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## Immunologic Suppression To Peanut During Immunotherapy Is Often Transient

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### Abstract

**Background**—Studies suggest that oral (OIT) and sublingual (SLIT) immunotherapy for food allergy hold promise; however, the immunologic mechanisms underlying these therapies are not well understood.

**Objective**—To generate insights into the mechanisms and duration of immunologic suppression to peanut during immunotherapy (IT).

**Methods**—Blood was obtained from subjects at baseline and at multiple timepoints during a placebo-controlled trial of peanut OIT and SLIT. Immunologic outcomes included spontaneous and stimulated basophil activity by automated fluorometry (histamine) and flow cytometry (activation markers, IL-4), allergen-induced cytokine expression in dendritic cell (DC)-T cell co-cultures by multiplexing technology, and expression of MHC II and costimulatory molecules on DCs by flow cytometry.

**Results**—Spontaneous and allergen-induced basophil reactivity (histamine release, CD63 expression, and IL-4 production) were suppressed during dose escalation and after 6 months of maintenance dosing. Peanut- and dust mite-induced expression of T<sub>H</sub>2 cytokines was reduced in DC-T cell co-cultures during IT. This was associated with decreased levels of CD40, HLA-DR, and CD86 expression on DCs, and increased expression of CD80. These effects were most striking in myeloid DC-T cell co-cultures from subjects receiving OIT. Many markers of

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immunologic suppression reversed following withdrawal from IT, and in some cases during ongoing maintenance therapy.

**Conclusion**—OIT and SLIT for peanut allergy induce rapid suppression of basophil effector functions, dendritic cell activation, and Th2 cytokine responses during the initial phases of IT in an antigen non-specific manner. While there was some inter-individual variation, in many patients, suppression appeared to be temporary.

### Keywords

Peanut allergy; Oral immunotherapy; Sublingual Immunotherapy; Sustained Unresponsiveness; Basophil Activation; Dendritic Cells; Food Allergy

## INTRODUCTION

Peanut allergy, a public health concern with substantial morbidity, affects 1% of the Western world.<sup>1,2</sup> Current clinical management focuses on avoidance and treatment of reactions following accidental exposures.<sup>3</sup> However, we and others have recently demonstrated<sup>4</sup> that oral (OIT) and sublingual (SLIT) immunotherapy may allow subjects to tolerate increased amounts of peanut compared to their baseline, although clinical reactivity often returns once subjects discontinue treatment, suggesting these therapies more likely induce transient desensitization rather than longer term tolerance.<sup>5</sup>

The immunologic mechanisms underlying the clinical effects of immunotherapy (IT) continue to be elucidated. Initial studies in peanut OIT and SLIT demonstrated decreases in peanut-specific IgE with concomitant increases in IgG4, as well as reduced T<sub>H</sub>2 cytokine responses to peanut and upregulation of T regulatory (T<sub>reg</sub>) cells, especially in OIT.<sup>6-10</sup> Hypomethylation of the FOXP3 locus as a result of peanut OIT, and subsequent remethylation with regained sensitivity to peanut, has also been proposed to be associated with clinical desensitization.<sup>10</sup> Changes in basophil reactivity during food IT have been another area of interest, as basophils express the high affinity receptor for IgE and are critical effector cells in allergic reactions via their release of histamine, cytokines and leukotrienes upon stimulation.<sup>11,12</sup> Decreased peanut-induced expression of basophil activation markers CD63 and CD203c has been demonstrated during peanut OIT.<sup>6,13</sup> One study also suggested that IT may induce basophil hyporesponsiveness in a non-antigen-specific manner.<sup>14</sup> Studies in milk and egg IT have demonstrated similar findings, although in some cases with less robust basophil suppression.<sup>14,15</sup>

Dendritic cells (DCs) are professional antigen presenting cells that direct T cell responses to food and other antigens, and therefore likely drive the changes in T cell responses during IT. Two major classes of DCs have been identified in the peripheral blood of humans: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs).<sup>16-18</sup> Both subtypes regulate food allergen-driven T<sub>H</sub>2 cytokine release by CD4<sup>+</sup> T cells, and have been shown to exhibit phenotypic changes during the course of venom IT.<sup>19,20</sup>

In this pilot study, we sought to evaluate the systemic effects of peanut OIT and SLIT<sup>4</sup> on the function of immune cells critical in allergic responses and tolerance, including basophils

and DCs, in order to gain insight into the immunologic changes exerted by these therapies and to explore whether cellular immune responses qualitatively correlate with the clinical effects of OIT and SLIT.

## RESULTS

### Spontaneous and peanut-induced basophil histamine release (HR) and CD63 expression

To investigate changes in basophil reactivity during OIT and SLIT, we measured spontaneous histamine release (SHR) and constitutive expression of basophil activation markers CD63 and CD203c in basophil-enriched suspensions (Fig 1 and E3 in the Online Repository). We also examined the same parameters in response to three different doses of peanut (Fig 2 and E4 in the Online Repository). At the end of dose escalation (T3) and after 6 (T4) and 12 (T5) months of maintenance IT, spontaneous CD63 expression and SHR were reduced markedly in the OIT group ( $p < 0.01$ ) compared to baseline (Fig 1A, B). A qualitatively similar decrease was seen in SLIT at T3 and T4, but did not reach significance (Fig 1A, B). Peanut-induced HR and CD63 expression were also suppressed versus baseline by the end of dose escalation in OIT (T3) and in both OIT and SLIT after 6 months of maintenance therapy (T4), especially at the higher doses of peanut (Fig 2).

After this initial suppression, constitutive CD63 expression increased in OIT subjects despite continued maintenance dosing such that CD63 expression was no longer significantly depressed compared to baseline by the end of the maintenance period (T6) (Fig 1B). A qualitatively similar pattern was evident for SHR (Fig 1A), and for both parameters in subjects receiving SLIT (Fig. 1A, B). Peanut-induced HR and CD63 expression also reverted in OIT subjects while they continued maintenance therapy (Fig 2). These parameters remained suppressed in the SLIT cohort (Fig 2). Of note, due to a crossover study design, all subjects in the SLIT group had OIT added between T5 and T6. This did not appear to inhibit SHR or constitutive CD63 expression, while peanut-induced HR and CD63 expression remained suppressed (Fig 1A, B; Fig 2). Additionally, three of seven patients receiving OIT were augmented with SLIT between T5 and T6. This addition of SLIT resulted in qualitatively greater suppression of both constitutive or stimulated basophil reactivity in 2 of 3 subjects, compared to 2 of 4 of those subjects that continued on OIT alone (data not shown).

Increases in both constitutive and peanut-induced CD63 expression and HR were also evident after a short period off therapy (T7), especially when compared to the point of maximal suppression (Fig 1A, B; Fig 2). Constitutive CD203c expression was not suppressed at any timepoint and actually increased at T6 versus baseline in both OIT and SLIT groups ( $p = 0.043$  and  $p = 0.018$  respectively), and after therapy withdrawal at T7 for SLIT ( $p = 0.011$ ; Fig 1C). CD203c upregulation in response to peanut was generally small and did not show significant changes during OIT or SLIT (Fig E4 in the Online Repository). Finally, CD63 and HR were strongly correlated ( $p < 0.001$ ,  $r = 0.92$ ), while this relationship was significant but weaker for HR and CD203c ( $p < 0.01$ ,  $r = 0.11$ ) (Fig. E5 in the Online Repository).

Despite some qualitative differences in spontaneous basophil activity during OIT and SLIT, direct comparisons between these two arms showed no significant differences overall. However, OIT resulted in more rapid suppression of basophil responses to peanut compared to SLIT (e.g. for rate of decrease in HR between T1 and T3:  $p = 0.001$  and  $p = 0.038$  for 0.1ng/ml and 1ng/ml peanut, respectively).

### **Peanut-induced basophil IL-4 expression**

To determine whether changes in basophil IL-4 expression may play a role in desensitization, we incubated whole blood samples with peanut, anti-IgE, dust mite and ionomycin. Peanut-stimulated basophil IL-4 expression was significantly reduced from the end of dose escalation (T3) through maintenance (T4-T6) compared to baseline (T1) in both OIT (T1 vs. T3-T6,  $p < 0.05$  at 0.1ng/ml and  $p < 0.001$  at 1ng/ml and 10ng/ml of peanut) and SLIT (T1 vs. T5,  $p < 0.05$  at 0.1ng/ml and  $p < 0.001$  for T1 vs. T3-T6 at 1 ng/ml and 10 ng/ml), but unlike CD63 expression and HR, expression of IL-4 did not revert to higher levels during maintenance therapy (Fig 3A). However, IL-4 expression did increase once subjects were taken off therapy (T7 vs. T6) in both cohorts (Fig 3A). Direct comparisons between SLIT and OIT cohorts revealed no significant differences in basophil IL-4 expression.

### **Specificity of basophil suppression during peanut OIT and SLIT**

To determine whether the changes we observed in basophil reactivity were specific for peanut, we evaluated responses from basophil-enriched suspensions (CD63, CD203c, HR) and whole blood samples (IL-4) incubated with a polyclonal anti-human IgE crosslinking antibody, two doses of dust mite, or ionomycin, an inducer of FcεRI-independent basophil degranulation. Suppression of basophil IL-4 expression during OIT and SLIT was not peanut-specific, as it was evident following stimulation with both dust mite and anti-IgE (Fig 3B, C, D). In both OIT and SLIT, IL-4 responses to anti-IgE and dust mite increased after the therapy withdrawal period (T7), although these changes did not always reach significance (Fig 3B-D). Basophil CD63 expression and HR to anti-IgE and dust mite were low and did not change significantly with therapy in either OIT or SLIT (Fig E6). No marker of basophil reactivity (IL-4, CD63 or HR) to the IgE-independent stimulus ionomycin changed during the course of OIT or SLIT (Fig. 3, E6). Finally, CD203c expression did not change significantly following stimulation with any of these stimuli in either cohort (data not shown).

### **Dendritic cell-driven T cell cytokine responses**

Since DCs play a central role in dictating T cell responses to allergens, we explored how SLIT and OIT affected cytokine responses by CD4<sup>+</sup> T cells co-cultured with either pDCs or mDCs and stimulated with peanut or dust mite (an allergen for which subjects have not received IT). As seen in Figure 4, T<sub>H</sub>2 cytokine responses (IL-13 and IL-5) to peanut were robust at baseline (T1), with significantly higher expression in mDC-T than pDC-T co-cultures (IL-13,  $p = 0.003$ , IL-5  $p = 0.007$ ). After 12 months of maintenance therapy (T5), T<sub>H</sub>2 cytokine release to peanut as well as dust mite was suppressed in both pDC-T and mDC-T cell co-cultures from OIT and SLIT subjects compared to baseline (T1; Fig 4). However, T<sub>H</sub>2 cytokine expression subsequently increased despite continued treatment in the OIT cohort such that levels at T6 were no longer significantly depressed compared to

baseline (T1; Fig 4). This reversion was less evident in the SLIT group, perhaps because OIT had been added to their treatment regimen between T5 and T6 (Fig 4). Although levels of T<sub>H</sub>2 cytokines produced in response to PN remained lower at T7 (after subjects discontinued treatment) compared to baseline in the SLIT cohort, neither PN-induced IL-5 nor IL-13 was significantly different between T7 and T1 in the OIT group (Fig 4A). OIT and SLIT also significantly altered peanut-induced expression of IFN- $\gamma$ , IL-10, and TNF- $\alpha$  in a manner similar to the changes in T<sub>H</sub>2 cytokine responses, with suppression followed by increased expression (Fig E7), while IL-17 expression was generally unchanged (data not shown). A similar pattern was also seen in cultures stimulated with dust mite (data not shown). For all co-cultures, media alone conditions resulted in low to non-detectable cytokine production (data not shown). Direct comparisons did not show any significant differences in cytokine responses between OIT and SLIT cohorts.

### Expression of HLA-DR and Co-stimulatory molecules

To evaluate whether the changes in DC-driven release of T<sub>H</sub>2 cytokines were associated with changes in expression of co-stimulatory molecules and HLA-DR by these cells over the course of IT, we stained the co-cultures described above following incubation with media alone, peanut, or dust mite for CD40, CD80, CD86, and HLA-DR. As shown in Figure 5, significant changes were seen in a number of co-stimulatory molecules over the course of OIT and SLIT. CD40 and CD86 were significantly suppressed on mDCs and pDCs from the OIT cohort following peanut and dust mite stimulation after 12 months of maintenance dosing (T5), and a similar trend was seen for HLA-DR (Fig. 5). This suppression was less evident in the SLIT cohort, particularly on pDCs (Fig 5). DCs cultured in media alone showed qualitatively similar trends to cultures treated with allergen, although levels of expression of co-stimulatory molecules were lower than those observed following antigen stimulation (Fig 5, E8).

The decline in expression of CD40, CD86, and HLA-DR on DCs during IT appeared to only be temporary in many subjects. Following the initial decline after 12 months of maintenance therapy (T5), expression of these markers on mDCs increased while maintenance dosing continued (T6) and following withdrawal of therapy (T7) in OIT (Fig 5, E8). A similar pattern was seen in SLIT (Fig 5, E8). While these changes were most robust with mDCs, the pattern was often visible in pDC cultures as well, but did not always reach significance (Fig. 5, E8). Direct comparisons between OIT and SLIT did not show any significant difference in HLA-DR or co-stimulatory molecule expression.

Expression of CD80 showed a very different pattern than HLA-DR and the other co-stimulatory molecules (Fig 6). While essentially not detectable on mDCs at baseline (T1), expression of CD80 was significantly elevated after 12 months of maintenance dosing in OIT, and SLIT to a lesser extent (T1 vs. T5; Fig 6). However, after 18 months of maintenance therapy (T6), expression was nearly completely lost and remained absent after withdrawal of treatment (T7; Fig. 6). Expression of CD80 on pDCs was extremely low and did not change significantly with therapy (data not shown).

### Correlation between mechanistic and clinical outcomes

Several biomarkers were significantly correlated with certain clinical outcomes (Table 1). A negative correlation was found between achievement of sustained unresponsiveness and baseline basophil CD63 expression, histamine release, and IL-4 production observed at the low dose (0.1ng/ml) of peanut. At baseline, 4 out of 5 patients with basophil CD63 expression of 10% or lower (when incubated with 0.1 ng/ml peanut) achieved sustained unresponsiveness, while all patients with basophil CD63 expression of greater than 10% did not ( $p=0.002$  Fisher's exact test). Average histamine release and IL-4 production at 0.1ng/ml peanut was lower at baseline in patients who achieved sustained unresponsiveness (28.3% vs. 15.3% of total for HR, and 5.3% vs. 1.7% of total basophils for IL-4) but these differences were not significant. Basophil IL-4 expression in response to all 3 doses of peanut correlated positively with peanut-specific IgE. Additionally, a consistent positive correlation was noted between  $T_H2$  cytokine production in mDC-T cell co-cultures, which showed the most robust changes during IT, and peanut-specific IgG4 levels, while peanut-specific IgE/IgG4 levels correlated negatively with this outcome (Table 1). Finally, correlation of specific number of reactions, and the severity of these at the time of DBPCFCs and OFCs, was evaluated for based on expression of certain markers such as expression of CD63. No specific correlations were seen.

Despite these correlations, a significant amount of inter-individual variation was seen, with some patients maintaining clinical desensitization while immunologic markers increased. For example, subjects who were able to maintain sustained unresponsiveness had generally lower peanut-induced CD63 expression at baseline but not while therapy continued, and in fact, some successful patients had a greater rise in CD63 after an initial suppression than non-successful patients (Fig. E9). A different pattern was evident with IL-4, where subjects who achieved sustained unresponsiveness generally demonstrated lower IL-4 expression to higher doses of peanut as therapy continued (Fig E9). Significant inter-individual variation was also seen in other outcomes including expression of  $T_H2$  cytokines and co-stimulatory molecules on DCs (Fig E10).

## DISCUSSION

IT trials have generated great excitement that a treatment for food allergy is on the horizon; however, this enthusiasm has been tempered by the knowledge that side effects are common, and only a minority of subjects achieve sustained unresponsiveness. How these clinical observations correlate with the degree and nature of immunologic suppression is not well understood. Here, we demonstrate that OIT (and to a lesser degree SLIT) for peanut allergy effectively suppresses basophil effector cell function and DC-driven  $T_H2$  cytokine responses to peanut. However, these parameters reversed in many subjects once they withdrew from therapy, and in some cases, while they continued on maintenance dosing. To our knowledge, this is the first study to demonstrate that systemic immunologic suppression mediated by IT for peanut allergy may not be long-lasting.

Stimulated expression of CD63 and CD203c on basophils has been studied as a potential biomarker of IgE-mediated food allergic responses.<sup>22</sup> SLIT and OIT potently suppressed peanut-induced expression of CD63 and HR from basophils, but not CD203c. HR strongly

correlated with CD63 and more weakly with CD203c, consistent with reports that CD63 is the best indicator of anaphylactic degranulation.<sup>22</sup> Expression of CD203c can be induced by IL-3, exhibits different kinetics than CD63, and may reflect piecemeal rather than anaphylactic degranulation.<sup>23</sup> While some previous studies have shown suppression in CD203c over the course of peanut OIT,<sup>13</sup> these studies were performed in whole blood with IL-3 stimulation, as opposed to washed basophil suspensions without IL-3 in our study. Basophils additionally support T<sub>H</sub>2 immune responses by producing IL-4 following activation through FcεRI. This function of basophils was also attenuated during IT, suggesting a novel mechanism by which these cells may contribute to immunologic suppression during IT.

Basophils from the majority of food allergic children have been shown to spontaneously release histamine.<sup>24</sup> While the clinical relevance and mechanisms responsible for this phenomenon are not well understood, high SHR appears to be IgE-dependent and may indicate more severe clinical reactivity to cow's milk.<sup>25,26</sup> Consistent with our previous findings in a trial of milk OIT,<sup>14</sup> peanut OIT significantly reduced both SHR and constitutive CD63 expression by the end of the dose escalation period. Collectively, these data support an overall decrease in IgE-dependent pathway activation in basophils early in the course of OIT.

Induction of T cell tolerance and/or anergy is purported to be central to the mechanisms of IT. Both pDCs and mDCs direct memory responses by CD4<sup>+</sup> T cells to food allergens, but in our study mDCs appeared to promote greater T<sub>H</sub>2 cytokine responses to peanut than pDCs. Interestingly, Ara h 1, a major peanut allergen, directly binds to Dendritic cell (DC)-specific ICAM-grabbing nonintegrin (DC-SIGN) on mDCs and acts as a T<sub>H</sub>2 adjuvant to activate T cells.<sup>27</sup> Peanut-induced levels of T<sub>H</sub>2 cytokines declined in DC-T cell co-cultures after 12 months of maintenance dosing, and were associated with reduced expression of co-stimulatory molecules CD86 and CD40 as well as HLA-DR on DCs. On the other hand, expression of CD80 increased on mDCs at the same time point. While the mechanisms of T-cell costimulation are highly complex,<sup>28</sup> some studies suggest that CD80 is the preferential ligand for the inhibitory T cell molecule CTLA-4,<sup>29</sup> suggesting the increase in CD80 on mDCs during IT may serve to dampen T cell responses. Nearly all of the changes in DC phenotype and function we observed during peanut IT were more prominent with mDCs than pDCs. Consistent with other IT trials for food allergy,<sup>9</sup> we did not find a switch from T<sub>H</sub>2 to T<sub>H</sub>1 cytokine responses during IT, but rather a generalized suppression of effector cytokine expression. It was recently demonstrated that the increase in IFN-γ during SLIT for grass pollen allergy is not mediated by T cells<sup>30</sup>; therefore, we cannot exclude the possibility that other cell types did indeed lead to increased Th1 and IL-10 responses in our study that we never detected since we only evaluated the T cell arm.

While peanut IT effectively suppressed basophil, DC and T cell reactivity by the end of dose escalation and after the first 6 months of maintenance dosing, remarkably almost all of the changes were only temporary in a majority of subjects. Peanut-induced and spontaneous HR and CD63 expression by basophils increased despite continued maintenance therapy compared to earlier time points, and IL-4 responses also reverted once subjects withdrew from therapy. IL-4 may have been more persistently suppressed than HR/CD63 as our IL-4

assay was performed in whole blood rather than washed cells, and therefore basophils in this assay had continued exposure to inhibitory serum factors. Even constitutive expression of CD203c, which hadn't changed during the early stages of IT, significantly increased after 1 year of maintenance dosing. This may have clinical relevance, as patients with lower levels of milk tolerance have been demonstrated to have elevated constitutive CD203C expression at baseline.<sup>31</sup> Likewise, the reduction in DC-driven T<sub>H</sub>2 cytokine responses to peanut, as well as expression of activation markers and HLA-DR on DCs, was often only transient.

The immunologic effects of IT were largely not antigen-specific. DC and T cell responses to dust mite were also reduced, and this suppression appeared to be transient as well in many subjects. This is consistent with previous findings suggesting IT promotes a pathway specific, antigen non-specific basophil energy.<sup>13</sup> While basophil HR and CD63 expression in response to anti-IgE and dust mite did not significantly change during treatment, neither of these stimuli evoked potent responses, perhaps because the doses used were more optimal for IL-4 expression than histamine release. Basophil expression of IL-4 to these stimuli was significantly attenuated, while no change in any measure of basophil reactivity to ionomycin, a non-IgE-dependent stimulant, was observed.

Our study did have several important limitations. First, we did not have a placebo group that received no intervention, although the study was placebo-controlled during the double blind treatment phase. Second, there was a high drop-out rate due to adverse effects, and mechanistic analyses were not performed on subjects who discontinued study participation, which may have excluded subjects with less robust immunologic suppression and skewed toward those better able to tolerate IT. An exception to this was data from histamine release, which was obtained at baseline and at one or two subsequent visits on patients who later dropped out. The addition of these data improves the significance obtained for the predictive value of baseline histamine release for achieving sustained unresponsiveness at 0.1ng/ml from 0.047 to 0.008 (Table 1), but otherwise does not alter the data. Detailed clinical information on the patients who dropped out can be found in Narisety et al.<sup>4</sup> Third, the crossover design<sup>4</sup> in which all patients on SLIT were augmented with OIT and some patients on OIT were augmented with SLIT created added complexity in analyzing the data, but remarkably revealed that add-on treatments generally did not prevent the increase in immunologic reactivity. For instance, we found that OIT augmentation on SLIT therapy seemed to be associated with continued suppression of stimulated CD63 expression, but most other immunologic markers showed reversion despite OIT augmentation. Finally, only subjects who completed the treatment and had no reaction at their T6 oral food challenge proceeded to T7, which resulted in an N of only 9 subjects at the T7 time point.

The degree of immunologic suppression achieved was qualitatively more pronounced in OIT than SLIT, consistent with our clinical observations that OIT subjects generally experienced greater clinical improvement than those receiving SLIT.<sup>4</sup> OIT subjects may have a more pronounced response due to overall larger dose of allergen received. SLIT subjects were more likely in some cases to exhibit persistent immunologic suppression, perhaps because they were augmented with OIT late in the maintenance phase due to the crossover design of the study. The high frequency of reactions and failure of most subjects to achieve sustained unresponsiveness to peanut is consistent with the transient nature of immunologic



suppression to peanut that we observed, although it is interesting to note that many subjects remained able to ingest peanut despite an apparent loss of immunologic suppression as mediated by both basophils and dendritic cells. This may be due to small sample size and the considerable inter-individual variation within the sample, or may suggest that other unknown mechanisms may play a role in induction and maintenance of desensitization. Although the study was not powered to identify predictors of clinical outcome, baseline basophil CD63 expression, HR, and Il-4 production at low doses of peanut did correlate significantly with achievement of sustained unresponsiveness, suggesting that those patients with the lowest baseline basophil responsiveness to peanut may have a better clinical outcome. The consistent correlation between T<sub>H</sub>2 cytokine production in mDC-T cell co-cultures, which showed the most robust changes during IT, and peanut-specific IgG4 levels suggests that further investigation into the role of IgG4 in modulating T cell responses during IT is warranted. A caveat in the interpretation of these data is the inherent risk of increased type 2 error when making multiple comparisons simultaneously. While larger clinical studies are needed to verify our findings and further inform their clinical significance, this pilot study raises the important possibility that current forms of IT for food allergy fail to elicit persistent immunologic suppression.

## METHODS

### Clinical Protocol Summary

Subjects aged 6–21 years with a diagnosis of peanut allergy (PA) were recruited from the Johns Hopkins Pediatric Allergy Clinic. Participants underwent a baseline evaluation including an oral food challenge (OFC) with up to 1,000 mg of peanut protein, after which eligible subjects were randomized 1:1 to receive either active SLIT with placebo OIT or active OIT with placebo SLIT. Over the next 16 weeks, subjects took daily home doses and returned to the research unit every 1-2 weeks for observed dose increases, with goal maintenance doses of 3.7 mg/day for SLIT and 2000 mg/day for OIT. A 10 gram OFC was completed after 6 months and 12 months of maintenance and, after the 12-month challenge, subjects and investigators were unblinded. Those subjects who completed the 12 month OFC with no more than mild symptoms were taken off treatment for 4 weeks and re-challenged. All other subjects proceeded to the unblinded phase of the study per protocol as follows: (1) those who tolerated between 5 and 10 grams before reacting continued on their prior SLIT or OIT maintenance for an additional 6 months, after which they underwent another 10 gram OFC; (2) those who reacted at < 5 grams were continued on their current active OIT or SLIT and had either active SLIT or OIT added for an additional 6 months. After that period, all subjects underwent a 10 gram OFC and those who tolerated the OFC were taken off therapy for 4 weeks and re-challenged.

### Basophil histamine release (HR) and expression of activation markers

Blood was collected in EDTA and subjected to double Percoll (Pharmacia Biotech, Piscataway, NJ) density centrifugation, as previously described.<sup>S1</sup> The lower fraction of cells using this protocol consists of basophil-enriched mononuclear cells (BECs) that were washed once in piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES)/albumin/glucose and then again in column buffer (PIPES containing 1% BSA and 2 mmol/L EDTA). Basophils

were counted after staining with Alcian blue. BECs (20,000 basophils per condition) were then cultured in a final volume of 50  $\mu$ L in AIM V medium (Invitrogen Life Technologies, Carlsbad, Calif). Cells were stimulated with media alone, crude peanut extract (Greer Laboratories) at 0.1ng/mL, 1ng/mL, and 10ng/mL, polyclonal goat anti-human IgE antibody at 10 ng/mL (generated in-house), dust mite (*D. Pteronyssinus*) extract (Greer Laboratories) at 1AU/mL or 10/AU/mL, or ionomycin (Sigma Aldrich, St. Louis, MO) at 500ng/mL. After a 45-minute incubation at 37°C, 1 mL of PIPES/albumin/glucose was added, and HR was measured in the cell-free culture supernatants by using automated fluorometry.<sup>S2</sup> The remaining cell pellet was fixed in 4% buffered paraformaldehyde (Sigma Aldrich, St. Louis, MO) and stored in 10% dimethyl sulfoxide (DMSO)/PBS (Fisher Scientific) at -80°C. Percentage histamine release under each condition was calculated relative to total histamine content, which was determined by treating an identical number of BECs with perchloric acid (1.6% final). Spontaneous HR was subtracted from values reporting HR in response to peanut, dust mite, anti-IgE, and ionomycin.

BECs from all time points were analyzed simultaneously for constitutive expression of CD63 and CD203c using flow cytometry. Cells were first washed in PBS and then blocked with human IgG (1 mg/mL, Sigma Chemical Co). The following antibodies were used: CD63-Phycoerythrin-Cyanine 7 (PE-Cy7; BD Pharmingen, San Jose, CA), CD203c-Phycoerythrin (PE; eBioscience, San Diego, CA), CD123-allophycocyanin (APC; eBioscience), and BDCA2-fluorescein isothiocyanate (FITC; eBioscience). Stained cells were analyzed with a FACSCalibur (BD PharMingen). Basophils were identified by gating on cells that were CD123<sup>+</sup> and BDCA2<sup>-</sup>, and activated CD63 expression was identified by gating on basophils demonstrating CD63<sup>high</sup> versus CD63<sup>low</sup> expression, while CD203c mean fluorescence intensity (MFI) was measured on all basophils. Appropriate isotype controls were used in all assays. Data was analyzed using Flowjo software (Tree Star, Inc., Ashland, OR).

### Measurement of basophil IL-4 expression

Heparinized blood was treated with stimulants exactly as described above for HR, with the addition of brefeldin A (Sigma Aldrich, St. Louis, MO) at a final concentration of 5  $\mu$ g/mL, in a 96 well round bottom plate in a final volume of 0.2 mL per condition. After a 120-minute incubation at 37°C, 0.1 mL of cell suspension was added to a 2 mL eppendorf containing 1.9 mL of pre-warmed Lyse/Fix solution (BD Pharmingen). The cells were incubated for 13 minutes in a 37° water bath, and then immediately spun. The cell pellet was washed in PBS and then stored in 10% DMSO/PBS at -80°C. Samples from all time points were analyzed simultaneously. Measurement of IL-4 protein expression was done by intracellular flow cytometry. Cells were first washed in PBS and then blocked with human IgG (1 mg/mL, Sigma-Aldrich) in PBS-0.1% saponin-5% non-fat milk buffer for 1 hour on ice (saponin; Sigma-Aldrich, non-fat dry milk; Nestle Carnation brand). Cells were then stained with the following antibodies for 30 minutes on ice: BDCA2-FITC (Miltenyi), CD123-PE (BD Pharmingen) and IL-4-APC (eBioscience). Basophils were identified by gating on cells that were CD123<sup>+</sup> and BDCA2<sup>-</sup>. Stained cells were analyzed with a FACSCalibur (BD PharMingen). Instrument variability was corrected for by using CaliBRITE allophycocyanin calibration beads (BD PharMingen).

## Preparation and culture of dendritic cells and T cells

Following double Percoll density centrifugation as described above, the upper fraction of cells consisted of basophil-depleted mononuclear cells and were used to isolate pDCs using BDCA4<sup>+</sup> magnetic bead selection (Miltenyi, Bergisch Gladbach, Germany). Cells not retained on this column were then used to isolate mDCs with BDCA1<sup>+</sup> selection (Miltenyi) after depletion of CD19<sup>+</sup> B cells. CD4<sup>+</sup> T cells were prepared by positive selection (Miltenyi) from remaining cells after DC removal.

DC subtypes ( $2.5 \times 10^4$  cells) were co-cultured with autologous CD4<sup>+</sup> T cells ( $1 \times 10^5$  cells) in a final volume of 200  $\mu$ L of AIM V and stimulated with media alone, 50  $\mu$ g/ml of crude peanut extract (Greer, Lenoir, NC, USA), or 100 AU/mL of dust mite extract (D. Pteronyssinus) for 5 days in a 96 well round bottom plate. CD4<sup>+</sup> T cells were also cultured alone. Cell supernatants were collected and stored at  $-80^\circ\text{C}$  for cytokine analysis. The cell pellets were combined, fixed in 4% buffered paraformaldehyde, and stored in 10% DMSO at  $-80^\circ\text{C}$ .

## Measurement of HLA-DR and activation markers in DC- T cell co-cultures

Samples from all time points were analyzed simultaneously. Cells were first washed in PBS and then blocked with human IgG (1 mg/mL, Sigma-Aldrich). The pDC-T cell fractions were stained with CD123-APC (eBioscience), and the aforementioned Fc $\epsilon$ R1-PE, CD3-APC-Cy7, CD80-PerCP-eF710, CD86-AF488, HLA-DR-eF450, and CD40-PE-Cy7. The mDC-T cell fractions were labeled with BDCA1-APC (eBioscience) and CD19-APC-Cy7 (Biolegend) along with the Fc $\epsilon$ R1-PE, CD3-APC-Cy7, CD80-PerCP-eF710, CD86-AF488, HLA-DR-eF450, and CD40-PE-Cy7. Samples were run on a BD FACSVerse (BD Biosciences, San Jose CA). pDCs were identified as CD123<sup>+</sup> CD3<sup>-</sup>, and mDCs as BDCA-1<sup>+</sup> CD3<sup>-</sup> CD19<sup>-</sup> cells. After gating on each cell type, the MFI of each activation marker and HLA-DR was determined. Appropriate isotype controls were included in each experiment.

## Cytokine measurements

Cytokines were measured using multiplex bead immunoassay (Millipore) according to the manufacturer's directions. A human x-plex panel consisting of IL-5, IL-13, IFN- $\gamma$ , IL-10, and IL-17, and TNF- $\alpha$  were used to evaluate supernatants from DC-T co-cultures. Limits of detection for this assay are IL-5 0.6 pg/mL, IL-13 0.5 pg/mL, IFN- $\gamma$  0.6 pg/mL, IL-10 0.6 pg/mL, IL-17 0.7 pg/mL, and TNF- $\alpha$  0.7pg/mL. Values obtained from culturing T cells alone were subtracted from the values obtained in the co-culture conditions.

## Statistical Analysis

Outcomes were evaluated by using linear regression models with generalized estimating equations to account for repeated measures over time or simple regression when appropriate. Difference in an outcome between a study timepoint (T3-T7) and baseline (T1) were considered significant only if the overall p-value for the GEE taking into account multiple comparisons was significant, and the p-value comparing values within the GEE model at one of the time points compared to baseline was also significant. Statistically significant p-values, defined as  $<0.05$ , are indicated. When appropriate, variables were log-transformed for analysis. Correlations between HR and CD63/CD203c expression were performed using

standard regression. All analyses were performed with STATA/SEv11 software (StataCorp, College Station, Tex). In the graphs, the box defines the 25<sup>th</sup> and 75<sup>th</sup> percentile; center line the median; whiskers the adjacent values; individual points the outliers

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>OIT</b>	Oral immunotherapy
<b>SLIT</b>	Sublingual immunotherapy
<b>DC</b>	Dendritic Cell
<b>pDC</b>	Plasmacytoid Dendritic Cell
<b>mDC</b>	Myeloid Dendritic Cell
<b>DBPCFC</b>	Double-Blind Placebo-Controlled Food Challenge
<b>OFC</b>	Open Food Challenge
<b>IT</b>	Immunotherapy

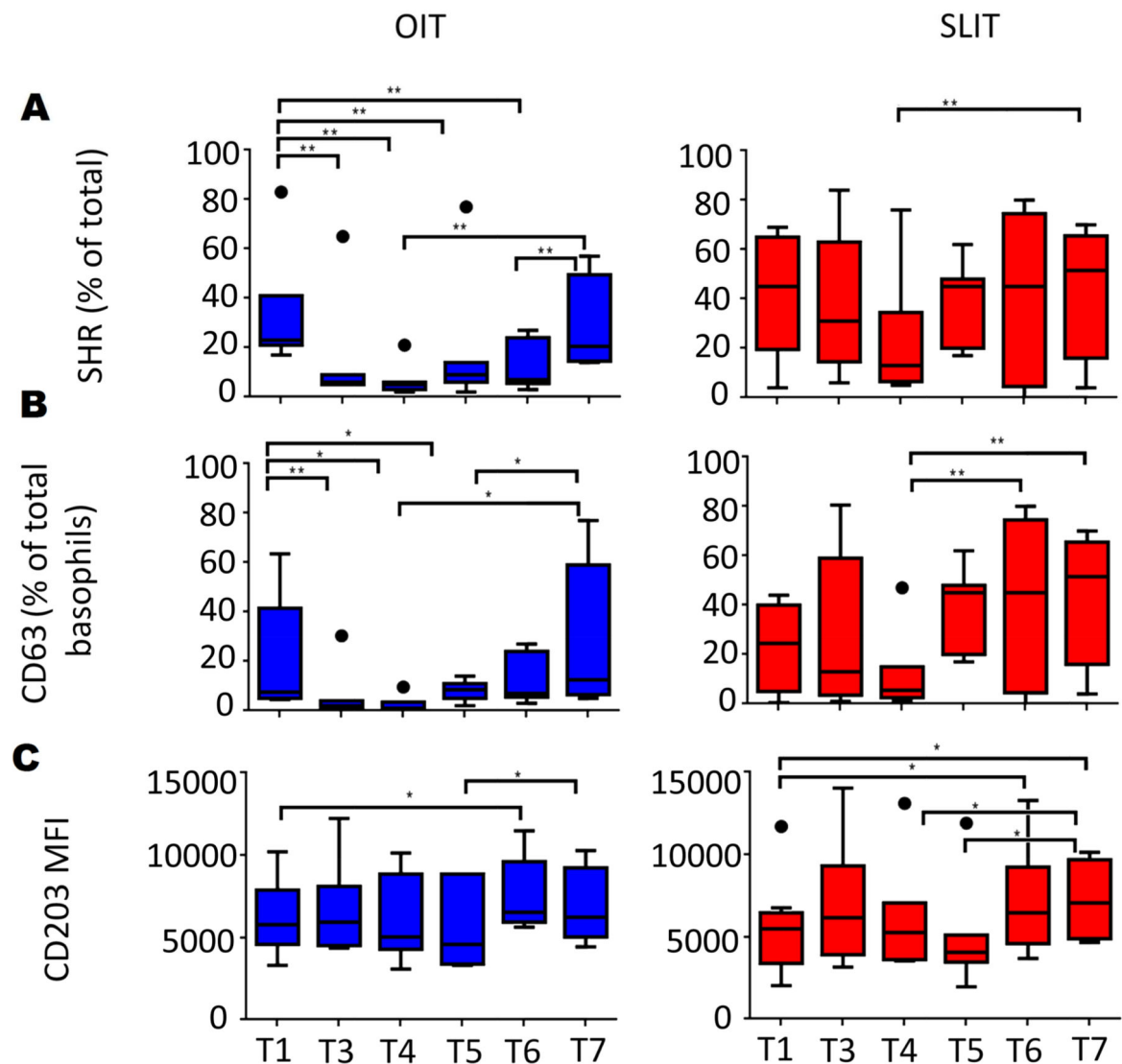
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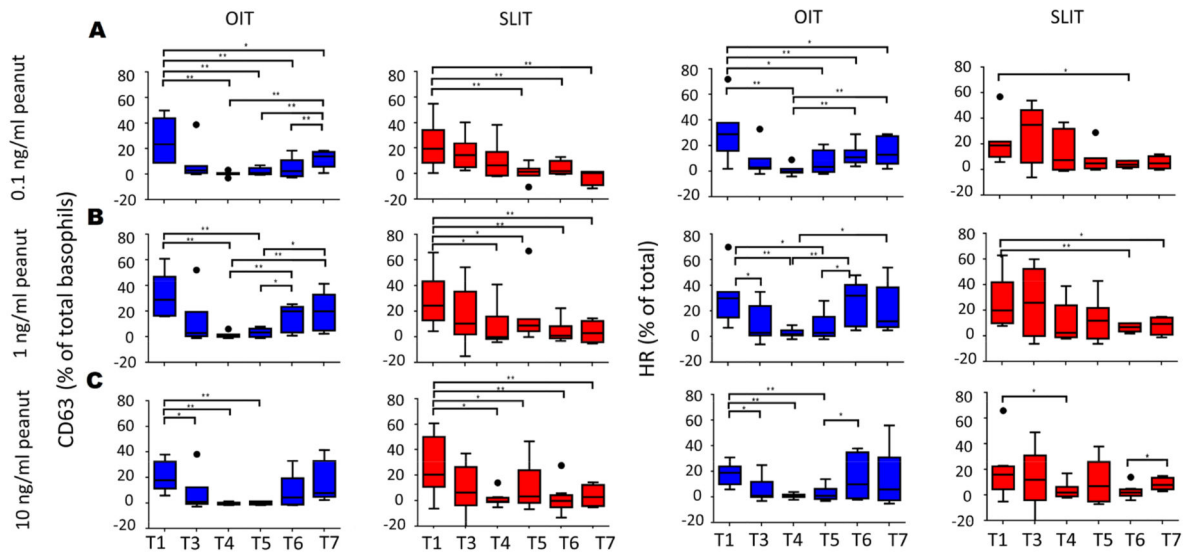
**Key messages**

- Oral and sublingual immunotherapy for peanut allergy suppress basophil and dendritic cell (DC)-driven T cell effector functions, although this inhibition is often transient.
- Although there was significant inter-individual variation and mechanistic outcomes did not always correlate with clinical outcomes, these findings may offer a mechanistic basis as to the relatively low rates of sustained unresponsiveness seen in immunotherapy trials for food allergy.

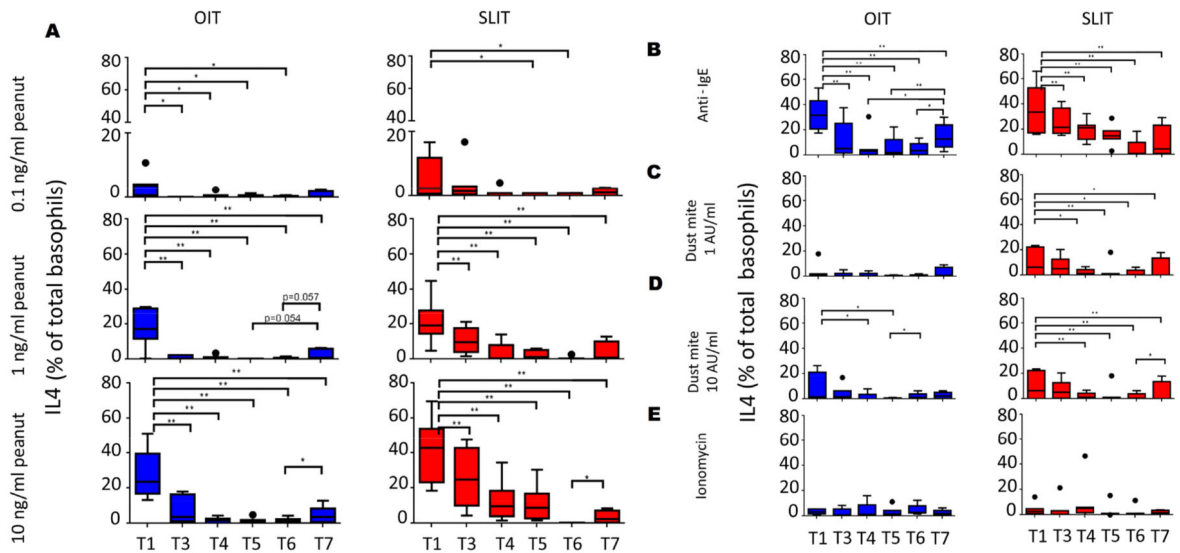
**FIG 1.**

Spontaneous histamine release (SHR) and constitutive expression of basophil activation markers during IT. SHR (A) and constitutive CD63 (B) and CD203c mean fluorescence intensity (MFI) (C) were measured in basophil-enriched suspensions from subjects undergoing OIT (left panels) and SLIT (right panels) for peanut. T1-T7 correspond to timepoints blood was collected. OIT: N=7 at T1-T5, 5 at T6, and 4 at T7; SLIT: N=8 at T1-T4, 7 at T5, 8 at T6 and 4 at T7. \* p<0.05; \*\* p<0.01

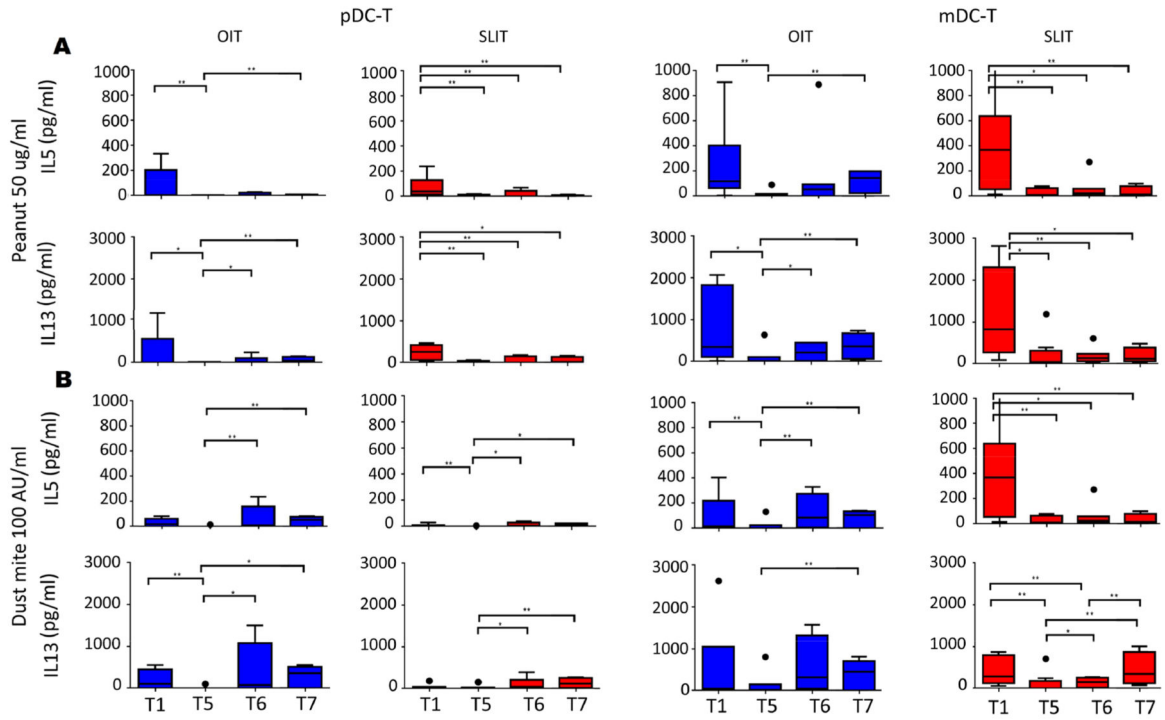


**FIG 2.**

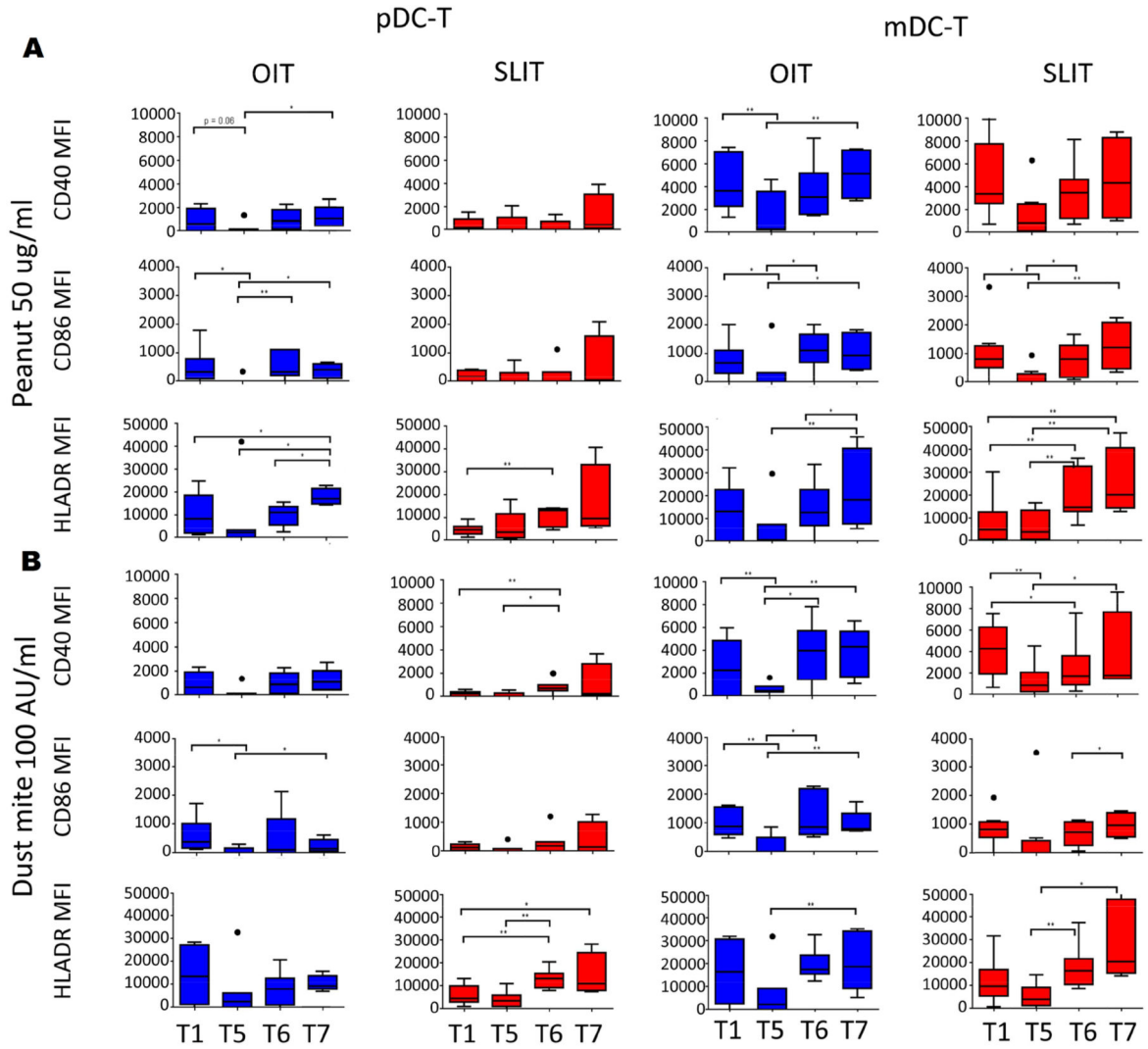
Effect of OIT and SLIT on basophil CD63 expression and histamine release (HR) in response to peanut. Basophil-enriched suspensions were stimulated with crude peanut extract at 0.1 ng/ml (A), 1 ng/ml (B) and 10ng/ml (C). Spontaneous histamine release and CD63 expression in media alone were subtracted to obtain stimulated values. T1-T7 correspond to timepoints blood was collected. OIT: N=7 at T1-T5, 5 at T6, and 4 at T7; SLIT: N=8 at T1-T4, 7 at T5, 8 at T6 and 4 at T7. \* p<0.05; \*\* p<0.01

**FIG 3.**

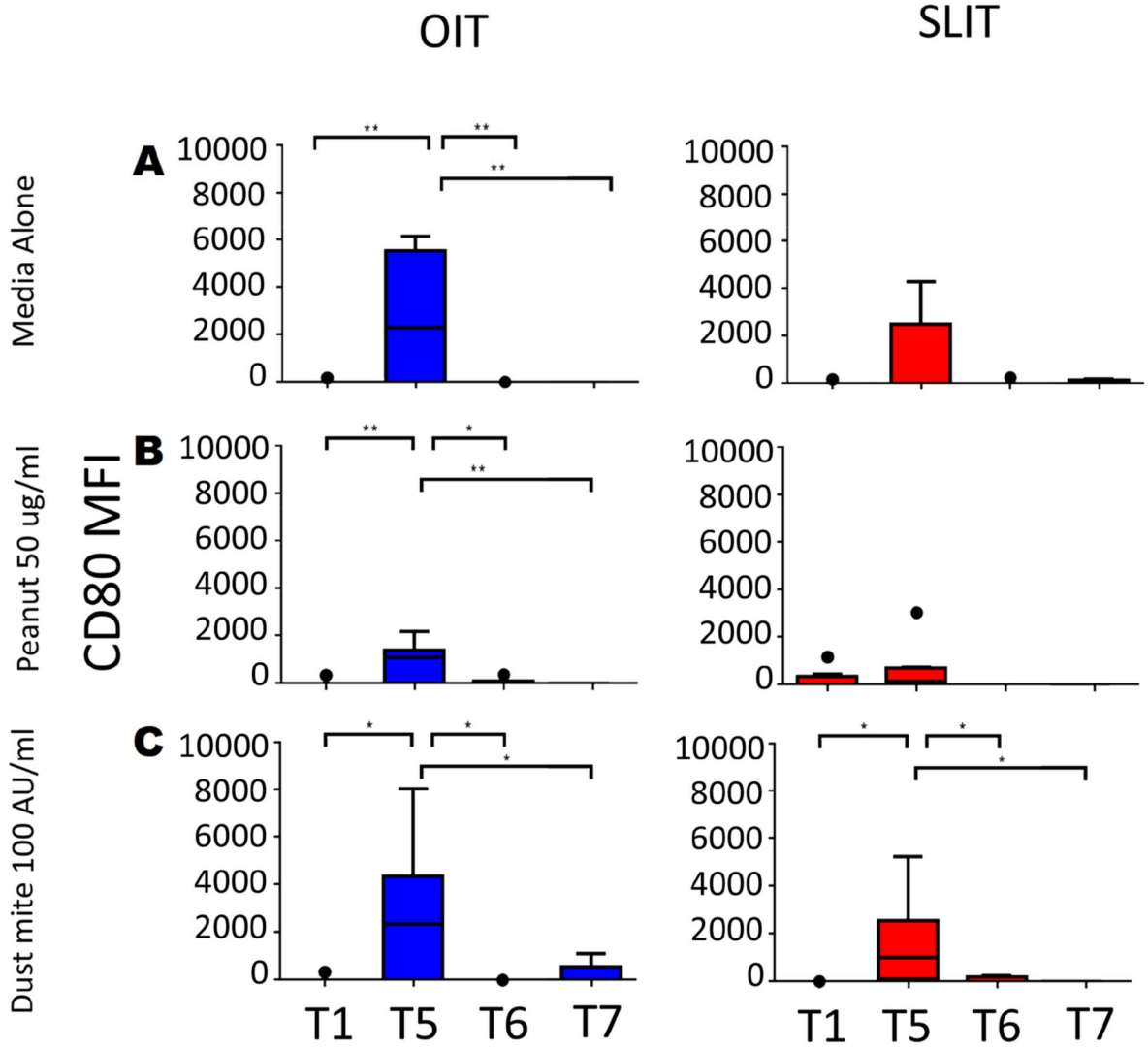
Changes in basophil IL-4 expression during peanut OIT and SLIT. Basophil expression of IL-4 was measured by intracellular flow cytometry after incubation of whole blood samples with peanut (A) anti-IgE (B), Dust mite at 1 AU/ml (C) or 10 AU/ml (D), and ionomycin (E). T1-T7 correspond to timepoints blood was collected. OIT: N=7 at T1-T5, 5 at T6, and 4 at T7; SLIT: N=8 at T1-T4, 7 at T5, 8 at T6 and 4 at T7. \*  $p < 0.05$ ; \*\*  $p < 0.01$



**Fig 4.** Effect of peanut OIT and SLIT on  $T_H2$  cytokine responses in DC-T cell co-cultures. IL-5 and IL-13 were measured in supernatants from co-cultures of pDCs and mDCs with autologous  $CD4^+$  T cells stimulated with 50 ug/ml crude peanut extract (A) or dust mite 100 AU/ml (B). Spontaneous cytokine secretion as measured in media alone was subtracted to obtain allergen-induced values. T1-T7 correspond to timepoints blood was collected. OIT: N=7 at T1-T5, 5 at T6, and 4 at T7; SLIT: N=8 at T1-T4, 7 at T5, 8 at T6 and 4 at T7. \*  $p < 0.05$ ; \*\*  $p < 0.01$



**Fig 5.** Effect of peanut OIT and SLIT on DC expression of co-stimulatory molecules and HLA-DR. Mean fluorescence intensity (MFI) of CD40, CD86, and HLA-DR on pDCs (left two columns) or mDCs (right two columns) following co-culture with CD4<sup>+</sup> T cells stimulated with peanut 50ug/ml (**A**) or dust mite 100 AU/ml (**B**) T1-T7 correspond to timepoints blood was collected. OIT: N=7 at T1-T5, 5 at T6, and 4 at T7; SLIT: N=8 at T1-T4, 7 at T5, 8 at T6 and 4 at T7. \* p<0.05; \*\* p<0.01



**Fig 6.** Effect of peanut OIT and SLIT on expression of CD80 on mDCs. CD80 mean fluorescence intensity (MFI) was measured on mDCs following co-culture with CD4<sup>+</sup> T cells stimulated with media alone (A), peanut 50ug/ml (B) or dust mite 100 AU/ml (C). T1-T7 correspond to timepoints blood was collected. OIT: N=7 at T1-T5, 5 at T6, and 4 at T7; SLIT: N=8 at T1-T4, 7 at T5, 8 at T6 and 4 at T7.. \* p<0.05; \*\* p<0.01

**Table 1**  
**Correlation of selected biomarkers with clinical outcomes**

Biomarker	Pass or Fail	Total dose	SPT	Log IgE	Log IgG4	Log IgE/IgG4
Spont. CD63 media	0.476	0.757	0.272	0.393	0.018	0.434
CD63 Peanut 0.1ng/ml	<0.001	0.673	0.411	0.959	0.058	0.226
CD63 Peanut 1 ng/ml	0.089	0.727	0.064	0.318	0.174	0.836
CD63 Peanut 10ng/ml	0.949	0.638	0.092	0.576	0.082	0.927
CD63 anti-IgE	NS	NS	NS	NS	NS	NS
CD63 ionomycin	NS	NS	NS	NS	NS	NS
CD63 Dust Mite 1AU/ml	NS	NS	0.041	NS	NS	NS
CD63 Dust Mite 10 AU/ml	0.017	0.305	0.075	NS	0.872	NS
IL4 Peanut 0.1ng/ml	0.013	0.851	0.591	0.047	0.742	0.152
IL4 Peanut 1ng/ml	0.053	0.536	0.079	0.001	0.524	0.057
IL4 Peanut 10ng/ml	0.980	0.051	0.147	0.011	0.510	0.099
IL4 anti-IgE	0.018	0.076	0.936	0.116	0.849	0.879
IL4 ionomycin	NS	NS	NS	NS	NS	NS
IL4 Dust Mite 1AU/ml	0.552	0.935	0.791	0.191	0.501	0.924
IL4 Dust Mite 10 AU/ml	0.485	0.125	0.703	0.038	0.605	0.650
Histamine media (SHR)	0.332	0.548	0.749	0.280	0.915	0.344
Histamine Peanut 0.1ng/ml	0.047	0.075	0.995	0.103	0.012	0.104
Histamine Peanut 1 ng/ml	0.233	0.556	0.262	0.330	0.083	0.739
Histamine Peanut 10ng/ml	0.892	0.930	0.248	0.332	0.090	NS
Histamine anti-IgE	NS	NS	NS	0.748	NS	NS
Histamine ionomycin	NS	0.388	NS	0.406	NS	NS
Histamine Dust Mite 1AU/ml	NS	0.116	0.018	NS	NS	NS
Histamine Dust Mite 10 AU/ml	NS	NS	NS	0.042	NS	NS
IL13 pDC Peanut 50ug/ml	0.412	NS	0.221	0.581	0.978	0.739
IL13 mDC Peanut 50ug/ml	0.492	0.745	0.667	0.209	0.049	0.029
IL13 pDC Dust Mite 100AU/ml	0.382	0.044	0.048	0.923	0.920	0.896
IL13 mDC Dust Mite100AU/ml	0.845	0.872	0.885	0.345	0.007	0.016
IL5 pDC Peanut 50ug/ml	0.409	0.660	0.458	0.225	0.861	0.377
IL5 mDC Peanut 50ug/ml	0.486	0.974	0.771	0.450	0.008	0.033
IL5 pDC Dust Mite 100AU/ml	0.757	0.171	0.013	0.823	0.635	0.646
IL5 mDC Dust Mite 100AU/ml	0.701	0.779	0.943	0.982	0.021	0.139

P values describing significant positive correlations are shown in green and negative correlations in red. NS indicates that the Wald test was > 0.05. Pass or Fail – success or failure in achieving sustained hyporesponsiveness, Total dose – mg of peanut protein ingested during each challenge without symptoms, SPT – peanut skin prick test wheal size in mm, Log IgE – log of peanut-specific IgE (kUa/L), Log IgG4 – log of peanut - specific IgG4 (mGa/L), Log IgE/IgG4 – ratio of the logs of peanut-specific IgE and IgG4. Pass or Fail outcomes were correlated to baseline biomarkers only, while all other outcomes were correlated over the entire course of the clinical trial.