDR3 region. Hence, heavy chain V gene (VH) lineage can greatly affect B-cell receptor specificity and affinity. In line with B-cell repertoires described in literature,[33] VH3 family genes were observed to dominate across all groups, with a shift to higher proportions (89% of sequences, range: 80–100%, mainly IGHV3-30, VH3-23 and VH3-72) in allergic donors ($X^2(2, n = 183) = 23.67$, $p < 0.0001$). This significant difference was still present upon neglecting redundant sequences from the same donor, excluding a bias by proliferation of certain 2S albumin-binding B cells. In turn, IGHV4 genes accounted for a larger part of incorporated VH genes in tolerant donors (21% of sequences, range: 7–50%) than in allergic ones (7% of sequences, range: 0–12%). In contrast, no significant differences were detected for light chain V (VL) family gene usage ($X^2(2, n = 248) = 22.90$, $p = 0.1165$, Table 2). Furthermore, pairs of VH genes and corresponding VL genes are shown in Figure 3C. Although certain VH-VL pairs (IGHV3-30 – IGKV1 family, IGHV3-72 – IGKV1-9, IGHV3-23 – IGHV3-11) emerged repeatedly in allergic and partly in non-atopic donors (IGHV3-72 – IGKV1-9), most VH-VL pairs appeared to be highly individual; particularly within tolerant donors. Overall, VH-family gene usage, especially the dominant VH3 family gene usage, differs between allergic and tolerant patients sensitized to Ara h 2 and/or 6, supporting the hypothesis of differences in allergen-specific antibody repertoires between allergic and tolerant patients.

3.8 | An IgE+ B-cell is largely clonally related to an IgG1+ B cell in allergic patients

In total, three VDJ gene transcripts were successfully amplified from IgE+ B cells, representing the smallest isotype group when disregarding IgG3 and IgG4. Where two VDJ gene transcripts of IgE+ B cells derived from one tolerant donor were not closely related to another amplified VDJ gene transcript, the VDJ gene transcript of

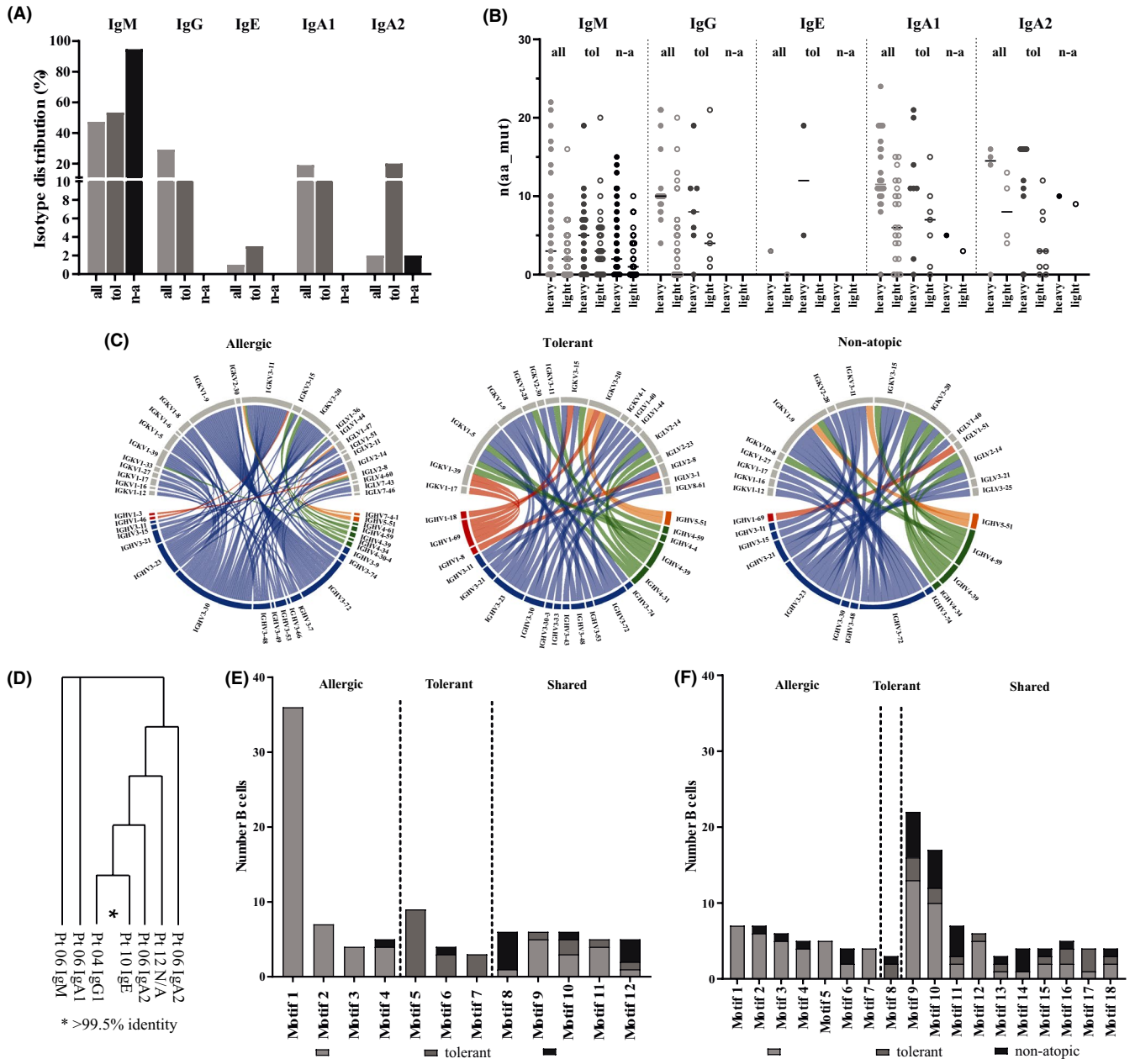


FIGURE 3 Descriptive gene analysis and motif analysis of HCDR3 regions. Gene lineage analysis of successfully sequenced heavy chain gene transcripts defined as productive (allergic *n* = 151; tolerant *n* = 68; non-atopic, *n* = 51), irrespectively of successfully sequenced corresponding light chain gene transcript. (A) Isotype distribution split for allergic (all), tolerant (tol) and non-atopic (*n*-a) donors. (B) Numbers of non-silent mutations located on the variable region of the heavy and light chain expressed with the median (95% CI) and split for allergic (all), tolerant (tol) and non-atopic (*n*-a) donors; filled dots: variable region heavy chain, open dots: variable region light chain. (C) V gene lineage of the heavy and corresponding light chain was visualized with circled diagrams showing the heavy chain at the bottom connected to the light chain (grey) at the top. VH1 family genes are shown in red, VH3 family genes in blue, VH4 family genes in green and VH5/7 family genes in orange. (D) Clonotypes were defined as identical V gene, J gene and HCDR3 length and related clones were aligned using ClustalW2. The IgE mAb of Pt-10 was highly related to an IgG1 antibody of Pt-4. (E) HCDR3 sequence motifs were analysed by Levenshtein distances and hierarchical clustering (detailed description in the method section), and sequences with distances ≤5 were grouped into one motif. More detailed description of the motifs is presented in Table 3. (F) LCDR3 sequence motifs were analyzed by Levenshtein distances and hierarchical clustering (detailed description in the method section), and sequences with distances ≤2 were grouped into one motif. More detailed description of the motifs is presented in Table S2

one IgE+ B-cell originating from an allergic donor (Pt-10) was nearly identical to the gene transcript of an IgG1+ B cell derived from an unrelated allergic donor (Pt-04). These two sequences differed only in one silent mutation within the FR4 region and one non-silent mutation within the FR2 region, pointing towards conserved clones between unrelated donors (≙ public antibody sequence, Figure 3C). Although it has to be emphasized that only a limited number of IgE+ B cells were selected, this finding may support that public antibody

TABLE 2 Comparison of the VH and VL family gene usage between the present study, healthy controls and Ara h 2-related datasets (OIT trails); (transcripts from allergic, *n* = 120; tolerant, *n* = 63; non-atopic, *n* = 52)

VH/L family	Allergic group	Tolerant group	Non-atopic group	Hoh 2016[18]	Patil 2015[15]	Goldstein 2019[43]	Graichy[a] 2020[42]
VH1 [%]	2	21	3.8	18	23	15–18	5–18
VH2 [%]	-	-	-	-	-	4	2
VH3 [%]	89[b]	54	63	60	62	46–50	40–65
VH4 [%]	6.3	21	29	15	15	22–25	25–35
VH5 [%]	1.3	2.9	3.8	-	-	5–7	1–2
VH7 [%]	1.3	-	-	-	-	0.05–1	0.5–1
VK1 [%]	54.5	52.6	46.2	-	-	-	-
VK2 [%]	3.3	10.5	2.6				
VK3 [%]	42.3	34.2	52.6				
VK4 [%]	-	2.6	-				
VL1 [%]	31	17	33	-	-	-	-
VL2 [%]	45	67	44				
VL3 [%]	3.5	8	22				
VL4 [%]	3.5	-	-				
VL7 [%]	17	-	-				
VL8 [%]	-	8	-				

[a]Age group between 25 and 40 years used for comparison, most closely related to the average age of the study population; data for different subsets were combined.

[b]Dominant usage VH3 family genes in all individuals.

sequences, attributed to peanut allergy, are shared between individuals and isotypes.[27,28]

3.9 | HCDR3 sequence motifs are related to allergy or tolerance

As HCDR3 regions have a great impact on antigen binding, their characteristics were more deeply analyzed. Detailed analysis of physiochemical properties, such as amino acid distribution, aliphatic index, Boman index and Kidera factors 1–10, is shown in Figures S6 and S7.[21] The Kidera factor 5, expressing the preference to form double-bended structures, was significantly higher for the HCDR3 region of allergic donors compared with that of tolerant donors (p = 0.0226, Bonferroni correction: non-significant). Nevertheless, the mean Boman index, describing the theoretical ability to bind proteins, was comparable between HCDR3 regions of allergic, tolerant and non-atopic donors. For deeper analysis, HCDR3 sequence motifs associated with peanut allergy and/or tolerance were evaluated by Levenshtein distances (≤5 replacements, deletions or insertions) and hierarchical clustering. Levenshtein distances were favoured over clonal relationship analysis because of individual genetic variation regarding haplotype diversity, single nucleotide polymorphisms, gene copy number and preferred gene lineage.[34] For this analysis, all successfully amplified and sequenced heavy chain gene transcripts were used irrespectively of successfully sequenced corresponding light chain gene transcripts. Overall, four unique HCDR3 sequence motifs were associated with peanut allergy and three with peanut tolerance (Figure 3E, Table 3). The most dominant motif was 'CARDSSALEIYNRFDPW' (motif 1), which was derived from 36 different B cells belonging to three different allergic donors (Pt-1 (n = 32), Pt-5 (n = 3), Pt-6 (n = 1)) including 32 nearly identical B cells of Pt-1 (monoclonal proliferation). This motif was formed by VH3-30, DH3-3 and JH5 genes. Besides motifs exclusively related to either allergy or tolerance, five motifs were shared between allergic, tolerant and non-atopic donors. It must be mentioned that one motif (CARNVFDGYWLVYW) associated with tolerance was only found in Pt-11 and no motif was shared between all allergic or tolerant donors. In comparison, LCDR3 motifs, defined as Levenshtein distances ≤2 based on reduced heterogeneity of LCDR3 sequences compared to HCDR3 sequences, were detected in allergic (n = 7) and tolerant donors (n = 1). However, more dominant motifs (n = 10) containing up to 24 LCDR3 sequences were shared between allergic, tolerant and non-atopic donors (Figure 3F, detailed description Table S2). Heterologously expressed mAbs corresponding to these motifs showed specific binding to Ara h 2 and/or 6, supporting the identification of public antibody sequences potentially attributed to peanut allergy or tolerance despite sIgE sensitization.

4 | DISCUSSION

Specific IgE levels to Ara h 2 and 6 between 0.1 and 1.8 kU/L overlap between peanut allergic and tolerant subjects,[6,7] risking inaccurate diagnosis and therefore indicating the need for new diagnostic strategies. In the present study, we observed a preferential usage of VH3

TABLE 3 HCDR3 sequence motifs associated with peanut allergy or tolerance

Motif	HCDR3 sequence	Antibodies	Isotype	Specificity
Allergy				
1	CARDSSALEIYNRFDPW	Pt-1-1-**Pt-1-32**[a], Pt-5-3, Pt-5-4, **Pt-5-8**[a], **Pt-6-43**[a]	IgG2	Ara h 2 + 6
2	CVKDRQQYSSRWLDSW	Pt-5-2, Pt-5-5, **Pt-6-32**[a], Pt-6-42, Pt-6-45, Pt-6-47, Pt-6-48	IgA1	Ara h2 + 6
3	CASMDILAANTHFGMDVW	**Pt-6-17**[a], Pt-6-37, **Pt-6-52**[a], **Pt-10-19**[a]	IgG2	Ara h2 + 6
4	CARG**LV**GA**NF**YYYMDVW	**Pt-4-10**[a], Pt-4-41, Pt-4-61	IgM	Ara h2
4	CARG**RS**GA**TY**YYYMDVW	**NA-4-5**[a]	IgM	Ara h2 + 6
4	CARG**RAGPSY**YYYMDVW	Pt-4-44	IgM	n. e.
Tolerance				
5	CARNVFDGYWLVYW	*Pt-11-3*, *Pt-11-4*, ***Pt-11-14***[a], *Pt-11-15*, *Pt-11-23*, *Pt-11-24*, *Pt-11-25*, *Pt-11-26*	IgA2	Ara h2 + 6
6	CAREGHYSSQFDYW	*Pt-8-3*, *Pt-8-4*, *Pt-8-5*, NA-2-3	IgM	n. e.
7	C**A**RD**YGG**YPHAAF**D**IW	*Pt-11-11*	IgM	n. e.
7	C**T**RD**T**G**T**YPHAAF**N**IW	*Pt–3-1*, ***Pt-3-5***	IgM, IgG2	Ara h2 + 6
Shared				
8	CTRPYRAFNWA**I**GHW	NA-1-15	IgM	n. e.
8	CTRPYRAFNWA**T**GHW	NA-1-17, **NA-5-2**[a](only Ara h2), **NA-5-5**[a], NA-5-11, Pt-6-19	IgM, IgA1	Ara h2 + 6
9	CARVSSSWHTEYW	*Pt–3-7*, Pt-6-33, **Pt-6-46**[a], Pt-6-53, Pt-6-60, Pt-6-61	IgA1	Ara h2 + 6
10	CAR**GIIDK**YGMDVW	Pt-6-38, *Pt-7-6*, Pt–10-13, Pt-10-14	IgG2	n. e.
10	CAR**EYY**YGMDVW	NA-4-1	IgM	n. e.
10	CAR**TLG**YGMDVW	*Pt-11-16*	IgM	n. e.
11	C**T**RGA**VS**YTRHFQ**F**W	**Pt-4-20**[a]	IgM	Ara h2
11	C**V**RGA**LA**YTRHFQ**Y**W	**Pt–4-34**[a]	IgM	
11	C**V**RGA**MS**NTRHFQ**Y**W	**Pt-4-28**[a]	IgM	Ara h2/6[b]
11	C**A**RGA**MS**YTRHFQ**Y**W	Pt-4-8, Pt-4-40, **Pt–4-63**[a], *Pt-8-6*	IgM	Ara h2/6[b]
12	CA**KAY**GSGSY**L**FDYW	*Pt-7-4*, **NA-1-10**[a]	IgM	Ara h2 + 6
12	CA**RGG**GSGSY**T**FDYW	NA-1-4, NA-1-6, NA-1-13	IgM	n. e.
12	CA**RSG**GSGSY**T**FDYW	**Pt-10-6**[a]	IgM	Ara h2 + 6

Bold: differences of HCDR3 sequences; italic: tolerant donors; bold patient numbers: indicate heterologous expression mAb, n. e., not expressed.

[a]specificity to Ara h2/6 proven.

[b]only specificity against mixture of Ara h2 and 6.

family genes in peanut 2S albumin-specific B cells from peanut allergic patients. Additionally, we identified 2S albumin-binding B cells carrying HCDR3 sequence motifs either related to peanut allergy or tolerance. These findings indicate the association of certain public antibody sequences to allergy or tolerance in sensitized patients with suspected peanut allergy.

Despite a large number of amplified heavy chain VDJ gene transcripts (n = 280), only three of them belonged to IgE class-switched B cells. This low abundance may be potentially explained by dominant class-switching to IgE in the tissue,[26] low BCR surface expression of plasmablast-like IgE+ B cells[23,35,36] and the extreme low abundance of IgE+ B cells within the circulation.[30] Nevertheless, the relevance of the B cells sorted within the present study is supported by the close relationship between one heavy chain gene transcript from an IgE+ B cell and a gene transcript from an IgG1+ B cell derived from an unrelated peanut allergic donor. Additionally, some generated mAbs were able to slightly inhibit patient-derived serum IgE-binding to Ara h 2 and 6 and to partly inhibit activation of basophils loaded with serum IgE upon stimulation with Ara h 2 and 6 (Figures S3 and S5). Correspondingly, there is evidence that human IgE+ B cells are predominately plasmablasts or plasma cells generated by sequential class-switching from IgG1+ B cells (γ switch region remnants) and that the humoral IgE memory is contained in IgG+ memory B cells.[28] Further evidence for this theory was obtained by clonal relationship analyses, since IgE+ B cells were dominantly clonally related to IgG1+ B cells, but also, to a lesser extent, to B cells of other isotypes.[23,26,27]

The majority of mAbs generated in the present study showed specific binding to peanut 2S albumins and two-thirds of these mAbs showed specific binding to both allergens Ara h 2 and 6, pointing towards a high degree of cross-reactivity. Strikingly, 67% of the mAbs were even able to bind, partly to a lesser extent, to the non-homologous peanut allergen Ara h 3. In current literature, a certain degree of serum IgE cross-reactivity has been reported between Ara h 2 and 6[37,38] and even serum IgE cross-reactivity between Ara h 2 and the non-homologous allergens Ara h 1 and 3 has been described by Bublin and colleagues.[24] Auxiliary, mAbs derived from peanut

allergic patients showed cross-reactivity between Ara h 2 and Ara h 3 but not to Ara h 1.[23]

Regarding isotype distribution of the selected 2S albumin-binding B cells, IgM+ B cells represented the largest isotype group, as detected in respectively 47, 53 and 94% of allergic, tolerant and non-atopic donors. Additionally, IgM+ B cells from non-atopic donors, all specific for Ara h 2 and/or 6, shared HCDR3 motifs (motif 4, 8, 10) with IgM+, IgG2+ and IgA1+ B cells from allergic donors. Clonal relationships between IgA/G/M+ and IgE+ B cells have been described for B cells derived from gut tissues by Hoh and colleagues.[26,39] Combining the findings from the present study with the finding of Hoh and colleagues leads to the suggestion that non-atopic donors can potentially possess non-IgE antibodies with required specificity or affinity to theoretically induce an allergic reaction.

Moreover, tolerant donors showed a higher proportion of IgA+ B cells and tended to have higher specific IgA serum levels than allergic donors (Figure S3). Allergen-specific IgA serum levels have been shown to be increased in peanut allergic subjects undergoing oral immunotherapy compared with their baseline levels.[29] Moreover, salivary IgA levels have been closely associated with the degree of tolerance induction by sublingual immunotherapy confirmed by DBPCFCs, pointing to a protective role of specific IgA against mucosal allergen absorption.[40] Such a protective role is supported by increased intestinal permeability in the absence of IgA in mice.[41] Taken together, these findings suggest a potential protective effect of allergen-specific IgA in the mucosa of sensitized but tolerant patients.

Regarding gene lineage, VH3 family genes were significantly more often used ($p < 0.0001$) in heavy chain gene transcripts of peanut 2S albumin-specific B cells from allergic donors (89%) than in those from tolerant (54%) and non-atopic donors (63%). Previous studies on VH gene usage of heavy chain gene transcripts in healthy donors showed VH3 family gene usage in 40 to 65% of the B cells, which was comparable to the usage in the tolerant and non-atopic groups in our study (Table 2).[42,43] These findings suggest a shift towards VH3 family gene usage in 2S albumin-specific B cells from allergic donors. Contrary to our findings, other data sets of Ara h 2-specific B cells did not observe a similar shift in VH gene usage.[15,18] These data sets, however, were generated from patients undergoing peanut oral immunotherapy with a focus on different time points during immunotherapy. This fact hampers the comparison between the present and previous Ara h 2-related data sets. Moreover, conflicting results regarding preferred VH gene usage were described for different food and respiratory allergies, with a shift to VH3 usage for anti-alpha Gal antibodies and anti-grass pollen Phl p6 and 11 antibodies,[44,45] indicating that the preferred VH family gene usage may be allergen dependent. Moreover, the preferred usage might depend on the status of allergy or tolerance and can potentially be used for diagnostic purposes upon validation in a larger patient cohort. A validation study will also provide information about the number of detected 2S albumin-binding B cells is sufficient for a diagnostic workflow.

Besides differences in VH-family gene usage, certain HCDR3 sequence motifs were associated with either peanut allergy or tolerance and appear to have the ability to discriminate between those two groups. The main HCDR3 sequence motif 'CARDSSALEIYNRFDPW' was associated with peanut allergy and derived from recombined VH3-30, DH3-3 and JH5 genes. Comparably, VH3-30*18 was incorporated in the VDJ gene transcript of clonally related IgE+B cells specific for Ara h 2 in the study of Croote and colleagues.[23] Additionally, a highly similar HCDR3 region (CAREGYESSGFDYW) to motif 6 (CAREG**HY**SS**Q**FDYW), associated with tolerance, has been described for peanut allergic subjects undergoing oral immunotherapy.[18] Oral immunotherapy may shape the antibody repertoire towards repertoires present in tolerant subjects. These comparisons support the identification of public antibody sequences potentially attributed to peanut allergy or tolerance.

In conclusion, peanut allergy is associated with dominant usage of VH3 family genes and certain public antibody sequences, expressed as HCDR3 sequence motifs, are attributed to either peanut allergy or tolerance despite sIgE sensitization. Validation of these findings in a larger patient cohort with greater ethnical diversity, leading to different gene allele usage, may be achieved using next-generated sequencing approaches, potentially combined with the sorting strategy of 2S albumin-binding B cells presented in this study.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

AE, CHJ and HO: experimental design, AE and AC: patient recruitment, AE and CHJ: experimental performance, AE, AC and HO: data analysis and interpretation, AE: drafting manuscript, CHJ, AC and HO: critical revision of the manuscript and all authors approved the final manuscript.

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SUPPORTING INFORMATION

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

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