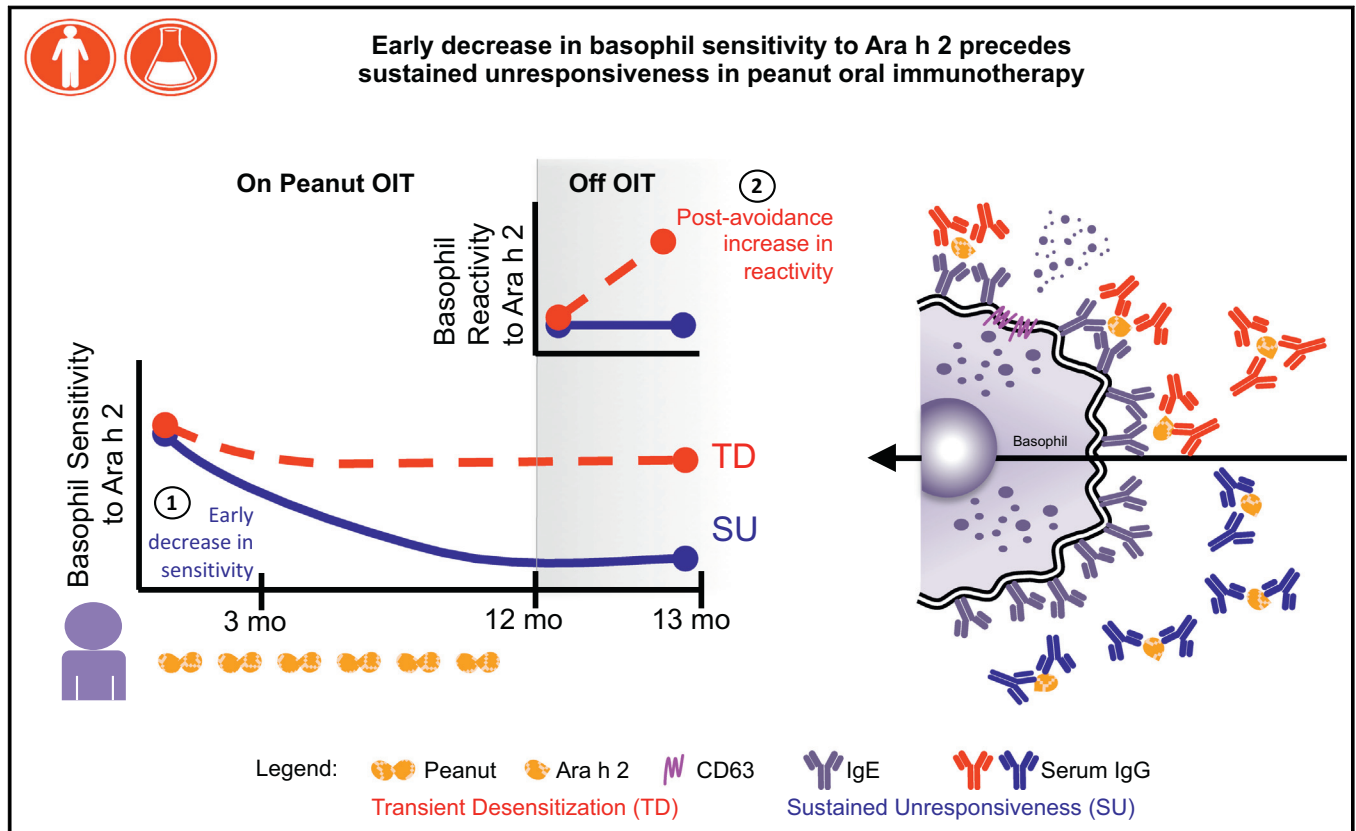


Early decrease in basophil sensitivity to Ara h 2 precedes sustained unresponsiveness after peanut oral immunotherapy



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GRAPHICAL ABSTRACT



Background: Only some patients with peanut allergy undergoing oral immunotherapy (OIT) achieve sustained clinical response. Basophil activation could provide a functional surrogate of efficacy.

Objective: We hypothesized that changes in basophil sensitivity and area under the curve (AUC) to the immunodominant allergen Ara h 2 correlate with clinical responses to OIT.

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This work was supported by the National Institutes of Health (NIH)/National Institute of Allergy and Infectious Diseases (grant K23AI121491 to S.U.P.); the American Academy of Allergy, Asthma & Immunology/Food Allergy Research Education; the 2012 Howard Gittis Memorial 3rd/4th Year Fellowship/New Faculty Research Award (to S.U.P.), and research support from BÜHLMANN Laboratories AG (to W.G.S.). The clinical trial work was performed in the Harvard Clinical and Translational Science Center supported by grants 1UL1TR001102 and 8UL1TR000170 from the National Center for Advancing Translational Sciences and 1UL1 RR025758 from the National Center for Research Resources. Cytometry performed in the MGH Department of Pathology Flow and Image Cytometry Research Core was supported

by the NIH Shared Instrumentation program with grants 1S10OD012027-01A1, 1S10OD016372-01, 1S10RR020936-01, and 1S10RR023440-01A1.

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

Received for publication April 30, 2019; revised July 5, 2019; accepted for publication July 17, 2019.

Available online August 1, 2019.

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0091-6749/\$36.00

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<https://doi.org/10.1016/j.jaci.2019.07.028>

Methods: Children with peanut allergy aged 7 to 13 years were enrolled in a single-center, open-label peanut OIT trial. Levels of specific immunoglobulins were measured throughout OIT. Peripheral blood from multiple time points was stimulated *in vitro* with peanut allergens for flow cytometric assessment of the percentage of CD63^{hi} activated basophils.

Results: Twenty-two of 30 subjects were successfully treated with OIT; after avoidance, 9 achieved sustained unresponsiveness (SU), and 13 had transient desensitization (TD). Basophil sensitivity, measured by using the dose that induces 50% of the maximal basophil response, to Ara h 2 stimulation decreased from baseline in subjects with SU (after OIT, $P = .0041$; after avoidance, $P = .0011$). At 3 months of OIT, basophil sensitivity in subjects with SU decreased from baseline compared with that in subjects with TD (median, 18-fold vs 3-fold; $P = .01$), with a receiver operating characteristic of 0.84 and optimal fold change of 4.9. Basophil AUC to Ara h 2 was suppressed after OIT equally in subjects with SU and those with TD ($P = .4$). After avoidance, basophil AUC rebounded in subjects with TD but not those with SU ($P < .001$). Passively sensitized basophils suppressed with postavoidance SU plasma had a lower AUC than TD plasma (6.4% vs 38.9%, $P = .03$). **Conclusions:** Early decreases in basophil sensitivity to Ara h 2 correlate with SU. Basophil AUC rebounds after avoidance in subjects with TD. Therefore, different aspects of basophil activation might be useful for monitoring of OIT efficacy. (J Allergy Clin Immunol 2019;144:1310-9.)

Key words: Basophil activation, immunotherapy, immunoglobulin, oral immunotherapy, food allergy, peanut allergy, Ara h 2, IgE, IgG₄

The increasing incidence and persistence of IgE-mediated peanut allergy^{1,2} highlight the growing need for effective therapies. Peanut oral immunotherapy (OIT) induces desensitization, raising the threshold of exposure required to cause a reaction in most patients with peanut allergy who successfully complete it; however, only about 30% achieve more durable clinical efficacy.^{3,4} Multiple immunologic changes occur during OIT,⁵ including suppression of basophil area under the curve (AUC),⁶ depletion of peripheral allergen-specific T_H2 effector cells,⁷ expansion of antigen-specific B cells,⁸ and induction of allergen-specific antibodies.^{8,9} However, the extent to which any of these changes are mechanistically related to clinical outcomes, in particular to durable efficacy, remains unclear.

Basophil activation to allergen stimulation has emerged as an informative surrogate marker of clinical allergic status.^{5,10} For example, Santos et al reported that among untreated allergic patients subjected to a graded oral peanut challenge, the clinical severity of the reaction correlates closely to the *in vitro* magnitude of basophil degranulation reflected by the maximal or peak response. The threshold dose provoking a clinical reaction also correlates to *in vitro* basophil sensitivity, as reflected by the dose that induces 50% of the maximal basophil response (ED50) of the dose response.¹¹ These 2 features of basophil responses, basophil AUC and sensitivity, are somewhat independently influenced by distinct features of the antigen-antibody-Fc receptor complexes that form, including the IgE/non-IgE clonality, affinity,¹² glycosylation, and intrinsic basophil reactivity.¹³⁻¹⁷ Measures of basophil activation have been shown to have additional diagnostic

Abbreviations used

AUC: Area under the curve
ED50: Dose that induces 50% of the maximal basophil response
OIT: Oral immunotherapy
SU: Sustained unresponsiveness
TD: Transient desensitization

utility for IgE-mediated food allergies,¹⁸ although not for evaluating for sustained unresponsiveness (SU).

In the context of OIT, several clinical trials have demonstrated suppression of *in vitro* basophil AUC in subjects with decreased clinical reactivity to peanut.^{6,19,20} Previous work on basophil suppression with post-OIT serum has shown that this suppression can be mediated *in vitro* by antigen-specific IgG₄ through inhibitory FcγRIIb engagement.¹⁴ However, sustained efficacy of OIT, as measured by sustained clinical unresponsiveness to peanut after strict allergen avoidance, has been repeatedly shown to not correlate with the concentration of allergen-specific IgG₄ induction in previous trials.^{3,4} On the other hand, in other forms of immunotherapy, functional antibody testing, as opposed to specific antibody levels, correlated better with clinical outcomes.¹⁷

Therefore we hypothesized that OIT-induced suppression of basophil sensitivity to the immunodominant antigen Ara h 2 is a biomarker of SU. After longitudinally studying basophil activation both during OIT and after avoidance, we conclude that basophil sensitivity and AUC can not only better inform clinical outcomes than bulk measurements of allergen-specific antibodies but might also provide a surrogate functional measure of the induction of protective antigen-specific antibodies.

METHODS

Peanut OIT trials

Peripheral blood was obtained after institutional review board–approved consent from participants with peanut allergy to an open-label randomized trial of peanut OIT (NCT01324401). Study inclusion criteria included participants aged 7 to 21 years with a diagnosis of peanut allergy based on a clinical history of reaction to peanut within 1 hour of ingestion and either an increased skin prick test response (>8 mm wheal) or increased peanut-specific IgE level (CAP FEIA >10 kU/L).

A total of 30 patients were enrolled, including 4 in an observational control arm who were monitored for 1 year, after which they also received active OIT with premeasured peanut flour (Medium Roast; Golden Peanut, Alpharetta, Ga). The protocol, as previously described,²¹ began with a 1-day modified rush phase, 44-week-long build-up phase, and 12-week maintenance phase with the daily dose of 4000 mg at the end of which desensitization was evaluated with an oral food challenge. After 4 weeks of peanut avoidance, subjects underwent a double-blind oral food challenge to peanut. Peripheral blood sampling was performed at baseline, every 4 weeks during the buildup phase from 4-24 weeks, at 36 weeks, and once during maintenance and then at both food challenges.

Before the start of active OIT, 2 subjects voluntarily withdrew and 1 subject was a screen fail because of failure to tolerate a minimal dose on the modified rush. During the study, 3 subjects were withdrawn by the investigator because of persistent reactivity to peanut, 3 subjects withdrew during the study (1 because of difficulty adhering to the timeline, 1 because of a dose reaction, and 1 because of concern about underlying peanut allergy), and 1 withdrew because of failure to tolerate the starting dose during the modified rush protocol. Twenty-three subjects reached the clinical end point, of whom 1 subject was not effectively desensitized. Of the remaining 22 subjects, 9 had SU after 4 weeks of peanut avoidance, and

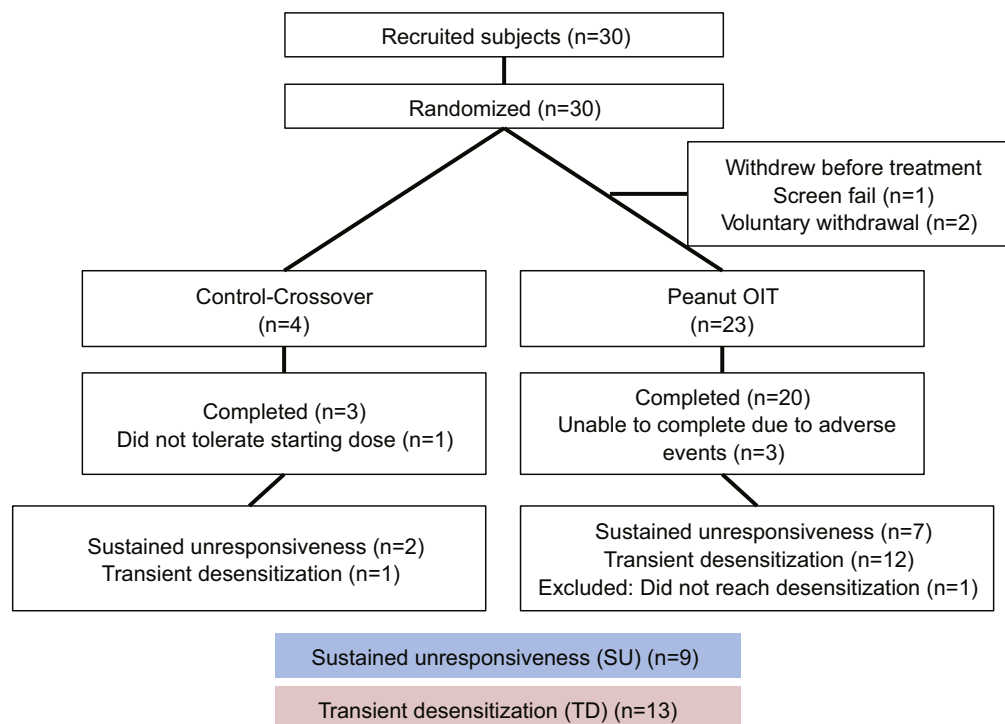


FIG 1. Peanut OIT clinical trial. CONSORT diagram of subjects enrolled in the peanut OIT clinical trial in both the control crossover and active OIT arms. Summary of clinical outcomes is located below the diagram, with the total number of patients who achieved SU (blue, $n = 9$) and those who achieved TD (red, $n = 13$) defined by peanut tolerance on double-blind placebo-controlled challenge after 1 month of peanut avoidance.

TABLE I. Clinical demographics by outcome after peanut OIT

	Subjects with SU ($n = 9$)	Subjects with TD ($n = 13$)	<i>P</i> value*
Age (mean $\pm$ SD)	9.33 $\pm$ 2.40	9.62 $\pm$ 2.22	.78
Sex (% male)	7 (77.8)	9 (69.2)	1.0
Ethnicity			1.0
Non-Hispanic (%)	9 (100.0)	12 (92.3)	
Race			.46
White	0	2 (15.4)	
Asian	1 (11.1)	1 (7.7)	
More than 1 race (%)	8 (88.9)	10 (76.9)	
History of allergic rhinitis (%)	7 (77.8)	5 (38.5)	.17
History of atopic dermatitis (%)	3 (33.3)	9 (69.2)	.22
History of anaphylaxis (%)	1 (11.1)	6 (46.2)	.20
History of other food allergies (%)	5 (55.6)	11 (84.6)	.31
Age of diagnosis	1.44 (0.46)	2.23 (0.82)	.02
Age of first reaction	2.11 (1.60)	3.36 (2.68)	.23

**P* values were calculated by using the Wilcoxon rank sum test. Boldface indicates $P < .05$.

13 regained responsiveness to peanut and were labeled as having transient desensitization (TD; Fig 1).

Specific immunoglobulin measurement

Measurement of peanut and antigen-specific immunoglobulin levels in the serum or plasma from peripheral blood of subjects undergoing OIT was determined by using the ImmunoCAP 1000 instrument (Phadia AB, Uppsala, Sweden), according to the manufacturer's instructions.

Basophil activation testing

By using the Flow CAST assay (BÜHLMANN Laboratories, Schönenbuch, Switzerland), basophils in whole blood were stimulated with allergens (whole

peanut extract, Ara h 1, Ara h 2, and Ara h 6) and stained with fluorescent CCR3 and CD63 antibodies, according to the manufacturer's protocol. Flow cytometric data were acquired on an LSRII or Fortessa (Becton, Dickinson and Company, Franklin Lakes, NJ) in FCS file format. As previously published,²² using Bioconductor tools in R software with basophil activation testing data from patients undergoing peanut OIT, we developed a data-driven automated gating strategy in which we used clustering approaches to define activated basophils as side scatter-low CCR3⁺CD63^{hi} cells. In each individual experiment, the CD63 cutoff to determine activated CD63^{hi} basophils was determined as the 97.5th percentile of CD63 expression of unstimulated basophils. Gate visualization on plots of all stimulation conditions provided additional quality control (R 3.2.1 and Bioconductor 1.22.3).²³⁻²⁵ Statistical characterization of basophils was performed and aggregated for additional analysis.

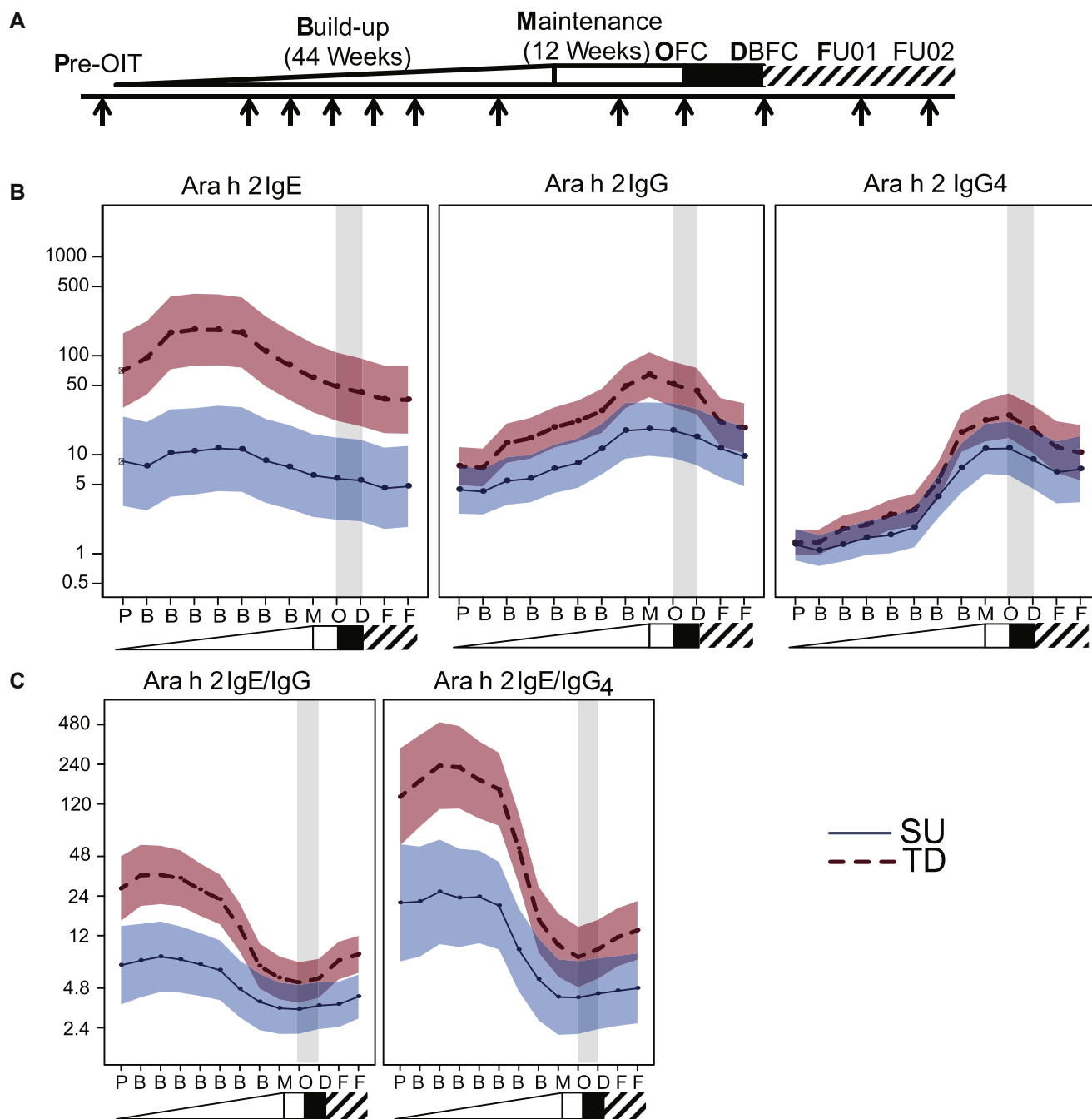


FIG 2. Serum immunoglobulins during peanut OIT. **A**, Clinical schema of the active arm of peanut OIT, including pre-OIT, build-up with increasing peanut doses, 3-month maintenance, postimmunotherapy oral food challenge (OFC), postavoidance double-blind food challenge (DBFC) after 1 month of peanut avoidance, and follow-up period (FU01 and FU02) with *ad hoc* peanut consumption. Black arrows mark peripheral blood and serum sampling. **B**, Serum Ara h 2 IgE, IgG, and IgG₄ levels in subjects with SU (blue) and subjects with TD (dashed line, red) over time determined by using a logistic regression model with random intercepts. **C**, Serum IgE/IgG and IgE/IgG₄ ratios in subjects with SU (blue) and those with TD (dashed lines, red) over time determined by using a logistic regression model with random intercepts. Lines indicate means, and shaded regions indicate SDs. Lettered labels on the x-axis refer to visits, pre-OIT (P), build-up (B), maintenance (M), post-OIT oral food challenge (O), postavoidance double-blind food challenge (D), and follow-up (F).

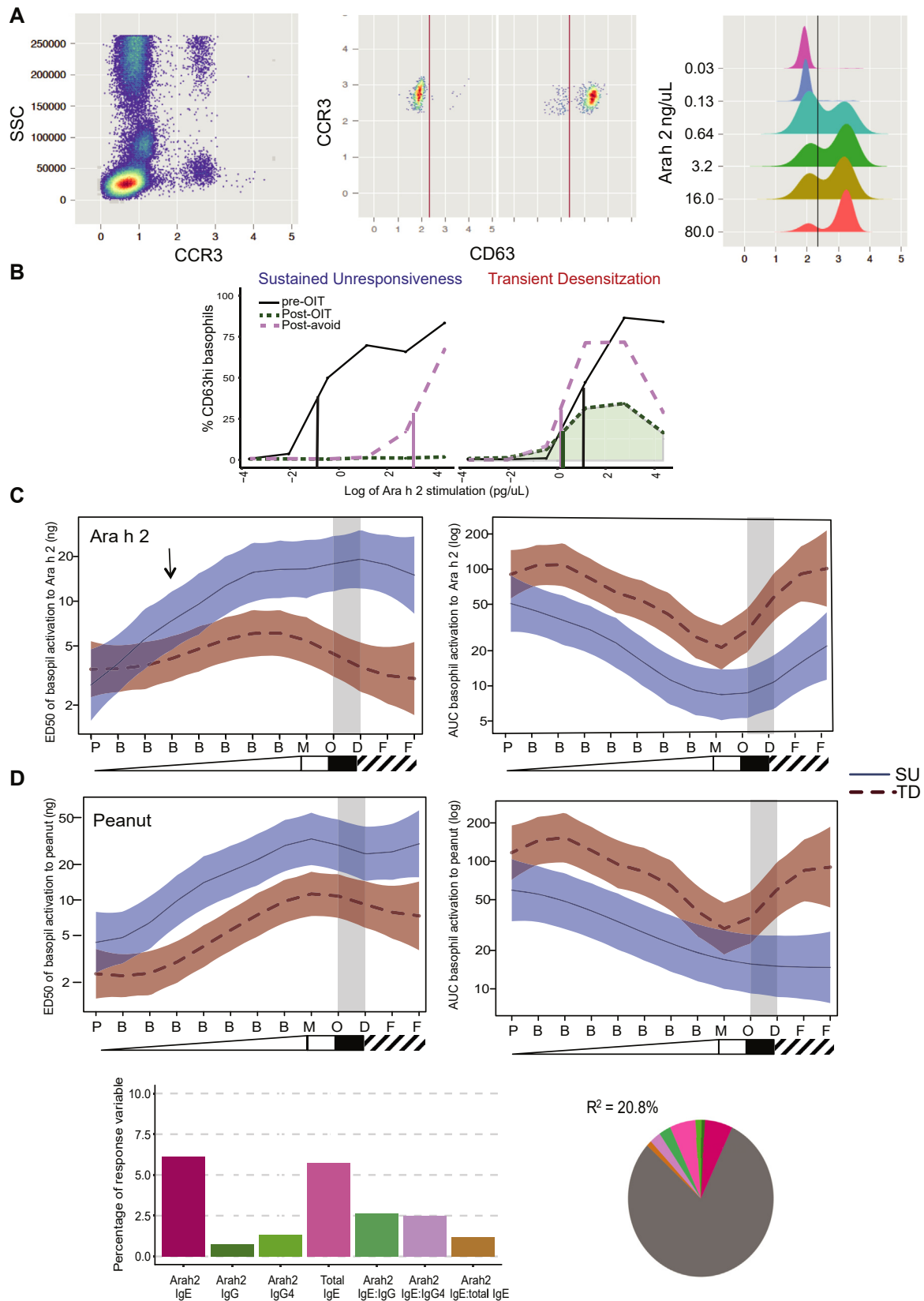


FIG 3. *Ex vivo* direct basophil activation during peanut OIT. **A**, Peripheral basophil activation testing to Ara h 2 to identify degranulation as a percentage of CD63^{hi} basophils by using flow cytometry. SSC, Side scatter. **B**, Basophil sensitivity (ED50) and AUC (solid green) in representative subjects with SU (left, blue) and TD (right, red). **C** and **D**, Longitudinal basophil sensitivity and AUC determined by using a

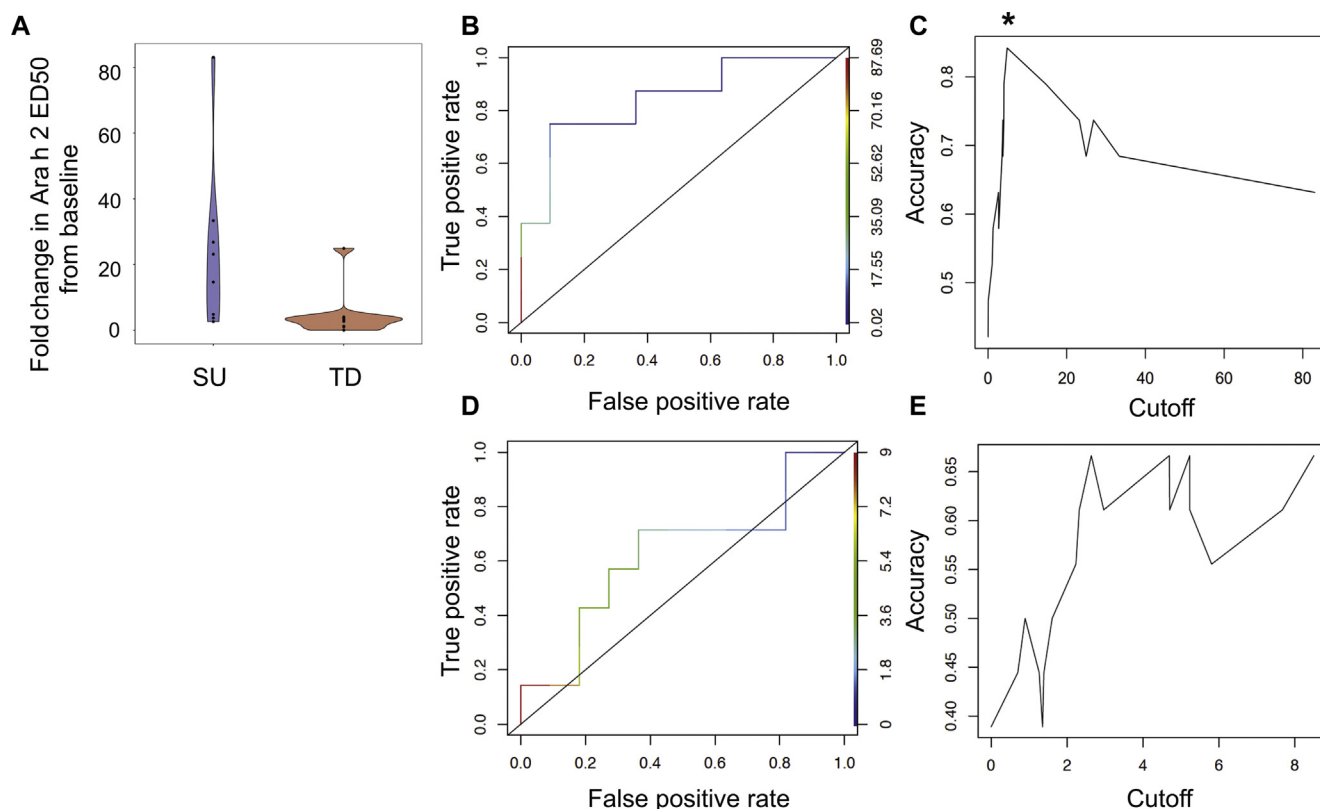


FIG 4. Early decrease in basophil sensitivity to Ara h 2 associated with SU after peanut OIT. **A**, Fold change in basophil sensitivity to Ara h 2 from baseline to 3 months (Fig 3) was compared in subjects with SU (blue) and those with TD (red). **B–E**, The receiver operating characteristic of the early change of basophil sensitivity to Ara h 2 (Fig 4, B) with an optimal cutoff (Fig 4, C) compared with the receiver operating characteristic of the early change of basophil sensitivity to peanut (Fig 4, D) with an optimal cutoff (Fig 4, E).

Basophil activation in passively sensitized basophils

PBMCs from healthy donors were isolated with a Ficoll density gradient and stripped of endogenous IgE by using a lactic acid-stripping protocol¹² followed by a 1-hour incubation in undiluted plasma from 1 to 2 months after the start of OIT. After washing the PBMCs, stimulation with Ara h 2 at various concentrations was performed in medium or in medium mixed with separate aliquots of autologous plasma at a 1:20 ratio from an early time point (1–3 months), at the end of immunotherapy, or after avoidance.

Statistical analysis

Longitudinal data were analyzed by using generalized, nonlinear, mixed modeling in R software. For analysis of serum immunoglobulins, natural log transformed data was used, and values were reported as means along with standard deviations. For basophil activation analysis, dose-response curves were fitted by using the R package *drc*,²⁶ assuming a 4-parameter log-logistic dose-response model, and the ED50 value was derived. The default, nonrobust, least-squares unconstrained estimation fitting procedure (ie, mean) was not always the optimal solution, and therefore we also fitted the same dose-response using robust

methods²⁶: median estimation (ie, median) and least median of squares (ie, *lms*). Because the percentage of CD63^{hi} basophils were bounded (they cannot be <0 or >100), we also extended the mean fitting procedure by using a “constrain” estimation,²⁶ restricting the lower and upper bounds (so-called box constraints) to those allowed by the data. For each of these 4 estimating procedures, we selected the dose-response curve with the smallest Akaike information criterion. Nondeterminable ED50 values were imputed by using multivariate imputation with chained equations,²⁷ with application of the random forest algorithm. The AUC was calculated by using trapezoidal approximation. Basophil activation analysis packages available at www.github/saritaupatil/AutoBAT as well as hub.docker.com/r/saritaupatil/autobot. Relative importance was calculated by using the *relimp* package.²⁸ Classifier performance was conducted by using the *RORC* package.²⁹ Passive basophil experiments were analyzed by using ANOVA and paired *t* tests. Data analysis was conducted in R 3.2.1 software.²⁵

RESULTS

Peanut OIT clinical outcomes

Of the 30 subjects recruited for the clinical trial, 27 received treatment with peanut (Fig 1 and Table I). During

generalized additive mixed model (with smoothing by Laplace approximate) to Ara h 2 (Fig 3, C) and peanut (Fig 3, D) in subjects with SU (blue) or TD (dashed line, red). The y-axis is logarithmically scaled for basophil AUC, the peanut avoidance period is shaded in gray, and the black arrow marks 3 months of OIT. Labeled visits on the x-axis include pre-OIT (B), build-up visits (B), maintenance (M), post-OIT oral food challenge (O), postavoidance double-blind food challenge (D), and follow-up visits (F). Lines indicate means, and shaded regions indicate SDs. Lettered labels on the x-axis refer to visits, pre-OIT (P), build-up (B), maintenance (M), post-OIT oral food challenge (O), postavoidance double-blind food challenge (D), and follow-up (F). **E**, The proportion of variance in Ara h 2 ED50 change from baseline to postavoidance explained by quantitative measurements of serum Ara h 2-specific immunoglobulins.

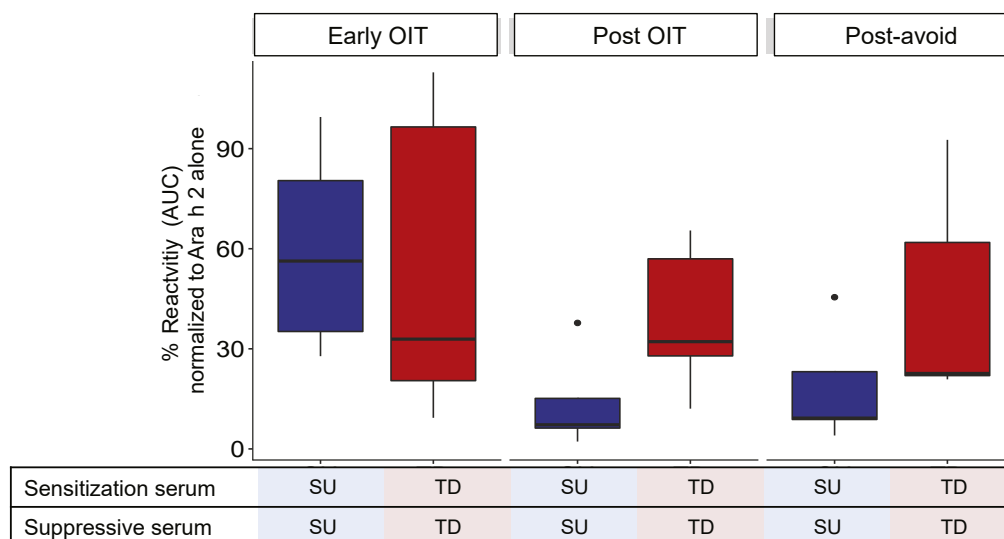


FIG 5. Basophil AUC suppression is increased after OIT and after avoidance in subjects with SU. Basophil reactivity, measured as AUC, of basophils stimulated with Ara h 2 and plasma from subjects with SU (blue) or TD (red) were normalized to Ara h 2 stimulation alone. The suppressive plasma was collected from 3 time points: during early OIT (1-3 months), after OIT, or during the postavoidance period. These indirect basophil activation assays were performed after stripping off endogenous IgE and passively sensitizing healthy donor PBMCs ($n = 5$).

immunotherapy, all subjects experienced at least 1 adverse event related to dosing. Of the adverse events, 96% were mild, only 4% were moderate, and there were no severe adverse events. A total of 17 events (0.5% of all reported adverse events) required epinephrine administration among a total of 10 of the subjects. Adverse events requiring epinephrine treatment occurred most often during the build-up phase (11 events, 65%), followed by food challenges (5 events, 30%) and maintenance (1 event, 5%). During the study, 3 subjects withdrew because of persistent adverse events, and 1 subject withdrew because of failure to tolerate the minimal dose of peanut at the beginning of dosing.

Of the 23 subjects who completed active immunotherapy and underwent an oral challenge to assess desensitization, 1 subject did not tolerate 5 g of peanut. Of the remaining 22 subjects who reached the primary end point, 9 achieved SU and tolerated 5 g of peanut on challenge after 4 weeks of peanut avoidance, and 13 regained responsiveness to peanut after avoidance and were designated as achieving TD.

Subjects with TD required more time to build up to their maintenance dose (434 ± 39 vs 394 ± 41 days, $P = .034$) because of reactions during that build-up phase requiring dose modifications. Patients with SU were given a diagnosis of peanut allergy earlier in life than those with TD (1.44 ± 0.46 vs 2.23 ± 0.82 years, $P = .02$); no other differences in baseline age, sex, or ethnicity were found between these subjects (Table 1).

Kinetics of specific immunoglobulins

Consistent with previously published studies,^{4,21,30} antigen-specific immunoglobulins changed over the course of OIT (Fig 2), and pretreatment serum peanut- and Ara h 2-specific IgE levels tend to be greater in patients with TD compared with those with SU (see Fig E1, A, in this article's Online Repository at www.jacionline.org). In this clinical trial we focused on Ara h 2, an immunodominant peanut antigen.³¹ Circulating Ara h

2-specific IgE levels increased transiently and early in all subjects, increasing from baseline for the first 20 weeks ($P < .001$) in subjects with TD. Total circulating IgE levels also increased from 6 to 20 weeks ($P < .05$) in subjects with TD, but a similar trend did not reach significance in subjects with SU (see Fig E1). A significant decrease in Ara h 2-specific IgE levels from pre-OIT levels occurred after OIT in subjects with TD (70.3 ± 1.6 to 42.7 ± 1.5 kU/L, $P = .004$), and although there was a similar trend in subjects with SU, it did not reach significance.

In this study, peanut-specific IgE levels at baseline correlated with clinical outcomes after OIT. Before the start of OIT, subjects with TD had significantly greater levels of Ara h 2-specific IgE than those with SU (70.3 ± 1.6 vs 8.6 ± 1.7 kU/L, $P = .002$; Fig 2, B). However, the ratio of Ara h 2-specific IgE to total IgE was not significantly different (0.2 ± 0.1 vs 0.3 ± 0.2 , $P = .5$).

Similar to previous studies, circulating Ara h 2-specific IgG and IgG₄ levels increased during OIT. Notably, the increase in Ara h 2-specific IgE and IgG levels occurred early in OIT, and Ara h 2-specific IgG levels peaked at the end of the build-up phase (Fig 2, B). In this study, after peanut OIT, subjects with TD had greater Ara h 2 IgG levels than subjects with SU (51.2 ± 1.3 vs 17.5 ± 1.4 ng/mL, $P = .01$), with persistence through the postavoidance period (43.8 ± 1.3 vs 15.1 ± 1.4 ng/mL, $P = .01$; Fig 2, B). There was a trend toward an increased Ara h 2-specific IgG₄ level in subjects with TD compared with those with SU after OIT (24.7 ± 1.3 vs 11.6 ± 1.4 ng/mL, $P = .07$) that disappeared after avoidance (18.1 ± 1.3 vs 8.9 ± 1.4 ng/mL, $P = .11$; Fig 2, B).

Ratios of Ara h 2 IgE to Ara h 2 IgG and ratios of Ara h 2 IgE to Ara h 2 IgG₄ were significantly increased in subjects with TD compared with those with SU at baseline (IgE/IgG: 27.4 ± 3.1 vs 3.0 ± 3.4 , $P = .003$; IgE/IgG₄: 135.8 ± 3.6 vs 21.4 ± 4.0 , $P = .006$) but were more similar after OIT (IgE/IgG: 5.3 ± 2.9

vs 3.4 ± 3.1 , $P = .1$; IgE/IgG₄: 8.2 ± 3.1 vs 4.0 ± 3.4 , $P = .09$) or 1 month of avoidance (IgE/IgG: 5.8 ± 2.9 vs 3.6 ± 2.9 , $P = .1$; IgE/IgG₄: 9.4 ± 3.1 vs 4.3 ± 3.4 , $P = .06$; Fig 2, C).

Basophil suppression during OIT

To study a functional correlate of the humoral response to immunotherapy, we monitored peanut allergen-induced basophil activation *ex vivo* in peripheral blood from subjects during the clinical trial (Fig 3, A). Basophil AUC can correlate with the degree of clinical symptoms on challenge (Fig 3, B).³² Stimulation with whole peanut protein was compared with Ara h 2 (Fig 3, C and D).

Despite the significant differences in Ara h 2 IgE levels, basophil AUCs before OIT were similar in subjects with SU and those with TD when basophils were stimulated with either Ara h 2 (68.2 ± 1.5 vs 85.3 ± 1.4 , $P = .7$; Fig 3, C) or peanut (85.3 ± 1.5 vs 112.2 ± 1.4 , $P = .6$; Fig 3, D). After OIT, basophil AUCs were significantly suppressed from baseline in subjects with SU (Ara h 2: 68.2 ± 1.5 vs 8.1 ± 1.5 , $P < .001$; peanut: 85.3 ± 1.5 vs 12.6 ± 1.5 , $P < .001$), as well as in subjects with TD (Ara h 2: 85.3 ± 1.4 vs 17.6 ± 1.4 , $P < .001$; peanut: 112.2 ± 1.4 vs 19.0 ± 1.4 , $P < .001$). Basophil AUC to both Ara h 2 and peanut was similar in subjects with SU and those with TD at the end of OIT. However, after 1 month of avoidance, basophil AUC in subjects with TD rebounded, as did clinical reactivity, as opposed to that seen in subjects with SU in whom both basophil AUC and clinical reactivity remained suppressed (Ara h 2: 12.0 ± 1.5 vs 93.4 ± 1.4 , $P = .0001$; peanut: 14.6 ± 1.5 vs 101.8 ± 1.4 , $P = .0005$). These patterns were also found with basophil activation to Ara h 6 (see Fig E2 in this article's Online Repository at www.jacionline.org).

Basophil sensitivity, measured as the ED₅₀ of the dose-dependent basophil activation curve, has been shown to change with other forms of immunotherapy.³³ Previous work suggests that basophil sensitivity can change because of the valency of allergen and surface IgE interactions,¹² which we expect to change with induction of an effective inhibitory antibody response. Therefore we sought to test whether basophil sensitivity was more suppressed in subjects with SU compared with subjects with TD.

At baseline, basophil sensitivity was similar in subjects with SU and those with TD to both Ara h 2 (SU: 1.9 ± 1.5 ng vs TD: 3.8 ± 1.4 ng, $P = .2$) and whole peanut (SU: 3.5 ± 1.6 vs TD: 2.4 ± 1.4 , $P = .5$; Fig 3, C and D). After OIT, basophil sensitivity to Ara h 2 significantly decreased in subjects with SU but not those with TD (SU: 22.4 ± 1.5 ng vs TD: 5.0 ± 1.4 ng, $P = .005$), which was persistent after avoidance (SU: 15.8 ± 1.5 ng vs TD: 2.8 ± 1.4 ng, $P = .001$). In comparison, the differences in basophil sensitivity to peanut after OIT and after avoidance were not significant (post-OIT SU: 29.1 ± 1.5 ng vs post-OIT TD: 12.1 ± 1.4 ng, $P = .1$; postavoidance SU: 23.1 ± 1.5 ng vs postavoidance TD: 9.0 ± 1.4 ng, $P = .08$). Basophil sensitivity and AUC to Ara h 6 were similar to those to Ara h 2 (see Fig E2).

To evaluate the influence of immunoglobulin levels on the OIT-induced change in basophil sensitivity to Ara h 2, we compared the change from before OIT to after OIT in bulk Ara h 2-specific serum IgE and IgG levels to the change in the ED₅₀ to Ara h 2. Using a multiple linear regression model to analyze the relative importance of antigen-specific immunoglobulin levels, we found that only about 20.8% of the change in

basophil sensitivity to Ara h 2 is accounted for by changes in bulk measurements of Ara h 2-specific serum immunoglobulins, with the largest contributions by the difference in Ara h 2-specific IgE and total IgE levels (Fig 3, E).²⁸ Our data suggest that additional qualitative factors of induced immunoglobulins, such as clonality or affinity, might be more relevant to the decrease in basophil sensitivity and the clinical efficacy of OIT.

Early changes in basophil sensitivity to Ara h 2 as a biomarker of SU

We have previously described early immunologic changes in the humoral response to Ara h 2.⁸ To test our hypothesis that the decrease in basophil sensitivity to Ara h 2 might be an effective biomarker of future SU, we used SU and TD in a binary classifier system. The fold change in early basophil sensitivity, as measured by the change in ED₅₀ to Ara h 2 from baseline to 3 months on OIT, was significantly greater in subjects with SU than in those with TD (median, 18.9- vs 3.1-fold change; $P = .01$; Fig 4, A). The receiver operating characteristic for the early change of basophil AUC to Ara h 2 was 0.84, with an optimal fold change cutoff of 4.9 compared with the receiver operating characteristic of the change in peanut-stimulated basophil AUC (0.66), which has an optimal cutoff of 8.5 (Fig 4, B-E).

Indirect basophil suppression to Ara h 2 by serum

We hypothesized that basophil suppression is persistent after avoidance because of serum immunoglobulins in subjects with SU. To test this hypothesis, we used an indirect basophil activation test with PBMCs from healthy donors. After removal of endogenous cell-bound IgE and passive sensitization using plasma from individual subjects with SU or subjects with TD at baseline, we stimulated cells with Ara h 2 alone or in the presence of plasma from individual subjects with SU or TD during or after OIT. We were able to compare suppression by using the same subject's plasma across 3 time points: early OIT (1-3 months after starting OIT), after OIT, or after avoidance (see Fig E3 in this article's Online Repository at www.jacionline.org).

Similar to the kinetics of basophil activation in *ex vivo* basophils, we demonstrated suppression of Ara h 2-stimulated basophils derived from healthy donors and sensitized with plasma from early OIT-treated subjects. Relative basophil AUC normalized to stimulation in medium decreased after OIT in the entire group of subjects ($62.5\% \pm 39.8\%$ vs $32\% \pm 36\%$, $P = .0005$) and remained similar through the postavoidance period ($32\% \pm 33.5\%$, $P = .98$). Moreover, basophil AUC was significantly lower when using plasma from subjects with SU than from subjects with TD after OIT (median, $6.4\% \pm 27.1\%$ vs $38.9\% \pm 39.3\%$; $P = .02$) and after avoidance (median, $12.3\% \pm 26.5\%$ vs $30.2\% \pm 37.2\%$; $P = .03$; Fig 5). Sensitivity to Ara h 2 tended to be lower in postavoidance plasma-treated basophils from subjects with SU compared with those from subjects with TD (ED₅₀ mean, 2.1 ± 2.2 vs 1.8 ± 1.7 ; $P = .06$; see Fig E4 in this article's Online Repository at www.jacionline.org).

DISCUSSION

In this study, we demonstrate that basophil sensitivity to Ara h 2 is a useful biomarker of sustained clinical efficacy after peanut OIT. In those with SU after avoidance, basophil sensitivity was

significantly suppressed, as evidenced by an increase in the ED50 of the Ara h 2–stimulated basophil activation. On the other hand, basophils from subjects who had only TD not only failed to change their basophil sensitivity, either early or later in OIT, but also had a significant rebound in their basophil AUC values. Hence monitoring basophil activation to Ara h 2 has 2 potential uses during OIT, not only as an early biomarker to identify those likely to have SU but also as a marker after OIT to evaluate when clinical reactivity to peanut might be likely to return.

Our findings underscore other recent studies on several points. First, the overall kinetics of basophil activation are similar to those in other studies, with suppression of the AUC, whereas the dose of peanut administered increases during immunotherapy.³⁴

Second, the mirroring of clinical reactivity and basophil AUC after avoidance is parallel to that in a previous study of patients with peanut allergy, showing that severity of clinical reactions correlates with basophil AUC.¹¹ On the other hand, basophil sensitivity correlated to the clinical threshold on oral challenge, which might be similar to our finding that basophil sensitivity decreases in patients who have long-term tolerance of higher amounts of peanut after OIT and avoidance. To facilitate reproducible basophil activation data analysis for future applications, we pioneered automated flow cytometric analysis on the commercially available basophil activation kit used in this study and refined a robust statistical analysis for measures of basophil activation.²²

Third, allergen-specific IgG or IgG₄ is consistently induced by OIT but without any significant correlation to clinical outcomes. Serum from those with clinical protection after avoidance demonstrated greater suppression of indirect basophil activation testing, suggesting that neutralizing antibody activity, rather than concentration, does correlate with clinical reactivity, specifically with sustained clinical protection.

Lastly, although not predictive, pretreatment specific IgE levels tend to be greater among those who do not attain persistent clinical tolerance, at least in the relatively short time frames that have been evaluated in trials of this nature. Although 4 weeks of avoidance does not indicate long-term tolerance, it does stratify the patients in a manner that correlates with differences in OIT-induced peanut-specific immune responses.

As allergic effector cells, basophils can serve as an integrative measure of clinical efficacy in allergen immunotherapy. The valency of the interaction between antigen and basophil surface IgE levels has been shown to directly influence basophil sensitivity.^{12,35} Correlation of basophil suppression with clinical tolerance after immunotherapy highlights its utility as a monitoring tool for the efficacy of peanut immunotherapy³⁶ without subjecting patients to repeat oral challenges. Moreover, the indirect basophil test captures the functional ability of specific antibodies to suppress basophil activation. In this study, qualitative rather than quantitative Ara h 2–specific immunoglobulin changes correlated with clinical tolerance after immunotherapy because subjects with SU had lower levels of induced Ara h 2–specific IgG with stronger suppressive ability. At baseline, those with TD have significantly greater levels of IgE, but by the end of active immunotherapy, the Ara h 2 IgE/IgG ratio is similar, again underscoring that qualitative rather than quantitative differences in sera of subjects with SU account for their stronger suppressive capabilities. Basophil sensitivity is modulated by the effective valency of antigen-immunoglobulin interactions, which in turn are affected by specific antibody

repertoire. Postimmunotherapy suppression of basophil activation by sera can be influenced by many factors, including inhibitory Fc receptor engagement,¹⁴ as well as antigen sequestration from IgE cross-linking on the basophil surface. Further study of the relative contributions of these diverse mechanisms might shed light on other regulatory mechanisms that could be important to clinical efficacy, including the affinity of immunoglobulin Fc for inhibitory receptors and modulation of inhibitory receptor signaling pathways.

In summary, the decrease in basophil sensitivity to the immunodominant antigen Ara h 2 is persistent in OIT-treated patients with SU and can be used as an early biomarker to identify those most likely to attain durable clinical efficacy. On the other hand, basophil AUC to Ara h 2 levels after OIT closely mirrors clinical reactivity to peanut in OIT-treated subjects. The OIT-induced suppression of basophil activation is influenced by qualitative characteristics of the induced immunoglobulin repertoire. Because humoral responses are likely an important component of long-lived tolerance to allergen induced by immunotherapy, refined measures of induced immunoglobulin responses will help us better understand, differentiate, and promote more effective clinical responses to immunotherapy.

The peanut OIT clinical trial results are available through [clinicaltrials.gov](https://clinicaltrials.gov/NCT01324401) NCT01324401. We would like to thank our clinical research coordinators, Lauren Tracy, Alanna Hickey, and Caroline Southwick, for their assistance with clinical trial administration. We thank Cecilia Washburn for her technical assistance. We also thank our dedicated patients and families for their participation and support.

Key messages

- The decrease in post-OIT basophil sensitivity to Ara h 2 in those with SU has utility as early as 3 months during OIT.
- Basophil AUC to Ara h 2 is a biomarker of clinical reactivity after OIT in patients with TD.

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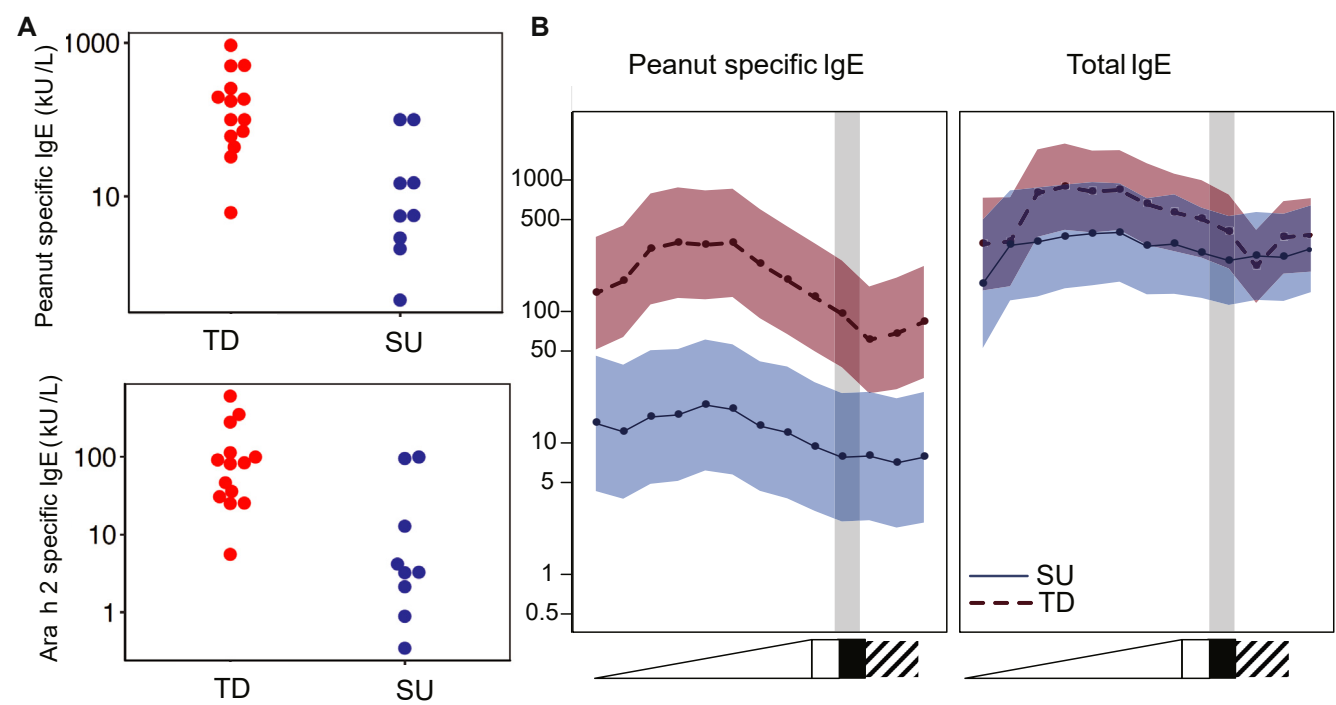


FIG E1. Serum antigen-specific IgE during baseline and peanut OIT. **A**, Serum peanut-specific IgE and Ara h 2 IgE in subjects with SU (blue) and those with TD (*dashed line*, red) at baseline. **B**, Serum peanut-specific IgE and total IgE in subjects with SU (blue) and TD (*dashed line*, red) over time determined by using a logistic regression model with random intercepts. *Lines* indicate means, and *shaded regions* indicate SDs.

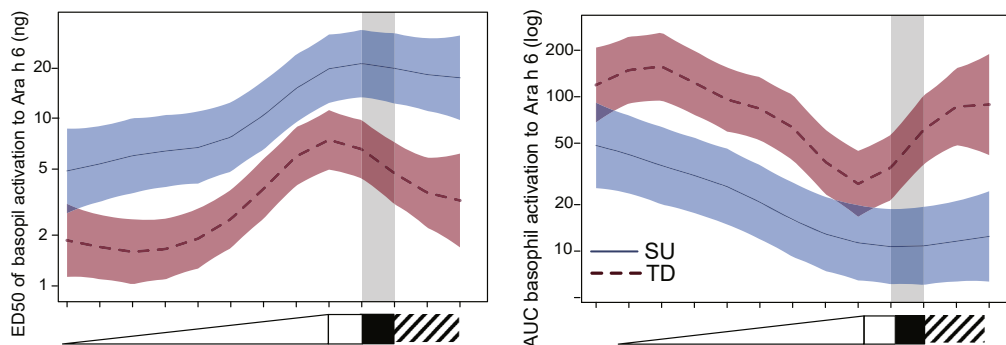


FIG E2. Direct *ex vivo* basophil sensitivity to Ara h 6. Direct *ex vivo* basophil sensitivity, as measured by ED50, and basophil AUC of the dose-response curve to Ara h 6 by using a generalized additive mixed model (with Laplace approximate smoothing) in subjects with SU (blue) and TD (*dashed line*, red). The *y-axis* is logarithmically scaled in the AUC graph, and the period of peanut avoidance is shaded in gray. *Lines* indicate means, and *shaded regions* indicate SDs.

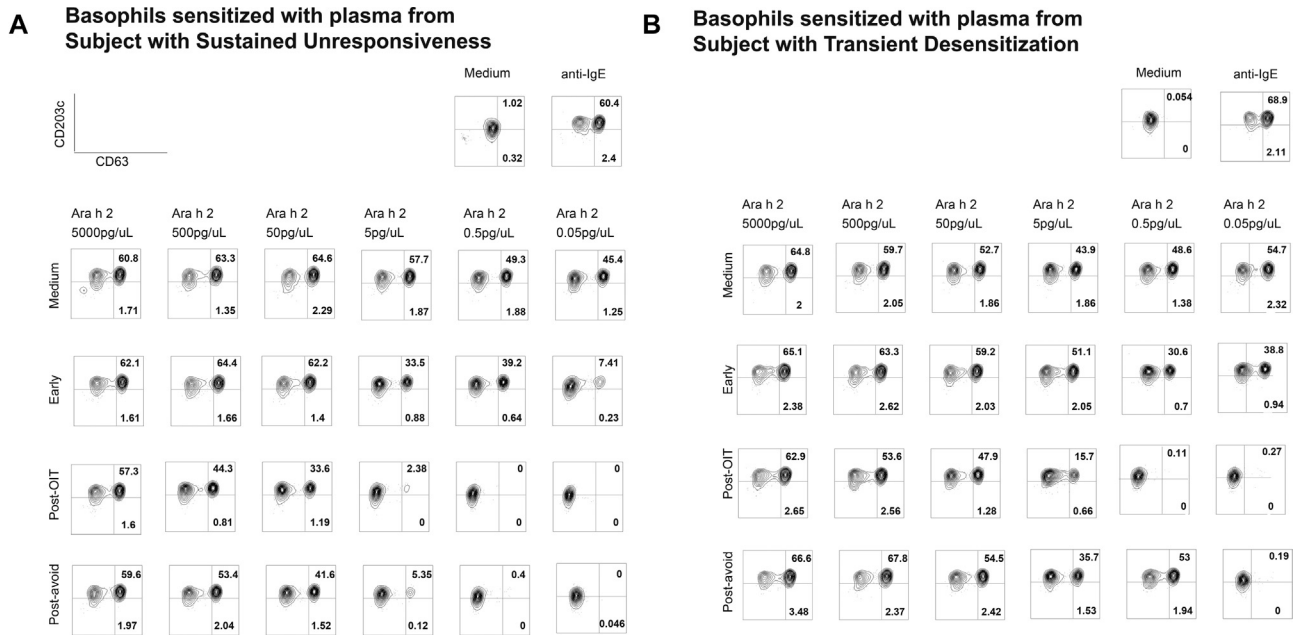


FIG E3. Suppression of basophil activation by plasma. Basophils from normal donors were sensitized with SU plasma (**A**) or TD plasma (**B**) activated with various doses of Ara h 2 and suppressed with early, post-OIT, and postavoidance serum from the same patient or with medium only. Experimental controls included sensitized basophil stimulation with medium or anti-IgE. Basophil activation was assessed by using flow cytometry, including CD203c (*y-axis*) and CD63 (*x-axis*). Percentages of CD63^{hi} activated basophils were quantified (*right-sided quadrants*) for calculation of the AUC or ED50.

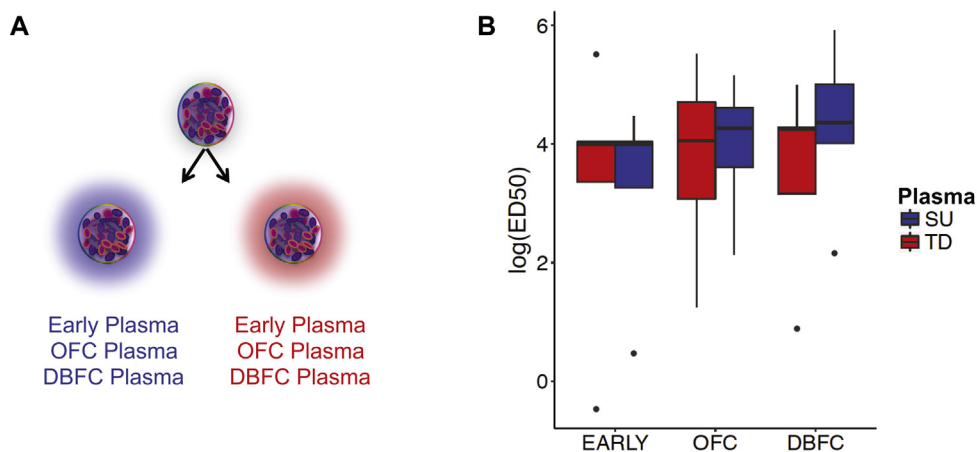


FIG E4. Plasma from post-OIT subjects with SU decreases basophil sensitivity to Ara h 2. **A**, Basophils from normal donors were sensitized with SU (blue) or TD (red) plasma and stimulated with Ara h 2 co-incubated with plasma from several time points. **B**, Basophil sensitivity measured as the ED50 of the dose-response curve of basophils suppressed in SU (blue, n = 5) or TD (red, n = 5) serum from early, post-OIT, and post-avoidance time points of basophils sensitized with subjects with SU (*top panel*) or TD (*bottom panel*) IgE on activation with Ara h 2. Comparison used paired Wilcoxon rank sum tests.