

ORIGINAL ARTICLE

Clinical Mechanisms in Allergic Disease

Blocking antibodies induced by peanut oral and sublingual immunotherapy suppress basophil activation and are associated with sustained unresponsiveness

Kelly Orgel^{1,2} | Caitlin Burk^{1,2} | Johanna Smeekens^{1,2}  | Jada Suber^{1,2} |
Lakeya Hardy^{1,2} | Rishu Guo^{1,2} | A. Wesley Burks^{1,2} | Michael Kulis^{1,2} 

¹Department of Pediatrics, UNC School of Medicine, Chapel Hill, North Carolina

²UNC Food Allergy Initiative, Chapel Hill, North Carolina

Correspondence

Michael Kulis, UNC Chapel Hill, Chapel Hill, NC.

Email: mike.kulis@unc.edu

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Abstract

Background: Oral and sublingual immunotherapies for peanut allergy have demonstrated efficacy in small clinical trials; however, mechanisms and biomarkers correlating with clinical outcomes remain elusive. Previous studies have demonstrated a role for IgG in post-OIT plasma in the suppression of IgE-mediated mast cell reactions.

Objective: The aim of this study was to characterize the role that peanut oral and sublingual immunotherapy-induced plasma factors play in the inhibition of *ex vivo* basophil activation and whether inhibitory activity is associated with clinical outcomes.

Methods: Plasma samples from subjects on placebo, peanut oral immunotherapy (OIT) or peanut sublingual immunotherapy (SLIT), and IgG-depleted plasma or the IgG fraction were incubated with sensitized basophils, and the inhibition of basophil activation following stimulation with peanut extract was measured. Basophil inhibition results were compared between the two routes of immunotherapy, time on treatment and clinical outcomes.

Results: Plasma from subjects after 12 months of active peanut OIT, but not placebo, inhibits basophil activation *ex vivo*. Depletion of IgG abrogated the blocking effect of OIT plasma, while the IgG fraction substantially blocked basophil activation. Basophils are inhibited to a similar extent by undiluted OIT and SLIT plasma; however, diluted OIT plasma from the time of desensitization challenge inhibited basophils more than diluted SLIT plasma from time of desensitization challenge. Plasma from subjects who experienced sustained unresponsiveness following OIT inhibited basophils to a greater extent than plasma from subjects who were desensitized, but this was not true for SLIT.

Conclusions and Clinical Relevance: Peanut immunotherapy induces IgG-dependent functional changes in plasma that are associated with OIT but not SLIT clinical outcomes. Understanding the mechanisms of peanut OIT and SLIT may help derive informative biomarkers.

KEYWORDS

basophil activation, food allergy, IgG, immunoglobulin, immunotherapy, peanut allergy

1 | INTRODUCTION

Peanut allergy is a major public health concern affecting 1% of the US and European populations, rising in prevalence, and outgrown in only 20% of those affected with the disease.¹⁻³ Although there are no FDA- or EMA-approved treatments for peanut allergies, extensive investigation has focused on the use of several immunotherapy approaches. Emerging peanut allergy therapies include oral immunotherapy (OIT),⁴⁻⁶ sublingual immunotherapy (SLIT)^{7,8} and epicutaneous immunotherapy (EPIT),^{9,10} among others.¹¹ While routes, doses and duration vary for each form of therapy, these therapies all expose the allergic subject to increasing quantities of peanut protein over a period of months to years. OIT and SLIT have been effective at inducing both short-lived desensitization (defined as an increased allergen threshold while taking therapy daily) and sustained unresponsiveness (SU) (defined as an absence of allergic symptoms during challenge after stopping therapy).^{5,12} However, no confirmed biomarkers exist to identify which subjects will achieve SU and which will be transiently desensitized.

Despite the promising clinical findings, the mechanisms by which OIT and SLIT alter the immune system and result in desensitization or SU remain unclear. Skin prick test data and mechanistic studies have previously shown that peanut immunotherapies promote mast cell and basophil hyporesponsiveness^{4,13,14} as well as an increase in regulatory T cells.^{4,14,15} Extensive work by our group and others has demonstrated that peanut-specific IgE (PN-sIgE) levels often transiently increase on OIT and SLIT within a few months of starting therapy but are significantly decreased after many months or years of therapy.⁴ It is important to note that PN-sIgE levels are uncoupled from desensitization as observed when PN-sIgE is increased from baseline but clinical reactivity and mast cell and basophil degranulation has diminished.¹⁶ OIT and SLIT both cause a significant increase in levels of peanut-specific IgG4 (PN-sIgG4) though these quantities alone have not been shown to be predictive of SU vs desensitization.^{6,12}

Oral immunotherapy and SLIT are known to result in increased antigen-specific IgG, and particularly IgG4, which are antibodies known to block IgE-mediated reactions, even in the presence of persistent levels of IgE. Previous work has shown that serum from subjects on peanut OIT inhibits facilitated antigen binding, suggesting that a plasma factor has antigen-specific blocking capabilities.⁶ Two recent studies have investigated the functional role of IgG in the context of effector cell inhibition. In the first, LAD2 mast cells passively sensitized with plasma from peanut-allergic subjects exhibited greater activation following peanut stimulation than those passively sensitized with plasma from peanut-sensitized but tolerant subjects, which had higher levels of PN-sIgG4.¹⁷ Furthermore, plasma from subjects on peanut OIT was able to block mast cell activation and removal of IgG partially abrogated these findings. Similarly, a second study demonstrated that sera from mice on OVA OIT or humans on peanut OIT suppressed activation of sensitized bone marrow mononuclear cells or basophils, respectively.¹⁸ Inhibition of these

effector cells was further shown to be dependent on the inhibitory receptor FcγRII.

In our present study, we definitively demonstrate that OIT-induced changes in IgG lead to suppression of basophil activation to peanut. We also compared the inhibitory effects of plasma from subjects on peanut OIT and SLIT and on different durations of therapy. Finally, we sought to determine whether basophil inhibition caused by plasma transfer can be used to distinguish subjects that experience desensitization from those that experience SU on peanut OIT or SLIT.

2 | METHODS

2.1 | Clinical trials

OIT and SLIT studies were IRB approved and study drug administered under INDs.¹⁹ OIT Trial #1 listed below was designed with the primary goal of assessing SU after several years on therapy, while OIT Trial #2 was focused on desensitization with a shorter treatment duration. The doses defining SU were chosen based on our understanding of the effectiveness of these therapies at the time of trial implementation. The details provided below are relevant to the present studies on blocking antibodies.

OIT Trial #1 (Clinical trial #: NCT00815035, NCT00597675): Peanut-allergic subjects were randomized to peanut OIT or placebo. Doses started at 0.1 mg and reached maintenance doses of 4000 mg protein. Subjects on placebo crossed-over to active treatment after 12 months. After 48 months of active treatment, subjects underwent an oral food challenge (OFC) to assess desensitization. Subjects were taken off therapy for up to 3 months before undergoing an OFC to assess SU. For the purpose of these experiments, a cut-off of 5000 mg peanut protein was used to define SU.

OIT Trial #2 (NCT01814241): Peanut-allergic subjects were given open-label peanut OIT up to 1450 mg. After 6 months of active treatment, subjects underwent an OFC to assess desensitization. Subjects were taken off therapy for up to 1 month before undergoing an OFC to assess SU. For the purpose of these experiments, a cut-off of 3750 mg peanut protein was used to define SU.

SLIT (NCT00597727): Peanut-allergic subjects received peanut SLIT. Maintenance doses reached 2 mg protein. After 60 months of active treatment, subjects underwent an OFC to assess desensitization. Subjects were taken off therapy for 1 month before undergoing an OFC to assess SU. For the purpose of these experiments, a cut-off of 1750 mg peanut protein was used to define SU.

2.2 | Plasma samples

For each of these studies, venous blood was drawn into sodium-heparin tubes. Whole blood was centrifuged and plasma collected. Within 24 hours of the blood draw, plasma samples were stored at -20°C until analysis. Peanut-specific IgE, IgG4 and IgG values were quantified using a Phadia ImmunoCAP100 (Thermo Scientific, Portage, MI, USA) according to manufacturer's instructions.

2.3 | IgG antibody depletion and enrichment

Pierce Protein A/G Agarose Beads (Pierce Biotechnology, Rockford, IL, USA) were washed three times with PBS prior to use. According to the manufacturer, these beads have high affinity to all human IgG subclasses and a weaker interaction with IgA, IgE and IgM. An equal volume of beads (suspended in PBS) and plasma or PBS and plasma were mixed and incubated on a rotator for 90 minutes for IgG depletion or sham depletion, respectively. Plasma was separated from the beads by centrifuging at 1000 *g* for 90 seconds and the supernatant collected. IgG fractions were eluted from the beads with 0.1 mol/L glycine (pH 2) and neutralized with 1 mol/L Tris (pH 8). The presence of IgG was assessed with a Total Human IgG ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA). Peanut-specific IgE, IgG4 and IgG values from the sham and IgG-depleted samples were quantified using a Phadia ImmunoCAP100 according to manufacturer's instructions.

2.4 | Basophil activation and inhibition assays

For basophil assays on blood from peanut-allergic donors, 200 μ L whole blood was centrifuged at 300 *g* for 10 minutes. Fourteen peanut-allergic donors were chosen based on failure of a peanut OFC conducted under IRB approved protocols at UNC, and had detectable PN-sIgE (median 96 kU/L). Plasma from the allergic donor was removed and replaced with an equal volume of plasma from a subject on OIT, plasma from a subject on placebo (Figure 1) or pooled plasma in the case of IgG-depleted plasma (Figure 2). Cells were incubated at 37°C and 5% CO₂ for 1 hour. For all experiments, following incubation with plasma, cells were stimulated with 200 μ L of peanut extract (final concentration of 0.01 μ g/mL) diluted in RPMI containing 2 ng/mL human IL-3 at 37°C and 5% CO₂ for 30 minutes. Peanut extract was prepared in-house using Golden Peanut flour (Alpharetta, GA) as described previously.²⁰ For each assay, a negative control consisting of blood from the allergic donor stimulated with RPMI and IL-3 was used to confirm that cells were not being activated non-specifically. Degranulation was stopped promptly at 30 minutes by adding cold 20 mmol/L EDTA to the sample. Cells were then stained with antibodies specific for CD63-FITC (BD Biosciences, San Jose, CA, USA), CD203c-PE (Beckman Coulter, Indianapolis, IN, USA) and CD123-PE-Cy5 (BD Biosciences). Following staining, red blood cells were lysed and remaining cells fixed with FACS Lysis Buffer (BD Biosciences) for 15 minutes. Samples were centrifuged at 800 *g* for 15 minutes, and isolated cell pellets were resuspended in staining buffer consisting of PBS, 2 mmol/L EDTA, 0.5% BSA. Samples were analysed on a CyAn ADP (Beckman Coulter, Indianapolis, IN) flow cytometer and gated using FlowJo V10 (FlowJo, LLC, Ashland, OR, USA) software. The gating strategy is shown in Figure S1.

For passive sensitization of basophils from a non-allergic donor (Figures 3 and 4), whole blood from a donor with no known allergies was centrifuged at 300 *g* for 10 minutes. The plasma from the non-allergic donor was removed and replaced with pooled plasma from

peanut-allergic subjects. These pools were created by adding equal parts of plasma from 2 to 3 subjects on OIT Trial #2 and had an average PN-sIgE of 327.98 kU/L and an average PN-sIgG4 of 0.50 μ g/mL. Cells were incubated with the pooled plasma from allergic subjects for 2 hours at 37°C and 5% CO₂ and mixed every 30 minutes. Following passive sensitization, blocking plasma from subjects on OIT or SLIT were applied and cells stimulated, stained and analysed as described above.

2.5 | Statistical analyses

GraphPad/Prism version 7.02 was used to analyse all data. Mann-Whitney *U*, Wilcoxon and paired and unpaired *t* tests were performed and a *P*-value < 0.05 was considered significant. Percent inhibition of basophil activation was calculated by subtracting the % CD63⁺ basophils in the presence of OIT or SLIT plasma from the % CD63⁺ basophils at baseline, dividing by the %CD63⁺ basophils at baseline and then multiplying by 100%.

3 | RESULTS

3.1 | Plasma from subjects on peanut OIT, but not placebo, inhibits peanut-stimulated basophil activation

Plasma from peanut-allergic subjects was removed and replaced with plasma from subjects on either 0 months or 12 months of peanut OIT as shown in the schematic in Figure 1A. Following stimulation with peanut extract, activated basophils (CD123⁺CD203c⁺ Lymphocytes) were identified by upregulation of cell-surface CD63 (Figure 1B). Incubation with 12-month active OIT plasma resulted in decreased basophil activation compared to incubation with autologous 0-month plasma (*P* < 0.0001); however, this blocking of basophil activation was not observed in basophils incubated with plasma from subjects on 12 months of placebo (Figure 1C). This inhibition capability was accompanied by small decreases in PN-sIgE (*P* < 0.05, Figure 1D) and larger increases in PN-sIgG4 (*P* < 0.05, Figure 1E). Similarly, we investigated the inhibition capabilities of plasma from subjects who started on 12 months of placebo before crossing over to active OIT. Basophils incubated with plasma from 12 months of active therapy inhibited basophil activation (*P* < 0.01), whereas plasma from the same subjects while on placebo had no effect on basophil activation compared to baseline plasma (Figure 1F). Taken together, these results demonstrate that OIT-induced changes in plasma can block basophil activation.

Due to limited plasma volumes for use in further experiments, we tested the ability of pooled 12-month OIT plasma to block basophil activation. Consistent with the findings for basophils incubated with individual OIT plasma, basophils incubated with pooled OIT plasma had decreased activation (*P* < 0.001, Figure S2). These findings show that pooled plasma can be used to further study the inhibitory effect on basophils. As a result, pooled plasma was used for the experiments in Figure 2.

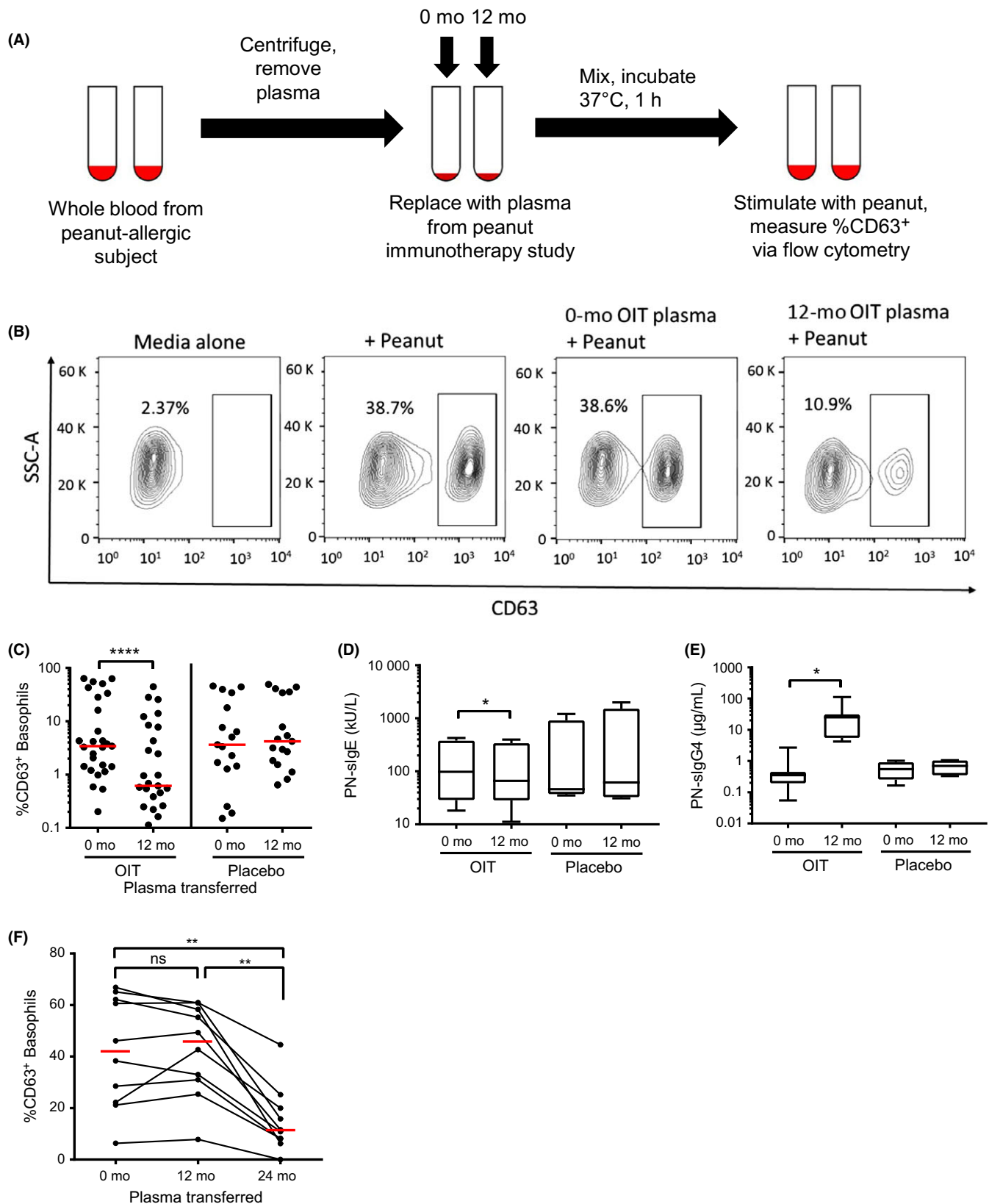


FIGURE 1 Basophil activation following pre- and post-immunotherapy plasma transfer. Assay schematic in which peanut-allergic plasma is replaced with either 0- or 12-mo oral immunotherapy (OIT) plasma then stimulated with peanut extract (A). Representative %CD63⁺ basophil results are shown (B). Basophil activation for cells incubated with plasma from subjects on 0 or 12-mo OIT, 0 or 12 mo of placebo (C) or 12 mo of placebo followed by 12 mo of OIT (F). PN-sIgE (D) and PN-sIgG4 (E) shown for subjects on 0 or 12 mo of OIT or placebo. Individual data are shown in C and F; red lines indicate medians; **P* < 0.05, ***P* < 0.01, *****P* < 0.0001

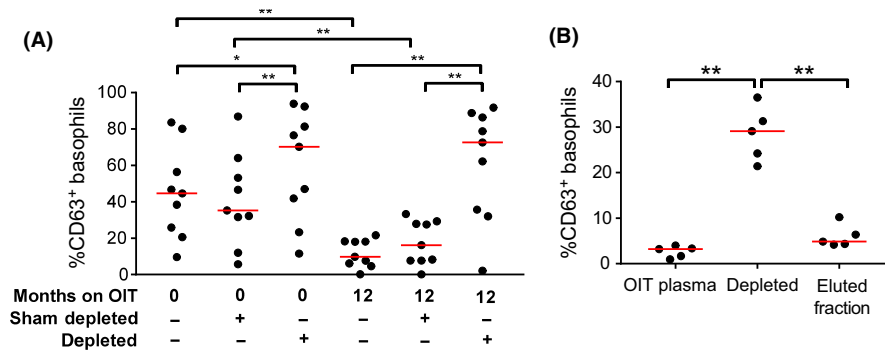


FIGURE 2 Inhibition capacity of IgG-depleted plasma or the IgG fraction isolated from plasma. Percent CD63⁺ basophils following incubation with undiluted, sham-depleted, or IgG-depleted 0- or 12-mo oral immunotherapy (OIT) plasma and stimulation with peanut extract (A). Percent CD63⁺ basophils following incubation with undiluted, IgG-depleted or IgG fractions from 18-mo OIT plasma and stimulation with peanut extract (B). Individual data are shown; red lines indicate medians; * $P < 0.05$, ** $P < 0.01$

3.2 | Peanut OIT-induced IgG is a major contributor for inhibiting basophil activation

Since peanut-specific IgG subclasses such as IgG1 and IgG4 have been shown to increase throughout peanut OIT, we hypothesized that the observed basophil inhibition was at least partly due to changes in IgG. To test this hypothesis, we added IgG-depleted plasma to basophils from peanut-allergic individuals. PN-sIgG (Figure S3A) and PN-sIgG4 ($P < 0.01$, Figure S3B) levels were decreased to $<1 \mu\text{g/mL}$ following depletion with Protein A/G beads compared to sham depletion. PN-sIgE levels also decreased with depletion, although IgE was readily detectable and decreases were modest (Figure S3C). The ratio of PN-sIgE to PN-sIgG4 increased with depletion compared to sham depletion, though changes were not significant (Figure S3D). These results suggest that the depletion had a greater effect on IgG and IgG4 than IgE, as expected. Basophils incubated with either undiluted or sham-depleted 12-month OIT plasma had decreased activation compared to baseline (0 mo) plasma ($P < 0.01$) (Figure 2A). This inhibition was abrogated by IgG depletion ($P < 0.01$, Figure 2A), demonstrating that OIT-induced IgG is critical for the blocking of basophil activation, consistent with the findings of others.^{17,18} We further demonstrated the importance of IgG by using the IgG fraction enriched from OIT subject plasma to block basophil activation. Incubating IgE-sensitized basophils with IgG-depleted plasma did not inhibit basophil activation, while incubating with the IgG fraction substantially blocked basophil activation ($P < 0.01$, Figure 2B).

3.3 | OIT and SLIT subjects' plasma have similar basophil inhibition capacity

Due to limited availability of basophils from peanut-allergic donors, we developed an assay to passively sensitize basophils from non-allergic donors (Figure 3A). In this assay, the plasma from a non-allergic subject was replaced by pooled plasma from peanut-allergic subjects. Prior to this passive sensitization protocol, the donor's basophils were not activated upon stimulation with peanut extract;

however, they can be activated with peanut stimulation following incubation with plasma from allergic subjects, and activation can be blocked with OIT plasma (Figure 3B). Using this tool, we sought to determine if the plasma from subjects on OIT and SLIT had similar effects on basophil activation. Surprisingly, plasma from a 6-month OIT regimen had a greater inhibition effect on basophils than plasma from a 48-month OIT regimen ($P < 0.01$, Figure 3C), although both had $>80\%$ median inhibition. Both had similar levels of PN-sIgG4, but the 48-month OIT plasma samples had significantly less PN-sIgE (Figure 3D,E). Similar inhibition of basophil activation was observed for plasmas from 6 months of OIT and SLIT (Figure 3F). These samples contained similar levels of PN-sIgE, but the OIT samples had higher levels of PN-sIgG4 (Figure 3G,H). Plasma from the time of desensitization challenge (6 months for OIT and 60 months for SLIT) while subjects were still on therapy demonstrated no difference in blocking ability when incubated undiluted with basophils (Figure 3I). Diluting the plasma 1:4 in PBS resulted in decreased basophil inhibition for SLIT plasma compared to OIT plasma ($P < 0.05$, Figure 3I). Despite these functional differences, PN-sIgG4 levels were higher and PN-sIgE samples were lower in SLIT samples compared to OIT samples (Figure 3J,K), indicating that functional blocking capacity is not strictly related to PN-sIgG4 and PN-sIgE quantities.

3.4 | Extent of basophil inhibition by OIT, but not SLIT, plasma is associated with clinical outcomes following therapy

Ex vivo basophil activation has previously been shown to decrease on OIT but does not discriminate subjects who achieve SU from those that are desensitized.²¹ We sought to determine whether the immunotherapy-induced plasma inhibition of donor basophils can be useful in distinguishing or predicting these clinical outcomes following either OIT or SLIT. When used undiluted, plasma from OIT subjects at the time of desensitization challenge who were later classified as SU did not induce a different percent inhibition than plasma from subjects who would later be identified as transiently desensitized (Figure 4A). Similarly, inhibition of basophil activation

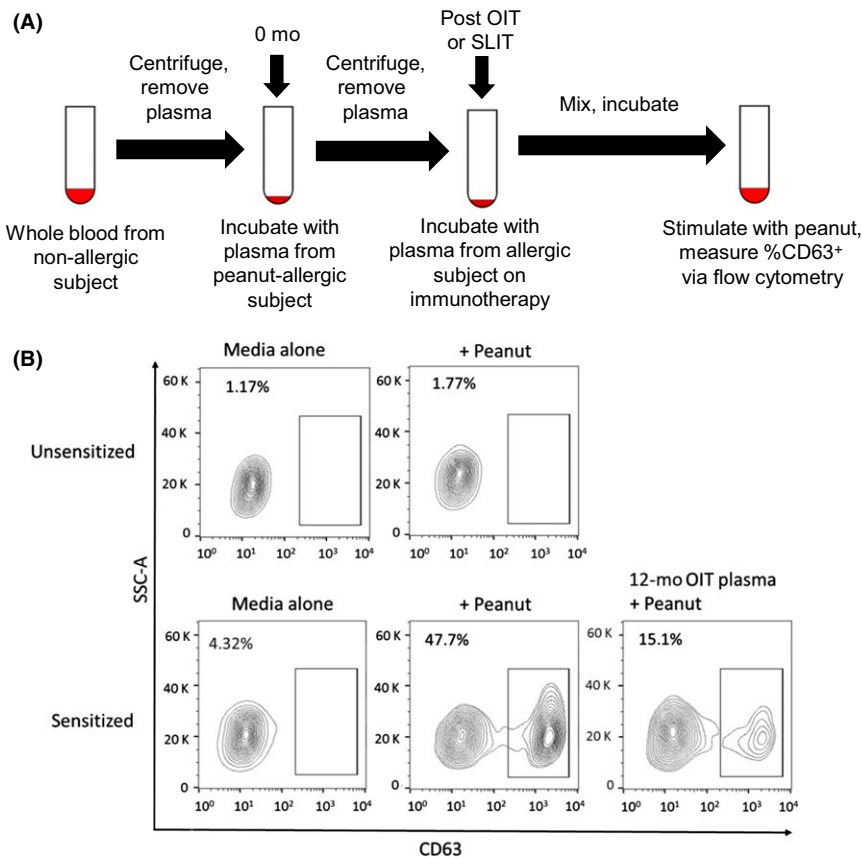


FIGURE 3 Oral immunotherapy (OIT) and sublingual immunotherapy (SLIT) plasma basophil inhibition capability. Schematic for use of non-allergic donor basophils incubated with peanut-allergic plasma in the basophil activation assay (A) and representative results (B). Percent inhibition following incubation with plasma at the time of tolerance challenge for a 6-mo OIT and 48-mo OIT regimen (C) and corresponding PN-sIgE (D) and PN-sIgG4 (E) levels. Percent inhibition following incubation with plasma following 6-mo OIT or 6-mo SLIT (F) and corresponding PN-sIgE (G) and PN-sIgG4 (H) levels. Percent inhibition following incubation with undiluted and diluted 6-mo OIT or 60-mo SLIT plasma (I) and corresponding PN-sIgE (J) and PN-sIgG4 (K) levels. Individual data are shown in C, F and I; red lines indicate medians; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

was not different between plasma from SU and desensitized subjects at the time of tolerance challenge after discontinuing OIT (Figure 4B). Nevertheless, when OIT plasma from the time of desensitization challenge was diluted 1:10 or 1:50, plasma from SU subjects had a greater percent inhibition than that from desensitized subjects, suggesting that this assay may be useful in predicting clinical outcomes after stopping therapy ($P < 0.05$, Figure 4C). Plasma PN-sIgE levels were not different between groups at either challenge time-point, though levels tended to be lower in the group that experienced SU (Figure 4D). Conversely, PN-sIgG4 levels were higher in the subjects who experienced SU than those who were desensitized (Figure 4E). Interestingly, these quantity differences were not significant at the time of desensitization challenge, when the functional differences were noted (Figure 4C). Despite these interesting findings from OIT samples, undiluted or diluted plasma samples from the time of desensitization challenge while on SLIT had no significant difference in percent inhibition of basophil activation for subjects who achieve SU compared to those that achieve desensitization; however, the sample size limits statistical power (Figure 4F). PN-sIgE and PN-sIgG4 levels were not different between SLIT outcomes

(Figure 4G,H). Together, these results suggest that inhibition of basophil activation may be a mechanism for SU following OIT, and that PN-sIgG4 is likely not the only isotype involved in the basophil inhibition process.

4 | DISCUSSION

Oral immunotherapy and SLIT are two promising investigational therapies for peanut allergy with a substantial number of subjects demonstrating desensitization, and in some cases SU, however key knowledge gaps remain. There is currently no way to predict which subjects will have success with OIT or SLIT or how long protection persists after subjects discontinue therapy. Further, immunotherapy-induced immune change(s) that can distinguish subjects who achieve SU from those that are transiently desensitized, have yet to be identified. Finally, the mechanisms by which OIT and SLIT induce desensitization and SU have not been fully elucidated. Here, we demonstrated that plasma from subjects on OIT and SLIT can inhibit basophil activation, a potentially important mechanism of desensitization and SU.

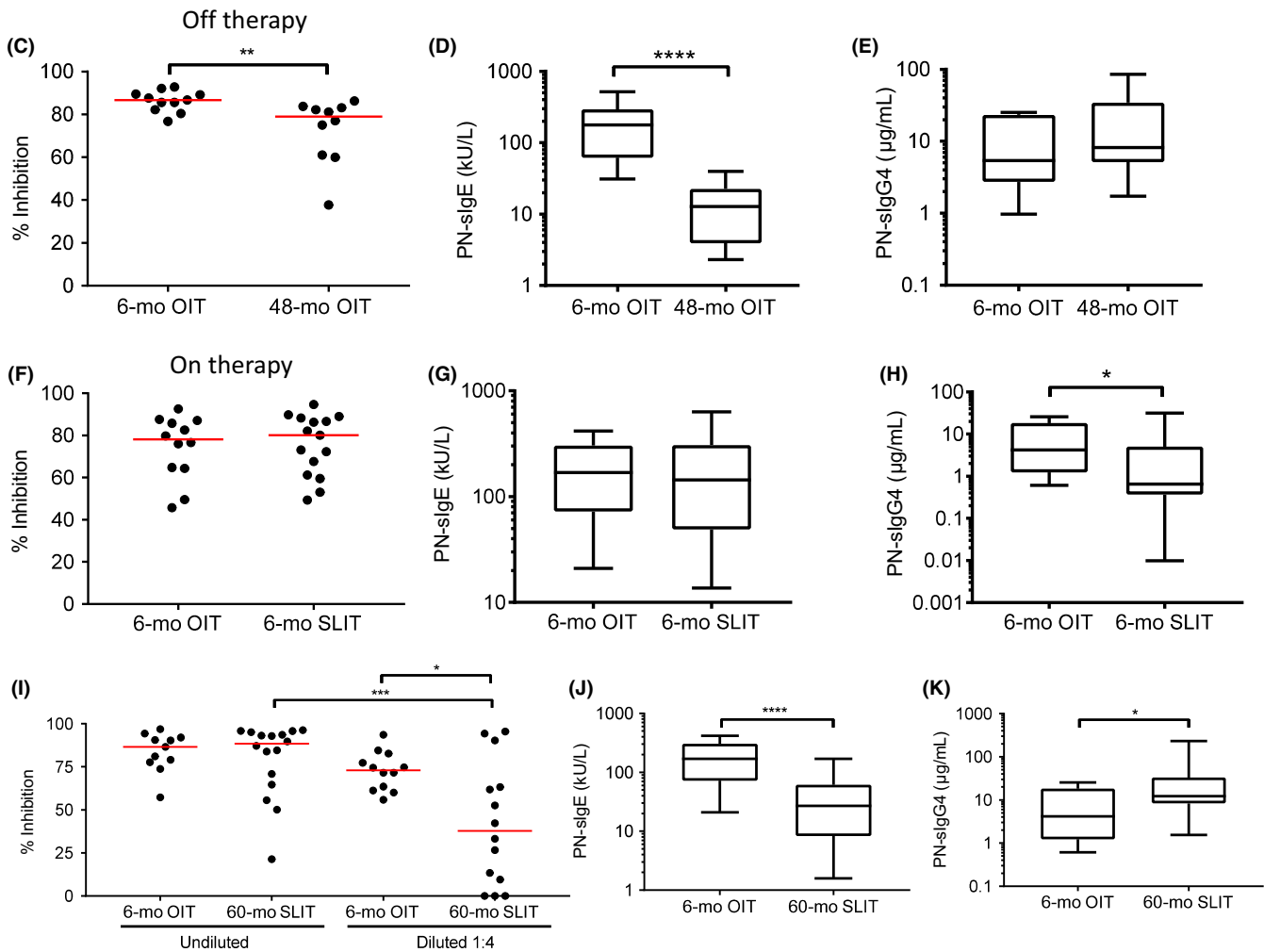


FIGURE 3 (Continued).

Several reports have described inhibition of *ex vivo* basophil activation following OIT for peanut, milk, and egg; however, it is not clear how this effector cell desensitization occurs. We used plasma from subjects on OIT or placebo to demonstrate that OIT-induced changes in plasma, that are at least partly IgG-dependent can inhibit basophil activation in response to peanut stimulation. We further confirmed the role of IgG in inhibiting basophil activation by demonstrating that incubating with the IgG-enriched fraction inhibits activation of IgE-sensitized basophils. Santos et al¹⁷ identified post-OIT PN-sIgG4 to be a factor in plasma responsible for inhibiting basophil activation. However, in their study, depletion of IgG4 only partially abrogated the inhibition effect, suggesting that additional plasma factors, such as other IgG subclasses, play a role. Our group has recently demonstrated that OIT causes increases in antigen-specific IgA, IgA1 and IgA2, in addition to increased IgG4 and that higher levels of these antibodies are associated with SU,²² indicating a potential role for several isotypes. Further investigation is required to determine the potential functional role of IgA, IgG and their subclasses, which are known to increase during OIT and SLIT, in inhibiting basophil activation.

This report is the first to compare the basophil inhibition ability of plasma from subjects on OIT and SLIT. When comparing plasma from subjects on either 6-months OIT or 6-months SLIT, we found that the ability to block basophil activation was similar, suggesting that blocking antibodies are induced early on in OIT and SLIT. Similarly, incubation with plasma from peanut OIT and SLIT at the time of desensitization challenge (60 months for SLIT, 6 months for OIT), resulted in comparable levels of basophil inhibition. Diluting these plasma samples 1:4 prior to incubation with basophils was able to substantially reduce the inhibition effect for SLIT, but not OIT. Interestingly, plasma from these time-points of OIT and SLIT contained equivalent levels of PN-sIgG4. These results could be explained by the hypothesis that both OIT and SLIT make enough IgG4 in excess to suppress basophil activation, but that there are differences in function. These differences could be related to epitope-specificity, avidity of the antibodies and post-translational modifications, such as glycosylation. Future experiments testing the ability of isolated peanut-specific IgA or IgG1 to inhibit basophil activation would prove useful in demonstrating their role or lack thereof following immunotherapy.²²

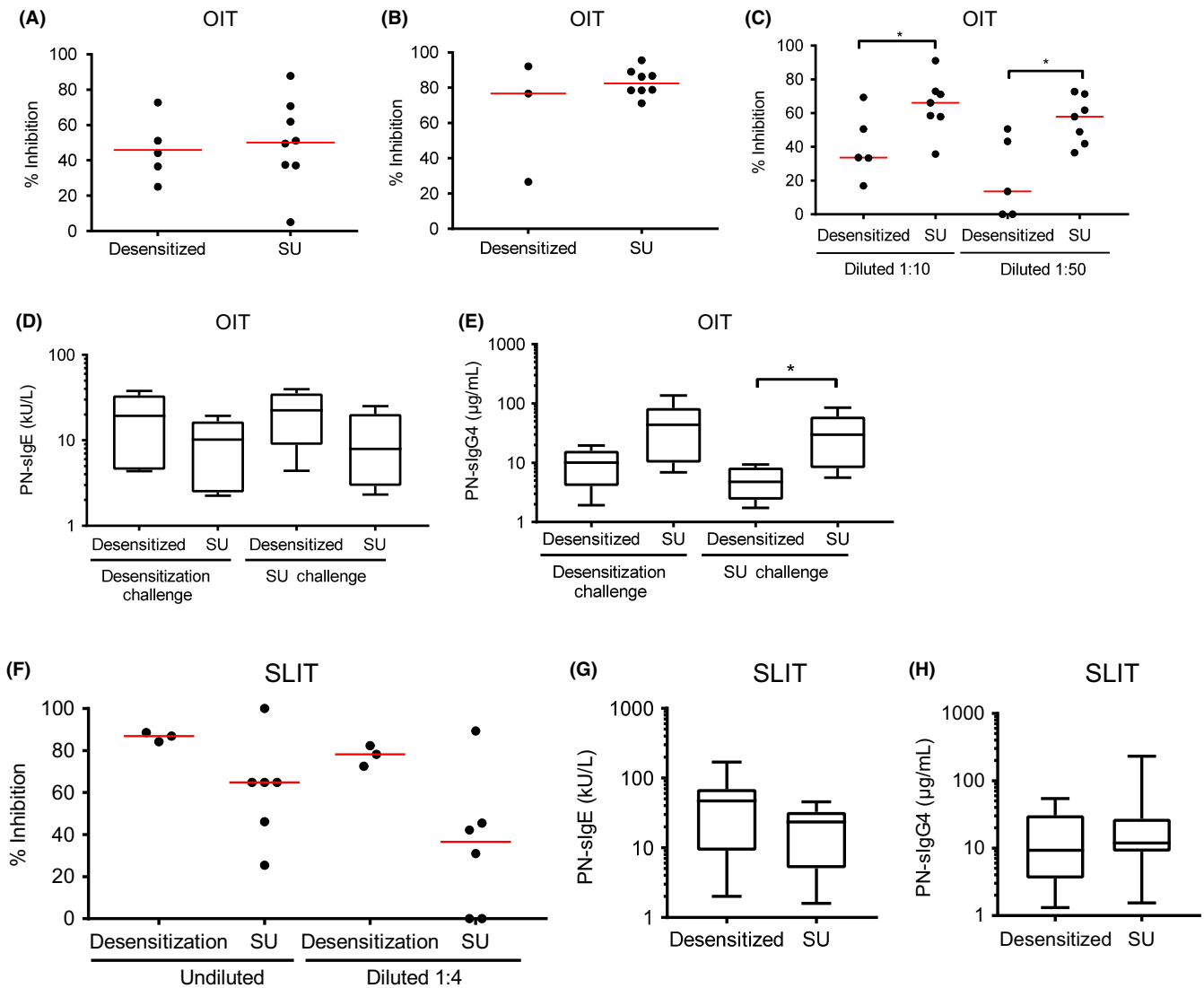


FIGURE 4 Oral immunotherapy (OIT) and sublingual immunotherapy (SLIT) clinical outcomes in relation to basophil inhibition. Percent inhibition of basophil activation for 48-mo OIT plasma (A) or tolerance challenge plasma after discontinuation of therapy (B) separated by clinical outcome. Percent inhibition of basophil activation following incubation with 48-mo OIT plasma diluted either 1:10 or 1:50 (C). Corresponding PN-sIgE (D) and PN-sIgG4 (E) levels. Percent inhibition of basophil activation following incubation with 60-mo SLIT plasma either undiluted or diluted 1:4 (F) and corresponding PN-sIgE (G) and PN-sIgG4 (H) levels. Individual data are shown in A-C and F; red lines indicate medians; * $P < 0.05$

This report is also the first to relate the degree to which plasma from OIT or SLIT inhibits basophil activation to clinical outcomes following completion of therapy. Basophil inhibition by undiluted plasma from subjects on 48 months of OIT was not predictive of which subjects would develop SU after time off therapy. In fact, even basophil inhibition by undiluted plasma at the time of tolerance challenge was not different between subjects who experienced SU from those who were transiently desensitized. However, the inhibition effect of diluted plasma from subjects at the time of desensitization challenge was indicative of which subjects would later be classified as SU and which would be classified as desensitized after discontinuing therapy. Interestingly, PN-sIgG4 quantities were not different at this challenge time-point between subjects who experienced SU from those who were desensitized, signifying that it is not

just the quantity of PN-sIgG4, but perhaps functional changes that are important for the development of SU. Importantly, these findings were true for plasma samples from the time of desensitization challenge, suggesting that the percent inhibition of basophils by diluted plasma while still on OIT may eventually be useful as a predictive marker for subjects that will develop SU. On the other hand, basophil inhibition by plasma from subjects on 60 months of peanut SLIT was not associated with clinical outcome even when samples were diluted. Taken together, these results suggest that the mechanisms for the induction of SU may differ between OIT and SLIT.

The possible applications for the assays and findings presented here have valuable potential for food allergy research and more broadly, allergen immunotherapy studies. The assay using passively sensitized cells is a useful tool to analyse the functional changes in

plasma while controlling for therapy-induced changes in the cells themselves. While these studies do not dispute alterations in the basophils and mast cells, they offer a means to isolate changes in the plasma alone. It has been proposed that IgG prevents basophil activation by either intercepting antigen before it can cross-link surface-bound IgE or by binding to inhibitory receptors on mast cells triggering an inhibitory rather than activating signal.^{23,24} Other forms of immunotherapy including subcutaneous immunotherapy for bee venom²⁵ and grass pollen^{26,27} induce an increase in IgG4 that intercepts antigen, preventing it from binding to cells. A previous study showed that peanut OIT-induced IgG acts through the IgG receptor, FcγRII on basophils to inhibit their activation.¹⁸ Future experiments are needed to determine intrinsic cellular changes in subjects actively undergoing immunotherapy in addition to the plasma changes observed in these studies. For example, we have previously demonstrated impaired calcium flux due to actin rearrangement following desensitization in model systems.²⁸ The findings also assist in the understanding of the changes that distinguish subjects that develop SU. As a result, if the findings are replicated in larger experiments we may be able use these basophil inhibition assays to determine if a subject needs to be on therapy for a longer period of time. More broadly, the improved understanding of immunotherapy mechanisms will allow for targeted therapies in the future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Johanna Smeekens  <http://orcid.org/0000-0001-9651-3852>

Michael Kulis  <http://orcid.org/0000-0001-8092-7444>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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