Evolution of epitope-specific IgE and IgG_4 antibodies in children enrolled in the LEAP trial

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Background: In the LEAP (Learning Early About Peanut Allergy) trial, early consumption of peanut in high-risk infants was found to decrease the rate of peanut allergy at 5 years of age. Sequential epitope-specific (*ses*-)IgE is a promising biomarker of clinical peanut reactivity.

Objective: We sought to compare the evolution of *ses*-IgE and *ses*-IgG_4 in children who developed (or not) peanut allergy and to evaluate the immunomodulatory effects of early peanut consumption on these antibodies.

Methods: Sera from 341 children (LEAP cohort) were assayed at baseline, 1, 2.5, and 5 years of age, with allergy status determined by oral food challenge at 5 years. A bead-based epitope assay was used to quantitate *ses*-IgE and *ses*-IgG_4 to 64 sequential epitopes from Ara h 1 to Ara h 3 and was analyzed using linear mixed-effect models.

Results: In children avoiding peanut who became peanut allergic, the bulk of peanut *ses*-IgE did not develop until after 2.5 years. Minimal increases of *ses*-IgE occurred after 1 year in consumers, but not to the same epitopes as those in children developing peanut allergy. No major changes in *ses*-IgE were seen in nonallergic or sensitized children. IgE in sensitized consumers was detected against peanut proteins. *ses*-IgG_4 increased over time in most children regardless of consumption or allergy status.

Conclusions: Early peanut consumption in infants at high risk of developing peanut allergy appears to divert the immunologic response to a presumably "protective" effect. In general, consumers tend to generate *ses*-IgG_4 earlier and in greater quantities than nonconsumers do, whereas only avoiders tend to generate significant quantities of *ses*-IgE. (J Allergy Clin Immunol 2021;■■■:■■■-■■■.)

Key words: *Peanut allergy, biomarkers, sequential epitope, antibody, IgE, IgG_4, bead-based epitope assay*

In 2015, a landmark clinical trial, LEAP (Learning Early About Peanut Allergy), showed that early consumption of peanut in high-risk infants was associated with a decreased rate of peanut allergy at 5 years of age.[1] Of 628 infants, only 3.2% of those who consumed ~6 g of peanut protein weekly developed peanut allergy compared with 17.2% in the peanut avoidance group.[1] These differences were retained at 6 years of age after both groups eliminated peanut from their diet for 1 year, with a peanut allergy prevalence of 4.8% in the consumption and 18.6% in the avoidance groups, respectively.[2]

The LEAP trial had a 98.4% retention rate of all participants returning at 60 months with 96.4% undergoing oral food challenges (OFCs). Importantly, regardless of peanut intake, both groups had comparable duration of breast-feeding, body mass index, and total energy intake.[3] However, in contrast with the marked reduction of peanut allergy in the consumption group, rates of eczema, asthma, or rhinoconjunctivitis were similar between groups over time.[4] While the LEAP trial demonstrated the importance of early introduction of peanut in preventing peanut allergy, the mechanisms of immunologic tolerance are still

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Abbreviations used
BBEA: Bead-based epitope assay
FDR: False discovery rate
ICC: Intraclass correlation coefficient
LEAP: Learning Early About Peanut Allergy
MFI: Median fluorescence intensity
nMFI: Normalized median fluorescence intensity
OFC: Oral food challenge
PN-s: Peanut-specific
ses-Ig: Sequential epitope-specific immunoglobulin
TBN: Tween and BSA

elusive. The analysis of standard immune markers demonstrated that both groups had detectable increases of peanut-specific (PN-s) IgE antibodies over time, which were more pronounced in children who developed peanut allergy. Higher peanut sIgG$_4$ to sIgE ratios, and lower peanut sIgE and Ara h 2 levels were observed in the consumption group at 5 years of age.[2,4]

Several groups have demonstrated that IgE specific to sequential epitopes (*ses*-IgE) can be used as a biomarker of clinical peanut reactivity,[5-8] with IgE epitope specificity even in children whose peanut sIgE is below the diagnostic decision level,[9] and higher epitope diversity in children with more severe allergic reactions.[10] In this study we sought to further investigate the evolution of humoral immunity in the development of peanut allergy in the LEAP cohort, specifically looking at IgE and IgG$_4$ binding to sequential epitopes from 3 major peanut allergens using a bead-based epitope assay (BBEA).[11]

MATERIALS

Study cohort

The LEAP trial was a randomized, open-label, controlled trial to determine whether early consumption of peanut in high-risk infants would reduce the prevalence of peanut allergy at 5 years of age.[1] Infants with severe eczema and/or egg allergy were enrolled between 4 and 11 months of age and randomly assigned to either avoidance or consumption groups. Subjects in the consumption group were fed at least 6 g of peanut protein weekly until the age of 60 months. Clinical assessments and blood draws were done at baseline, and at 12, 30, and 60 months of age, as detailed previously.[2]

Peanut allergy status at the 5-year visit was based on the outcome of the peanut OFC except in 2 children. Children were considered peanut allergic at the 60-month visit if they reacted during the OFC or experienced clear-cut clinical symptoms following a known ingestion of peanut (designated "allergic"). Those who tolerated the OFC were considered nonallergic but sensitized (designated "sensitized") if they had PN-sIgE levels >0.1 kU$_A$/L and/or they had a skin prick test $\geq$1 mm or "nonallergic" if PN-sIgE < 0.1 kU$_A$/L and a negative skin prick test.

This mechanistic cohort consisted of a subset of 341 children (172 avoiders, 169 consumers) from the per-protocol population (n = 589) who had sufficient aliquots of plasma from all LEAP trial time points. All children known to be allergic or sensitized to peanut at the 5-year visit were profiled, while only 100 nonallergic children, randomly selected in 1:1 ratio from avoiders and consumers were used for epitope-specific antibody profiling. Sensitized participants were further evaluated based on the time when their sensitization was first detected, with those recording PN-sIgE >0.1 kU$_A$/L at baseline distinguished from those exhibiting it at a later visit.

BBEA protocol and signal processing

Sixty-four 15-mer peanut epitopes from major peanut allergens (34 from Ara h 1, 16 from Ara h 2, and 14 from Ara h 3) (see Table E1 in this article's Online Repository at www.jacionline.org) were identified and commercially synthetized (CS Bio, Menlo Park, Calif). These informative epitopes were selected after screening 15-mer overlapping peptides (13-mer overlap) covering the entire sequences of Ara h 1, Ara h 2, Ara h 3, and Ara h 7, as well as the nonhomologous region of Ara h 6 proteins (data not shown). The BBEA was run as described previously;[11] in brief, peptides were coupled to xMAP microspheres (Luminex Corporation, Austin, Tex) and stored in PBS-TBN buffer (1x PBS with 0.02% Tween-20 and 0.1% BSA). A master mix of microspheres (100 μL/well) was added to 96-well filter plates. Plates were washed with PBS-TBN and 100 μL of a 10-fold diluted plasma sample was added to the wells in triplicates and incubated on a shaker (300 revolutions/min) for 2 hours at room temperature. Plates were washed twice and incubated for 30 minutes at room temperature. Excess plasma was removed, plates were washed, and 50 μL/well of mouse anti-human IgE-phycoerythrin (2 μg/mL; cat. MA1-10375, ThermoFisher Scientific, Waltham, Mass) or IgG$_4$-phycoerythrin (0.25 μg/mL; cat. 9200-09; Southern Biotech, Birmingham, Ala) secondary antibody was added and plates were incubated for 30 minutes at room temperature. After a final wash, PBS-TBN was added, and microspheres were transferred to fixed-bottom 96-well reading plates. For every microsphere (epitope) and sample, median fluorescence intensity (MFI) was quantified with the xPONENT software on Luminex200 instrument (Luminex Corporation). For nonspecific signal detection, each plate included 3 wells with only PBS-TBN buffer. The peanut-BBEA assay was previously shown to have high reproducibility and sensitivity.[11]

Statistical analysis

All data processing, quality control, and analyses were performed in R (version 3.5.1; R Foundation, Vienna, Austria). BBEA's MFI was normalized and converted to nMFI. Differences arising from multiple microplates were assessed as previously defined.[11] Plate effect was estimated using mixed-effects linear models and subsequently subtracted from the nMFI values. Overall scores for each sample were calculated by taking the average of the z-scores of all 64 epitopes.

Changes in the ImmunoCap (ThermoFisher Scientific) measures (sIgE and sIgG$_4$) were reported for the LEAP cohort[4] and are analyzed here using mixed-effects models on the subcohort with BBEA profiles (after $\log_{10}$ transformation).

Agreement among triplicates—assessed via the 2-way intra-class correlation coefficient (ICC)—improved with higher nMFI values, which increased with age. For example, ICC > 0.75 for most IgG$_4$-specific epitopes (considered excellent according to ICC/κ guidelines[12]) and increased to >0.95 in measures taken after 12 months. For IgE, most epitopes had a fair agreement (ICC > 0.4) overall but excellent (ICC > 0.75) after 2.5 years.

Epitope-specific antibody changes were modeled using linear mixed-effect models in the *limma* framework.[13] *P* values of the hypotheses of interest were adjusted for multiple comparisons (across epitopes) using the Benjamini-Hochberg approach, which controls the false discovery rate (FDR).

RESULTS

Study population

In this study, we used a subset of the per-protocol population from the LEAP trial[1] cohort as defined in the methods. Of the 341 high-risk infants 4 to 11 months of age with severe eczema and/or egg allergy, 172 were randomized to the avoidance group and 169 to the peanut consumption group. Plasma samples from the baseline (4-11 months) and 12-, 30-, and 60-month visits were assayed for IgE and IgG$_4$ binding to 64 informative peanut epitopes using BBEA.

Baseline serological measures were comparable in the avoidance and consumption groups (Table I). Baseline PN-sIgE levels were greatest in the children who developed peanut allergy by 5 years of age, followed by the sensitized group ($P < .001$). A similar relationship was observed with sIgE to peanut

TABLE I. Characteristics of the BBEA cohort at study initiation, by intervention, and allergy status at 60 months

Baseline characteristics	Avoidance	Consumption	Peanut avoidance			Peanut consumption		*P**
			Not allergic	Sensitized	Allergic	Not allergic	Sensitized	
No. of children	172	169	75	59	38	75	94	
Age, mo	7.81 ± 1.75	7.62 ± 1.75	7.59 ± 1.71	8.15 ± 1.67	7.70 ± 1.91	7.59 ± 1.74	7.64 ± 1.77	.334
Ethnicity								.235
White	127 (73.8)	112 (66.3)	60 (80.0)	41 (69.5)	26 (68.4)	55 (73.3)	57 (60.6)	
Black	19 (11.0)	16 (9.5)	6 (8.0)	9 (15.3)	4 (10.5)	9 (12.0)	7 (7.4)	
Asian	4 (2.3)	9 (5.3)	2 (2.7)	1 (1.7)	1 (2.6)	1 (1.3)	8 (8.5)	
Mixed	20 (11.6)	29 (17.2)	7 (9.3)	7 (11.9)	6 (15.8)	9 (12.0)	20 (21.3)	
Other	2 (1.2)	3 (1.8)	0 (0.0)	1 (1.7)	1 (2.6)	1 (1.3)	2 (2.1)	
Eczema	168 (97.7)	167 (98.8)	72 (96.0)	58 (98.3)	38 (100.0)	74 (98.7)	93 (98.9)	.525
SCORAD	35.48 ± 19.3	35.82 ± 18.5	31.67 ± 18.8	37.11 ± 18.5	40.44 ± 20.8	31.07 ± 18.2	39.62 ± 18.1	.005
SPT	0.49 ± 1.1	0.37 ± 0.9	0.11 ± 0.6	0.78 ± 1.4	0.79 ± 1.3	0.17 ± 0.6	0.53 ± 1.1	<.001
Peanut component proteins								
PN-sIgE	0.10 (0.01, 0.51)	0.05 (0.01, 0.43)	0.02 (0.01, 0.17)	0.12 (0.02, 1.14)	0.52 (0.18, 2.85)	0.02 (0.01, 0.08)	0.10 (0.02, 1.51)	<.001
PN-sIgG$_4$	70 (70, 70)	70 (70, 70)	70 (70, 70)	70 (70, 70)	70 (70, 70)	70 (70, 70)	70 (70, 70)	.009
PN-sIgG$_4$ = 70 (LOD)†	159 (92.4)	155 (91.7)	73 (97.3)	53 (89.8)	33 (86.8)	74 (98.7)	81 (86.2)	.009
Ara h 1-sIgE	0.02 (0.01, 0.12)	0.01 (0.01, 0.05)	0.01 (0.01, 0.04)	0.02 (0.01, 0.12)	0.04 (0.01, 0.14)	0.01 (0.01, 0.02)	0.02 (0.01, 0.08)	.001
Ara h 2-sIgE	0.04 (0.03, 0.07)	0.04 (0.03, 0.05)	0.03 (0.03, 0.04)	0.04 (0.03, 0.05)	0.05 (0.03, 0.13)	0.03 (0.02, 0.05)	0.04 (0.03, 0.06)	.021
Ara h 3-sIgE	0.02 (0.01, 0.05)	0.01 (0.01, 0.04)	0.01 (0.01, 0.02)	0.02 (0.01, 0.07)	0.03 (0.01, 0.09)	0.01 (0.01, 0.02)	0.02 (0.01, 0.06)	<.001
Ara h 8-sIgE = 0.01†	118 (88.1)	118 (87.4)	35 (94.6)	53 (89.8)	30 (78.9)	40 (95.2)	78 (83.9)	.089
Ara h 9-sIgE = 0.01†	109 (81.3)	114 (85.1)	32 (86.5)	49 (83.1)	28 (73.7)	37 (90.2)	77 (82.8)	.377
Sensitized at 4-11 months	86 (50.0)	64 (37.9)	24 (32.0)	31 (52.5)	31 (81.6)	17 (22.7)	47 (50.0)	<.001

LOD, Limit of detection; *SCORAD*, Scoring Atopic Dermatitis.
Values are mean ± SD, n (%), or median (interquartile range) unless otherwise indicated.
**P* values for testing the differences across intervention/outcomes groups. Chi-square test was used to determine percentages. Analysis of variance was used to determine mean ± SD. Kruskal-Wallis was used to determine median (interquartile range).
†Median (interquartile range) were equal to the minimum value, as such we report the proportion with this value. The Wilcoxon test was used to compare the median lead to similar results as were reported by the chi-square test comparing proportion.

component proteins, Ara h 1 to Ara h 3, but the majority of children had minimum detectable values for Ara h 8 and Ara h 9 at baseline.

***ses*-IgE expansion occurred mainly in avoiders while *ses*-IgG$_4$ increased in all patients.** Because oral exposure to peanut protein in high-risk infants often results in production of peanut-specific antibodies, we evaluated both intervention groups for changes in *ses*-IgE and *ses*-IgG$_4$ from baseline (4-11 months) to 12, 30, and 60 months in both intervention groups, which is summarized in Fig 1. There were no baseline differences in either *ses*-IgE or *ses*-IgG$_4$ profiles at baseline between intervention groups.

In the avoidance group, peanut *ses*-IgE antibodies appeared primarily after 2.5 years of age and bound most of the informative epitopes evaluated, with few Ara h 1 and Ara h 2 *ses*-IgE antibodies showing as early as 12 months (Fig 1, *B*). Of note, although not reaching the FDR cutoff, the consumption group, but not the avoidance group, had detectable changes ($P < .05$) in 4 IgE-binding epitopes on Ara h 1 as early as month 12.

On the other hand, *ses*-IgG$_4$ levels increased earlier in consumers, with significant *ses*-IgG$_4$ expansion (epitope spreading) by 12 months in epitopes found on Ara h 1 and Ara h 2 (Fig 1, *C*). *ses*-IgG$_4$ expansion occurred after 12 months for avoiders, but by 5 years, *ses*-IgG$_4$ expansion was comparable in both groups.

Children sensitized to peanut at baseline experienced marked *ses*-IgE expansion in the avoidance group, but not in the consumption group. We compared the evolution of *ses*-IgE and *ses*-IgG$_4$ among children who were sensitized (PN-sIgE >0.1 kU$_A$/L) or not at the randomization (baseline) visit (Fig 2). In this cohort, 50% (n = 86) of avoiders and 38% (n = 64) of consumers were sensitized at baseline (4-11 months) (Table I).

Subjects who were not sensitized at the 4- to 11-month visit did not show any increase in *ses*-IgE regardless of the peanut consumption (Fig 2). Among baseline-sensitized patients, a large expansion of *ses*-IgE was observed for all 3 peanut proteins among avoiders, while sensitized consumers showed a low-level transient expansion in *ses*-IgE up to 30 months, returning to baseline thereafter. This expansion occurred first in regions of Ara h 1, with *ses*-IgE to 1 epitope significantly increased at 12 months and a broader expansion and diversity (5 Ara h 1 epitopes) at 30 months, whereas *ses*-IgE to only 1 each Ara h 2 and Ara h 3 epitope showed an increase. After 30 months, *ses*-IgE levels decreased, returning close to baseline levels (see Fig E1 in this article's Online Repository at www.jacionline.org).

These findings were in contrast to the PN-sIgE, which includes IgE to both conformational and sequential epitopes (beyond the 64 evaluated in this study), where children in the avoidance arm

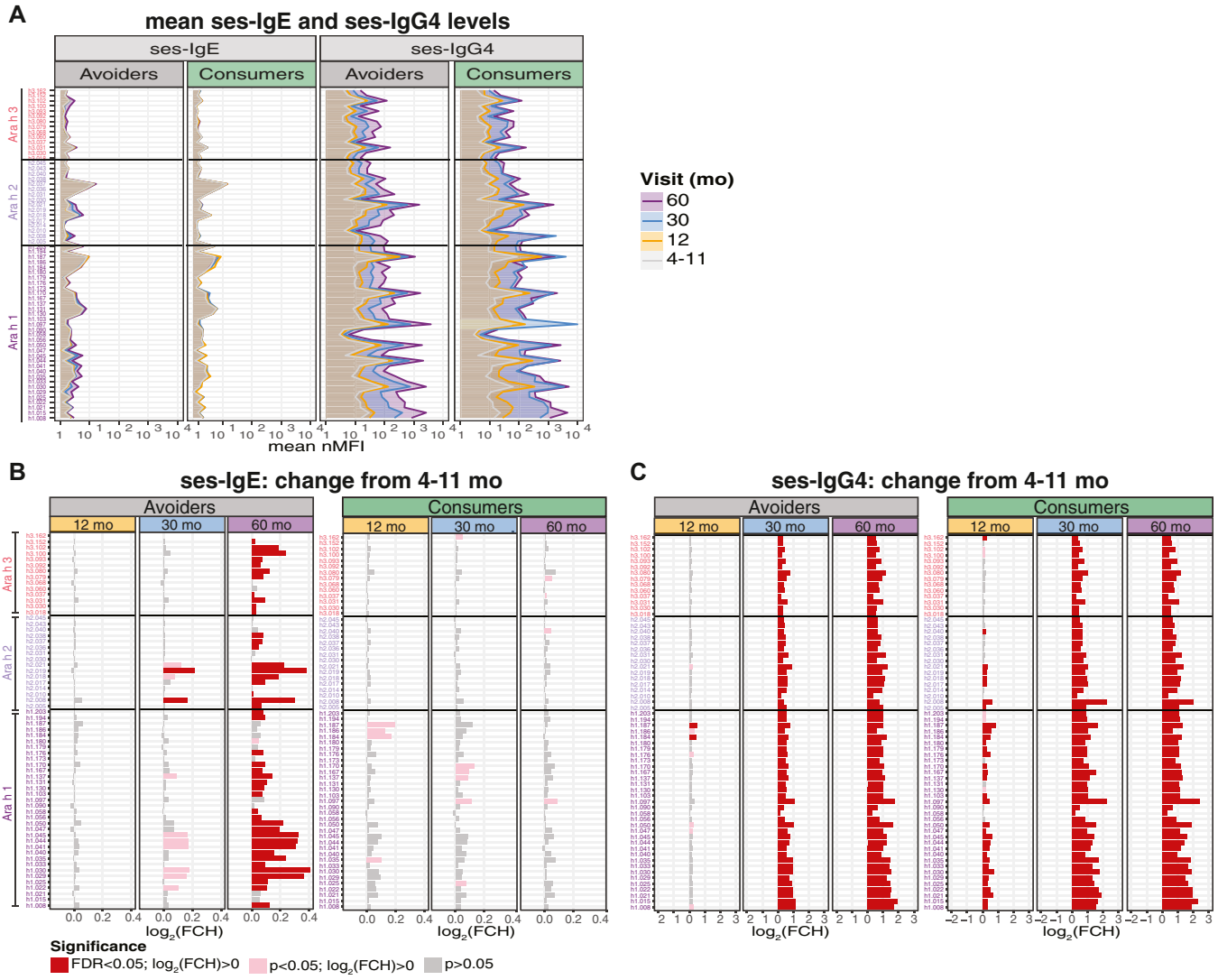

FIG 1. Epitope expansion over time in peanut avoiders and consumers from the LEAP trial. **A,** Mean nMFI expansion for *ses*-IgE and *ses*-IgG$_4$ at each visit by peanut exposure. **B and C,** Barplots representing mean changes (expressed as log$_2$[FCH] on the x-axis) in *ses*-IgE **(B)** and *ses*-IgG$_4$ **(C)** from baseline to each visit, for avoiders (*left*) and consumers (*right*). Color bar indicates the strength of the increase based on FDR (*dark red*) or nominal *P* values (*pink* or *gray*). Individual peptides are represented on the y-axis from botton to top by their position on the Ara h 1 to Ara h 3 proteins. Significant *ses*-IgE expansion occurred primarily in the avoidance group between 2.5 and 5 years of age. *ses*-IgG$_4$ expansion was significant among avoiders and consumers and began earlier in the consumer group. *FCH,* Fold-change.

that were sensitized at baseline did not have a sustained increase in PN-sIgE and only minimal increase in Ara h 2 sIgE (Fig 2). For sensitized children randomized to peanut consumption, however, the transient increase in IgE was observed primarily for peanut component proteins. As depicted in Fig 1, virtually no IgE directed at sequential epitopes was generated during the first 2.5 years of life.

Regardless of baseline sensitization status, the consumers generated increasing peanut sIgG$_4$ and *ses*-IgG$_4$ in the first year of life that appeared to peak at about 30 months of age with no significant increase in peanut *ses*-IgG$_4$ beyond 30 months of age (Fig 2 and Fig E1). Among avoiders, those who were not sensitized at baseline had a higher *ses*-IgG$_4$ expansion than those who were sensitized with no difference between those groups in PN-sIgG$_4$. Interestingly, in those children who were not sensitized, *ses*-IgG$_4$ expansion at 12 months was broader in avoiders than in consumers, with significant increases in 37 epitopes from all 3 proteins (Fig E1).

***ses*-IgE expansion was limited to avoiders who became allergic at the 60-month visit.** To further elucidate the specific humoral differences occurring during the development of peanut allergy, we investigated the association of *ses*-IgE antibody expansion with the allergy outcome at the end of the trial (60 months). Baseline *ses*-IgE and *ses*-IgG$_4$ profiles were similar regardless of the allergy outcome at year 5 (Fig 3, *A*, and see Fig E2, *A* in this article's Online Repository at www.jacionline.org).

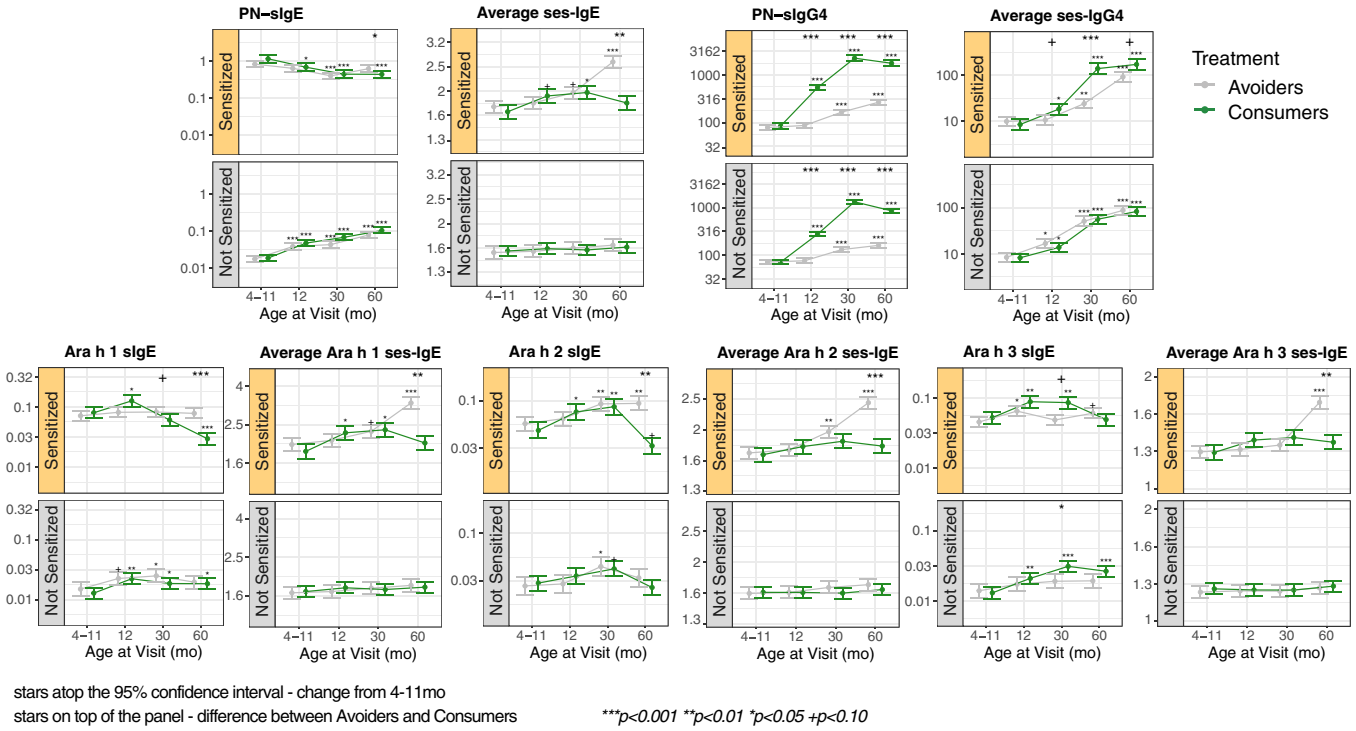

FIG 2. Changes in *ses*-IgE and *ses*-IgG$_4$ binding by peanut exposure and baseline sensitization status. Changes in overall *ses*-IgE and *ses*-IgG$_4$ z-score and sIgE and sIgG4 from baseline to 5 years for subjects who were found to be sensitized at the 4- to 11-month visit, by peanut exposure, presented as mean ± 95% CI. Stars atop upper confidence limit represent significant change from baseline while those atop black bars represent differences between the 2 treatment groups. +$P < .1$; *$P < .05$; **$P < .01$; ***$P < .001$.

As shown in Fig 3, *A* and *B*, the overall *ses*-IgE expansion observed in the avoiders group was exclusively among children with OFC-confirmed allergy at the 60-month visit, with no change among avoiders who had a negative OFC. Allergic avoiders had consistent significant increases in their *ses*-IgE over time, with IgE binding to 19 different epitopes at 30 months and further broad expansion to 64 different epitopes by the 60-month visit (Fig 3, *B*, and see Fig E3 in this article's Online Repository at www.jacionline.org). The greatest increase in *ses*-IgE occurred in the region of Ara h 1.029, Ara h 1.030, Ara h 1.041 to Ara h 1.050, Ara h 2.008, Ara h 2.017 to Ara h 2.021, and Ara h 3.100, which began after the 12-month visit. Among consumers, *ses*-IgE antibodies to 3 epitopes on the Ara h 1 protein (Ara h 1.184, Ara h 1.186, Ara h 1.187) were significantly increased among sensitized children by the 12-month visit (Fig E3), but these epitopes were not recognized by IgE of allergic avoiders. As noted previously for peanut component proteins,[14] peanut *ses*-IgE levels are significantly lower in sensitized children than in those who are allergic.

These findings are in contrast to the PN-sIgE changes reported over time for the LEAP cohort,[4] which are reproduced in Fig E6 in this article's Online Repository (available at www.jacionline.org) using the same modeling approach and patient population used for the epitope analyses. While PN-sIgE levels remained unchanged in nonallergic children in both intervention arms (with a trend to decrease) and increased early in allergic avoiders, sensitized children had increased PN-sIgE levels regardless of the intervention (see Fig E4, *A* in this article's Online Repository at www.jacionline.org). Consumers who became sensitized showed significantly increased sIgE to Ara h 1 to 3 component proteins primarily in the first 30 months, while both sensitized consumers and avoiders demonstrated significant increases in sIgE to Ara h 8 and to a lesser extent to Ara h 9 (Fig E4, *B*) from months 30 to 60, which accounts for a significant proportion of the increase in whole PN-sIgE during this period. PN-sIgE measures antibody binding to both conformational and sequential epitopes while the BBEA measures IgE to sequential epitopes only. Therefore, our results show that sensitized children in both intervention arms lacked an increase of IgE antibodies to sequential epitopes while increasing IgE to peanut protein and its components, suggesting that the increase in IgE may be due to an increase of IgE to conformational epitopes. Importantly, there was no increase in *ses*-IgE to sequential epitopes in consumers, contrasted with an increase in sIgE to Ara h 1 only at 12 months and to Ara h 2 and Ara h3 component proteins up to the 30-month visit.

In the oral peanut consumption group, children who were sensitized by 60 months had a larger *ses*-IgG4 expansion than nonallergic children did. In contrast to *ses*-IgE, *ses*-IgG$_4$ antibodies increased significantly in all groups by the 30- and 60-month visits (Fig E2). However, only the consumers who were sensitized at the end of the trial had broad diversity at the 12-month visit, with expansion in the majority of Ara h 1 peptides (31 peptides or 91.2%) but also to 7 epitopes (43.8%) and 5 epitopes (35.7%) of the Ara h 2 and Ara h 3 proteins, respectively (Fig 4, *B*). Whether or not IgG$_4$ is protective or merely reflects changes in immune responses that are associated with IgG$_4$

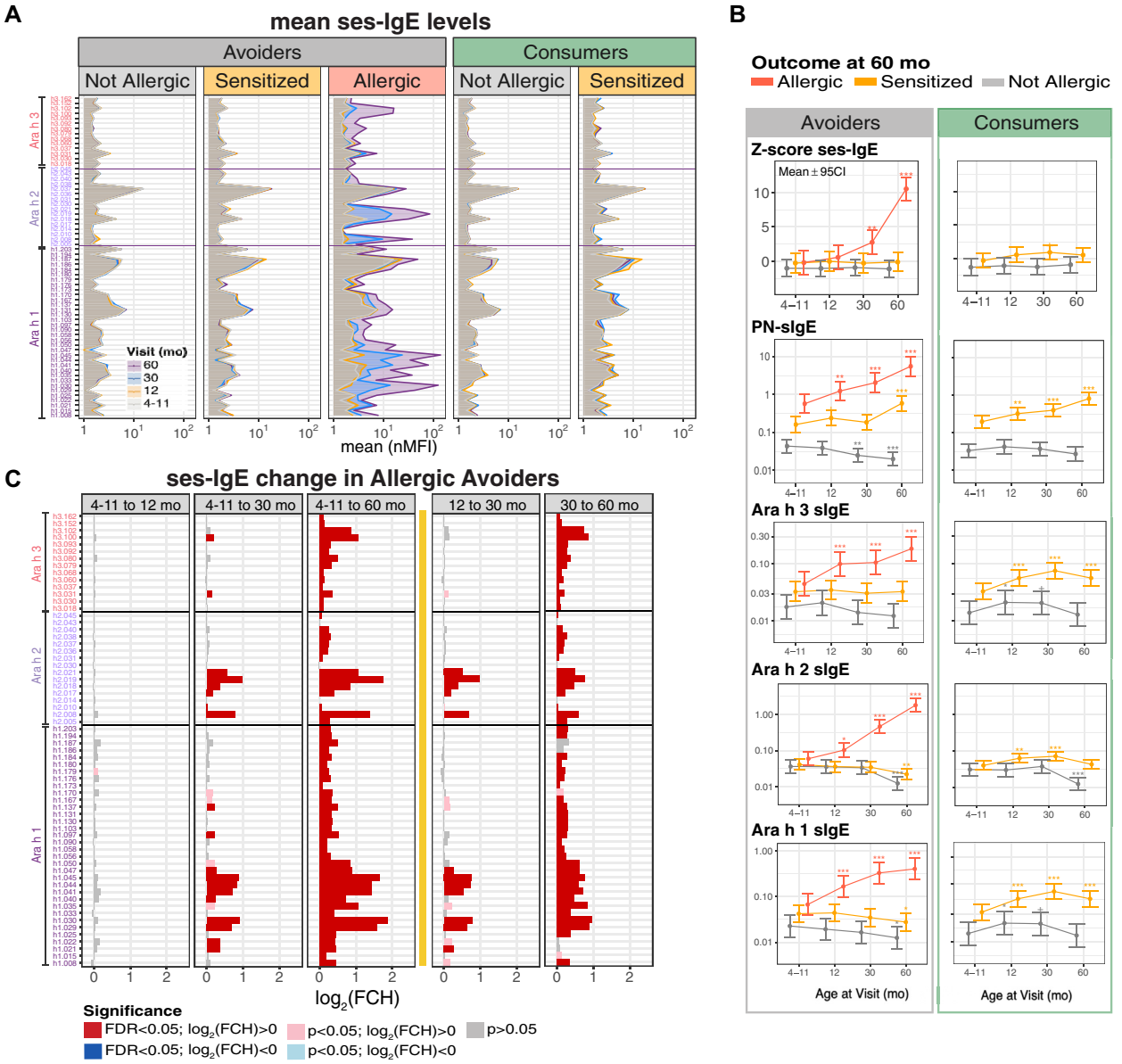

FIG 3. Changes in *ses*-IgE by peanut exposure and allergy status at the 60-month visit. **A** and **B,** Mean nMFI expansion in *ses*-IgE from baseline to 5 years by peanut exposure and outcome groups by epitopes **(A)** and overall *ses*-IgE z-score, with mean and 95% CI **(B)**. **C,** *ses*-IgE of allergic avoiders showing changes from baseline and between various time points. Color bar indicates the direction of the change (*red*: increase, *blue*: decrease) and strength of the changes based on FDR (*dark*) or nominal *P* values (*light*). +*P* < .1; **P* < .05; ***P* < .01; ****P* < .001.

expansion, this early IgG_4 expansion occurred only in consumer children. *ses*-IgG_4 antibodies continued to expand rapidly at subsequent visits and by the 30-month visit, the sensitized consumers had greater increases in binding than any other group by 5 years of age.

DISCUSSION

The LEAP trial demonstrated that early introduction of peanut protein into the diet of infants at high risk could prevent peanut allergy.[1] In this mechanistic study of 341 patients, a subset of the LEAP trial cohort, we sought to understand the evolution of humoral responses in children developing peanut allergy compared with those who remained tolerant by investigating the evolution of *ses*-IgE and *ses*-IgG_4 antibody repertoires in each group.

At enrollment (baseline), children who developed peanut allergy by 60 months of age had the greatest levels of sIgE to peanut and Ara h 1 to Ara h 3 at the baseline visit (Table I). Over the trial's 5 years, nonallergic children, regardless of consumption status, showed no significant changes in peanut or Ara h 1 to Ara h 3 sIgE, while both sensitized and allergic children showed consistent increases in peanut sIgE over time (Fig E4).

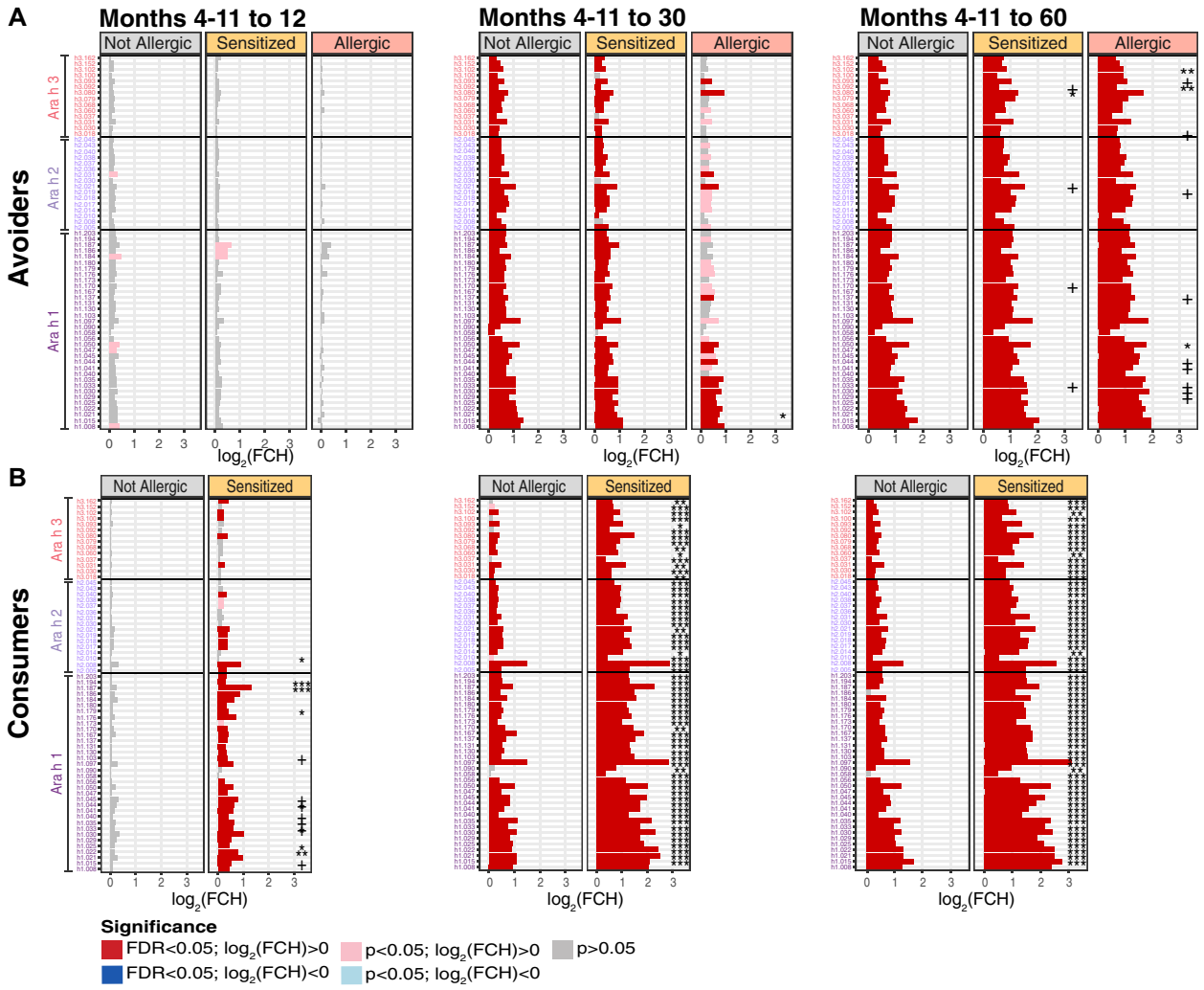

FIG 4. Changes in *ses*-IgG$_4$ by peanut exposure and allergy status at the 60 months visit. Barplots representing log$_2$(FCH) (mean ± SD) between peanut exposure and outcome groups with changes from baseline and between subsequent time points for avoiders **(A)** and consumers **(B)**. Color bar indicates the direction of the change (*red*: increase, *blue*: decrease) and strength of the changes based on FDR (*dark*) or nominal *P* values (*light*). Stars indicates significant differences between avoiders and consumers within the same allergy status at 5 years. +$P < .1$; *$P < .05$; **$P < .01$; ***$P < .001$.

It was previously shown that *ses-I*gE is associated with clinical peanut allergy.[5-8] We therefore evaluated the relative quantity and changes in *ses*-IgE and *ses*-IgG$_4$ to 64 informative peanut epitopes at 4 time points during the LEAP trial. The relative quantities of *ses*-IgE and *ses*-IgG$_4$ (Fig 1) were similar in both the avoider and consumer groups at baseline. While *ses*-IgG$_4$ expansion occurred naturally in all groups, it appeared to a greater degree earlier in children who consumed peanut, especially in those who were sensitized, and primarily in the first 30 months of life. At 5 years of age, *ses*-IgG$_4$ levels were comparable in both groups (see Fig E5 in this article's Online Repository at www.jacionline.org).

Although both avoiders and consumers had minimal quantities of *ses*-IgE to some informative sequential epitopes at baseline, significant increases of *ses*-IgE to most of these epitopes were observed only in the avoiders, and predominantly after 2.5 years of age (Fig 1, *B*). However, as shown in Fig 2, *ses*-IgE expansion occurred exclusively in avoiders who were diagnosed with clinical peanut allergy at 5 years of age, whereas sensitized avoiders instead began generating *ses*-IgG$_4$ (Fig 4, *A*).

While increases in sIgE to whole peanut and peanut component proteins were detected in both sensitized avoiders and consumers (Fig E4), no significant *ses*-IgE expansion to sequential epitopes was observed in these sensitized children (Figs 3, *B*, and E3). In addition, there was no significant increase in *ses*-IgE to sequential epitopes in consumers even though there was an increase in sIgE to Ara h 1 only at 12 months and to Ara h 2 and Ara h3 component proteins up to the 30-month visit. Assuming that conformational epitopes are contributing to the observed increases in sIgE levels, this would support the hypothesis that IgE antibodies against conformational epitopes are generated early in life and are less indicative of persistent, systemic clinical reactivity.

We also compared the evolution of *ses*-IgE and *ses*-IgG$_4$ in children who were found to be sensitized at baseline (ie, before 1 year of age) and those who were not sensitized. Consumers who were sensitized to peanut at baseline experienced significant increases in peanut sIgE and generated very small amounts of *ses*-IgE to a limited number of epitopes primarily on Ara h 1 until ~30 months of age (Fig 2). No significant increases were seen before

the first 30 months in peanut sIgE or *ses*-IgE in avoiders who were sensitized at baseline. Regardless of baseline sensitization status, the consumers generated increasing peanut $sIgG_4$ and *ses*-IgG_4 in the first year of life that appeared to peak at about 30 months of age with no significant increase in peanut *ses*-IgG_4 beyond 30 months of age (Fig 2). In contrast, peanut avoiders had delayed increases in peanut $sIgG_4$ and *ses*-IgG_4 antibodies compared with consumers; primarily until after the first year of life. Interestingly, in those children who were not sensitized at 4 to 11 months, avoiders generated more peanut *ses*-IgG_4s earlier than did those in the consumer group (Fig E1). This suggests that early generation of *ses*-IgG_4 protects or is associated with protection against the development of peanut allergy, irrespective of intervention.

One limitation of this study is that children randomized to the consumer group who were reactive to peanut at baseline (n = 7) or who experienced allergic reactions and therefore stopped consuming peanut (n = 9) were not included in this analysis. However, the intent of this study was to compare the development of *ses*-IgE and *ses*-IgG_4 in children who were nonreactive at baseline and who successfully consumed or avoided peanut for the full 5 years of the trial (ie, the per protocol cohort). Evolution of *ses*-IgE and *ses*-IgG_4 in this reactive group may be highly informative and will be the subject of further study. Although the 64-plex library of informative epitopes used in this study was selected by screening a whole set of overlapping 15-mer peptides covering entire sequences of Ara h 1 to Ara h 3 and Ara h 7, as well as nonhomologous regions of Ara h 6 proteins, it is possible that additional epitopes from Ara h 6 or other peanut allergens could contribute to peanut allergy or sensitization.

In summary, this study suggests that young infants who develop persistent peanut allergy begin generating substantial quantities of IgE antibodies to sequential epitopes primarily after 2.5 years of age, whereas such epitope spreading does not occur in peanut-tolerant children who are only sensitized or never sensitized. The early oral introduction of peanut in both sensitized and non-sensitized infants appears to inhibit expansion of *ses*-IgE antibodies and to promote progressive expansion of *ses*-IgG_4 antibodies that become apparent at about 12 months of age. While *ses*-IgG_4 expansion occurs in all children regardless of peanut consumption, it is delayed and occurs to a lesser extent in infants who avoid peanut consumption. Whether this early introduction of peanut into the diet leading to the greater induction of *ses*-IgG_4 is responsible for the prevention of peanut allergic reactivity or whether it is associated with other immunologic changes that prevent the development of clinical reactivity remains to be determined.

Interestingly, over 30% of the infants studied in this cohort had measurable IgE to peanut at enrollment in both study arms and initially exhibited an increase in IgE to whole peanut and its major protein components in the absence of any significant increases *ses*-IgE antibodies to sequential epitopes in these major component allergens. This suggests that IgE to peanut and its major components may be generated against conformational epitopes in the first 2.5 years of life, and that the early oral introduction of peanut into the diet could redirect the immune response from generating *ses*-IgE, which is associated with persistent peanut allergy. This further supports the concept that there is a potential "window of opportunity" during which peanut allergy can be prevented or possibly successfully treated.

Key messages

- **Consumption of peanut in high-risk infants blocked the induction of IgE to sequential epitopes that occurs in those who avoided peanuts. Instead, early consumption of peanut increased IgG_4 epitope-specific antibodies.**
- **Infants sensitized to peanut who consume peanut generate IgE to whole peanut and peanut component proteins, but not to sequential epitopes, whereas sensitized infants who avoid peanuts and become allergic generate IgE initially to peanut component proteins and subsequently to sequential peanut epitopes.**
- **Most of the *ses*-IgE in children developing peanut allergy occurred after the second year of life, suggesting that there may be an "early window" where introduction to peanut protein could prevent the development of persistent peanut allergy.**

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