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ORIGINAL ARTICLE

Food Allergy and Gastrointestinal Disease

Oral desensitization therapy for peanut allergy induces dynamic changes in peanut-specific immune responses

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Abstract

Background: The PALISADE study, an international, phase 3 trial of peanut oral immunotherapy (POIT) with AR101, resulted in desensitization in children and adolescents who were highly allergic to peanut. An improved understanding of the immune mechanism induced in response to food allergen immunotherapy would enable more informed and effective therapeutic strategies. Our main purpose was to examine the immunological changes in blood samples from a subset of peanut-allergic individuals undergoing oral desensitization immunotherapy with AR101.

Methods: Blood samples obtained as part of enrollment screening and at multiple time points during PALISADE study were used to assess basophil and CD4+ T-cell reactivity to peanut.

Results: The absence of clinical reactivity to the entry double-blinded placebo-controlled peanut challenge (DBPCFC) was accompanied by a significantly lower basophil sensitivity and T-cell reactivity to peanut compared with DBPCFC reactors. At baseline, peanut-reactive TH2A cells were observed in many but not all peanut-allergic patients and their level in peripheral blood correlates with T-cell reactivity to peanut and with serum peanut-specific IgE and IgG4 levels. POIT reshaped circulating peanut-reactive T-cell responses in a subset-dependent manner. Changes in basophil and T-cell responses to peanut closely paralleled clinical benefits to AR101 therapy and resemble responses in those with lower clinical sensitivity to peanut. However, no difference in peanut-reactive Treg cell frequency was observed between groups.

Conclusion: Oral desensitization therapy with AR101 leads to decreased basophil sensitivity to peanut and reshapes peanut-reactive T effector cell responses supporting its potential as an immunomodulatory therapy.

KEYWORDS
basophils, CD4+ T cells, oral immunotherapy, peanut allergy, Th2A cells

Abbreviations: BAT-EC50, concentration of allergen corresponding to 50% of maximal activation of basophils in the basophil activation test; CCR6, C-C motif chemokine receptor 6; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; DBPCFC, double-blinded placebo-controlled peanut challenge; FOXP3, forkhead box P3; freq, frequency; GATA3, GATA binding protein 3; HPGDS, hematopoietic prostaglandin D synthase; IFNG, interferon gamma; IL, interleukin; PALISADE, Peanut Allergy Oral Immunotherapy Study of AR101 for Desensitization in Children and Adults; POIT, peanut oral immunotherapy; PPARG, peroxisome proliferator-activated receptor alpha; pTeff, peanut-reactive T cell; RORC, RAR-related orphan receptor C; ST2, suppression of tumorigenicity 2.

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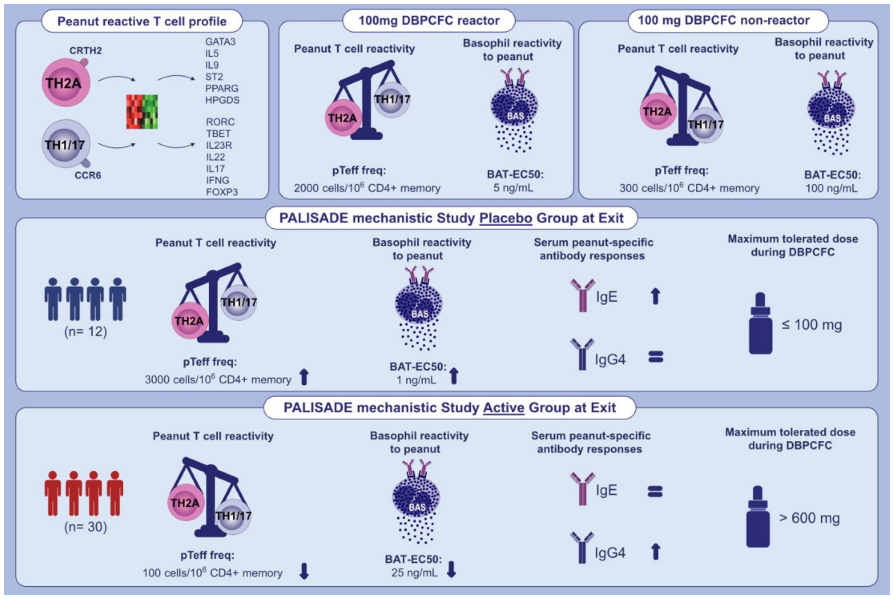

GRAPHICAL ABSTRACT

CRTH2+ pTeff cells and CCR6+ pTeff cells represent two mutually exclusive, nonoverlapping cellular and molecular entities involved in food-allergic diseases. Circulating CRTH2+ pTeff cells are mostly restricted to peanut-allergic individuals who react to the 100 mg DBPCFC compared to those with lower clinical sensitivity to peanut. Changes in basophil and T-cell responses to peanut closely parallel clinical benefits to POIT and resemble responses in those that did not react to the baseline 100 mg DBPCFC.
Abbreviations: BAT-EC50, concentration of allergen corresponding to 50% of maximal activation of basophils in basophil activation test; CCR6, C-C motif chemokine receptor 6; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; DBPCFC, double-blinded placebo-controlled peanut challenge; FOXP3, forkhead box P3; freq, frequency; GATA3, GATA binding protein 3; HPGDS, hematopoietic prostaglandin D synthase; IFNG, interferon gamma; IL, interleukin; PALISADE, Peanut Allergy Oral Immunotherapy Study of AR101 for Desensitization in Children and Adults; POIT, peanut oral immunotherapy; PPARG, peroxisome proliferator activated receptor alpha; pTeff, peanut-reactive T cell; RORC, RAR related orphan receptor C; ST2, suppression of tumorigenicity 2

1 | INTRODUCTION

Peanut allergy is increasingly prevalent among children in the United States and other industrialized countries and is now estimated to affect approximately 2% of children.[1–4] While there are currently no approved treatment options, peanut allergy usually persists into adulthood, can be life-threatening, and accounts for most deaths related to food allergy.[5–8] Previously published academic studies suggest that oral immunotherapy (OIT), in which a preparation of food allergen is mixed into a vehicle and then ingested in gradually increasing quantities, may hold promise as a treatment of peanut allergy.[9,10] Goals for OIT-induced desensitization include reduced reactivity to an extent that can provide reliable protection against a potentially severe or life-threatening reaction after an accidental exposure.

The PALISADE (Peanut ALlergy Oral Immunotherapy Study of AR101 for DEsensitization in Children and Adults) study was an international, randomized, double-blinded, placebo-controlled phase 3 trial of AR101 in 554 peanut-allergic (PA) patients aged 4–55 years (ClinicalTrials.gov identifier NCT02635776). AR101 (now approved by the US Food and Drug Administration and the European Commission as Palforzia®) is an oral biologic drug product with a characterized peanut–protein profile for use in OIT in subjects with peanut allergy.[11] Following 1 year of treatment, 67% of those in the active treatment group, compared with 4% receiving placebo, were able to tolerate 600 mg of peanut protein,[12] a level of peanut protein that exceeds the amount of peanut typically triggering a reaction with accidental ingestion.[13] Although clinical efficacy is the final test of these approaches, a better understanding of immunological responses to treatment could play an important role in patient selection and treatment monitoring. We established an optional substudy of deep immune profiling among PALISADE participants to pursue these aims. The success of OIT is attributed to the modulation of allergen-specific cellular and humoral immune responses, but the specific mechanisms by which OIT provides a desensitization state remains unclear. Such mechanistic insight can help in the improvement of current therapeutic approaches, in the prediction of therapy success, and in the identification of companion biomarkers of therapeutic responses. Basophils are important effector cells in IgE-mediated allergy and have emerged as a useful biomarker reflecting the clinical threshold for eliciting symptoms and for the evaluation of clinical efficacy in allergen immunotherapy.[14–17] The number of basophils that respond to a given dose of stimulus is defined as basophil reactivity and can be measured by using activation markers such as CD203c or CD63.[18] Moreover, by stimulating the basophils *in vitro* with decreasing doses of allergen, the smallest amount of allergen able to activate the basophils is presented as basophil allergen threshold sensitivity.[19] In this study, we hypothesized that PA individuals who underwent AR101 oral desensitization

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therapy would be shifted toward lower basophil allergen threshold sensitivity. Antigen-specific T cells play a central role in mediating specific immune responses and in the formation of immunological memory. Previous studies have shown that the peripheral CD4+ T-cell responses elicited by food allergen in allergic subjects are transcriptionally and functionally heterogeneous.[20–22] In this study, we reasoned that the therapeutic benefit that results from POIT may depend on the inherent qualities of the immune response of an individual that influences the frequency and functional phenotype of responsive allergen-specific T cells. This emphasizes the need for assays that accurately detect and quantify T-cell-mediated, antigen-specific immune responses with minimal *in vitro* manipulation.

The aim of this substudy was to define key mechanistic correlates of the reduction in clinical reactivity to peanut observed in PA children and young adults during PALISADE. We also investigated the immune basis underlying reactivity to ≤100 mg peanut protein among PA participants who underwent DBPCFC prior to therapy. As patients progress on active desensitization therapy, we observed a subset-dependent drop in peanut-specific T effector (pTeff) frequency along with attenuated basophil responses, compared with placebo. Our findings suggest that a shift in pTeff cell subset immunodominance is related to, and possibly indicative of, the immunomodulatory potential of POIT.

2 | MATERIALS AND METHODS

2.1 | Clinical study

Individuals aged 4–55 years who had a clinical history of peanut allergy were eligible for participation in the trial if they had a serum peanut-specific IgE level of ≥0.35 kUA/L (allergen-specific unit) according to ImmunoCAP (Thermo Fisher Scientific), a mean wheal diameter that was at least 3 mm larger than the negative control on skin prick testing for peanut, or both. Participants were then required to experience allergic dose-limiting symptoms at a challenge dose of ≤100 mg of peanut protein in a double-blind, placebo-controlled food challenge (DBPCFC) conducted during the screening period in accordance with modified PRACTALL guidelines.[23] Participants with challenge-confirmed IgE-mediated peanut allergy were randomly assigned, in a 3:1 ratio, to receive AR101 or matched placebo in an escalating-dose program. Subjects initiated the study with a single-day dose–escalation from 0.5 mg to 6 mg of study product; if tolerated, they returned to clinic the following day and were orally administered 3 mg/d for the next 2 weeks. Doses were escalated biweekly over the course of about 22 weeks to the target maintenance dose of 300 mg/day, which then continued daily for approximately 6 months before undergoing an exit DBPCFC to measure the primary endpoint. The study design and clinical outcomes have been reported previously.[12] Briefly, in the intent-to-treat analysis, 250 of 372 participants (67.2%) who received active treatment, as compared to 5 of 124 participants (4.0%) who received placebo, were able to ingest a dose of 600 mg or more of peanut protein, without dose-limiting symptoms, at the exit food challenge.

2.2 | Mechanistic study participant

This mechanistic study was an optional substudy of the PALISADE trial intended to provide a more comprehensive assessment of the immunological effects of oral immunotherapy with AR101. As participation in study was optional for PALISADE participants, it had no prespecified sample size and was not prospectively powered. Blood samples (10–20 ml) were collected before the screening DBPCFC, at the end of up-dosing and before the exit DBPCFC from PALISADE study. Additional informed consent for participation in this mechanistic substudy was obtained from all subjects or parents/guardians according to local ethical guidance, and all procedures were approved by the ethics committees at each clinical site. Participants who experienced allergic dose-limiting symptoms at a challenge dose of ≤100 mg of peanut protein were categorized as DBPCFC reactors, while those who tolerated the challenge were categorized as DBPCFC nonreactors. The nonreactor cohort included a mixture of patients who were tolerant to peanut and those who were allergic but not sensitive to small amounts of peanut. Subject demographics are detailed in Table 1. Coded samples were provided to the laboratory, which performed all assays in a blinded fashion.

2.3 | Basophil activation test

A CD203c-based basophil activation test (BAT) was performed within 24 h of blood draw. Whole heparinized blood was prepared as previously described[24–28] and stimulated for 30 min with a pool of 8 distinct allergen extracts (grass pollen, tree pollen, milk, egg, walnut, house dust mite, cat, and mold) as a positive control, media alone as a negative control, or serial dilution of peanut crude allergen extract (Stallergenes Greer). PE-Cy7-conjugated anti-CD3, fluorescein isothiocyanate-conjugated anti-chemoattractant receptor-homologous molecules expressed on TH2 lymphocytes (CRTH2), and phycoerythrin-conjugated anti-CD203c antibodies were added during the reaction. Basophils were detected based on the forward- and side-scatter characteristics, and negative CD3 and positive CRTH2 results. Up-regulation of CD203c on basophils was determined using a threshold that was defined by the fluorescence of unstimulated cells (negative control). Basophil allergen threshold sensitivity (EC-50) represents the concentration at which basophil activation is half of the maximum activation.[19]

2.4 | Blood processing

Blood samples were obtained in sodium heparin Vacutainer tubes and shipped overnight from participating sites in temperature-controlled boxes (GreenBox, ThermoSafe) assembled according to standard operating procedures. PBMCs were isolated by density centrifugation with Ficoll–Paque Plus (GE Healthcare).

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TABLE 1 PALISADE trial subject demographics for mechanistic studies

	Active POIT	Placebo	Screen failure	Overall
Number	30	12	14	56
Sex				
Male	14 (47%)	4 (33%)	11 (79%)	29 (52%)
Female	15 (50%)	7 (58%)	2 (14%)	24 (43%)
Age				
4–11 years	6 (20%)	5 (42%)	5 (36%)	16 (29%)
12–17 years	16 (53%)	6 (50%)	2 (14%)	24 (43%)
>17 years	7 (23%)	0 (0%)	6 (43%)	13 (23%)
Race				
Non-Hispanic Caucasian	22 (73%)	7 (58%)	13 (93%)	42 (75%)
Other	7 (23%)	4 (33%)	0 (0%)	11 (20%)
Baseline peanut sensitivity				
Median IgG4	0.39	0.85	NA	0.41
Median IgE	31.5	84.2	NA	44.7
Median SPT	12	14.5	NA	12.5
Median max tolerated dose	10	30	NA	10
History of anaphylaxis	22 (73%)	9 (75%)	9 (64%)	40 (71%)
History of asthma	17 (57%)	7 (58%)	6 (43%)	30 (54%)

2.5 | *Ex vivo* analysis of peanut-reactive CD4+ T cells

Peanut-reactive CD4+ T effector (pTeff) cells were tracked using the CD154 up-regulation assay.[29,30] Briefly, 10–20 × 10^6 freshly isolated PBMCs in culture medium at a concentration of 10 × 10^6 cells/ml were stimulated with a pooled library of 20-mer peptides derived from Ara h 1, Ara h 2, Ara h 3, Ara h 6, and Ara h 8 peanut-allergic components and 1 μg/ml of anti-CD40 blocking mAb (clone HB14, Miltenyi Biotec). After 14-h stimulation at 37°C, cells were harvested and labeled with PE-conjugated anti-CD154 mAb for 10 min at 4°C. Cells were then washed, labeled with anti-PE magnetic beads, and enriched by using a magnetic column, according to the manufacturer's instructions (Miltenyi Biotec). Magnetically enriched cells were next stained with antibodies against markers of interest and analyzed on a FACSAria™ II flow cytometer (BD Biosciences). Live memory CD45RA- CD154+ CD4+ T cells were considered as pTeff cells. A combination of the vital dye Via-Probe (BD Pharmingen) as a viability marker, CD19 (eBioscience), and CD14 (eBioscience) was used to exclude dead cells, B cells, and monocytes from the analysis, respectively. Frequency was calculated as previously described.[31] Data were analyzed with FlowJo software (Tree Star, Inc.).

2.6 | Peanut-specific T regulatory (Treg) assay

Peanut-reactive CD4+ T regulatory (pTreg) cells were tracked using the CD137/OX-40 up-regulation assay.[32–34] Briefly, unbound fractions from CD154 assay were collected and labeled with PE-Cy7-conjugated anti-CD137 mAb for 10 min at 4°C. The cells were then washed, labeled with anti-PE magnetic beads, and enriched using a magnetic column, according to the manufacturer's instructions (Miltenyi Biotec). Magnetically enriched cells were next stained with antibodies against markers of interest and analyzed on a FACSAria™ II flow cytometer (BD Biosciences). Live CD25+ CD127- FOXP3+ CD154- CD137+ OX-40+ CD4+ T cells were considered as pTreg cells.

2.7 | RNA-seq library preparation and analysis

A total of 100 sorted pTeff cells were sorted directly into reaction buffer from the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara), and reverse transcription was performed followed by PCR amplification to generate full-length amplified cDNA. Sequencing libraries were constructed using the Nextera XT DNA Sample Preparation Kit with unique dual indexes (Illumina) to generate Illumina-compatible barcoded libraries. Libraries were pooled and quantified using a Qubit® Fluorometer (Life Technologies). Sequencing of pooled libraries was carried out on a HiSeq 2500 sequencer (Illumina) with paired-end 53 base reads and a target depth of 5 million reads per sample. Base calls were processed to FASTQs on BaseSpace (Illumina), and a base call quality-trimming step was applied to remove low-confidence base calls from the ends of reads. The FASTQs were aligned to the University of California Santa Cruz (UCSC) human genome assembly version 19, using TopHat (v1.4.1), and gene counts were generated using htseq-count. QC and metrics analysis was performed using the Picard family of tools (v1.134). In total, 19 samples were sequenced. 16 (84%) of samples passed the quality criteria. To detect differentially expressed genes between sorted cell subsets, the RNA-seq analysis functionality of the linear models for microarray data (Limma) R package was used.[35] Expression counts were normalized using the TMM algorithm.[36] A

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false discovery rate adjustment was applied to correct for multiple testing. A FDR <0.05 and a fold change of at at least were used to define differentially expressed genes. The datasets described in this manuscript have been deposited in the NCBI Gene Expression Omnibus under accession number GSE196495.

2.8 | Immunoglobulin measurements

Blood was collected for assessment of peanut-specific antibody (IgE and IgG4) at baseline, the end of up-dosing, and study exit visits. Total IgE and peanut-specific IgE and IgG4 were measured using a commercial (ImmunoCAP) automated immunoassay system.[37]

2.9 | Statistical analysis

Prism software (GraphPad) and R were used for statistical analysis of flow cytometry data. No randomization or exclusion of data points was used. The nonparametric Mann–Whitney *U* test was used for unpaired comparisons between groups. The Spearman rank correlations were used to measure association between variables. Basophil sensitization curves were analyzed using R software (version 3.5.2; Vienna, Austria) (R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/), and MCMC was performed using Stan.[38] A hierarchical Bayesian nonlinear 3-parameter logistic growth model was fit using MCMC to estimate the parameters of each dose–response curve. This hierarchical approach allows for shrinkage of parameter estimates to occur to the extent that subjects and observations within subjects are similar to each other. Weakly informative priors were used to constrain parameters to lie within ranges reasonable for each parameter; for scale parameters, these take the form of half-Cauchy distributions; otherwise, normal distributions were assumed. Convergence of the MCMC algorithm was assessed visually using traceplots and using numeric diagnostics such as the potential scale reduction factor R. Estimates for parameters of interest were computed as the posterior medians, and where given, 95% posterior intervals were the 2.5th and 97.5th percentiles. Analysis code is shared online at https://github.com/BenaroyaResearch/Bajzik_et_al_AR101OralDesensitizationTherapy.

3 | RESULTS

3.1 | Basophil reactivity to peanut between DBPCFC reactors patients with peanut allergy and those with peanut sensitization who did not react to the entry DBPCFC

AR101 is indicated as oral immunotherapy to reduce the incidence and severity of allergic reactions that may occur with accidental exposure to peanut in patients aged 4–17 years with a peanut allergy. The baseline DBPCFC obtained as part of enrollment screening for PALISADE was first used to assess the performance of BAT as an indicator distinguishing between peanut-allergic individuals and those with lower clinical sensitivity to peanut in the absence of therapy. Since the screening DBPCFC stopped at 100 mg of peanut protein, the nonreactor population included a mixture of patients who were not allergic and those who were allergic but not sensitive to small amounts of peanut. Expression of the marker CD203c was used to quantify the magnitude of basophil activation in response to in vitro stimulation of whole blood with peanut allergen extract. Of the 40 peanut-sensitized subjects (30 DBPCFC reactors and 10 DBPCFC reactors) tested in the study, 2 DBPCFC reactors showed nonresponder basophils and were excluded from the analysis. Basophil allergen threshold sensitivity (EC-50) and maximal reactivity (CD-max) to peanut obtained during BAT were calculated from the subjects' dose–response curves to peanut allergen extract. As shown in Figure 1A, most patients exhibited positive basophil activation test to peanut extract. However, the dose–response for peanut-induced basophil activation of DBPCFC nonreactor individuals was shifted to higher concentrations compared with the dose–response of DBPCFC reactors (Figure 1A,B and Figure S1). Accordingly, DBPCFC nonreactors showed a significantly lower proportion of activated basophils at concentrations of peanut extract ranging from 10 to 100 ng/ml and a correspondingly lower basophil sensitivity, as expressed by higher EC-50 value compared with patients who clinically reacted to peanut during entry challenge (Figure 1A–C). Our data also indicate that nonreactors tended to have lower CD-max to peanut compared with DBPCFC reactors (Figure 1D and Figure S1). However, we did not observe any correlation between basophil reactivity and the serum peanut-specific IgE or serum peanut-specific IgG4 (data not shown). Clinically, our CD203-based BAT results in participants with challenge-confirmed peanut allergy showed no explicit correlation with the maximum tolerated dose (MTD) (Figure 1E,F), severity (Figure 1G,H), or symptoms experienced during entry DBPCFC (Figure S2).

3.2 | Peanut-reactive T-cell responses between participants with challenge-confirmed peanut allergy and those with peanut sensitization who did not react to the entry DBPCFC

Peanut-reactive CD4+ T effector (pTeff) cell responses were assessed *ex vivo* using the CD154 up-regulation assay following short stimulation (14 h) of freshly isolated PBMCs with a pool of overlapping peptide library spanning the entire Ara h 1, Ara h 2, Ara h 3, Ara h 6, and Ara h 8 peanut major allergens. As shown in Figure 2A, DBPCFC reactors had significantly higher pTeff cells compared to those with peanut sensitization who did not react to the 100 mg peanut protein entry challenge. Since antigen-specific Foxp3+ regulatory T cells do not selectively up-regulate CD154 upon stimulation,[33] the nonenriched cell fraction was reused following the CD154 magnetic bead enrichment step to assess peanut-reactive CD4+ T regulatory (pTreg)

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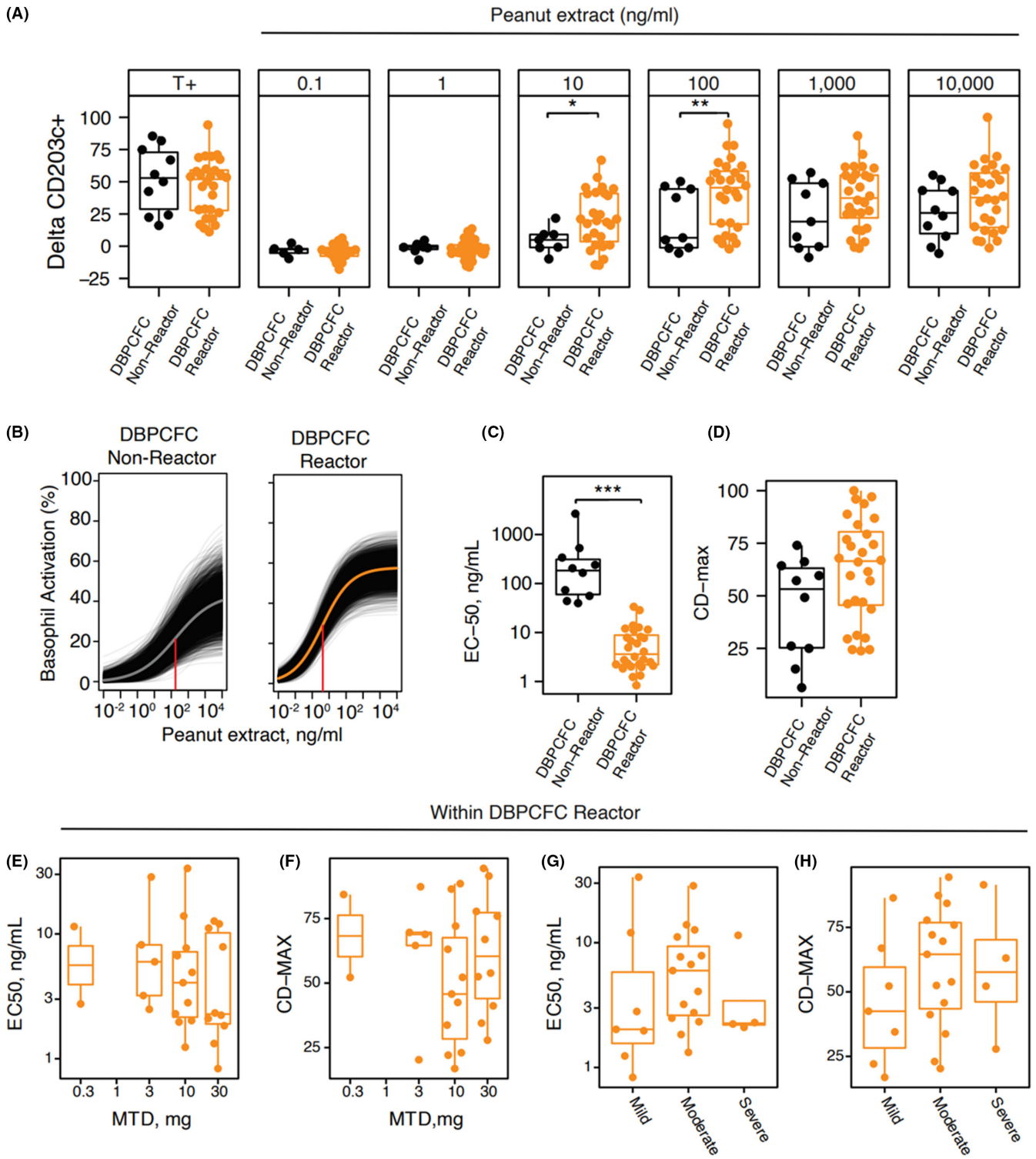

FIGURE 1 DBPCFC nonreactors have lower basophil sensitivity to peanut allergen extract. (A) Peanut dose–response of basophil activation between DBPCFC nonreactors (black dots; $n = 10$) and reactors (orange dots; $n = 28$) at baseline. (B) A Bayesian modeling approach was used to obtain logarithmic fits to the data and determine the concentration at which basophil activation is half of the maximum activation (EC-50) for each patient. (C, D) Statistical summary showing BAT EC-50 (C) and CD-max (D) values to peanut at baseline between DBPCFC nonreactors and reactor individuals. (E, F) Statistical summary showing BAT EC-50 (E) and CD-max (F) values to peanut based on threshold sensitivity to peanut during baseline DBPCFC in reactor individuals. (G, H) Statistical summary showing BAT EC-50 (E) and CD-max (F) values to peanut based on severity of allergic reaction during baseline DBPCFC in reactor individuals. Each dot represents distinct individuals. Differences between groups were analyzed by using the two-sided Mann–Whitney test. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$

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cell responses according to CD137 and OX-40 co-expression within FOXP3+ Treg cell subset.[32–34] Gating strategy is shown in Figure S3A. There were no significant differences between groups in the frequencies of pTreg (Figure 2B). In subjects not reacting to baseline DBPCFC, we observed higher expression of CD27 and of the TH17-associated surface marker CCR6 within pTeff cells relative to DBPCFC reactor (Figure 2C). In contrast, pTeff cells from DBPCFC reactors were characterized by higher expression of the TH2-associated surface marker CRTH2. Most CRTH2+ pTeff cells felt into the CD161+CD27- T-cell subset (Figure S3B), allowing us to consider them primarily TH2A cells. Although restricted to DBPCFC reactors, CRTH2 expression on circulating pTeff cells was observed in many but not all PA patients and seems to inversely correlate with CCR6 expression on pTeff cells (Figure 2D). For instance, subjects with the highest percentage of CCR6+ pTeff cells had lowest percentage of pTH2A cells and vice versa. Strikingly, the expression of CRTH2 and CCR6 within pTeff cells was noted to be mutually exclusive suggesting two distinct phenotypes in the pTeff populations (Figure S2B). We also observed an inverse correlation between proportion of CRTH2+ pTeff cells and proportion of CD27+ pTeff cells, whereas CCR6 expression within pTeff cells positively correlate with CD27 expression (Figure 2D).

To better understand this dichotomous pattern of CRTH2+ and CCR6+ pTeff cells in PA individuals, we next assessed the expression levels of transcripts associated with TH2A and TH1/TH17 signaling both in sorted CRTH2+ and in CCR6+ pTeff cell subset. Gene transcripts involved in a TH2A network such as *IL-17RB*, *IL-1RL1*, *IL-4*, *IL-5*, *IL-13*, *IL-9*, and *PPARG* were significantly up-regulated in CRTH2+ pTeff cells (Figure 3A–C). Conversely, Th1/Th17-related genes such as *IFNG*, *RORC*, *IL-17A*, *IL-17F*, *IL-23R*, and *IL-22* were enriched in CCR6+ pTeff cells implying a putative role of this pathway in food allergy. CCR6+ pTeff cells also contrasted transcriptionally with CRTH2+ pTeff cells, having a central memory differentiation state (CCR7 and CD27) and higher expression level of *FOXP3*, which suggest survival benefit during chronic antigen exposure and potential regulatory properties (Figure 3B, Table S1).

Consistent with a key role of pTH2A cells in driving peanut sensitization,[39–43] the expression of CRTH2 within pTeff cells correlated positively with baseline serum peanut-specific IgE level and with global pTeff cell frequency (Figure 4A,B). In contrast, the expression of CD27 and of CCR6 within pTeff cells in PA individuals tended to correlate negatively. Interestingly, we also observed a positive

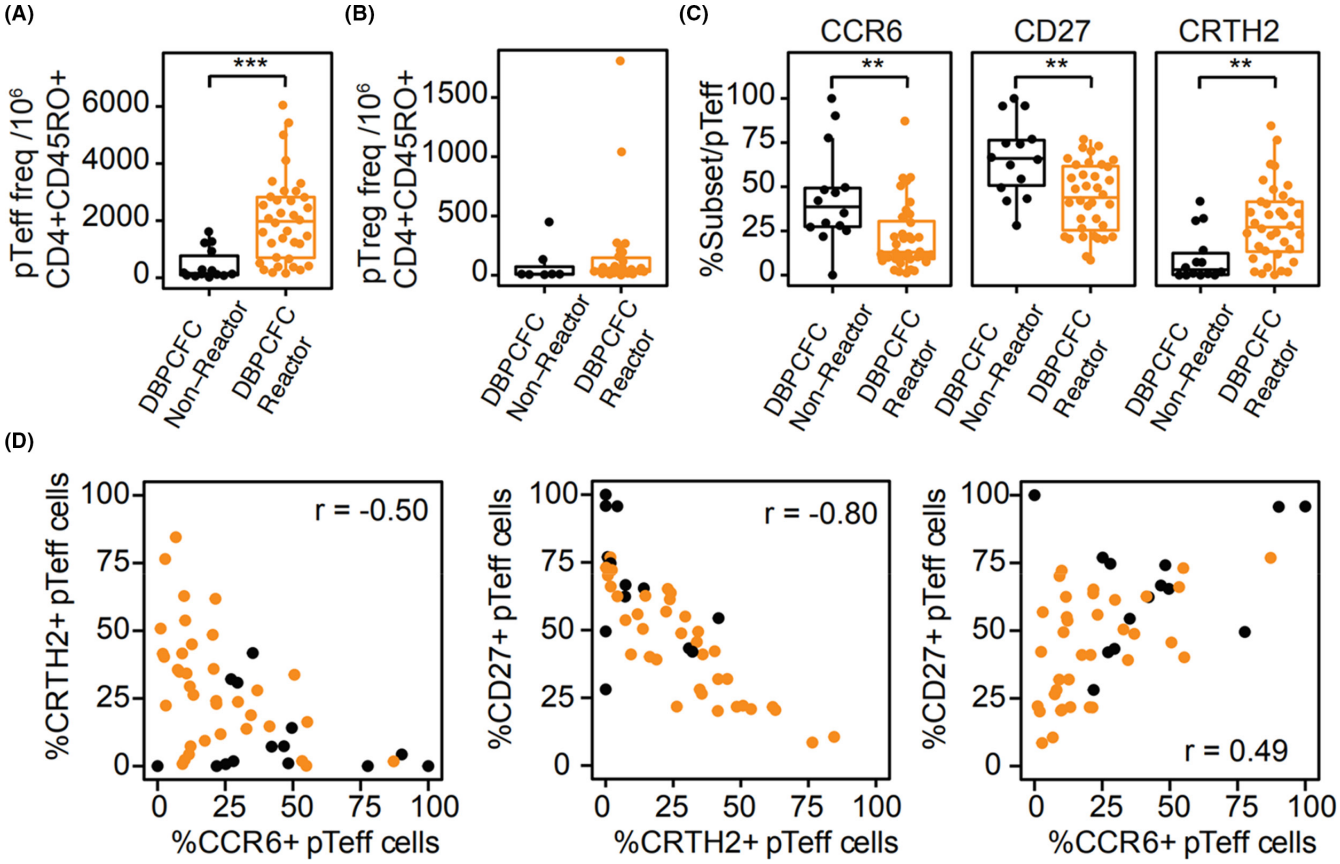

FIGURE 2 Ex vivo peanut-reactive T-cell profile between DBPCFC reactor and nonreactors. (A, B) *Ex vivo* frequencies of circulating pTeff cells (A) and pTreg cells (B) in DBPCFC reactor (orange box) and nonreactors (black box). (C) Percentages of CCR6; CD27, and CRTH2 expression within pTeff cells between DBPCFC reactor (orange dots) and nonreactor individuals (black dots). (D) Correlation between CRTH2+; CCR6+, and CD27+ pTeff cells in DBPCFC reactor (orange dots) and nonreactor individuals (black dots). (A–D) Each dot represents distinct individuals. Differences between groups were analyzed by using the two-sided Mann–Whitney test. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$

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correlation between the proportion of CRTH2+ pTeff cells and baseline serum peanut-IgG4 level suggesting a potential functional connection in B-cell-derived shifts from IgE to IgG4 (Figure 4C). However, no explicit correlation was observed between pTeff cell profile and skin prick test to peanut (Figure 4D), the severity of allergic reaction (Figure 4E), maximum tolerated dose (Figure 4F), or symptoms experienced (Figure S4) during DBPCFC. Altogether, our data suggest that peanut-allergic subjects can be divided into individuals that exhibit high level of circulating pTH2A cells (TH2A high) and those with low level of pTH2A cells (TH2A low) with distinct immunological and clinical characteristics (Figure 4G).

3.3 | POIT decreased peripheral basophil sensitivity to peanut

In this study, a subset of PA patients undergoing POIT were examined to characterize short- and longer-term (~1 year) effects of POIT on peripheral immune responses. Compared with placebo, all but one POIT-treated patient who participated in the mechanistic study were clinically desensitized based on higher doses of peanut protein that could be ingested without dose-limiting symptoms during exit DBPCFC relative to baseline (Figure 5A). While no significant change in serum peanut-specific IgE level was observed between screen and exit visit (Figure 5B), treatment with AR101 resulted in significantly higher serum peanut-specific IgG4 levels (Figure 5C). In this context, we observed that subjects in the active group showed significantly lower basophil threshold sensitivity to peanut extract post-therapy, resulting in a shift of the dose–response curve for peanut-induced basophil activation to the higher concentration (Figure 5D,E). Conversely, at exit visit the placebo group showed a higher basophil sensitivity compared with baseline, and a corresponding lower threshold concentration of peanut triggering the basophil reactivity. However, POIT-elicited decreased basophil reactivity to peanut did not reach the level in those with peanut sensitization who did not react to the entry DBPCFC (Figure 5F).

3.4 | POIT reshaped circulating peanut-reactive T-cell responses in a subset-dependent manner

The effect of POIT on profile and breadth of circulating pTeff and pTreg cell responses during the PALISADE study was also evaluated. The up-dosing phase was marked by a significant decrease in circulating pTeff cell frequencies in the active arm of the trial (Figure 6A). This downward trend continued in the active group when receiving the maintenance therapy, although differences between the end of up-dosing and the exit visit did not reach statistical significance. No significant variation was observed in the circulating pTreg compartment between the baseline and exit visits (Figure 6B). As individuals progressed on therapy, a marked decrease in the frequency of circulating CRTH2+ pTeff cell subset was observed in the active group, whereas the frequency of CCR6+ pTeff cells remained relatively constant (Figure 6C). Accordingly, such subset-dependent drop of pTeff frequency during active therapy led to a dramatic change in the pTeff cell immunodominance hierarchy reshaping initial immunotypes toward an immunotype that mirrored the one observed at baseline in DBPCFC nonreactor (Figure 6D–H and Figure S5). However, no clear correlation was observed between the exit MTD and tested immunological parameters among participant in the active group (Figure S6). Meanwhile, no significant variation in the pTeff frequency and in the pTeff cell immunodominance hierarchy was observed between baseline and exit visits in the placebo group (Figure 6C,D and Figure S7A) or in the only active nonresponder of this study (Figure S7B).

How inherent qualities of pTeff cell responses at baseline may influence response to POIT was also addressed. We observed a direct correlation between the level of CRTH2+ pTeff cells at baseline and POIT-induced peanut-specific IgG4 production (Figure 6I). We also observed a direct correlation between percentage of CRTH2+ pTeff cells at baseline and POIT-induced changes in frequency of circulating pTeff cells (Figure 6J) and in the proportion of CD27+ pTeff cells (Figure 6K). Specifically, a threefold to 10-fold decrease in pTeff cell frequency and a twofold to fivefold increased proportion of CD27+ pTeff cells were observed post-therapy in patients that had a high proportion of pTH2A cells at baseline.

4 | DISCUSSION

In clinical trials, POIT with AR101 was shown to be efficacious, with an acceptable safety profile in children and adolescents who were highly allergic to peanut, supporting its potential as an immunomodulatory therapy. In this study, we investigated the mechanistic correlates of clinical responses to POIT in a subset of participants from a recently completed placebo-controlled phase 3 trial.[12,44] We also investigated whether POIT-induced immune responses resemble those in patients with lower clinical sensitivity to peanut in the absence of therapy. This study follows a previously published proof of concept from an earlier phase 2 trial[11] and expands upon those findings as the most comprehensive study to date establishing the immunomodulatory potential of POIT. Notably, this study evaluated both short- and longer-term (~1 year) effects of AR101 therapy on peripheral peanut-reactive T-cell responses and on peripheral basophil reactivity. We also investigated the immune basis underlying the threshold of reactivity ≤100 mg peanut protein among PA participants who underwent DBPCFC prior to therapy.

Basophil activation testing has emerged as a reproducible and informative measure of clinical allergic status and clinical efficacy in allergen immunotherapy.[14,45,46] The CD203-based BAT results were consistent with prior studies in which the basophil threshold sensitivity to peanut (EC-50) clearly differentiated DBPCFC reactors from peanut-sensitized patients who did not react at the 100 mg entry food challenge threshold. POIT was accompanied by marked change in basophil sensitivity mirroring the increased level of serum peanut-specific IgG4 competing with IgE. In contrast,

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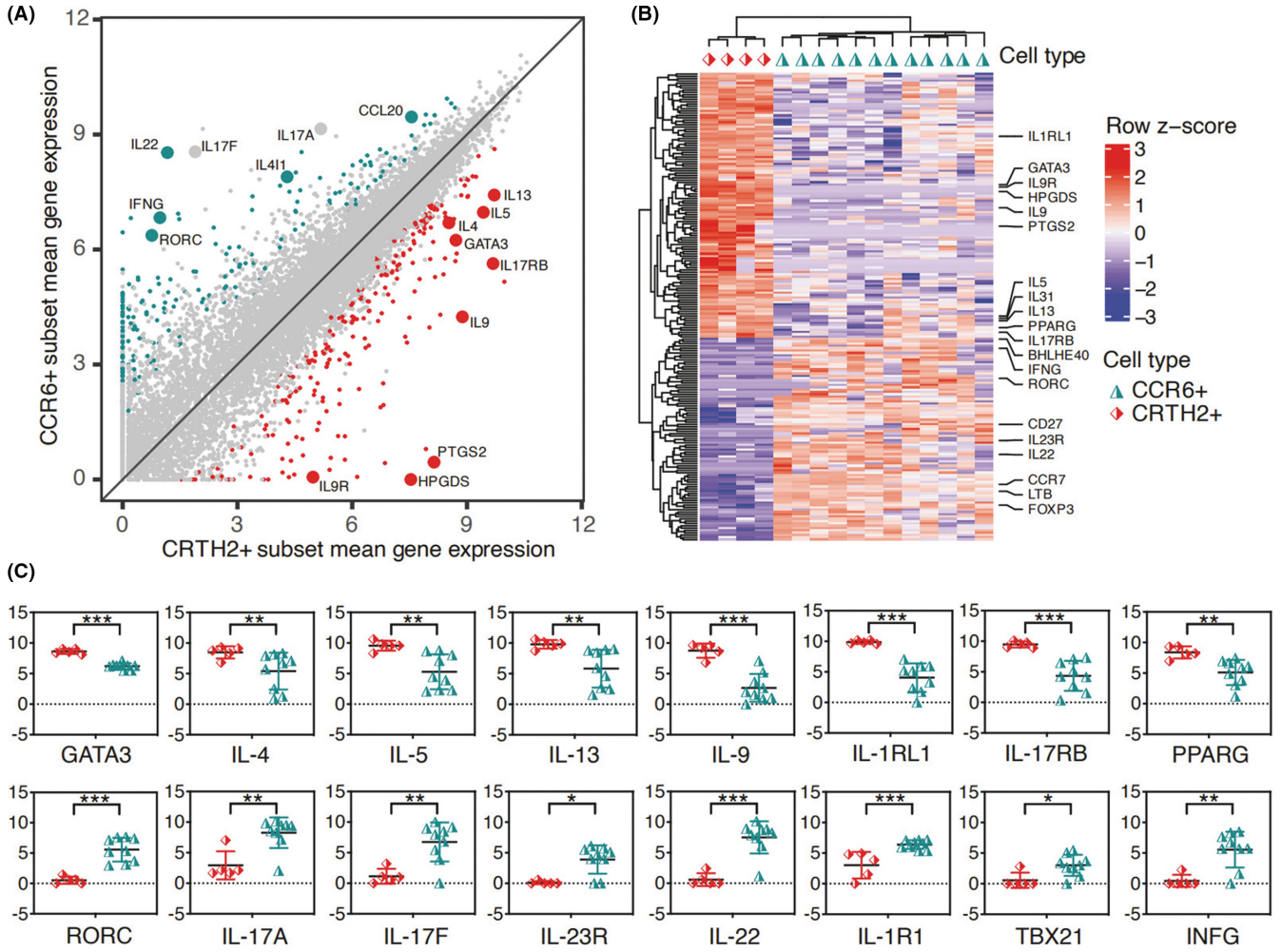

FIGURE 3 Dichotomous pattern of CRTH2+ and CCR6+ pTeff cells in PA individuals. (A) Scatterplot of the average signal of CRTH2+ pTeff cell vs. CCR6+ pTeff cell gene expression. Shown are genes whose transcription has been up-regulated (red) or down-regulated (blue) by a factor of 2. (B) Heatmap of the top differentially expressed genes between the sorted pTeff cell subset. Data are shown in z score-scaled values. (C) Expression levels of transcripts associated with TH2A and TH1/TH17 signaling in CRTH2+ (red) and CCR6+ (blue) pTeff cells. Each dot represents distinct individuals. Differences between groups were analyzed using the two-sided Mann–Whitney test. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$

basophil sensitivity to peanut tended to increase in the placebo-treated patients likely due to the two peanut challenges performed during the trial in the absence of therapy. It remains to be determined whether such decreases in basophil sensitivity to peanut in POIT-treated PA patients will inform sustained unresponsiveness after therapy as recently suggested.[15,47] However, POIT-elicited decrease in basophil sensitivity to peanut did not reach the level in those with peanut sensitization who did not react to the entry DBPCFC suggesting that 1-year desensitization therapy may not be sufficient to sustain unresponsiveness. It should be noted that the flow cytometry assessment of activated basophils in our study was determined by measuring only the allergen-induced CD203c expression and may display variations compared with CD63 to reflect disease severity and threshold of allergic reaction to peanut during DBPCFC.[48] However, CD203c-based BAT has been described to be as reliable as CD63-BAT for the in vitro diagnosis of patients with IgE-mediated allergy.[27,28,49]

It is generally accepted that enumeration and characterization of antigen-specific T cells provide essential information about the potency of the immune response.[50,51] In this study, we used an *ex vivo* approach to map the peanut-specific CD4+ T-cell landscape in a well-characterized cohort of PA patients and to search for POIT-driven CD4+ T-cell changes. This information could inform efforts to improve current therapeutic approaches, by identifying distinct patient populations who might most benefit from specific targeted treatments and for defining potential clinically meaningful biomarkers. Compared with DBPCFC reactors, we found that the absence of a clinical response to the 100mg peanut challenge in peanut-sensitized participants was associated with significantly lower pTeff cell responses. Growing evidence now suggests that TH2A cells act as key pathogenic TH2 cells in multiple atopic contexts.[40–43,52] These cells can be identified by co-expression of the prostaglandin D2 receptor (CRTH2) and CD161 in the absence of CD27 expression on CD4+ T cells and characterized cells with enhanced IL-5

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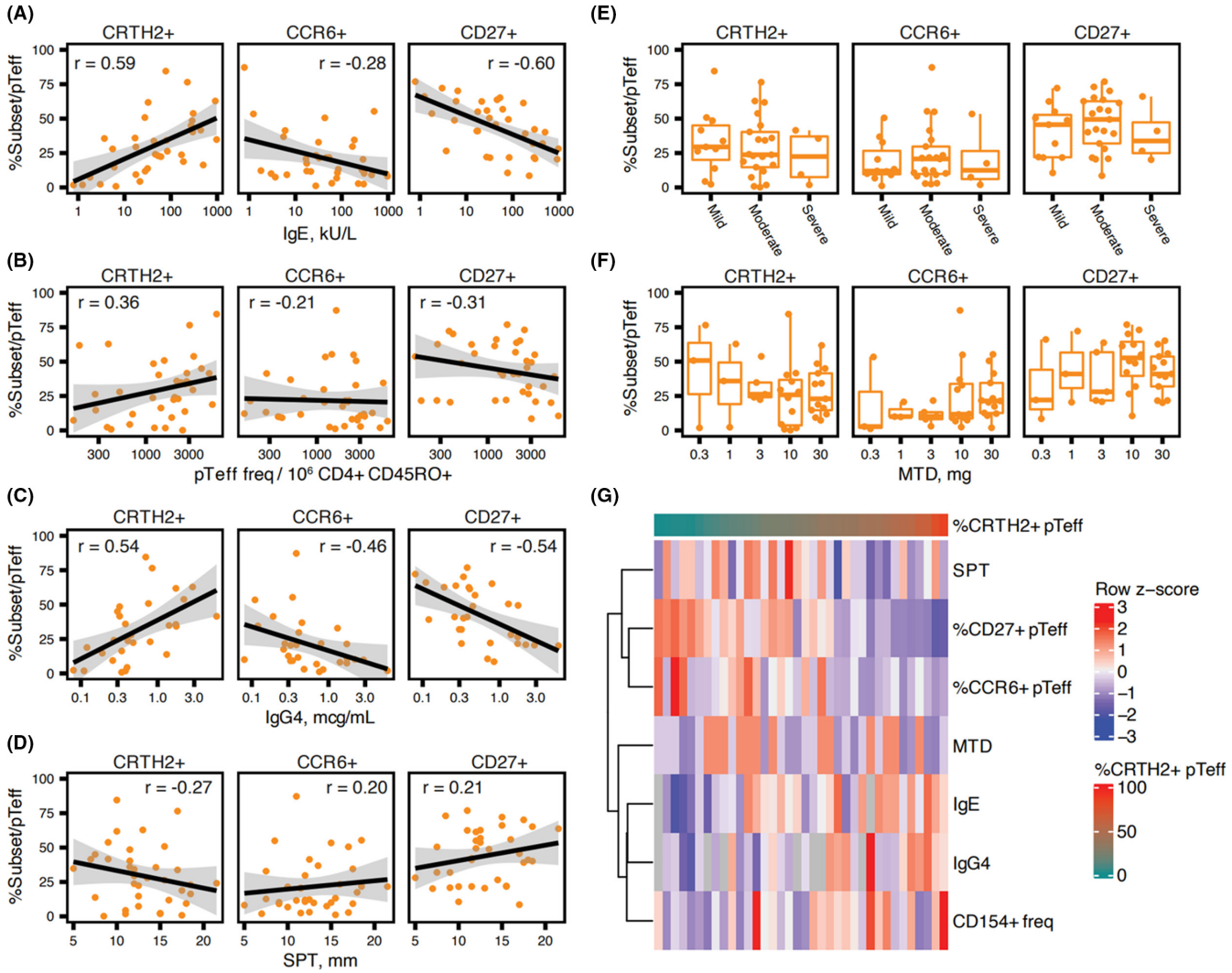

FIGURE 4 Level of circulating peanut-reactive TH2A cells characterizes peanut-allergic subjects with distinct immunological and clinical characteristics. (A–F) Correlation between proportion of pTeff cell subsets and baseline serum peanut-specific IgE level (A), global pTeff cell frequency (B), serum peanut-specific IgG4 level (C), skin prick test to peanut (D), severity of allergic reaction during DBPCFC (E), and threshold sensitivity to peanut during DBPCFC (F). (G) Heatmap of indicated baseline clinical and immune parameters by row; and proportion of CRTH2+ pTeff cells in each tested DBPCFC reactor are indicated across the top. Data are shown in z score-scaled values. (A–F) Each dot represents distinct individuals. Differences between groups were analyzed using the two-sided Mann-Whitney test. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$

and IL-9 producing potential in response to allergen.[39] The data presented herein emphasize the heterogeneity of pTeff cell responses in PA subjects. One of the noteworthy findings in this study is that CRTH2+ pTeff cells and CCR6+ pTeff cells represent two mutually exclusive, nonoverlapping cellular and molecular entities involved in food-allergic diseases. In this study, CRTH2+ pTeff cells were characterized by cellular (CD161+, CD27-) and molecular signature enriched in pathogenic type 2 inflammatory response (*GATA-3*, *IL-1RL1*, *IL-5*, *IL-9*, *IL-17RB*, *PPARG*), allowing us to consider them primarily TH2A cells. Overall, our findings suggest that a critical frequency of Th2a cells is required to perpetuate the immune cascade that leads to type 1 hypersensitivity reaction and clinical symptoms. First, we observed that circulating CRTH2+ pTeff cells were mostly restricted to DBPCFC reactors compared to those with peanut sensitization who did not react to the 100mg DBPCFC. Second, pTH2A cells in DBPCFC reactors emerged as the subpopulation with the highest positive correlation to baseline serum peanut-specific IgE level along with global pTeff cell frequency. Finally, as individuals progress through the desensitization process, a marked decrease was observed in the frequency of circulating pathogenic CRTH2+ pTeff cells in the absence of significant changes in frequency of CCR6+ or CD27+ pTeff cell subset. Hence, skewing of allergen-specific effector T cells away from the TH2A cell responses may represent a key event in the development of long-lasting peripheral tolerance to allergen.

In contrast to pTH2A cells, the percentage of CCR6+ pTeff cell subsets in PA individuals tended to correlate negatively with baseline serum peanut-specific IgE level. The nature and role of CCR6+

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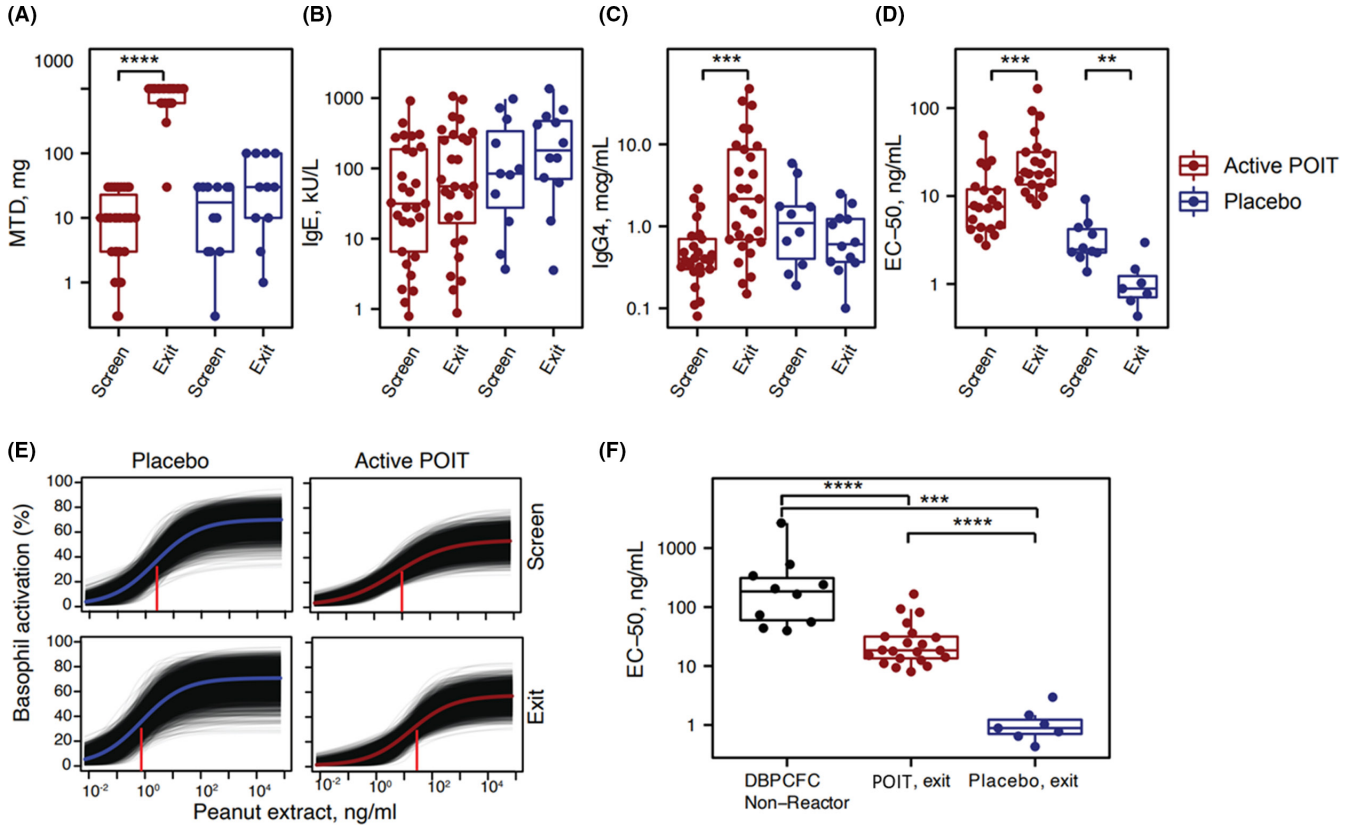

FIGURE 5 POIT decreased peripheral basophil sensitivity to peanut. (A–D) Maximum tolerated dose (A), serum peanut-specific IgE level (B), serum peanut-specific IgG4 level (C), and basophil sensitivity to peanut (D) at screen and exit DBPCFC between active (red dots; $n = 30$) and placebo (blue dots; $n = 12$) arm during PALISADE trial. (E) Peanut dose–response curves of basophil activation at screen and exit visit between placebo (blue line) and active arm (red line). A Bayesian modeling approach was used to obtain logarithmic fits to the data and determine the concentration at which basophil activation is half of the maximum activation (EC-50) for each patient. (F) Basophil sensitivity to peanut between peanut-sensitized individuals who did not react to the entry DBPCFC (black dots), POIT-treated participants at exit visit (red dots), and placebo-treated participants at exit (blue dots). Each dot represents distinct individuals. Differences between groups were analyzed by the two-sided Mann–Whitney test. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$

pTeff cell subset defined in this study remains unclear. CCR6 is a well-established marker of T effector cells prone to acquire Th17 effector functions.[53–55] In our study, transcript analysis of sorted CCR6+ pTeff cells highlights the existence of a second molecular signature in food allergy related to the expression levels of TH1/TH17 and Treg-related genes (IFN-γ, RORγt, IL-17A, IL-17F, IL-22, IL-23R, CCL20, FOXP3). CCR6+ pTeff cells also display a central memory differentiation state (CCR7 and CD27), which may have implication for durability upon antigen re-exposure[56,57] and explain different fate between CCR6+ and CRTH2+ pTeff cells during immunotherapy. Although restricted to peanut-allergic individuals, CRTH2+ pTeff cells were observed in many but not all peanut-allergic patients and their proportion in peripheral blood seems to inversely correlate with CCR6+ pTeff cells. In this study, we observed that the presence of pathogenic peanut-reactive TH2A cell subset at baseline not only correlates with baseline serum peanut-specific IgE level but also with POIT-driven induction of serum peanut-specific IgG4 level. Intriguingly, explicit differences in frequencies of circulating pTreg cells were neither observed between DBPCFC reactors and nonreactors nor between baseline and exit visits in the active arm. Instead, our data suggest that a process of selective T-cell exhaustion/deletion specifically occurs in pathogenic TH2 cells during OIT regardless of the balance of other pTeff cell subsets. Thereafter, if treatment is not continued long enough to further trigger selective TH2A cell deletion, the initial pathogenic feature of these cells may gradually recover after discontinuation. Alternatively, there may be a role for other immune cells such as regulatory B cells or tolerogenic dendritic cells that were not assessed in this study. The specific mechanisms of subset-dependent decreases in pTeff cells during desensitization remain unclear. Recent studies demonstrated that a high ratio of serum specific to total IgE correlated with the effectiveness of AIT.[58,59] It was previously shown that allergen-specific TH2 cells exhibit greater susceptibility to activation-induced cell death (AICD).[60–62] Moreover, it has been shown that IL-10-producing T cells may arise from fully differentiated T effector cells that have lost the ability to secrete their hallmark cytokines as a result of chronic antigenic stimulation.[63,64] Given that the POIT involves the repeated administration of escalating doses of the peanut allergen over months, it is possible that desensitization therapies act through this mechanism. Whether the dose–escalation and maintenance phase during OIT preferentially

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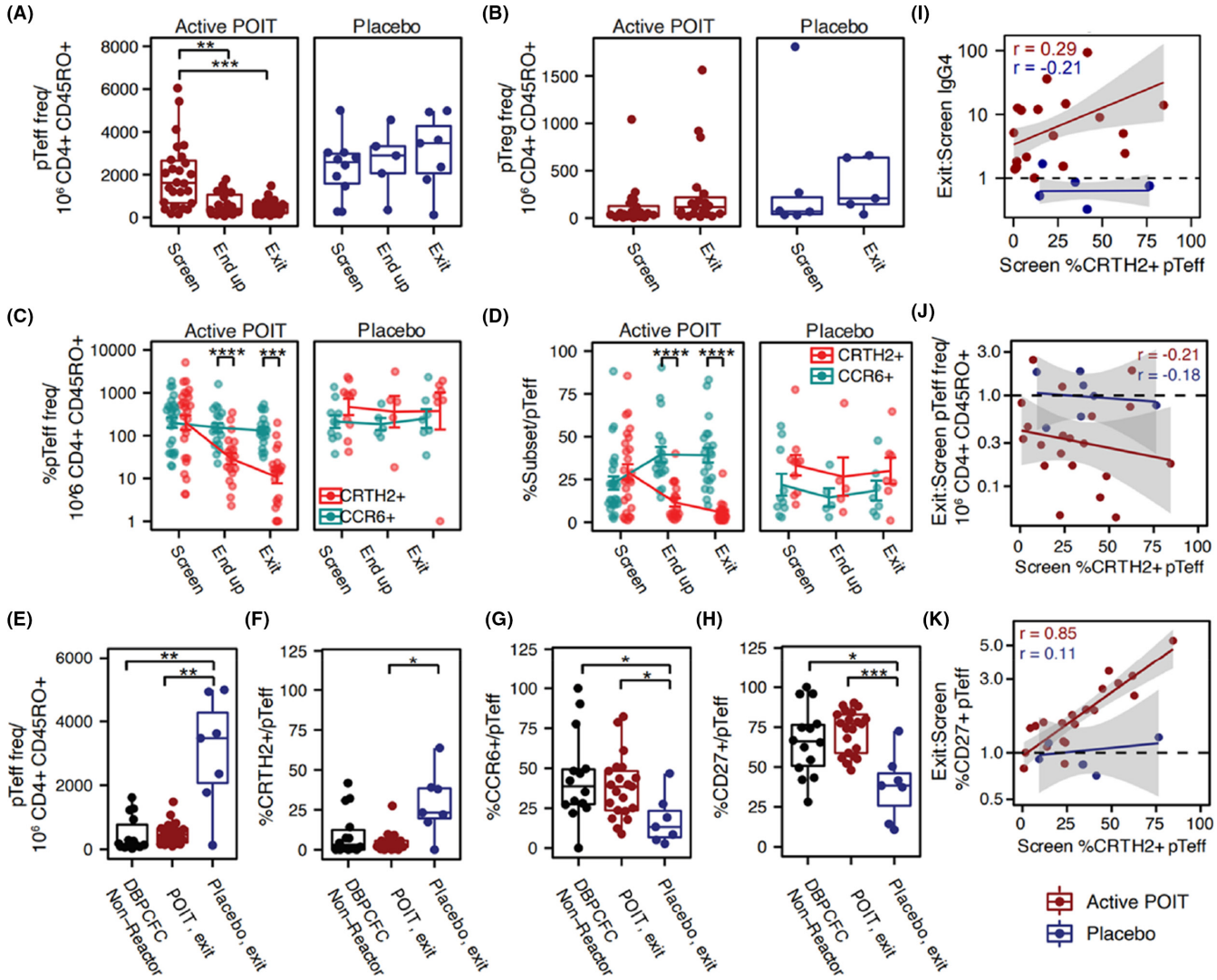

FIGURE 6 POIT reshaped circulating peanut-reactive T-cell responses in a subset-dependent manner. (A, B) Dynamic of circulating pTeff (A) and pTreg (B) cell frequencies between active and placebo arms during PALISADE trial. (C, D) Dynamic of CRTH2+ (red dots) and CCR6+ (blue dots) pTeff cell frequencies (C) and proportion (D) between active and placebo arm during PALISADE trial. (E–H) Frequency of pTeff cells (E), proportion of CRTH2+ (F), CCR6+ (G) and CD27+ (H) pTeff cells between peanut-sensitized individuals who did not react to the entry DBPCFC (black dots), POIT-treated participants at exit visit (red dots), and placebo-treated participants at exit (blue dots). (I–K) Correlation between proportion of CRTH2+ pTeff cells at baseline and exit:screen ratio peanut-specific IgG4 level (I), exit:screen ratio pTeff cell frequency (J), and exit:screen ratio proportion of CD27+ pTeff cells (K) between active and placebo arm during PALISADE trial. Each dot represents distinct individuals. Differences between groups were analyzed by the two-sided Mann–Whitney test. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$

and transiently potentiates the CRTH2+ pTeff cells and/or CCR6+ pTeff to produce IL-10 as a feedback loop to prevent excessive pro-allergic immune responses still needs to be determined.

There were several important limitations to this study. This study was in a small subset of patients from the parent phase 3 study, and thus, the sample size is small and may not be fully representative of the larger population of trial participants. Specifically, the absence of significant number of patients who did not benefit from desensitization limited our ability to define a variable that may screen out those candidates in whom such therapy could potentially result only in exposure to unnecessary risks. It is known that a relatively small subset of PA individuals undergoing POIT will not achieve sustained clinical response[65]; therefore, understanding the underlying cellular and molecular mechanisms may also be particularly important to appreciate the basis for long-term immunomodulation and disease remissions following POIT treatment discontinuation. It is recognized that TH2 cells associated with food allergy are heterogeneous,[20,66] and hence, it remains to be determined whether a subset of residual pTeff cells, distinct from the TH2A cell subset, which did not decrease during POIT, could serve as reservoir to replenish pathogenic TH2A cells post-therapy. Finally, it is unknown whether the proportion of CRTH2+ pTeff cells of PA individual at baseline may vary over time depending on recent exposures to food allergen or the age of the patient. However, the absence of

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significant variation in the pTeff cell profile in the placebo group demonstrated the stability of a PA immunotype over time and thus the robustness of our T-cell assay to track and monitor immunological changes longitudinally.

In summary, blood samples collected during entry DBPCFC and over time in patients undergoing POIT were used to determine how POIT modulates both basophil and T-cell responses to peanut antigen. Our study showed that desensitization immunotherapy dramatically reshapes basophil and pTeff cell response toward a response that mirrored the one observed in participant with peanut sensitization who did not react to the 100 mg peanut protein entry challenge. The data presented here complement the mechanistic insights gained to date for prevention studies and highlight the potential use of basophil and TH2A cells as response-monitoring biomarkers. As a key driver of immunoglobulin class switching and antibody responses, T helper cells may reflect upstream events in the allergic cascade not captured by the basophil activation test. Our data suggest that inherent qualities of pTeff cell responses at baseline cluster PA individuals with distinct immunological and clinical characteristics that may influence response to POIT. These results also highlight the importance of further understanding the regulation and interplay between CRTH2+ and CCR6+ pTeff responses for developing and optimizing therapeutic interventions strategies.

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CONFLICT OF INTEREST

EW receives grant support from NIAID, Food Allergy Research and Education (FARE), and Immune Tolerance Network (ITN); and research sponsorship from Regeneron Pharmaceuticals, Astellas, COUR Pharma, and Aimmune Therapeutics. DCA is a former employee of Aimmune Therapeutics and currently chairs the company's scientific advisory board. AS is an active employee of Aimmune Therapeutics. BPV receives grant support from NIH-NIAID and Immune Tolerance Network and Food Allergy Research and Education (FARE); and research sponsorship from Genentech. BPV is also an advisory board member for Aimmune Therapeutics, AllerGenis, LLC, FARE, and Reacta Biosciences.

AUTHOR CONTRIBUTIONS

EW conceptualized the overall study and wrote the manuscript. DCA and BPV designed the clinical trial. EW, VB, HAD, NG, BJR, and VHG designed the immunological studies. VB, NG, BJR, KKO, and QAN performed experimental work and data acquisition. VB, HAD, NG, BJR, COR, AS, AHW, CQ, VHG, and EW analyzed and interpreted the data. MF, DJ, BPV, and DCA were responsible for clinical oversight. VB, HAD, NG, BJR, BPV, and DCA critically reviewed and edited the manuscript.

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