

MS. MARIA SUPRUN (Orcid ID : 0000-0001-9161-4021)

DR. JEAN-MARC BUSNEL (Orcid ID : 0000-0001-6651-2437)

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A novel approach to the Basophil Activation Test for characterizing peanut allergic patients in the clinical setting

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To the Editor,

The basophil activation test (BAT) has been reported to increase the accuracy of diagnosing peanut allergy and decrease the need for oral food challenges¹⁻³. However, BAT has been largely restricted to research settings because basophils are unstable *ex vivo* and the activation assay is ideally done within 3-4 hours of sample acquisition⁴. If activation could be performed easily in the clinical setting and sent to a processing facility for cytometry, BAT could become more broadly accessible. In this pilot study, we evaluated a novel peanut-BAT (P-BAT) method that preserves basophil activation to determine its feasibility as a diagnostic method of peanut allergy.

The main objective was to determine whether basophils from peanut allergic patients, stimulated with P-BAT reagents, maintain consistent activation read-outs measured up to 5 days after initial activation. Basophils in whole blood were stimulated with six peanut concentrations (0.0001-10 mg/ml) on day 0 via a novel method developed by Beckman-Coulter and activation was measured by CD63⁺CD203c⁺ expression via flow cytometry on days 0, 1, 3 and 5 (**Figure S1**). One non-allergic subject was included and had no activation on any day (**Figure 1A**). Peanut and epitopes IgE and epitope-sIgG4 were measured (**Appendix S1**).

Thirty peanut allergic subjects (median age of 11.8 years and peanut sIgE of 22.9 kU_A/L) were enrolled (**Table S1**). Twenty six (87%) were BAT responders, with >5% of CD63⁺CD203c⁺ basophils

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in response to peanut extract⁵, similar torates found by others⁵⁻⁷. “Non-responders” had lower peanut-, Ara h 1- and Ara h 2-sIgE; 3/4 patients’ basophils did not respond to anti-IgE stimulation.

Responders demonstrated basophil activation at each peanut concentration on the initial day of P-BAT (day 0) that was sustained on days 1, 3, and 5 without further stimulation (**Figure 1A**), with a median coefficient of variation of 9.7%. The mean AUC of the activation curve was comparable between day 0 and days 1 and 3 (**Figure 1B**). Day 5 AUC was slightly but significantly lower than day 0 ($p=0.004$), still with a consistent positive mean activation. Similar results were observed for other BAT outcomes (**Figure S2**). Excellent consistency (intra-class correlation coefficients >0.9) was observed for all outcomes, especially for days 0 to 3 (**Figure 1C**). The ability of P-BAT to generate similar dose-response patterns for samples from the same patient was assessed by functional principal components (FPCs). Clustering of FPCs misclassified only 4/103 responses, assigning those to the neighboring clusters (**Figure 1D**).

Since IgE diversity likely results in greater basophil activation due to increased cross-linking of the high-affinity FcεRI receptors⁸, we examined P-BAT association with the Ara h 1, Ara h 2 and Ara h 3 epitope sIgE. Median antibody diversity across all subjects was 36% (IQR 13-54%) for IgE and 88% (73-95%) for IgG4 (**Figure 2A**). P-BAT non-responders demonstrated similar IgE diversity. Among responders, as CD-max increased, both IgE diversity ($p=0.029$) and peanut sIgE ($p=0.056$) tended to decrease (**Figure 2B**). No association was observed for IgG4 diversity.

The novel P-BAT method differs from current methods by eliminating the immediate need for flow cytometry. P-BAT activates basophils via a relatively simple process using whole blood, which can be easily performed within the initial 4 hours of sample collection. P-BAT maintains the stability of activated basophils for several days at room temperature, allowing for transportation to a dedicated facility for flow cytometry. This bypasses complex preparations in clinic and makes BAT more realistic as a diagnostic method, especially when compared to conventional BAT methods. Future studies including the use of more allergic patients, diagnostic markers and control variables (shipping methods and duration) are needed to further validate the assay. In conclusion, the P-BAT is a novel method of basophil activation that could be widely adopted to aid in clinical decision making.

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Figure Legends

Figure 1. Basophil activation is preserved with P-BAT. **A.** Basophil activation dose-response curve for each patient on day 0, 1, 3, and 5 after collection, summarized by area under the curve and CD-max at day 0. **B.** Marginal means of the AUC for responders show no difference between days 0, 1 and 3 ($p > 0.05$). **C.** ICC demonstrates high consistency of measured activation between days. **D.** FPC clusters demonstrate samples from the same patient assigned to the same cluster (each cluster within colored circles represents a drug-response pattern) are reliable.

Figure 2: Diversity of peanut epitope IgE expressed in allergic patients is proportional to their sensitivity to basophil activation. **A.** The heatmap shows each (by column) subjects' epitope-specific IgE diversity, ordered by their CD-max. **B.** CD-max is significantly decreased with the increase in the proportion of epitope-specific IgEs ($p < 0.05$), but not with the epitope-specific IgG4 diversity. CD-max 0.01: $n=8$, CD-max 0.1: $n=8$, CD-max 1: $n=5$, CD-max 10: $n=4$, NR: $n=4$, non-allergic: $n=1$.

Authors: Amanda Agymang, MD^{1*}, Maria Suprun, MPH^{1*}, Mayte Suárez-Fariñas, PhD¹, Faouria Boina, MSc², Rihane Arif-Lusson, MSc², Alexander Grishin, PhD¹, Jean-Marc Busnel, PhD², Hugh A Sampson, MD¹

¹ Icahn School of Medicine at Mount Sinai, New York, New York, USA

² Beckman Coulter Life Sciences Research, Marseille, France

Amanda Agymang and Maria Suprun equal contribution as first authors.

Correspondence

Hugh A Sampson, MD
Kurt Hirschhorn Professor of Pediatrics
Department of Pediatrics; Box 1089
Icahn School of Medicine at Mount Sinai
New York, NY 10029-6574

Phone: (212) 659-9426

Fax: (212) 241-7153

E-mail: hugh.sampson@mssm.edu

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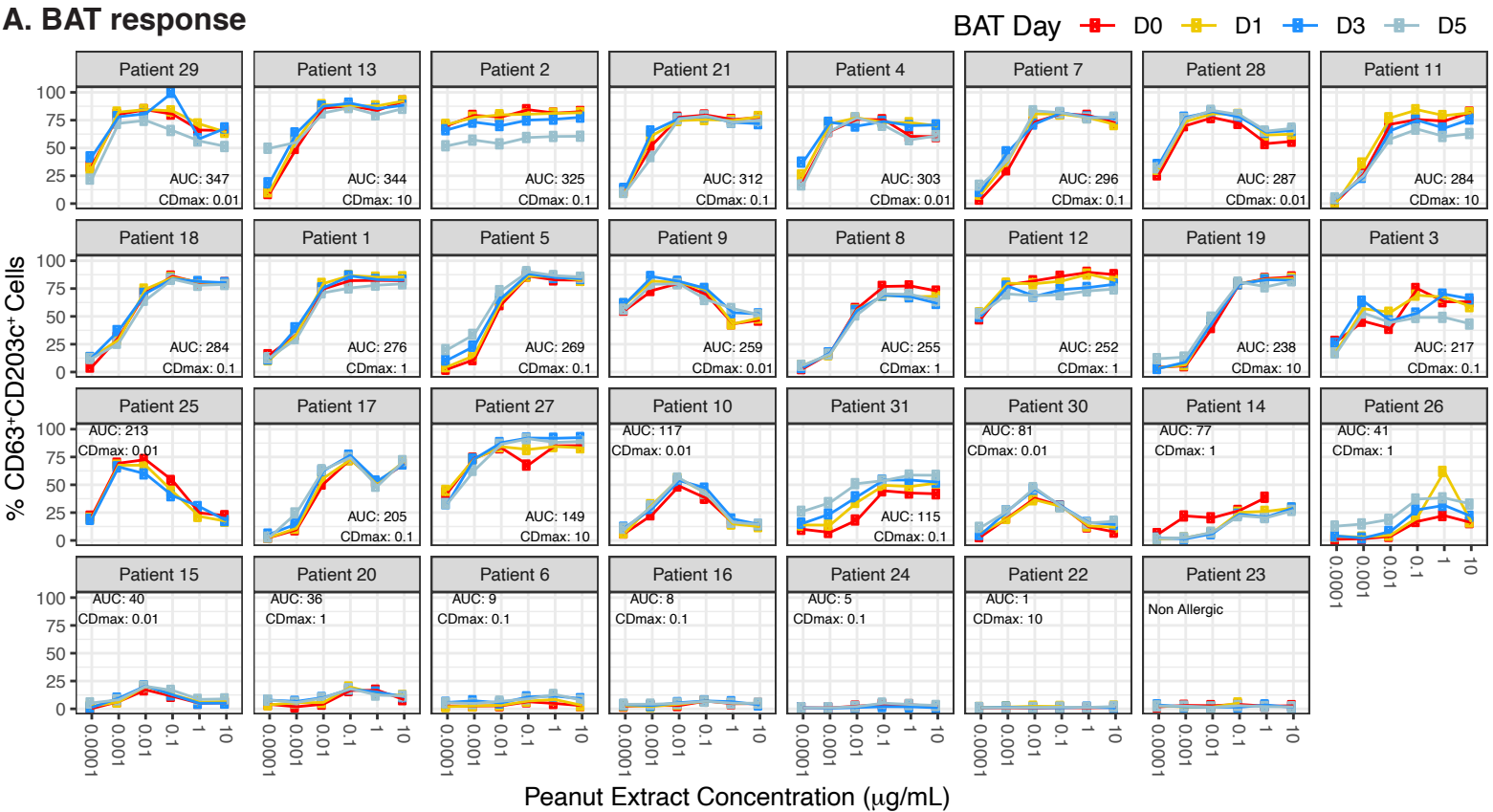
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Conflict of Interest

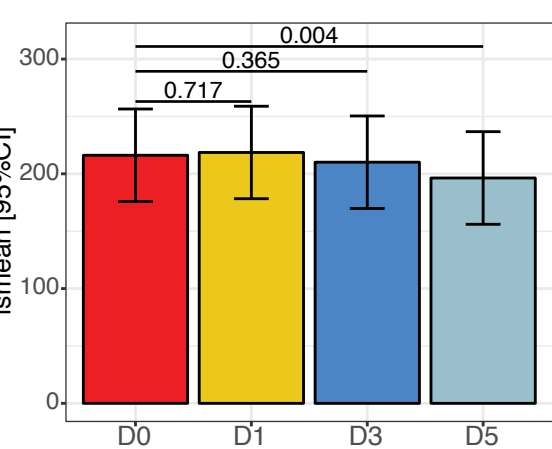
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Figure 1

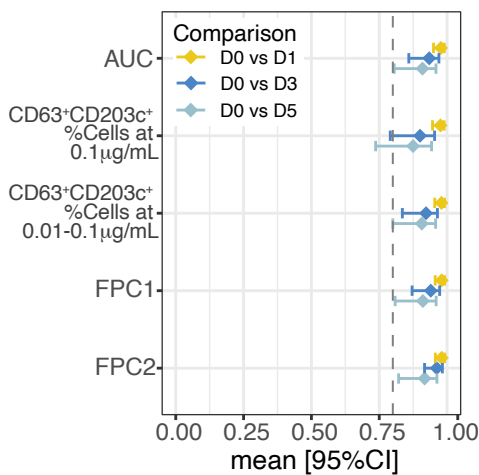
A. BAT response



B. AUC



C. ICC



D. FPCA

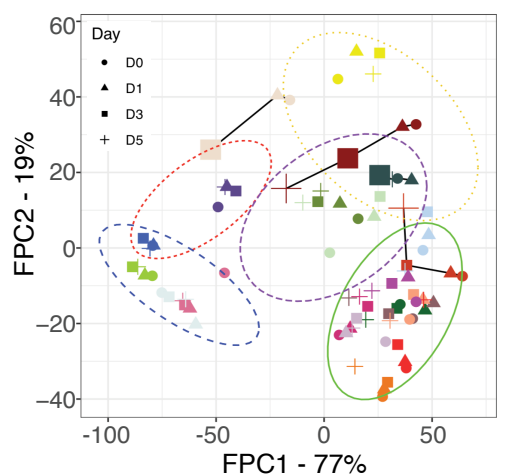
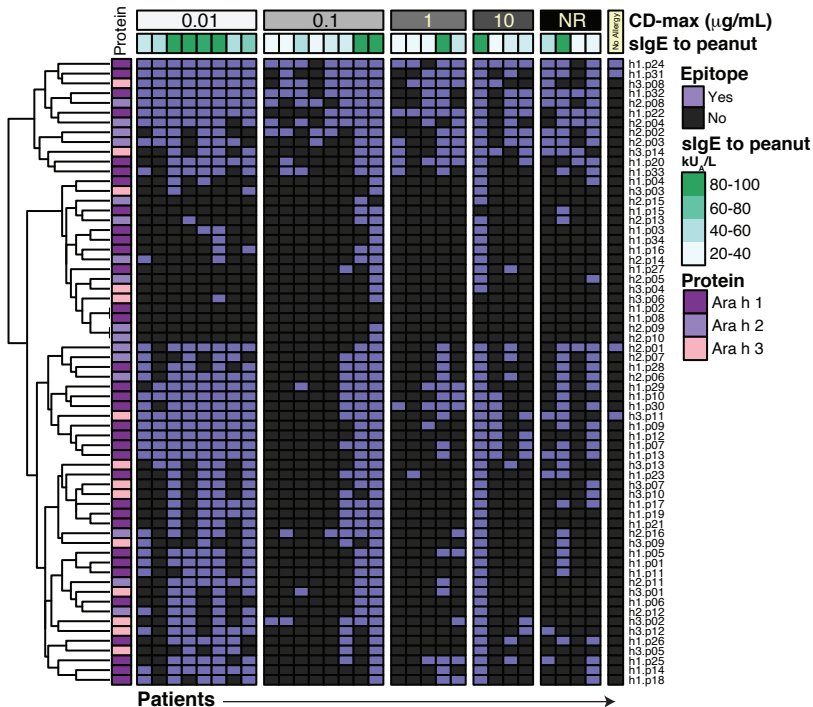
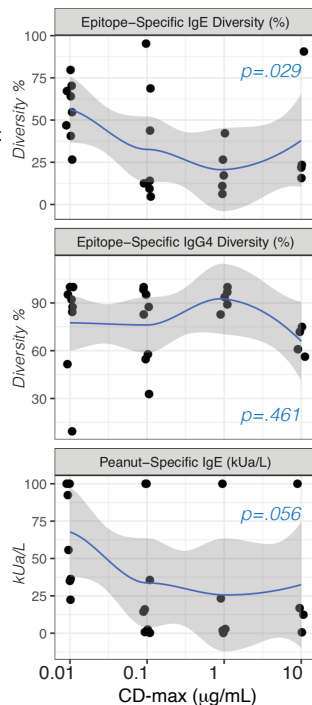


Figure 2

A. Epitope-Specific IgE Diversity



B. CD-max vs Ig



APPENDIX S1**A novel approach to the Basophil Activation Test for characterizing peanut allergic patients in the clinical setting**

Amanda Agyemang, MD¹, Maria Suprun, MPH¹, Mayte Suárez-Fariñas, PhD¹, Faouria Boina, MSc², Rihane Arif, MSc², Alexander Grishin, PhD¹, Jean-Marc Busnel, PhD², Hugh A Sampson, MD¹

¹ *Icahn School of Medicine at Mount Sinai, New York, New York, USA*

² *Beckman Coulter Life Sciences Research, Marseille, France*

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Supplementary Methods

Study population

Peanut allergic patients were recruited from the Jaffe Food Allergy Institute Pediatric Allergy Clinic at Mount Sinai from February to June 2018. Eligible subjects included pediatric patients of all ages with peanut allergy as defined by a convincing history of an allergic reaction to peanut and a positive peanut specific (s)IgE (> 0.35 kU_A/L) or positive skin prick test (3 mm $\geq$ than the negative control to peanut); or a positive oral food challenge to peanut with a history of positive testing. Subjects were excluded if they had concurrent milk allergy per history since it could result in a false positive BAT response (reagents for this assay contain bovine serum albumin, which potentially could produce positive responses that are actually due to milk sensitized basophils instead of peanut). However, all other co-allergens were acceptable. One non-peanut allergic patient was included in the analysis as a control. The Mount Sinai Institutional Review Board reviewed and approved this study and informed consent was obtained prior to enrollment.

Skin prick test and specific IgE

Skin tests and sIgE were obtained as previously described¹. Specific IgE to peanut and peanut component proteins (Ara h 1-3, 8 and 9) were quantified using the ImmunoCAP system (Thermo Fisher Scientific; Portage, Michigan) according to the manufacturer's instructions.

Basophil Activation Test

Per a standard operating procedure, whole blood samples were collected from patients at the clinical site and transported to the laboratory at room temperature for incubation with peanut within 4 hours of obtaining the sample. 50 μ l aliquots of whole blood from each subject were incubated in the pre-prepared Beckman-Coulter P-BAT reagent tubes containing serial 10-fold dilutions of peanut extract (0.0001, 0.001, 0.01, 0.1, 1, or 10 μ g/mL), negative control (PBS) or positive control (anti-IgE antibody, Clone E124.2.8, Beckman Coulter, Marseille, France) at 1 μ g/mL, for 20 minutes at 37 C. The peanut extract was confirmed to contain major allergens Ara h 1, 2, 3, and 6 (Figure S3). Red blood cells were subsequently lysed with 250 μ l of Optilyse C (Beckman Coulter, Marseille, France) in each test tube for 10 minutes. The lysis reaction was halted after an additional 10 minutes with 250 μ l of PBS. Samples were subsequently analyzed by flow cytometry without the implementation of any washing/centrifugation step prior to analysis. A proprietary dry reagent formulation technology was used to prepare ready-to-use and room temperature stable reagents. As a function of the experiments, flow cytometry staining reagents (CD45-KrO, CD3-PC7, CRTH2-A647, CD203c-PE and CD63-PB all from Beckman Coulter, Marseille, France) alone or in combination with either anti-IgE or peanut extract² were dried together in 12x75 mm test tubes.

Flow Cytometry

Flow Cytometry was performed³ on the same day of the activation test using a plate loader equipped flow cytometer (Cytoflex, 3 lasers, 13 colors, RUO, Beckman Coulter, Indianapolis, Indiana, USA). Activated basophils were identified by CD63⁺CD203c⁺ expression on the cell surface. The preservation of basophil activation over time was assessed by performing serial flow cytometry measurements of CD63⁺CD203c⁺ upregulation again at 1, 3 and 5 days after the initial basophil activation test. The gating of cell surface marker expression is described in the supplemental Figure S1 and analyses of the flow cytometry data were conducted with Kaluza software (RUO, Beckman Coulter, Indianapolis, Indiana, USA).

Peanut Extract Preparation

Whole peanut protein extract was prepared as previously described⁴ with modifications. Approximately 50 g of freshly ground raw peanut paste was defatted with ~20 volumes (milliliters per gram of peanut

paste) of ice-cold acetone under a chemical hood. First, peanut paste was resuspended in ~ 10 volumes of ice-cold acetone by stirring for ~30 min. in a glass beaker. Next, the paste suspension was filtered through two layers of filter paper Whatman No 1 placed in a glass funnel. The pellet on the filter was washed by rinsing with another ~ 10 volumes of ice-cold acetone and the filter with the pellet was dried under the chemical hood until there was no detectable acetone odor. The filter with the dried defatted paste was placed in a tray and was kept overnight in a cold room at ~4°C for complete desiccation. The dried defatted peanut paste pellet was collected off the filter on the next day and was placed in a 50 mL conical tube at ~20 mL of visible powder volume per tube.

The extraction was performed as follows: Approximately 10 ml or more as necessary of phosphate buffered saline (PBS) with proteinase EDTA-free inhibitor cocktail (Roche-Sigma-Millipore, cat. no. 11873580001) were added to ~ 20 mL (1:2 volume ratio) of the defatted powder in a 50 mL conical tube. The powder was completely re-suspended in the added buffer solution and placed on a rocking platform for overnight extraction at 4°C.

On the next day, the suspension was centrifuged at 13,000 g, for 10 min. at 4°C. The supernatant was collected avoiding taking any of the pellet and was centrifuged one more time under the same conditions. The supernatant was collected, and the protein concentration was measured by Micro BCA assay (Thermo Scientific, cat. no. 23235). An aliquot of the final peanut protein extract was analyzed by SDS polyacrylamide gel electrophoresis for protein band appearance (Figure S3). The peanut protein extract used in this study was also subjected to an endotoxin removal procedure since it was used for PBMCs stimulation in sterile cell culture conditions in another study.

Peanut sequential epitope sIgE and sIgG4

The peanut epitope library consisted of 64 sequential 15-mer peptides (CS Bio, Menlo Park, CA, USA) from Ara h 1 (n=34), Ara h 2 (n=16), and Ara h 3 (n=14) allergens (Table S2). The sequential epitopes were selected based on the previously published SPOTs membrane and peptide microarray studies⁵⁻¹⁰. The Bead-Based Epitope Assay (BBEA) was carried out as described previously¹¹, with minor modifications. A master mix of peptides, conjugated to the LumAvidin beads (Luminex Corporation, Austin, TX, USA) was prepared in PBS-TBN buffer (1xPBS with 0.02% Tween-20 and 0.1% BSA), and 100 µL/well of mix was added to 96-well filter plates. After washing the microspheres with PBS-TBN, 100µL of plasma samples diluted 1:10 was added to the wells in triplicates according¹² to the randomization scheme generated with the *PlateDesigner* application¹³. Plates were incubated on a shaker for 2 hours at 600 RPM at room temperature. Excess plasma was then removed, and the plate was washed. 50 µL/well of mouse anti-human IgE-PE (2 µg/mL, Cat. No MA1-10375, Thermo-Fisher Scientific) or IgG4-PE (0.25 µg/mL, Cat. No 9200-09, Southern Biotech) secondary antibody was added and plates were incubated for 30 minutes at room temperature, then washed and 100 µL of PBS-TBN was added to each well. Plates were read on the Luminex200 instrument (Luminex Corporation, Austin, Texas, USA).

Statistical analysis

Demographic and clinical characteristics were summarized as either mean and standard deviation, or median and interquartile range (IQR), or frequency and percent. Patients were considered BAT reactive if at least 5% of CD63⁺CD203c⁺ cells were activated for any of the six peanut concentrations on day 0. Only reactive subjects were used in the subsequent analyses. For the hypothesis testing, two-sided tests were used, and P-values (p) < 0.05 were considered statistically significant. All analyses were performed in R v3.4 software.

Analysis of BAT outcomes

For each sample and dose, the percentage of basophil activation was calculated by subtracting the percentage of CD63⁺CD203c⁺ unstimulated basophils to account for baseline activation. Assessment of

the appropriate parameters to interpret and compare activation amongst subjects was required because of the high variability of dose-response curve shapes and the presence of bell-shaped curves, which are commonly observed in BAT¹⁴, independent of the laboratory in which basophils are processed. Response curve shape is dependent on many variables, some of which include the affinity of epitope sIgE to variable antigen epitopes, epitopes available in the allergen extract, and antigen structure complexity. Each of these variables can differ from subject to subject or within the same subject at differing time points¹⁴. Previously published studies have commonly utilized the EC₅₀ value (effective dose at which 50% of maximal basophil response occurs) in describing basophil activation. However, EC₅₀ better describes dose response curves that are sigmoid in shape¹⁴. Given the high variability and presence of many bell-shaped curves, the AUC best describes both the reactivity of the basophils and their sensitivity to stimulation, and CD-max can be used to compare peak activation in subjects¹⁴.

Several measures were used to summarize the BAT dose-response curves for each sample as a single number: area under the curve (AUC), maximal effective concentration (CD-max, calculated as the concentration of peanut extract at which the maximum proportion of cells were activated), and functional principal components (FPC). For the calculation of these statistics, the dose response curve utilized peanut concentrations in the log₁₀ scale. FPCs are a common tool for dimensionality reduction of sparse curves and were calculated using the *fdapace* package¹⁵. FCP decomposes dose-response curve into the eigenfunctions (i.e., FPCs) of the estimated covariance, in such a way that the first few eigenfunctions explain most of the total variation¹⁶ in the functional data. In addition, we also included other BAT metrics previously reported by Santos et al^{16,17} as being predictive of the OFC outcome: the percent of CD63⁺CD203c⁺ cells at 0.1 µg of peanut extract /mL and the combined average percentage of activated CD63⁺CD203c⁺ cells found at both 0.01 and 0.1 µg of peanut extract/ml.

Several analyses were used to evaluate the differences in using BAT within 4 hours (day 0) versus 1, 3, or 5 days post-sample collection and activation. First, for each defined BAT outcome, a linear mixed-effect model with the day of analysis as a fixed effect and a random intercept for every patient was fit using the *lme* function. From this model, differences between days were estimated using *emmeans* functions. Additionally, the consistency among days for the BAT outcomes were assessed using the intraclass correlation coefficient (ICC) with a two-way model. According to the ICC and Kappa guidelines¹⁸, the values between 0.75-1.00 denote excellent agreement. We also applied an unsupervised hierarchical clustering (Manhattan distance and Ward agglomeration algorithm) to the first three FPCs. The ultimate number of clusters (k=5) was selected by a majority of metrics in the *NbClust* package^{19,20}.

Analysis of sequential epitope sIgE and sIgG4

Median Fluorescence Intensity (MFI) was obtained directly from the xPONENT® software. Each plate included 3 wells with only PBS-TBN buffer to estimate non-specific binding (NSB). An epitope was considered “present” if its MFI was above the upper limit of a 95% confidence interval of the NSB samples. For each sample, epitope diversity was then estimated as the proportion of epitopes “present”. An association between epitope-specific IgE or IgG4 diversity and BAT’s CD-max was modeled using a robust linear regression (*rlm* function from the *MASS* package). The robust regression was chosen as it allows an M-weight function that includes a special weight estimation for the data with outliers and/or influential data points.

Table S1

Demographic and clinical characteristics of subjects in the PBAT cohort. Means and standard deviations are presented for continuous variables, median and 1st and 3rd quartiles for the specific IgE. Categorical variables are presented as a frequency and percent. Control without peanut allergy is not included in this table.

	All Subjects (N=30)	Non-Responders (N=4)	Responders (N=26)
Age	11.8 (5.7)	6.3 (1.6)	12.7 (5.6)
Male sex (n, %)	15 (50.0)	1 (25.0)	14 (53.8)
Medical History (n, %)			
Asthma	12 (40.0)	0 (0.0)	12 (46.2)
Allergic Rhinitis	21 (70.0)	2 (50.0)	19 (73.1)
Eczema	22 (73.3)	3 (75.0)	19 (73.1)
SPT to Peanut, mm	11.4 (3.3)	16.0 (.)**	11.2 (3.1)
Specific IgE, kU_A/L			
sIgE Peanut	22.9 [2.6, 98.1]	17.7 [2.8, 48.9]	22.9 [2.6, 98.1]
sIgE Ara h 1	1.4 [0.1, 14.3]	0.4 [0.3, 5.3]	2.0 [0.1, 14.3]
sIgE Ara h 2	11.2 [0.8, 61.6]	8.3 [1.1, 32.1]	11.5 [0.8, 61.6]
sIgE Ara h 3	0.4 [0.1, 4.2]	2.6 [0.1, 6.9]	0.4 [0.1, 2.5]
sIgE Ara h 8	0.2 [0.1, 10.2]	0.3 [0.1, 3.6]	0.2 [0.1, 10.2]
sIgE Ara h 9	0.1 [0.1, 0.1]	0.5 [0.4, 1.6]	0.1 [0.1, 0.1]
Clinical Reaction to Peanut (n, %)			
History of Prior Reactions	21 (70.0)	2 (50.0)	19 (73.1)
History of Anaphylaxis	8 (26.7)	0 (0.0)	8 (30.8)
Oral Swelling	14 (46.7)	0 (0.0)	14 (53.8)
Hives	16 (53.3)	1 (25.0)	15 (57.7)
Vomiting	3 (10.0)	0 (0.0)	3 (11.5)
Respiratory Distress	3 (10.0)	0 (0.0)	3 (11.5)

** 3 Non-Responder patients did not have SPT performed.

Table S2

Amino acid sequences of 64 peanut epitopes.

Epitope	Amino Acid Sequence
ara h 1.p1	ATHAKSSPYQKKTEN
ara h 1.p2	LQSCQQEPDDLKQKA
ara h 1.p3	RCTKLEYDPRCVYDP
ara h 1.p4	KLEYDPRCVYDPRGH
ara h 1.p5	YDPRGHTGTTNQRSP
ara h 1.p6	RSPPGERTRGRQPGD
ara h 1.p7	PGERTRGRQPGDYDD
ara h 1.p8	PGDYDDRRQPRREE
ara h 1.p9	DRRQPRREEGGRWGP
ara h 1.p10	AGPREREREEDWRQP
ara h 1.p11	REREREEDWRQPRD
ara h 1.p12	RQPREDWRRPSHQQP
ara h 1.p13	REDWRRPSHQQPRKI
ara h 1.p14	PSHQQPRKIRPEGRE
ara h 1.p15	RPEGREGEQEWGTPG
ara h 1.p16	REETSRNPNFYFPSR
ara h 1.p17	NNPFYFPSRRFSTRY
ara h 1.p18	SGFISYILNRHDNQN
ara h 1.p19	SMPVNTPGQFEDFFP
ara h 1.p20	RDQSSYLQGFSRNTL
ara h 1.p21	SEEEGDITNPINLRE
ara h 1.p22	EGDITNPINLREGEP
ara h 1.p23	NNFGKLFVKKPDKKN
ara h 1.p24	RYTARLKEGDVFIMP
ara h 1.p25	DVFIMPAAHPVAINA
ara h 1.p26	PVAINASSELHLLGF
ara h 1.p27	LHLLGFGINAENNHHR

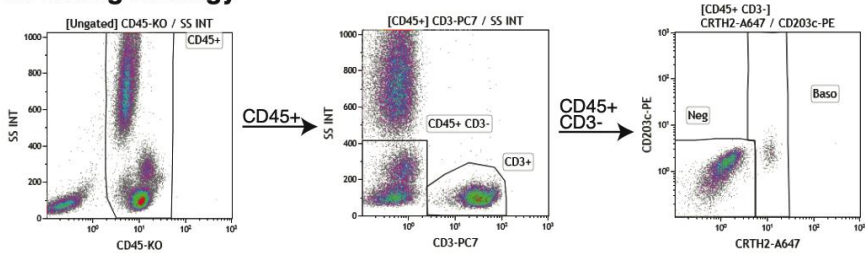
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ara h 1.p29	NHRIFLAGDKDNVID	ara h 2.p14	QGRQQEQQFKRELRN
ara h 1.p30	VIDQIEKQAKDLAFP	ara h 2.p15	KRELRNLPQQCGLRA
ara h 1.p31	KQAKDLAFPGSGEQV	ara h 2.p16	LPQQCGLRAPQRCDL
ara h 1.p32	KDLAFPGSGEQVEKL	ara h 3.p1	LRRNALRRPFYSNAP
ara h 1.p33	SHFVSARPQSQSQSP	ara h 3.p2	HYEEPHTQGRRSQSQ
ara h 1.p34	QEEENQGGKGPLLSI	ara h 3.p3	EPHTQGRRSQSQRPP
ara h 2.p1	AAHASARQQWELQGD	ara h 3.p4	QGEDQSQQQRDSHQK
ara h 2.p2	WELQGDRRCQSQLER	ara h 3.p5	NTEQEFLRYQQQSRQ
ara h 2.p3	RRCQSQLERANLRPC	ara h 3.p6	PYSPQSQPRQEEREF
ara h 2.p4	RPCEQHLMQKIQRDE	ara h 3.p7	EGGNIFSGFTPEFLE
ara h 2.p5	KIQRDEDSYERDPYS	ara h 3.p8	NIFSGFTPEFLEQAF
ara h 2.p6	RDEDSYERDPYSPSQ	ara h 3.p9	AIVTVRGGRLRLSPD
ara h 2.p7	DSYERDPYSPSQDPY	ara h 3.p10	TVRGGLRLISPDRKR
ara h 2.p8	PYSPSQDPYSPSPYD	ara h 3.p11	EYDEDEYEYDEEDRR
ara h 2.p9	CCNELNEFENNQRCM	ara h 3.p12	YEYDEEDRRRRGRGSR
ara h 2.p10	ELNEFENNQRCMCEA	ara h 3.p13	IANLAGENSVIDNLP
ara h 2.p11	LQQIMENQSDRLQGR	ara h 3.p14	RQLKNNNPFFKFFVPP
ara h 2.p12	IMENQSDRLQGRQQE		

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Figure S1

The gating strategy for measuring basophil activation with flow cytometry.

A. Gating Strategy



B. CD63+CD203c+ Controls

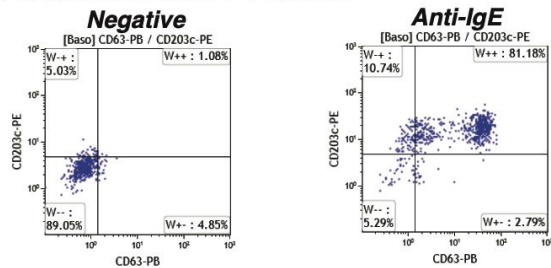


Figure S2

No significant difference in basophil activation between day 0, 1 and 3 ($p < 0.05$), as quantified by previously published threshold values for peanut allergy.

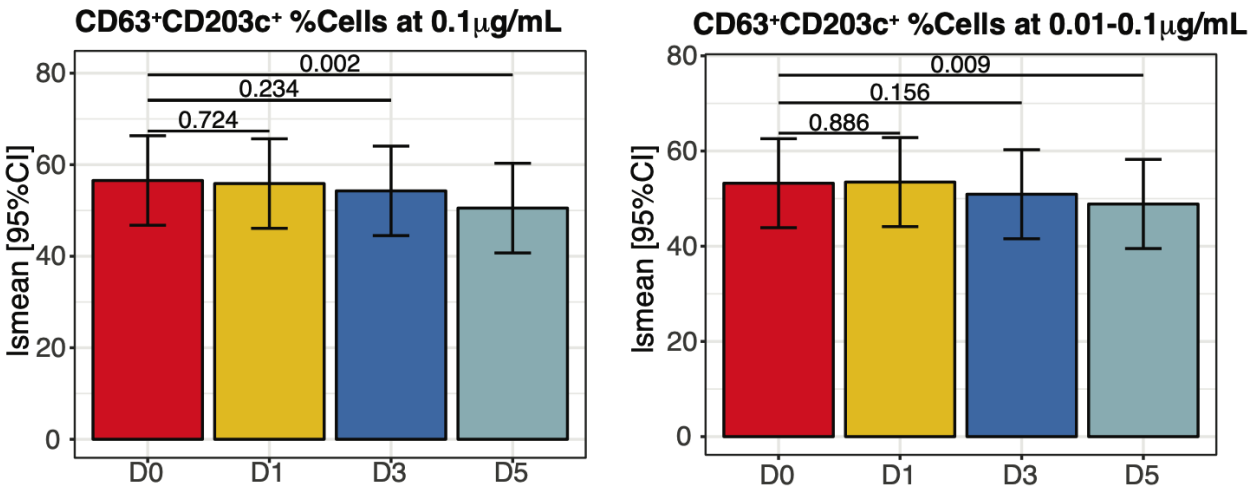
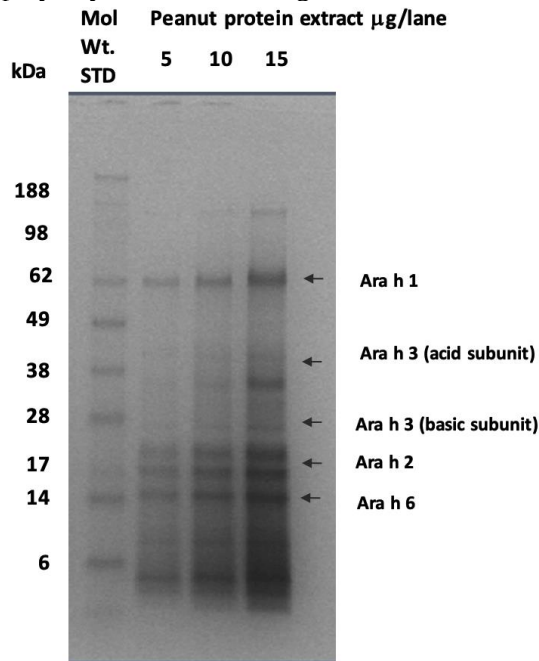


Figure S3

Protein band corresponding to the major peanut allergen Ara h 1, Ara h 2 and Ara h 3 are indicated by arrows. Indicated amounts of protein from peanut extract were separated on Nupage 4-12% polyacrylamide Bis-Tris gel and stained with SimpyBlue SafeStain (both by ThermoFisher Scientific).



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