

Letter to the Editor

Peanut oral immunotherapy induces blocking antibodies but does not change the functional characteristics of peanut-specific IgE

To the Editor:

Peanut allergy is one of the most common and severe food allergies, affecting about 2% of school-aged children in the United Kingdom and United States. Current management of peanut allergy consists of strict allergen avoidance and carrying emergency medication to treat acute allergic reactions that can develop after accidental exposure.

Peanut oral immunotherapy (POIT) is reported in various studies to induce desensitization (ie, the ability to increase the threshold of reactivity to peanut while patients are receiving daily therapy), but a recent systematic review of the literature has not found evidence that POIT induces long-term oral tolerance, which is defined as the ability to eat peanut *ad libitum*, regardless of the amount and frequency of peanut consumption.¹ Some patients have shown “sustained unresponsiveness,” which is defined by uneventful peanut consumption during a medically supervised challenge in the hospital after a period of discontinuation of treatment; however, the question remains as to whether the ability to eat peanut after POIT during peanut challenge reflects underlying immunologic changes compatible with long-term oral tolerance. At the effector cell level, we expected to see a suppression of basophil and mast cell responses to peanut after POIT but wanted to examine whether this suppression was due to blocking antibodies, changes in the characteristics of IgE, or both.

In a previous publication in the *Journal*, we showed that plasma from patients with peanut allergy treated with POIT was able to suppress activation of mast cells sensitized with plasma from a patient with peanut allergy and stimulated with peanut extract and that this suppression was partially mediated by IgG₄ present in the plasma of patients receiving POIT.² We also showed that the effect was similar to that of samples from patients who were peanut tolerant despite having IgE to peanut and the peanut major allergens Ara h 1, Ara h 2, and/or Ara h 3 in some cases at levels predictive of peanut allergy.² To assess whether suppression in mast cell activation observed after POIT was due to blocking antibodies (as previously shown in the inhibition of mast cell activation test [iMAT]) or also to changes in the characteristics of IgE induced by treatment, we tested samples of patients with peanut allergy before and after POIT in the mast cell activation test (MAT) and in the iMAT to peanut extract.

Plasma samples from patients with peanut allergy treated with POIT as part of the Study of Tolerance to Oral Peanut (STOP) I trial (registered at <http://ClinicalTrials.gov> with identification no. NCT01259804; for more details about the study, see the Methods section in this article's Online Repository at www.jacionline.org)³ collected before and after treatment were tested to peanut extract and the recombinant peanut allergens rAra h 1, rAra h 2, and rAra h 3 by using an immunoenzymatic assay (ImmunoCAP; Thermo Fisher, Waltham, Mass). IgG₄/IgE ratios were determined after conversion of kilounits per liter (IgE) and milligrams per liter (IgG₄) to nanograms per milliliter, as previously described.²

For mast cell assays,^{2,4} LAD2 cells were placed in culture with rIL-4 for 5 days before overnight sensitization with patients' plasma from before or after POIT. Sensitized cells were stimulated with peanut extract, anti-IgE, ionomycin, or 0.04% BSA RPMI alone for 30 minutes at 37°C and stained with the viability dye eFluor 450 and CD107b–fluorescein isothiocyanate (eBioscience, San Diego, Calif), CD203c–phycoerythrin, CD107a–peridinin-chlorophyll-protein complex–Cy5.5, CD63–allophycocyanin, and IgE–phycoerythrin–Cy7 (BioLegend, San Diego, Calif) and analyzed by using flow cytometry. Mast cell activation was expressed as the percentage of CD63⁺ LAD2 cells. For mast cell inhibition experiments, 20 µL of plasma from after POIT or stimulation buffer as negative control was incubated with an equal volume of stimulation buffer and 10 µL of allergen at 37°C for 1 hour. LAD2 cells were first sensitized with pre-POIT or post-POIT plasma and then washed before being added to allergen (in the MAT) or to allergen-plasma mixtures (in the iMAT; see Fig E1 in this article's Online Repository at www.jacionline.org). Washed cells do not exhibit receptor-bound IgG but only receptor-bound IgE on their surface (see Fig E2 in this article's Online Repository at www.jacionline.org), and thus MAT measures the function of IgE in its ability to induce mast cell activation after allergen stimulation. On the other hand, iMAT, because of the presence of plasma around the LAD2 cells, measures the effect of blocking antibodies in the ability of the IgE–allergen interaction to induce mast cell activation.

Flow cytometry was performed on a FACSCanto II with FACSDiva software (BD Biosciences, San Jose, Calif), and data were analyzed with FlowJo software (version 7.6.1; TreeStar, Ashland, Ore).

Peanut-specific IgE and IgG₄ levels did not significantly change with POIT, whereas IgG₄/IgE ratios increased over time with treatment (see Table E1 in this article's Online Repository at www.jacionline.org). Levels of specific IgE to the peanut major allergens Ara h 1 and Ara h 2 did not change, but Ara h 3–specific IgE levels were reduced over the course of treatment (see Table E2 in this article's Online Repository at www.jacionline.org). The small sample size and variable duration of treatment at the post-POIT time point studied are limitations of our study and could explain the lack of observed differences in allergen-specific IgE levels over the course of treatment; however, comparable levels of Ara h 2–specific IgE before and 12 months after treatment have been reported previously.⁵

When tested on the iMAT, peanut-induced activation of mast cells previously sensitized with the corresponding plasma samples that had been collected at baseline was reduced in the presence of post-POIT samples (Fig 1, A), which is similar to what we reported previously for iMAT when testing post-POIT samples against a standard indicator plasma from a patient with peanut allergy.² The post-POIT plasma abrogates the activation of mast cells after stimulation not only with allergen but also with anti-IgE, possibly because of binding of anti-IgE to IgE present in the plasma, leaving less anti-IgE available to cross-link receptor-bound IgE on the surfaces of mast cells (Fig 1, B).

In the MAT, which includes a washing step between sensitization with plasma and stimulation with allergen, the proportion of activated mast cells in response to peanut was similar before

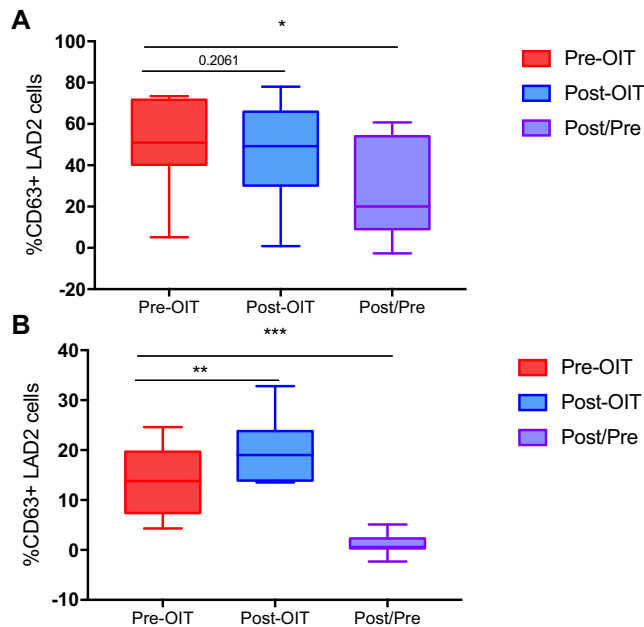


FIG 1. iMAT using LAD2 mast cells sensitized with plasma collected before POIT and stimulated with peanut extract or anti-IgE in the presence of plasma collected after OIT (in purple; *Post/Pre*) are shown together with results for the MAT using LAD2 mast cells sensitized with plasma collected before (in red; *Pre-OIT*) or after (in blue; *Post-OIT*) POIT. The 11 participants for whom there were both pre-POIT and post-POIT samples and who had peanut-specific IgE levels of 2 KU/L or greater were tested by using the iMAT. Results are shown for MAT and iMAT experiments performed in parallel. **A**, MAT to peanut extract (1000 ng/mL) before (in red) and after (in blue) OIT and iMAT to peanut extract (1000 ng/mL) after OIT (in purple) tested against pre-OIT plasma. **B**, MAT to peanut extract (1000 ng/mL) before (in red) and after (in blue) OIT and iMAT to anti-IgE (1 µg/mL) after OIT (in purple) tested against pre-OIT plasma. * $P < .05$, ** $P < .01$, and *** $P < .001$.

and after treatment (Fig 2, A), indicating that the functionality of IgE did not change after POIT, as opposed to what we have previously observed using the same assay to test samples of patients with peanut allergy and peanut-sensitized but tolerant patients.⁴ The mast cell response to polyclonal anti-IgE was greater after POIT (Fig 2, B), suggesting that the IgE-mediated pathway was primed after continued allergen exposure, possibly because of the increase in IgE characteristics such as IgE affinity, given that the cells were the same but sensitized with different plasma in pre- and post-POIT conditions.

Our findings support the induction of blocking antibodies with POIT, as previously reported by us² and others,^{6,7} and demonstrate, for the first time, that POIT did not change the functional characteristics of IgE, as determined by the ability of effector cells, such as mast cells and basophils, to respond to peanut allergens with cellular activation, degranulation, mediator release, and consequent acute allergic reactions to peanut. The enhanced mast cell response to anti-IgE after POIT suggests that continued exposure to the allergen can potentiate IgE-mediated mast cell responses. The fact that the underlying IgE function did not change and was possibly enhanced is consistent with the frequent allergic reactions that patients undergoing POIT experience during treatment, as highlighted in a recently published systematic review of POIT studies,⁸ and with the recurrence of symptoms after treatment withdrawal and the low proportion of treated

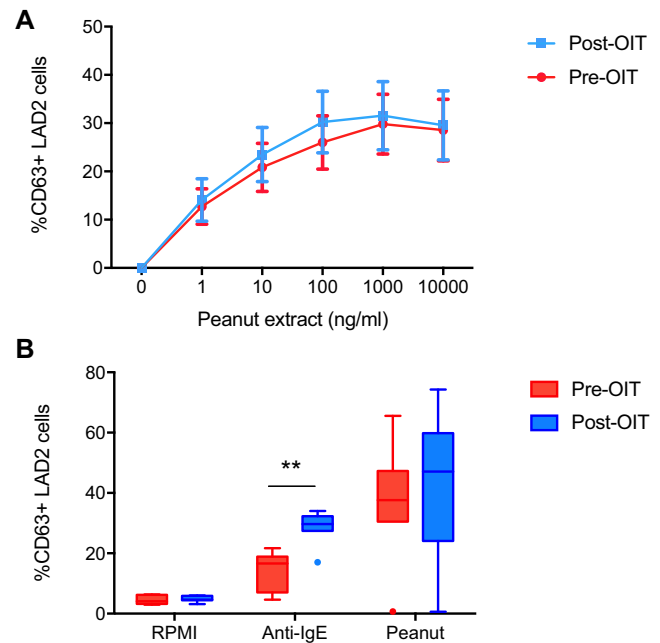


FIG 2. **A**, MAT after stimulation with peanut extract using cells previously sensitized with plasma before and after POIT. The 14 participants for whom there were samples from both pre-POIT and post-POIT time points were tested. P values were nonsignificant across peanut extract concentrations. **B**, MAT after stimulation to a negative control (RPMI), positive control (anti-IgE), or allergen (peanut extract) using cells sensitized with plasma before and after POIT. The 14 participants for whom there were both pre-POIT and post-POIT samples were tested. ** $P = .008$.

patients who show sustained unresponsiveness after a short period of allergen avoidance observed across studies.¹ The need for continued allergen consumption to maintain the increased threshold of clinical reactivity during oral food challenges is also consistent with our findings because continued allergen consumption is needed for induction of blocking antibodies. Others have shown reduced response of basophils to allergen after oral immunotherapy (OIT) similar to what we show here for mast cells but using patients' own basophils in the whole blood basophil activation test (BAT).^{6,7} Contrary to the MAT (in which cells have been washed after sensitization with plasma, removing potential blocking antibodies, and in which the mast cells are not from the patient but a cell line, which is used to test different patients), in the BAT the abrogation of response to allergen could be due to a combined effect of blocking antibodies with the intracellular effect of continued exposure to suboptimal allergen concentrations during POIT. Interestingly, this basophil suppression has previously been shown not to be allergen specific and to possibly be caused by basophil anergy, which seems to result from intracellular mechanisms.⁹

In summary, in the STOP I trial, POIT induced blocking antibodies that suppressed mast cell activation, as seen in the iMAT, but did not change the functional characteristics of allergen-specific IgE, as seen in the MAT. Doing a whole blood BAT in parallel would have allowed assessment of the intracellular effects of POIT alongside the antibody changes seen in the iMAT (for blocking antibodies) and MAT (for IgE). Thus the MAT, iMAT, and BAT can be used in combination to assess the different components of the allergen-induced effector cell response during immunomodulatory treatments, such as OIT.

A better understanding of the mechanisms underlying the patient's clinical response to treatment will help address the question of whether OIT induces long-term oral tolerance and help in the decision of whether treated patients can eat the food to which they were previously allergic *ad libitum* or need to follow a specific OIT regimen in the long term.

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This manuscript refers to the STOP I trial (registered at <http://ClinicalTrials.gov> with identification no. NCT01259804).

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METHODS

Detailed background to the STOP I trial

The STOP I trial was an open-label, investigator-led, proof-of-concept OIT trial conducted in a single center (Cambridge University Hospitals NHS Foundation Trust) in the United Kingdom by using characterized peanut flour (partially defatted peanut flour [12% fat and 50% protein]; product no. 521271; lot no. 108FA00806; Golden Peanut Company, Dawson, Ga) and retail products (whole roasted peanuts or peanut butter). The study was conducted in 22 patients with peanut allergy aged 4 to 18 years.^{E1,E2}

At the time of designing the STOP I trial, there were few data available to guide the choice of maintenance dose. Regimens in other small published studies used maintenance doses of 125 and 300 mg of protein.^{E3,E4} The STOP I trial was intended to explore the effect of a greater maintenance dose, with the aim of improved efficacy. Considering the balance of feasibility of dosing (greater doses being less palatable), 800 mg of protein was chosen as the maintenance dose. During development of the STOP I trial, it was recognized that, when presented in the form of peanut flour, the 800-mg daily protein dose was difficult for subjects to consume because of it being a large amount of flour (1.6 g) with an unpleasant texture and flavor. Therefore an approximately 800-mg dose was administered in the form of whole roasted peanuts (4–5 whole roasted peanuts) or peanut butter (2.5 mL), depending on the subject's choice.

The updosing phase increments were 0.5, 1, 2, 5, 12, 25, 50, 100, 200, 400, and 800 mg of peanut protein. Because this was an exploratory study, the starting doses for immunotherapy were individualized and started at less than the subject's own pre-OIT threshold, as determined by screening double-blind, placebo-controlled food challenge. Two-week dosing intervals were used. All 22 subjects had a positive screening double-blind, placebo-controlled food challenge result, after which updosing commenced, with 19 (86%) of 22 subjects tolerating updosing to the planned maximum dose of 800 mg of protein per day. Therefore patients treated with 200 mg of protein in peanut

flour were within a short period of time (approximately 14 days) able to tolerate twice as much peanut protein (400 mg of protein), and patients treated with 400 mg of protein in peanut flour were within a short period of time (approximately 14 days) able to tolerate around twice as much peanut protein (approximately 800 mg of protein) ingested in a retail product (whole roasted peanut or peanut butter).

After 6 weeks of maintenance treatment, 19 (86%) of 22 subjects underwent challenge to 2600 mg of protein in the form of whole peanut. Eighteen (82%) of 22 subjects ingested the full challenge dose; of those, 12 (55%) of 22 had no symptoms, and 7 (32%) of 22 had mild-to-moderate symptoms. After completing 30 weeks of the maintenance phase, 18 (81%) of 22 subjects underwent a 6600-mg protein challenge in the form of whole peanut. Fourteen (64%) of 22 subjects did not have any symptoms during the challenge. The STOP I trial proved the principle that OIT could increase the reactive threshold of treated patients and demonstrated good safety of the dosing regimen. It also demonstrated that after 6 weeks to 6 months of OIT, subjects' reactive thresholds (eg, 6600 mg of protein) could be increased many times greater than the treatment maintenance dose (800 mg of protein).

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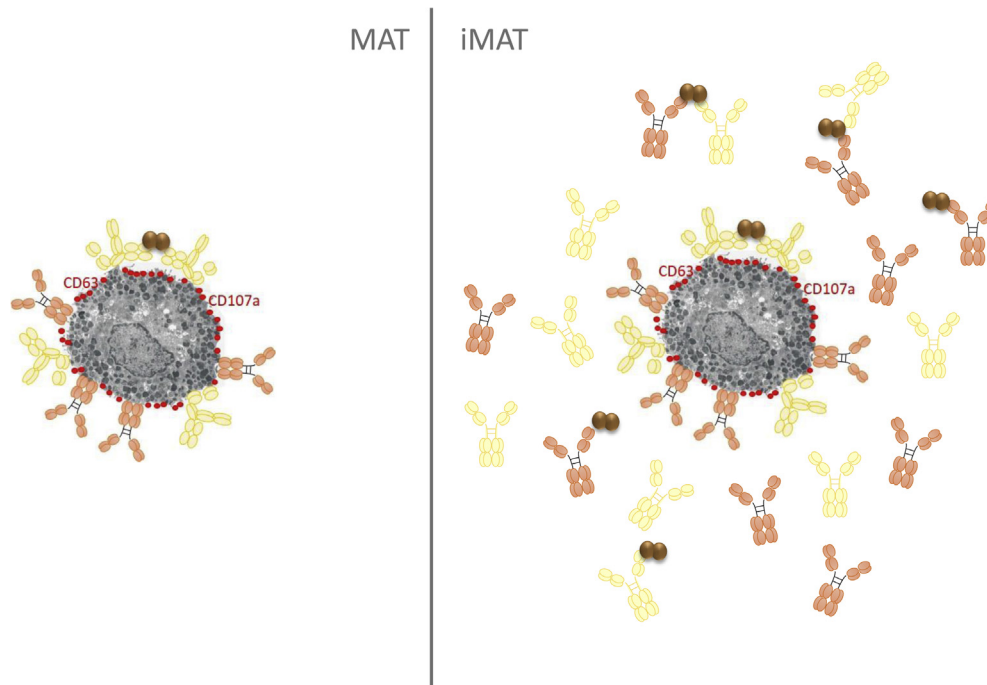


FIG E1. Schematic representation of the MAT and iMAT. In both assays LAD2 cells are stimulated with peanut extract, and expression of activation markers (eg, CD63) is measured by using flow cytometry. The key difference between the assays is that in the MAT cells are washed after sensitization with plasma from a patient with peanut allergy (collected before immunotherapy) and before stimulation with peanut extract, whereas in the iMAT the mast cells, which were previously sensitized with plasma from the same patient with peanut allergy (collected before immunotherapy), are stimulated with peanut in the presence of plasma collected after immunotherapy. The different experimental design allows assessment of the functional ability of IgE to induce mast cell activation in the MAT and assessment of the effect of blocking antibodies on mast cell activation in the iMAT.

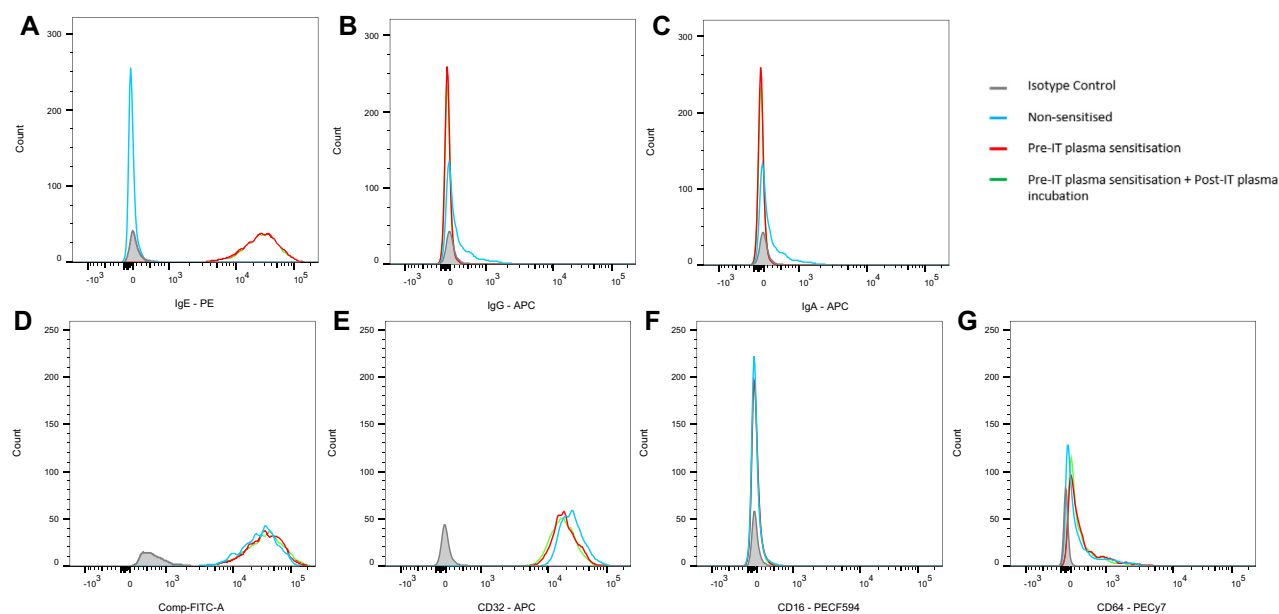


FIG E2. Expression of receptor-bound immunoglobulins and their respective receptors on sensitized LAD2 cells. LAD2 cells were sensitized with pre-POIT plasma from patients with peanut allergy before allergen stimulation (in red). Plasma samples from the same subjects obtained after immunotherapy treatment were incubated with allergen for 1 hour before addition to sensitized cells (in green). Surface expression of immunoglobulins and receptors was compared with that of the isotype control antibody (gray) and nonsensitized LAD2 cells (blue) by using flow cytometry. Receptor-bound IgE was highly expressed on sensitized LAD2 cells (A) but not IgG (B) or IgA (C). FcεRI (D) and CD32 (E) were constitutively expressed in sensitized and nonsensitized LAD2 cells. No surface expression of CD16 (F) or CD64 (G) was detected under the same conditions. APC, Allophycocyanin; FITC, fluorescein isothiocyanate; IT, immunotherapy; PE, phycoerythrin.

TABLE E1. Clinical characteristics of participants in the STOP I trial

Male/female ratio	1	
Age at first peanut reaction (y)	2 (0.3-7)	
Age at enrollment (y)	11 (4-18)	
Severity of worst peanut reaction before enrollment	Mild = 23%	
	Moderate = 36%	
	Severe = 14%	
	Unclassified = 27%	
Other allergic disease	Active (%)	Outgrown (%)
Asthma	64	9
Rhinitis	45	5
Eczema	36	45
Egg allergy	9	36
Milk allergy	5	23
Duration of treatment (mo)	18 (5-24)	
Cumulative threshold dose at baseline (mg of peanut protein)*	6 (5-256)	
Cumulative threshold dose after treatment (mg of peanut protein)*	800 (25-6570)	

Numbers and percentages and medians and ranges are represented for qualitative and quantitative variables, respectively.

* $P < .001$ for comparison between the cumulative dose before and after POIT using the Wilcoxon signed-rank test.

TABLE E2. Levels of specific IgE and IgG₄ to peanut and peanut major allergens in patients with peanut allergy undergoing POIT before and after treatment (n = 14)

	Before POIT	After OIT	<i>P</i> value
Peanut-specific IgE (KU/L)	25.5 (3.0-100.0)	9.43 (1.40-182.10)	.826
Ara h 1-specific IgE (KU/L)	0.81 (0.35-40.2)	0.57 (0.01-49.39)	.778
Ara h 2-specific IgE (KU/L)	18.7 (1.27-72.85)	1.28 (0.38-11.01)	.161
Ara h 3-specific IgE (KU/L)	0.35 (0.35-17.47)	0.09 (0.01-0.19)	.012
Peanut-specific IgG ₄ (μg/L)	500 (268-3518)	2585 (693-5480)	.674
IgG ₄ /IgE ratio	17.64 (4.10-416.67)	154.70 (33.82-512.07)	.017

Medians and interquartile ranges are represented. *P* values refer to the comparison between pre- and post-POIT levels by using the Wilcoxon signed-rank test.