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ORIGINAL ARTICLE

Validation of inducible basophil biomarkers: Time, temperature and transportation

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Abstract

Background: The short stability window of several hours from blood collection to measuring basophil activation has limited the use of flow cytometry-based basophil activation assays in clinical settings. We examine if it is possible to extend this

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window to 1 day allowing for shipment of samples between laboratories. Several options exist for reporting the results including reporting all the measured values directly, calculating ratios and reporting a single value covering all measured results. Each of these options have different stability and value to the physician.

Methods: Whole blood samples from peanut allergic patients were stimulated with four different peanut concentrations at Day 0, Day 1, and Day 2. Samples were stored under temperature-controlled conditions. Flow cytometry was used to analyze the samples. The basophil activation and degranulation were measured as percentage of positive CD63 basophils and CD203c MFI fold change. Shipped samples were transported under ambient conditions.

Results: The results show that CD63 is a stable marker at Day 1. The CD203c ratio decreases significantly at Day 1. Calculating the CD63/IgE ratio proves to be more stable than CD63 alone. The most stable readouts are the semi-quantitative results and the trajectory of the dose response curve. Finally, we confirmed that the stability can be extended to samples shipped overnight to the laboratory.

Conclusions: It is possible to extend the stability of the basophil activation assay to 1 day for samples stored at 18–25°C as well as samples shipped under ambient conditions as long as the temperature is within the 2–37°C range.

KEYWORDS
allergy, basophil activation test, basophils, CD63, peanut allergy

1 | INTRODUCTION

Upregulation of basophil surface markers CD63 and CD203c upon exposure to allergens was first shown almost two decades ago (Sainte-Laudy et al., 1998). The strong correlation between these inducible biomarkers and outcomes of food allergen challenge is proving to be a valuable clinical tool (Czechowska et al., 2019; Koplin et al., 2019; Santos et al., 2014). The requirement for fresh whole blood and reports on the time sensitivity and stability of the blood sample used in the basophil activation test (BAT), however, have limited its clinical use because samples had to be processed within 4 h of collection when stored at room temperature (Hoffmann et al., 2016; Sturm et al., 2009). A recent, small-scale study concluded that the stability could be extended to 24 h by storing samples at 4°C (Mukai et al., 2017). Within the last few years several other in vitro diagnostic tests utilizing stripped donor basophils, basophil cell lines, mast cell lines, or cultured primary human mast cells have been developed in research labs to overcome the limited stability of the BAT as well as diagnosing the approximately 10% of the patients that have non-responding basophils (Bahri et al., 2018; Elst et al., 2020; Falcone et al., 2018; Larsen et al., 2018; Mueller-Wirth et al., 2020; Puan et al., 2017; Santos et al., 2018). All these newer tests rely on IgE from isolated patient serum binding to a basophil or mast cell from another individual which comes with other limitations and challenges.

We revisited the issues associated with the whole blood sample stability of the basophil activation test for several reasons. First, even if a flow cytometry laboratory is readily accessible to a clinic, the handling and processing of samples will require batching to achieve a functional workflow because blood samples arrive in the laboratory at different times preventing processing of each sample immediately after a phlebotomy. Second, the storage temperature, both in the laboratory and during transportation can impact the stability of the blood sample (Mukai et al., 2017; Sturm et al., 2009). Third, the emergence of new therapies, such as oral food immunotherapy (OIT) as well as newly formed national organizations, such as Food Allergy Support Team and Global Food Therapy, have increased the demand for further evaluating and monitoring food allergy patients, hence the need for assays to help guide management (Chinthrajah et al., 2019). Fourth, there is an increasing number of Clinical Laboratory Improvement Amendments (CLIA) accredited clinical flow cytometry laboratories as extensions of allergy/immunology clinics in the United States that want to evaluate the basophil response in their allergic patients without having to rely on methods requiring culturing primary cells or cell lines. Real-world data collection from basophil-based assays will not be possible without resolving the stability question (Alpan et al., 2019).

Evaluating the stability of the allergen and of the anti-IgE induced basophil biomarkers is directly related to how the results are reported. The consensus is that CD63 is the best marker because it is directly related to histamine release and it easy to gate since it is expressed on a distinct positive population (Amano et al., 2001; Dvorak et al., 1983; Hoffmann et al., 2016; MacGlashan Jr, 2010; MacGlashan Jr, 2012). Another evaluable marker is CD203c but it

does not form a distinct positive population and is a more general basophil activation marker, not directly correlated with histamine release (MacGlashan Jr, 2010; MacGlashan Jr, 2012). When reporting the results of a basophil activation test, the most commonly used option is the percentage of CD63 positive at one to several different allergen concentrations, however, semi-quantitative analysis, CD63/Anti-IgE ratio, maximum histamine secretion, histamine ED50, concentration optimum, CDsens (50% of max response) and area under the curve (AUC) also have been used. Some reportable results depend on an ideally shaped dose response or bell-shaped curve whereas others rely on a high degree of reproducibility of exact values for CD63 or CD203c. In this paper we have addressed the question of post-collection stability by showing that basophils are stable 1 day post collection and we extended the stabilization studies to samples shipped in a cost-efficient way. Additionally, we discuss the best approach to reporting the data in a way that can be useful to the clinician.

2 | METHODS

2.1 | Basophil phenotyping

Lyophilized, defatted whole peanut extract (cat# F171, Stallergenes Greer, Cambridge, MA) was resuspended in PBS. Whole blood was collected in heparin tubes and allowed to adjust to room temperature for at least 1 h before mixing with titrating concentrations of peanut between 10 and 10,000 ng/ml. Anti-IgE (BD Bioscience, San Jose, CA) stimulation was used as the positive control while unstimulated blood functioned as the negative control. The samples were incubated for 20 min at 37°C followed by 10 min at 4°C (Ebo et al., 2020; Hoffmann et al., 2015; Mukai et al., 2017; Sousa et al., 2010; Sturm et al., 2009). Each sample was stained with the following antibodies anti-CD63-PE (Clone H5C6), anti-CD203c-PECY7 (Clone NP4D6), anti-CD45-AF700 (Clone 2D1), anti-IgE-FITC (Clone Ige21), anti-CD123-PerCPCy5.5 (Clone 6H6), and anti-CD193-APC

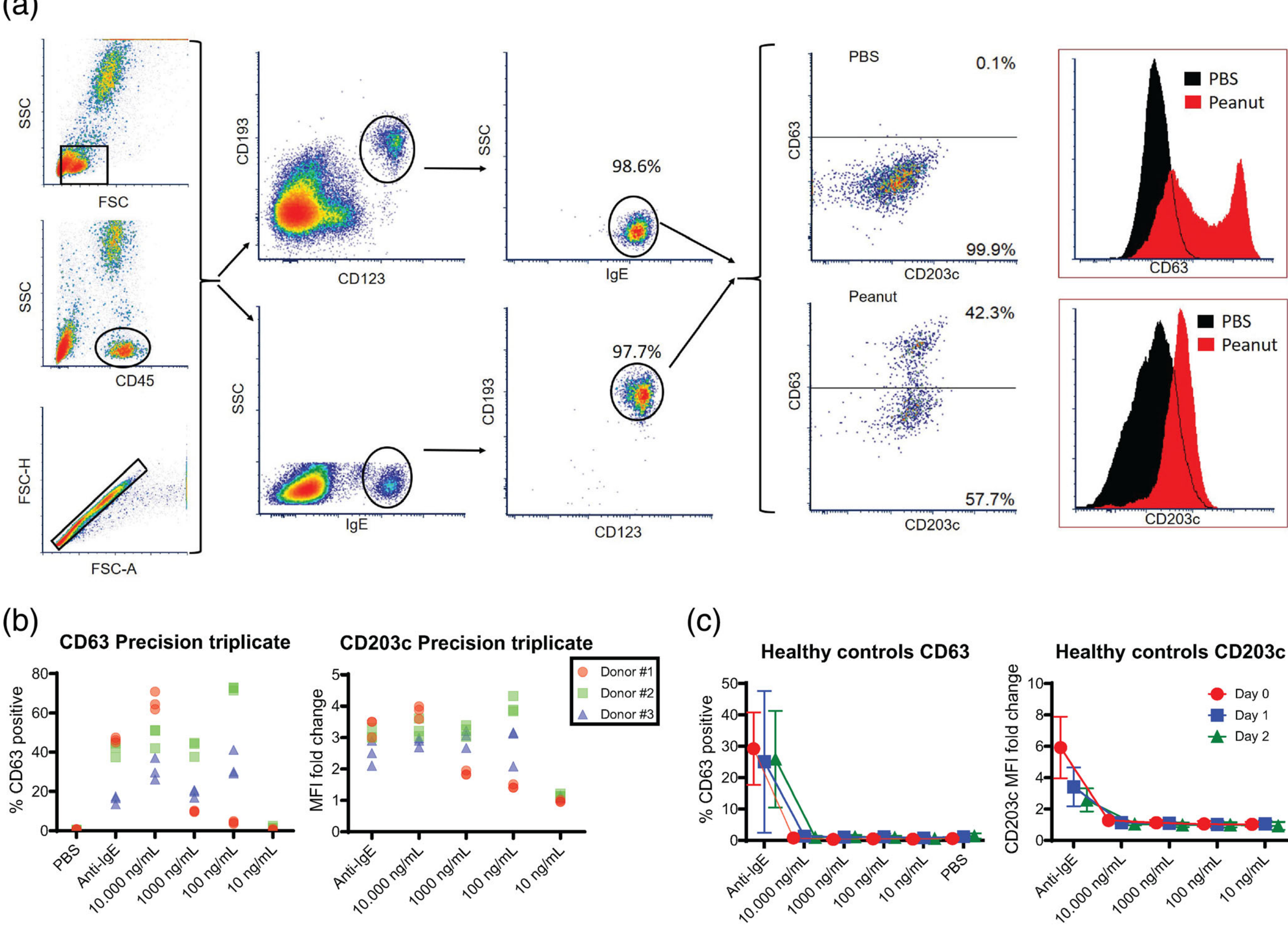

FIGURE 1 Design of the basophil assay. (a) Gating strategy. Initially an FCS/SSC, singlet (FCS-A/ FCS-H) and CD45/SSC are applied. This is followed by gating on basophils using both a CD123+/CD193+ and an IgE+/SSClow gate. Basophil activation is determined by measuring percentages of CD63 positive basophils as well as the fold change in CD203c MFI compared to the negative control. (b),(c) Whole blood was stimulated with peanut allergen concentrations as indicated in the figure or anti-IgE as a positive control or unstimulated negative control (PBS). After processing, the samples were analyzed by flow cytometry. (b) Assay precision in triplicate assays. The CD63 and CD203c activation markers were measured in samples from three donors processed in triplicate. (c) Whole blood from healthy controls were stimulated with PBS, Anti-IgE and peanut allergen concentrations as indicated in the figure at Day 0, Day 1, and Day 2 after blood collection [Color figure can be viewed at wileyonlinelibrary.com]

(Clone 5E8) (Thermo Fisher, Waltham, MA) for 30 minutes at 4°C. Each antibody was titrated to obtain the best separation (Ryherd et al., 2018). The red blood cells were lysed using BD FACS lysis solutions (BD Bioscience, San Jose, CA). A minimum of 500 basophils, defined as CD123/CD193/IgE positive cells, see Figure 1(a), were collected. The basophil assay used for these studies has been previously validated for clinical use in our laboratory (https://www.amerimmune.com/publications) and (Alpan, Kim, et al., 2020) and it has been implemented for clinical diagnostics at other CLIA/CAP laboratories.

2.2 | Instrumentation

The samples were acquired on a 3 laser/10 color BD FACSCanto. CS&T beads (BD Bioscience, San Jose, CA) were acquired daily to ensure consistent performance of the cytometer. The secondary site used in the shipping part of the study utilized a 4 laser/14 color Attune NxT Flow Cytometer where the daily consistency was monitored using Performance Tracking beads (Thermo Fisher, Waltham, MA). Both instruments have been CAP and CLIA validated for clinical diagnostic studies.

2.3 | Patients and controls

The studies involving human participants were reviewed and approved by Western Institutional Review Board (WIRB) Protocol #20121950. Written, informed consent and, when appropriate, assent was obtained from the individuals and minors' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article. A total of 31 peanut allergic patients age 4–32 and 6 healthy controls age 29–49 were consented for the study. One healthy control and one patient were non-responders in that their CD63 and CD203c response to anti-IgE antibody was not above the detection threshold. Therefore, they were excluded from the study. Of the 30 peanut allergic patients 17 were on OIT.

2.4 | Data analysis

Data analysis was performed using FCS Express software (De Novo software, Glendale, CA). CD63 was measured as the percentage of positive basophils and CD203c is reported as fold change in the median fluorescent intensity (MFI) compared to the unstimulated negative control (PBS). We defined a detection threshold for a CD63 response as when the percentage of CD63 positive cells in a sample is <1% and less than two times the value of the negative control rather than using healthy controls (Chirumbolo, 2014). In the CD203c assay, we defined the detection threshold as an MFI fold ratio < 1.1. Graphs were generated as scatter plots, and statistical analysis was performed using GraphPad Prism. All data comparisons were analyzed as paired, two tailed, two-sample unequal variance using the students *t* test to determine significance. A *p*-value less than 0.05 is considered significant, * $p < 0.05$, ** $p < 0.01$. Correlation and Bland–Altman analysis and plots were performed using GraphPad Prism (Bland & Altman, 1986; Giavarina, 2015).

3 | RESULTS

3.1 | Establishing the basophil assay

Whole blood was stimulated with four different peanut concentrations ranging from 10 to 10,000 ng/ml (Hoffmann et al., 2016; Santos et al., 2014). Samples stimulated with anti-IgE antibody were used as positive controls and an unstimulated (PBS) sample was used as negative control.

We used an FSC/SSC, CD45/SSC and singlet (FSC-A/FSC-H) gate for our initial gating. This was followed by a two-way approach to identify basophils using both a CD123/CD193 and an IgE/SSC gate ensuring a very pure basophil population (Kim et al., 2016). The degranulation of basophils was measured using CD63 and activation was measured by CD203c. CD63 staining results in two clearly distinct cell populations after degranulation and the result for CD63 was measured as the percentage of positive basophils. Following activation, the CD203c shifts up for the whole basophil population. We therefore reported CD203c as fold change in the MFI compared to the unstimulated negative control (PBS) (Figure 1(a)).

We first measured the precision of the assay by stimulating and staining in triplicate. The intra assay precision had a CV <15% for all concentrations above the cutoff which is within recommended guidelines for flow cytometry assays (O'Hara et al., 2011; Selliah et al., 2019; Wood et al., 2013) (Figure 1(b)).

We assayed the response to peanut allergen in five healthy controls using blood stored for up to 2 days under temperature-controlled conditions at 18–25°C. The results clearly showed positive CD63 and CD203c results after IgE stimulation and consistently negative results in the peanut allergen stimulated samples. None of the healthy controls were scored as a responder to any peanut concentration at any time point (Figure 1(c)).

3.2 | Post-collection stability

We compared basophil surface expression of CD63 and CD203c in response to anti-IgE or peanut allergen stimulation in blood stored under temperature controlled conditions at 18–25°C for 0–4 h (Day 0), 20–28 h (Day 1) and 44–52 h (Day 2) post collection. We evaluated 30 peanut allergic patients, age 4–32 (demographics are shown in Table 1 and for clinical history, see Supplementary Figure S1).

For each peanut concentration, we initially determined if the patient responded at the given time points. We defined a detection threshold for a CD63 response as when the percentage of CD63 positive cells in a sample is <1% and less than two times the value of the negative control. In the CD203c assay, we defined the detection threshold as an MFI fold ratio < 1.1.

TABLE 1 IgE, CD63, and CD203c values for individual peanut allergic patients

Sex/age	OIT	IgE/kU/L Total	Peanut	Ara h2	Marker	Response at: Day 0/day 1/day 2 10,000 ng/ml	1000 ng/ml	100 ng/ml	10 ng/ml
F4	No	193	1.71	1.23	CD63	+/+/+	+/+/+	+/+/+	−/−/−
					CD203c	+/+/+	+/+/+	+/+/+	**−/−/+**
F13	No	309	0.45	0.3	CD63	+/+/+	**+/+/−**	**+/+/−**	−/−/−
					CD203c	+/+/+	**+/+/−**	**+/+/−**	−/−/−
M6	No	1479	>100	>100	CD63	+/+/+	+/+/+	+/+/+	−/−/−
					CD203c	+/+/+	+/+/+	+/+/+	**+/+/−**
F9	No	210	0.92	0.63	CD63	+/+/+	+/+/+	+/+/+	−/−/−
					CD203c	+/+/+	+/+/+	+/+/+	−/−/−
F13	No	1114	>100	n/a	CD63	+/+/+	+/+/+	+/+/+	**+/+/−**
					CD203c	+/+/+	+/+/+	+/+/+	**+/+/−**
M10	No	455	5.54	n/a	CD63	+/+/+	+/+/+	+/+/+	−/−/−
					CD203c	+/+/+	+/+/+	+/+/+	**+/−/−**
F5	No	n/a	>100	n/a	CD63	+/+/+	+/+/+	+/+/+	+/+/+
					CD203c	+/+/+	+/+/+	+/+/+	+/+/+
F32	No	n/a	n/a	n/a	CD63	+/+/+	+/+/+	+/+/+	**−/−/+**
					CD203c	+/+/+	+/+/+	+/+/+	**+/−/+**
F3	No	309	1.25	1.17	CD63	+/+/+	**+/+/−**	+/+/+	**+/−/−**
					CD203c	+/+/+	+/+/+	+/+/+	**+/−/−**
M8	No	956	>100	>100	CD63	+/+/+	+/+/+	+/+/+	+/+/+
					CD203c	+/+/+	+/+/+	+/+/+	+/+/+
M5	No	48	0	0	CD63	+/+/+	**+/+/−**	−/−/−	−/−/−
					CD203c	+/+/+	**+/+/−**	−/−/−	−/−/−
F5	Yes	n/a	67	65	CD63	+/+/+	+/+/+	+/+/+	−/−/−
					CD203c	+/+/+	+/+/+	+/+/+	−/−/−
F14	Yes	1561	>100	>100	CD63	+/+/+	+/+/+	+/+/+	**+/+/−**
					CD203c	+/+/+	+/+/+	+/+/+	**+/+/−**
M6	Yes	8096	>100	>100	CD63	+/+/+	+/+/+	+/+/+	−/−/−
					CD203c	+/+/+	+/+/+	+/+/+	**−/−/+**
M5	Yes	314	52.3	41.6	CD63	+/+/+	+/+/+	+/+/+	−/−/−
					CD203c	+/+/+	+/+/+	+/+/+	**−/+/−**
M7	Yes	1290	>100	99.1	CD63	+/+/+	+/+/+	+/+/+	−/−/−
					CD203c	+/+/+	+/+/+	+/+/+	**+/+/−**
M6	Yes	839	>100	>100	CD63	+/+/+	+/+/+	+/+/+	−/−/−
					CD203c	+/+/+	+/+/+	+/+/+	**−/+/−**
M10	Yes	575	7.67	5.11	CD63	+/+/+	**+/+/−**	**+/+/−**	−/−/−
					CD203c	+/+/+	+/+/+	**+/+/−**	−/−/−
M8	Yes	1907	>100	>100	CD63	+/+/+	+/+/+	+/+/+	−/−/−
					CD203c	+/+/+	+/+/+	+/+/+	**+/−/−**
M8	Yes	1183	9.82	4.43	CD63	+/+/+	+/+/+	**+/+/−**	−/−/−
					CD203c	+/+/+	+/+/+	**+/−/−**	−/−/−
M10	Yes	511	>100	>100	CD63	+/+/+	+/+/+	+/+/+	**+/+/−**
					CD203c	+/+/+	+/+/+	+/+/+	−/−/−
F9	Yes	723	12.8	13.7	CD63	+/+/+	+/+/+	−/−/−	−/−/−
					CD203c	+/+/+	+/+/+	−/−/−	−/−/−
M21	No	n/a	n/a	n/a	CD63	+/+/+	+/+/+	+/+/+	+/+/+
					CD203c	+/+/+	+/+/+	+/+/+	**+/+/−**

TABLE 1 (Continued)

		IgE/kU/L				Response at: Day 0/day 1/day 2			
Sex/age	OIT	Total	Peanut	Ara h2	Marker	10,000 ng/ml	1000 ng/ml	100 ng/ml	10 ng/ml
F16	No	140	67.7	n/a	CD63	+/+/+	+/+/+	+/+/+	+/+/+
					CD203c	+/+/+	+/+/+	+/+/+	+/+/+
F14	Yes	985	8.5	9.43	CD63	+/+/+	+/+/+	**−/−/+**	−/−/−
					CD203c	+/+/+	+/+/+	+/+/+	−/−/−
F14	Yes	n/a	>100	>100	CD63	**+/+/−**	**+/+/−**	−/−/−	−/−/−
					CD203c	+/+/+	+/+/+	**−/+/−**	**−/+/−**
M10	Yes	190	35.9	10.7	CD63	+/+/+	+/+/+	+/+/+	+/+/+
					CD203c	+/+/+	+/+/+	+/+/+	+/+/+
M14	Yes	n/a	23.6	10.3	CD63	+/+/+	+/+/+	**−/−/+**	−/−/−
					CD203c	+/+/+	+/+/+	**−/−/+**	**−/−/+**
M16	Yes	162	3	1.49	CD63	+/+/+	+/+/+	**−/+/+**	−/−/−
					CD203c	+/+/+	+/+/+	**−/+/+**	−/−/−
M5	Yes	2173	>100	79	CD63	+/+/+	+/+/+	**−/−/+**	−/−/−
					CD203c	+/+/+	+/+/+	**+/−/+**	**+/−/−**

Note: Table 1 shows the age, sex, OIT information, total IgE, peanut specific IgE and Ara h2 (an allergenic peanut protein) specific IgE (Ara h2 IgE is the strongest predictor of anaphylaxis on peanut exposure) together with the individual patient's positive and negative reaction to the different concentrations of peanut allergen based on CD63 and CD203c on Day 0, Day 1 and Day 2 (Day 0/Day 1/Day 2). Discordant results are represented as bold values. For a list of allergic symptoms and exact CD63 and CD203c values see Supplemental Figure S1.

For CD63 we observed a discordant result, defined as a change from responding to not responding or from not responding to responding, at one allergen concentration for two patients between Day 0 and Day 1 and in 10 patients between Day 0 and Day 2. Four patients had discordant results for two allergen concentrations between Day 0 and Day 2. For CD203c there were discordant results at one allergen concentration for eight patients between Day 0 and Day 1 and in 15 patients between Day 0 and Day 2. Two patients had discordant CD203c results for two allergen concentrations between Day 0 and Day 1 and two patients had discordant results between Day 0 and Day 2. The discordant results were due to small changes in borderline percentage or fold changes at the lowest responding concentration rather than reversal of a clear positive or negative result (Table 1 and Supplemental Figure S1).

Next, we examined the stability of the CD63 percentage positivity and CD203c MFI fold change values at Day 1 and Day 2 compared to Day 0. We observed no statistical difference between Day 0 and Day 1 in the percentage of CD63 positive basophils by peanut or anti-IgE stimulation. Between Day 0 and Day 2 we observed a significant decrease in the response to anti-IgE and three of the four peanut concentrations. In the negative control sample, the percentage of CD63 positive basophils was increased significantly at Day 1 and Day 2. The fold changes in CD203c MFI were statistically lower on Day 1 and Day 2, compared to Day 0 for all samples due to an increase in CD203c MFI in the negative sample at Day 1 and Day 2 compared to Day 0 (Supplementary Figure S1 and Supplementary Figure S2). A lack of significant difference for the CD63 results between Day 0 and Day 1 does not mean the data was compatible. To analyze the correlation between the different days a R^2 correlation and Bland–Altman analysis for absolute change were used to compare Day 0 against Day 1 and Day 2. We included data from 17 peanut allergic patients independently performed twice within 2–4 h of blood draw for comparison purposes (Day 0 vs. Day 0b). The data, from two assays a few hours apart, showed the variations expected for this type of assay. The bias, SD, and R^2 for CD63 are similar for Day 0 versus Day 0b and Day 0 versus Day 1 whereas there was an increased bias, a slight increase in SD as well as decrease in R^2 at Day 0 versus Day 2. The bias for CD203c MFI was already higher at Day 0 versus Day 1 than at Day 0 versus Day 0b (Table 2 and Supplementary Figure S2).

3.3 | Stability of the dose response curve

Having the observed variations in the individual patient responses to a given peanut concentration we wanted to see if the dose response curve changed shape at different time points. We plotted the individual patient peanut, PBS, and anti-IgE dose response curves for Day 0, Day 1, and Day 2. The results showed that not all patients' responses follow an ideal bell curve or a declining dose response curve. The shape of the curves for both CD203c MFI fold change and CD63 positive basophils were extremely well preserved at Day 1 and for some patients at Day 2 as well (Figure 2 and Supplementary Figure S1).

3.4 | Stability of alternative reportable values

There are several different ways to report inducible basophil biomarkers in response to a positive control and allergens. Most patients have a

TABLE 2 R^2 correlation and Bland–Altman analysis for CD63 and CD203c values

Stimulation	Reportable	Comparing	Bland–Altman		Correlation R^2
			Bias	SD	
PBS	%CD63	Day 0 versus Day 0b	−0.22	0.37	0.29
		Day 0 versus Day 1	−0.25	0.32	0.08
		Day 0 versus Day 2	−0.44	0.43	0.21
Anti-IgE	%CD63	Day 0 versus Day 0b	−4.74	11.08	0.70
		Day 0 versus Day 1	4.51	12.67	0.57
		Day 0 versus Day 2	7.22	15.49	0.47
	CD203c ratio	Day 0 versus Day 0b	0.21	1.08	0.06
		Day 0 versus Day 1	1.05	1.26	0.26
		Day 0 versus Day 2	1.60	1.31	0.22
Peanut 10,000 ng/ml	%CD63	Day 0 versus Day 0b	0.69	13.35	0.74
		Day 0 versus Day 1	3.24	13.25	0.71
		Day 0 versus Day 2	9.38	14.82	0.65
	CD203c ratio	Day 0 versus Day 0b	0.33	1.15	0.08
		Day 0 versus Day 1	1.02	1.19	0.40
		Day 0 versus Day 2	1.74	1.39	0.20
Peanut 1000 ng/ml	%CD63	Day 0 versus Day 0b	2.6	12.52	0.73
		Day 0 versus Day 1	3.29	16.20	0.54
		Day 0 versus Day 2	9.23	16.17	0.49
	CD203c ratio	Day 0 versus Day 0b	0.26	1.22	0.00
		Day 0 versus Day 1	1.02	1.24	0.27
		Day 0 versus Day 2	1.64	1.41	0.12
Peanut 100 ng/ml	%CD63	Day 0 versus Day 0b	−4.63	12.23	0.84
		Day 0 versus Day 1	3.14	14.71	0.67
		Day 0 versus Day 2	8.74	15.66	0.62
	CD203c ratio	Day 0 versus Day 0b	0.06	1.16	0.26
		Day 0 versus Day 1	0.94	1.51	0.49
		Day 0 versus Day 2	1.57	1.65	0.45
Peanut 10 ng/ml	%CD63	Day 0 versus Day 0b	1.06	9.43	0.27
		Day 0 versus Day 1	1.05	4.07	0.91
		Day 0 versus Day 2	−0.77	10.92	0.25
	CD203c ratio	Day 0 versus Day 0b	0.09	0.53	0.31
		Day 0 versus Day 1	0.22	0.63	0.82
		Day 0 versus Day 2	0.29	0.70	0.63

Note: Table 2 shows a summary of the correlation (R^2) and Bland–Altman analysis (Bias and SD) for % CD63 positive basophils and CD203c MFI fold increase following stimulation of whole blood from 30 peanut allergic patients with PBS, Anti-IgE or the four peanut concentrations. The individual graphs are shown in Supplemental Figure S2. The Day 0 versus Day 0b data are from 17 patients analyzed independent of the stability experiment and is included for comparison.

positive correlation between the anti-IgE and the CD63 response at a given timepoint (Figure 2 and Supplementary Figure S1). This prompted us to calculate the CD63/Anti-IgE ratio (Santos et al., 2015). The results showed that the stability of the CD63/Anti-IgE ratio is not significant different at Day 1 or Day 2 compared to Day 0 making the CD63/IgE ratio a more stable readout than CD63 alone. The R^2 correlation and Bland–Altman analysis showed very similar results between Day 0 versus Day 0b and Day 0 versus Day 1 with respect to bias, SD and R^2. Day 0 versus Day 2 had an increase in bias and SD as well as a decrease in R^2 (Table 3 and Supplemental Figure S2).

Another method commonly used in reporting the results of a dose response experiment is the AUC. The AUC measurement has been a readout in several of the larger trials of peanut oral immunotherapy, hence it is important to address in this setting (Chinthrajah et al., 2019). AUC compiles all the results for the different concentrations into a single value (Patil & Shreffler, 2012). We calculated

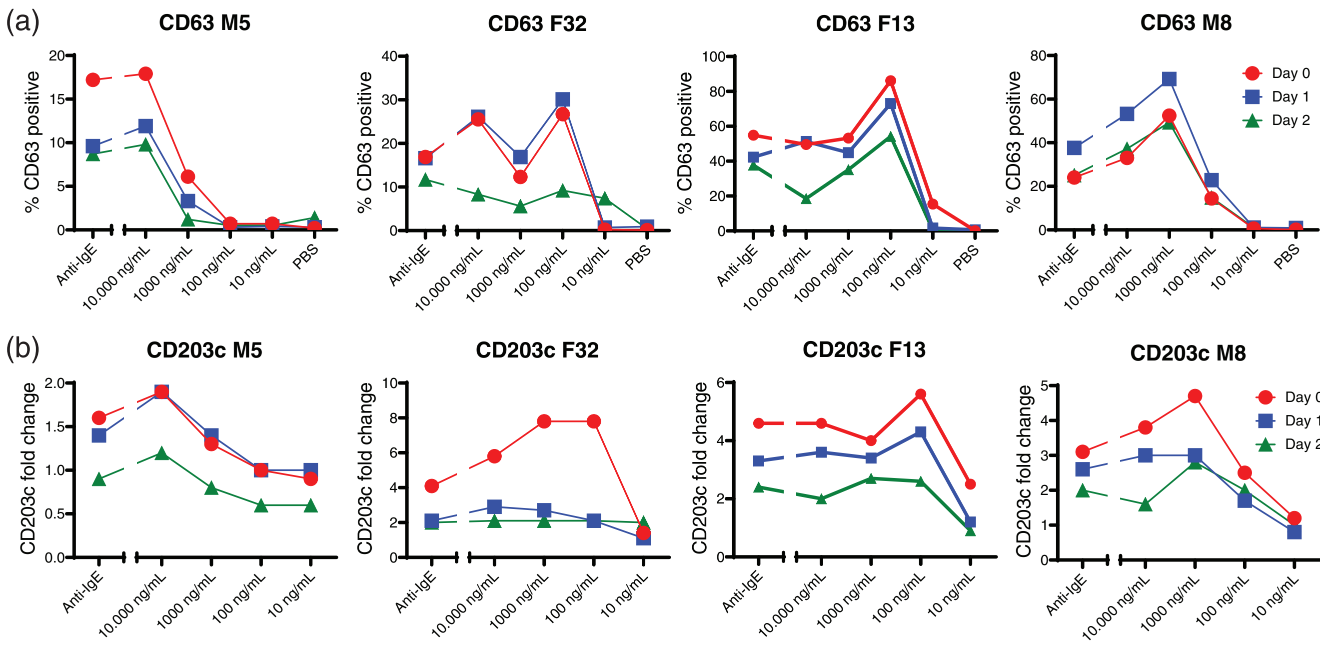

FIGURE 2 Representative dose response from four patients. The individual % positive CD63 and CD203c MFI fold change dose response curves for each day were created by connecting the data points for the various peanut concentrations, PBS and anti-IgE at Day 0 (red circles), Day 1 (blue squares) and Day 2 (green triangles). Four representative patients have been shown. The dose response curves for all 30 patients are included in Supplementary Figure S1. (a) % positive CD63 basophils. (b) CD203c MFI fold change [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 R^2 correlation and Bland–Altman analysis for alternative reportable values

Stimulation	Reportable	Comparing	Bland–Altman		
			Bias	SD	Correlation R^2
Peanut 10,000 ng/ml	CD63/Anti-IgE	Day 0 versus Day 0b	0.07	0.44	0.66
		Day 0 versus Day 1	0.03	0.68	0.46
		Day 0 versus Day 2	0.23	0.94	0.05
Peanut 1000 ng/ml	CD63/Anti-IgE	Day 0 versus Day 0b	0.17	0.57	0.71
		Day 0 versus Day 1	0.05	0.49	0.77
		Day 0 versus Day 2	0.25	0.91	0.26
Peanut 100 ng/ml	CD63/Anti-IgE	Day 0 versus Day 0b	−0.02	0.23	0.87
		Day 0 versus Day 1	−0.02	0.34	0.80
		Day 0 versus Day 2	0.18	0.63	0.32
Peanut 10 ng/ml	CD63/Anti-IgE	Day 0 versus Day 0b	0.06	0.45	0.16
		Day 0 versus Day 1	−0.05	0.22	0.90
		Day 0 versus Day 2	−0.09	0.26	0.45
AUC	%CD63	Day 0 versus Day 0b	−2.93	26.82	0.83
		Day 0 versus Day 1	8.58	34.68	0.60
		Day 0 versus Day 2	22.28	35.45	0.57
	CD63/Anti-IgE	Day 0 versus Day 0b	0.21	0.88	0.70
		Day 0 versus Day 1	0.02	0.95	0.67
		Day 0 versus Day 2	0.50	1.42	0.27

Note: Table 3 shows a summary of the correlation (R2) and Bland–Altman analysis (Bias and SD) for CD63/Anti-IgE ratio and CD63 AUC for 30 peanut allergic patients. The individual graphs are shown in Supplemental Figure S2. The Day 0 versus Day 0b data are from 17 patients analyzed independent of the stability experiment and is included for comparison.

the AUC for both the CD63 and CD63/anti-IgE results. The CD63 AUC results showed no significant difference between Day 0 and Day 1 but a significant decrease between Day 0 and Day 2. The R^2 correlation and Bland–Altman analysis showed very similar results between Day 0 versus Day 0b and Day 0 versus Day 1 with respect to both bias, SD and R^2. Day 0 versus Day 2 showed an increased bias and SD as well as a decreased R^2 (Table 3 and Supplemental Figure S2). The CD63/IgE AUC showed similar trends but was not significant decreased at Day 2 (Table 3 and Supplemental Figure S2).

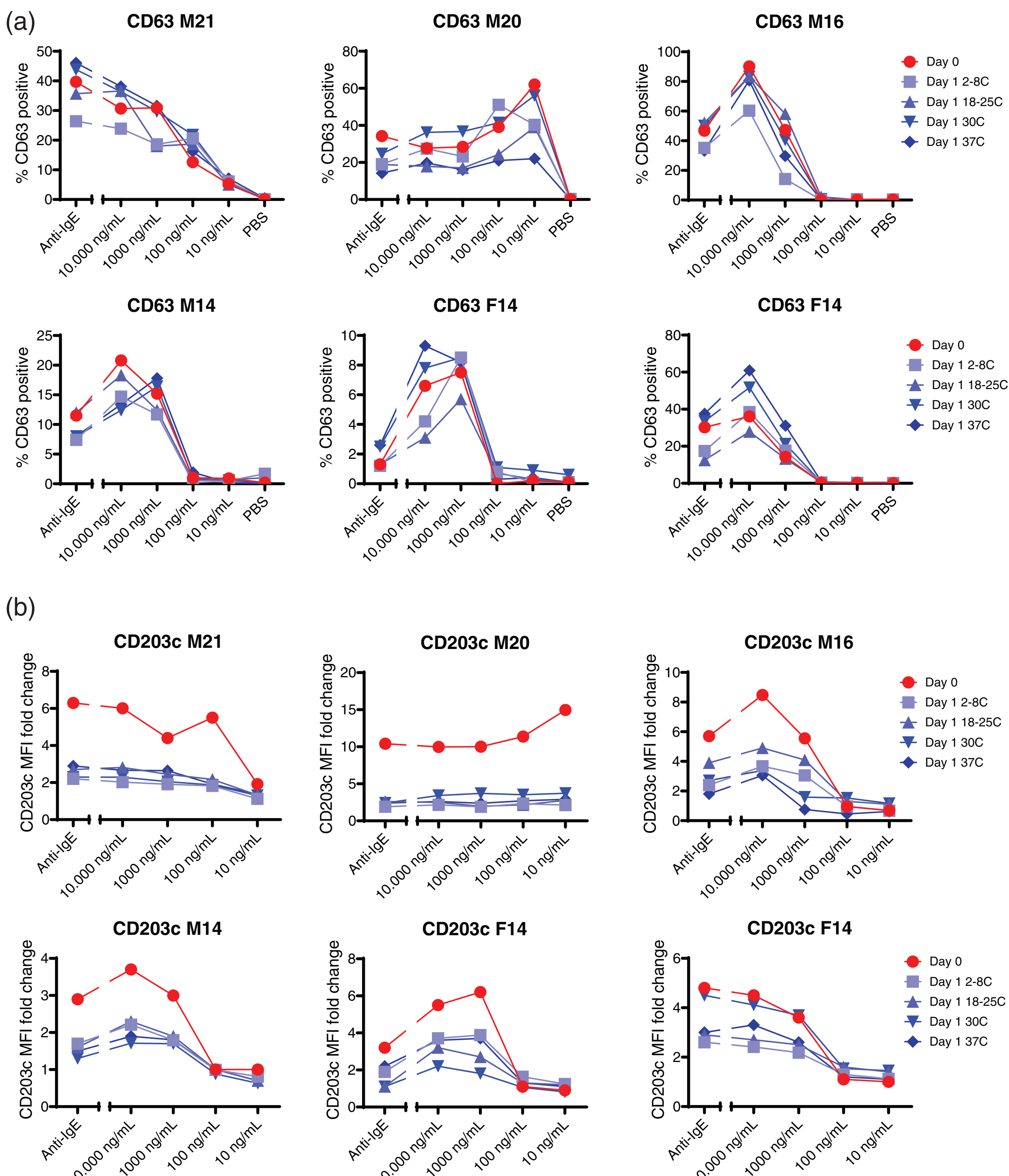

FIGURE 3 Temperature stability. Blood collected from six peanut allergic patients was stored under temperature-controlled conditions for 22–26 h at 2–8, 18–25, 30, and 37°C before stimulated with PBS, Anti-IgE and peanut allergen concentrations as indicated in the Figure (a) sample from the same patient that was stimulated within 4 hours of collection is included as Day 0. The expression of (a) CD63, measured as percentage of positive basophils, and (b) CD203c, measured as fold change in median fluorescent intensity (MFI) of the basophil population, was determined by flow cytometry [Color figure can be viewed at wileyonlinelibrary.com]

3.5 | Temperature stability and shipment of samples

To better understand the effect of temperature on basophil stability we stored bloods samples at 2–8, 18–25, 30, and 37°C for 22–26 h and compared the results to a sample assayed within the first 4 h post collection. The results showed a very high degree of CD63 stability for all the tested temperatures in that even blood stored at 2–8 and 37°C was still able to respond fully to stimulation and showed a response similar to what was observed within 4 h post collection. The CD203c MFI ratio was substantially lower at Day 1 with the four temperature conditions being similar (Figure 3).

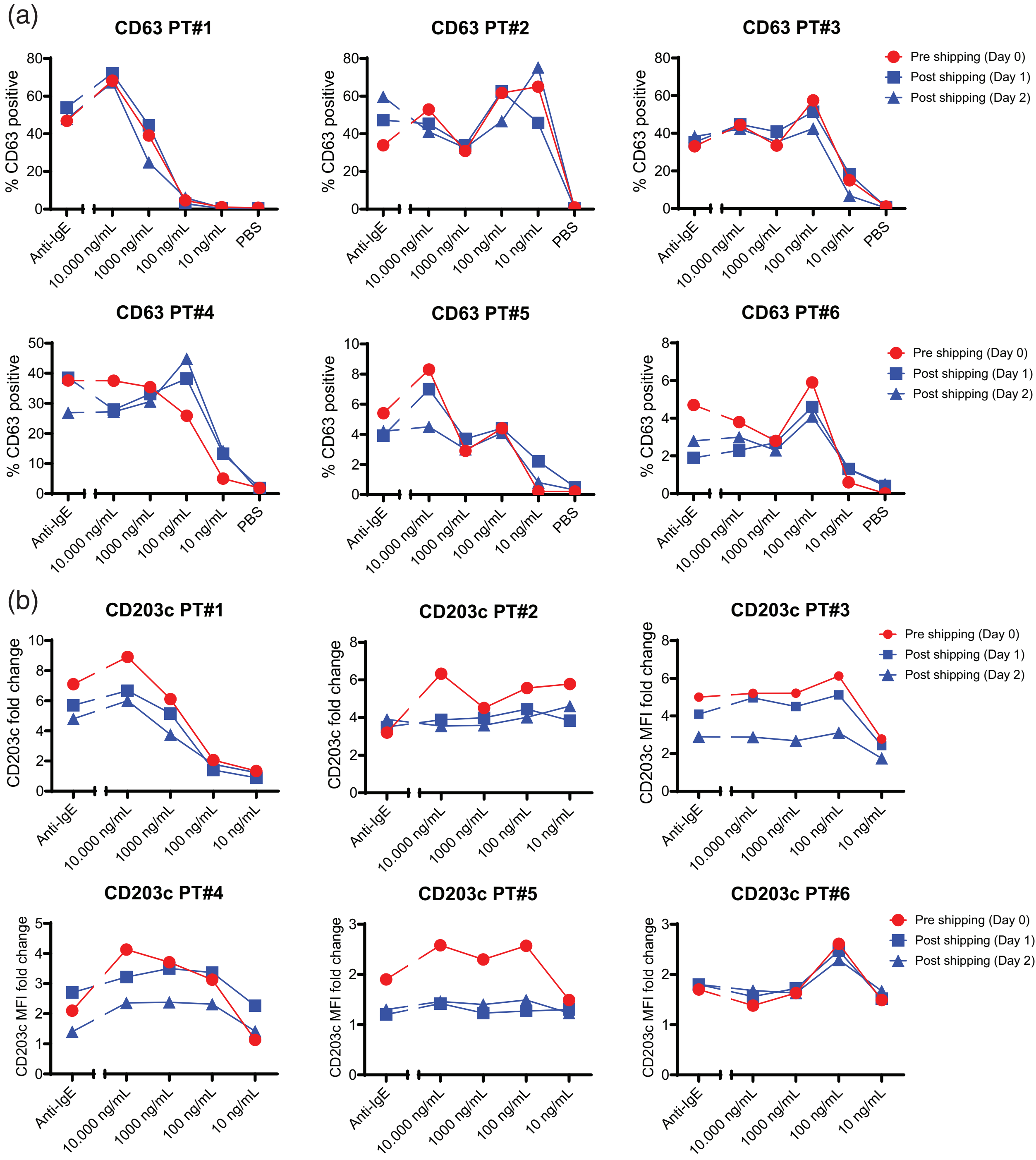

FIGURE 4 Stability of shipped samples. Blood collected from six peanut allergic patients was stimulated with PBS, Anti-IgE and peanut allergen concentrations as indicated in the figure. The expression of (a) CD63, measured as percentage of positive basophils, and (b) CD203c, measured as fold change in median fluorescent intensity (MFI) of the basophil population, was determined by flow cytometry at an out of state collection site validated to run the basophil test (Day 0). The samples were then shipped overnight to our central laboratory at ambient conditions where the basophil responses were measured on the day the samples arrived (Day 1) and the following day (Day 2) [Color figure can be viewed at wileyonlinelibrary.com]

Next, we wanted to assess the impact on the blood samples of shipping where the sample will be transported to at least one central facility and a local facility before being delivered the following day. During shipping, samples will be subject to changes in temperature as well as potentially other stress factors such as radiation and shaking. We will be referring to that as ambient conditions. We chose multiple geographic locations over four time zones in the United States to ship blood from peanut allergic patients for assaying basophil response at a central location. One site performed the basophil phenotyping on the Day 0 before shipping the sample. The results showed that the CD63 and CD203c dose response curves from the two sites equipped to perform flow cytometric evaluation of basophils are highly reproducible and only exhibit minor changes from Day 0 to Day 2 (Figure 4). For shipped samples where it was not possible to do the assay at Day 0 we compared to the averages of non-shipped samples from Supplemental Figure S1. We did not observe any background activation of the negative control in these samples nor significantly lower results in the positive control. The assay results showed similar patterns to the corresponding days of the samples that were not shipped (Supplementary Figure S3).

4 | DISCUSSION

The difficulties in accessing a validated, commercially available, functional basophil assay and the mistaken belief that blood samples need to be processed very quickly for an accurate result have been major barriers to the widespread use basophil activation testing in the United States and elsewhere. In this study we show that the post-collection stability of basophils can be extended to 1 day at room temperature, defined as 18–25°C without compromising the quality of the results. We took the study a step further and showed that samples shipped overnight at ambient conditions give stable results as long as the temperature is within 2–37°C, as can be monitored by a simple temperature strip.

The stability assessment is closely linked to how the results are reported. Reporting basophil activation test results as a single value that captures all the information from the assay would be simple, but such readouts would have to be based on an ideal bell shaped dose response curve (MacGlashan Jr, 2013; Santos et al., 2015). Our results show that although the shape of dose response curves is well preserved, most are not bell shaped or near perfect dose response curve. AUC does not require a specific curve shape but a very strong response to the two highest peanut concentrations and no response to the lowest two results in a higher AUC value than a low stable response to all four peanut concentrations. Therefore, using the AUC can result in the loss of important information that may have implications for interpreting the results.

We recommend against reporting basophil activation test results that attempt to capture all the information in a single number.

The values for %CD63 positive basophils show no significant change between analysis on Day 0 and Day 1 and but a significant decrease in CD63 positive basophils is seen on Day 2. This is confirmed by the Bland–Altman graphs. A careful study of the datasets shows the difference between Day 0 and Day 1 to be random in nature rather than reflecting a systematic bias in that the Day 1 bias is close to 0. For comparison purposes, we included data from two assays performed 2–4 h apart on the same day. The results show similar values with regards to bias, SD, and R^2 on Day 0 and Day 1. Although the optimal result would be if the values were identical or almost identical on different days, but as seen in the Bland–Altman plots, there are a few instances where the %CD63 positive cells changes substantially (for two patients more than 40%). A carefully study of these patients shows that there is a general decrease in the signal on Day 1 but that the semi-quantitative result is the same and the shape of the dose response curve is similar. Finally, the anti-IgE response is also lower, making the CD63/Anti-IgE ratio a more stable readout than %CD63 alone. This emphasizes the importance for the clinician of utilizing all the information provided by the laboratory.

The observed fluctuations in CD63 are similar to other, but not often cited reports on CD63 in basophils as well as ADP activated platelets, suggesting that active vesicle trafficking can induce a baseline "noise" in the CD63 expression which can explain the differences in results observed at Day 0 and Day 1 (Huskens et al., 2018; Mukai et al., 2017; Sainte-Laudy et al., 1998). The significant decrease observed at Day 2 is most likely due to basophil cell death.

The results for CD203c are significantly lower on Day 1 and Day 2 due to an increase in CD203c background expression rather than a lower fold upregulation resulting in an apparent lower response to stimulation confirming previous observations (Ebo et al., 2008). We conclude that CD203c is not as stable a marker as CD63 and it should not be used alone more than 4 h post collection.

The semi-quantitative results are very reproducible on Day 1 compared to Day 0 especially for CD63 but also for CD203c. While this approach is good for screening purposes, most clinical questions require a more comprehensive answer.

Our recommendations for reporting the results are that the semi-quantitative results, the trajectory of the dose response curve, the CD63/anti-IgE ratio, the percentage of CD63 positive cells, and the CD203c MFI ratios should all be made available to the clinician accompanied by a suggested interpretation provided by the laboratory. Such an interpretation would be similar to a pathologist's report on the histology of a surgical specimen.

Shipping blood across the country to a central laboratory can expose the sample to harsh conditions. The most obvious are extreme temperatures but also radiation and prolonged vigorous shaking that can potentially affect the outcome of the test. One option to overcome this problem is tight control of shipping conditions which would not be economically viable. Our investigation of temperature stability showed that samples stored at 2–8 and 37°C for 22–26 h provide very similar results. This is contrary to the currently accepted dogma in the field that basophils in a blood sample are extremely unstable and temperature sensitive. The results from our shipping experiments using ambient conditions showed a very high degree of reproducibility. Based on these observations, it is far better to ship samples under ambient conditions

and include a temperature strip to monitor if the min/max temperatures are within an acceptable range. Choosing the right controls, including a PBS control to measure background activation and an anti-IgE control to verify functional signaling through the IgE/IgE receptor pathway are essential for the interpretation of the results. Approximately 10% of all patients are IgE/IgE receptor pathway non-responders (Puan et al., 2017). Therefore, to distinguish non-responder samples from non-viable samples we suggest adding an extra tube stimulated with fMLP (f-Met-Leu-Phen), an IgE independent basophil activator, as an additional positive control (Ebo & Bridts, 2009; Knol et al., 1990).

The two most often used arguments against using the BAT are that it does not provide an answer for the 10% of the patients that are non-responders and the limited post collection stability. Other diagnostic options such as skin testing and sIgE are limited by frequent false positive results. Recently reported approaches in which IgE from patient serum is allowed to bind to a mast cell or basophil cell line or stripped using primary basophils avoids the stability and non-responder problems of the BAT, but these tests come with their own challenges such as difficulties in establishing and validating relevant controls for the cell line response. Furthermore, important information about the magnitude of the patient's own basophil response to both anti-IgE and allergen which can be strong indicators of treatment outcomes are lost. In a recent study of peanut oral food immunotherapy, basophil non/low responders were found to have better clinical outcomes compared to those with a strong basophil response to both the peanut allergen and positive control anti-IgE (Alpan, Layhadi, et al., 2020; Chinthrajah et al., 2019). The non-responder basophils are also referred to as anergic basophils (Puan et al., 2017). It has been postulated that this anergic state can be a protective response toward severe anaphylactic reactions. Further clinical correlations, especially in the food allergies, are necessary. Finally, information derived from other cells, such as IgG4 cells, that can affect the allergic reaction will be lost when not stimulating whole blood from the patient.

This study not only refutes the notion that whole blood samples used for basophil assays are unstable after a few hours, but also brings together 17 private practices across the United States to develop a network we call AmeriBAT, following the European counterpart, EuroBAT. The mission of AmeriBAT is centered around clinical collaborations, assay development to improve diagnostics of food allergies, proficiency testing and quality control network among in-office flow cytometry laboratories. In the United States, the landscape of diagnostic flow cytometry is changing with increased access to flow cytometry in the clinical care of patients (Alpan et al., 2019). The physical proximity of the flow cytometry laboratories to the clinicians is facilitating discussions around the use of flow cytometry in clinical care, as well as setting the stage for translational discoveries. Improvements in sample stability, especially for functional (input/output) assays, will broaden the spectrum of flow-cytometric testing. Even though there will be variations in assay design among laboratories, which is in the spirit of laboratory developed testing, real-world collection of these data along with clinical correlations will have an invaluable impact on our understanding of food allergies helping to improve food allergy therapies.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potentially conflicts of interest.

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SUPPORTING INFORMATION

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

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