Re-visiting the stability of inducible biomarkers on basophils

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Upregulation of basophil surface CD63 and CD203c on exposure to allergens was first shown almost two decades ago [1]. The strong correlation between these inducible biomarkers with

outcomes of food allergen challenge is proving to be a valuable clinical tool [2, 3]. Requirements for fresh whole blood and reports on the time sensitivity and stability of whole blood in the assay have limited its clinical use [4].

There are several reasons why the whole blood sample stability for the basophil assay question is important, hence the reason for our re-visit. First, even if a flow cytometry laboratory is very accessible to a clinic, samples generally arrive to the laboratory at different times and the handling and processing will require batching. This delay processing of each sample immediately after a phlebotomy. Second, the type of preservatives that are used to collect blood samples can impact stability of the blood sample [5, 6]. Third the emergence of new treatment modalities, such as oral food immunotherapy (OIT) as well as newly formed national organizations such as Food Allergy Support Team (FAST) and Global Food Therapy (GFT) have increased the demand for evaluating and monitoring food allergy patients. Fourth, with the increasing number of Clinical Laboratory Improvement Amendments (CLIA) / College of American Pathologists CAP) accredited clinical flow cytometry laboratories as extensions of allergy/immunology clinics in the United States, real world data collection of biomarker data involving basophil-based assays would not be possible without resolving the stability question[7].

In our study, we compared basophil surface expression of CD63 and CD203c in response to peanut allergen stimulation in blood collected in heparin tubes and stored at room temperature 0-4 hours (Day 0), 20-28 hours (Day 1) and 44-52 hours (Day 2) post collection. We chose heparin tubes over EDTA because basophils did not show good stability in the later ([6] and data not shown). We evaluated 22 peanut allergic patients, age ranging from 4-32 years old, half of whom were being treated with OIT. All patients were consented before blood sample collection (Protocol NCT01981785). To be comparable with prior published data, we chose peanut concentrations ranging from 20 ng/ml to 20,000 ng/ml when incubating whole blood samples [2, 8]. For gating, we used a two-way approach allowing the capture of a high percentages of the basophils and at the same time ensuring a clean population (Supplemental Figure E1).

The results show that basophils, incubated with peanut allergen or anti-IgE antibody, upregulate CD63 and CD203c at all timepoints. The percentage CD63 positive basophils varies at the different timepoints but the pattern of response, either a bell shaped or a positive dose response curve, remains constant (Figure 1). We found no statistical difference between Day 0 and Day1 in the percentage of CD63 induced on basophils by peanut or anti-IgE. Changes in CD203c, however, were statistically lower on Day1 and Day2, compared to Day 0.
Even though there is no change in CD63 from Day 0 compared to Day 1, the results in figure 1 and supplemental table E2 (one with all the patient data) show substantial changes in the percentages of CD63 positive basophils without a clear pattern. To exclude that it is a time dependent effect, we assayed CD63 expression in the same sample twice on Day 0 three hours apart and observed CD63 patterns identical to what is observed at Day 0/ Day 1 (supplemental Figure E3). This agrees with already published, but not often cited literature on CD63 in basophils as well as ADP activated platelets, suggesting that active vesicle trafficking can induce

a baseline "noise" in the CD63 expression [1, 9].

Next, we wanted to examine if the individual patients' positive or negative response would change over time. For that purpose, we defined a positive CD63 response as when the CD63 positive basophils are above 1% and above two times the value for the negative control. CD203c expression at least 1.1 times the MFI of the unstimulated control. Since the focus of this study is assay reliability, we did not perform any correlations to calculate cut-offs for clinical utility. For CD63 we observed a discrepancy in one allergen concentration for one patient between Day 0 and Day 1. For CD203c there were discrepancies in one allergen concentration for seven patients. No patient had more than one discrepancy for any marker and the discrepancies 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). Basophil surface CD63 is generally considered to be a better marker compared to CD203c due to its correlation with histamine release and the ease of gating on the positive population. Basophil surface CD203c is reported as change in MFI compared to the unstimulated control and can be impacted by background activation [8, 10, 11].

The samples assayed at different timepoints were stored at room temperature within the laboratory. To assess the impact of shipping on blood samples from peanut allergic individuals, we chose multiple geographic locations over 4 time zones in the United States and assayed the ability of anti-IgE and peanut allergen to induce CD63 and CD203c. Even though we didn't have a Day 0 for these samples, the results are statistically no different than Day 1 and Day 2 for non-shipped samples (Supplemental Figure E4).

Our experiments show allergen induced biomarkers on basophils can be successfully assayed and analyzed the day after the blood is collected in a heparin tube even after overnight shipment. The results for Day 2 show similar trends but can be problematic for individual laboratories to validate this timepoint due to variability?. The results are more consistent for CD63 than CD203c, but we recommend using both markers as they are supportive of each other allowing the clinical utilization of the assay with more confidence.

In the United States, the landscape of diagnostic flow cytometry is changing with increased access to flow cytometry [7] Improvements in sample stability, especially for functional (input/output) assays, will broaden the spectrum of diagnostic testing. Even though there will be variations in assay design among the in-office and reference laboratories, which is in the spirit of laboratory developed tests, real-world data collection of what comes out of these laboratories along with clinical correlations will have invaluable impact on our understanding of food allergies and how to improve food allergy therapies.

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Figure 1:

Figure 1: CD63 and CD203c expression on the surface of basophils.
Whole blood was stimulated with peanut allergen concentrations as indicated in the figure at Day 0, Day 1 and Day 2 after blood collection. The expression of CD63, measured as percentages positive basophils, and CD203c, measured as fold change in MFI of the basophil population, was determined by flow cytometry. * $p<0.05$, ** $p<0.01$ student's t-test (paired).

Table 1

		IgE / kU/L				Response at: day 0 / day 1 / day 2			
Sex/Age	OIT	Total	Peanut	ARAH2	Marker	20000 ng/ml	2000 ng/ml	200 ng/ml	20 ng/ml
F4	No	193	1.71	1.23	CD63	+/+/+	+/+/+	+/+/+	-/-/-
					CD203c	+/+/+	+/+/+	+/+/+	-/-/+
F13	No	309	0.45	0.3	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	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		5.54		CD63	+/+/+	+/+/+	+/+/+	-/-/-
					CD203c	+/+/+	+/+/+	+/+/+	+/-/-
M20	No	45			CD63	+/+/+	+/+/-	-/-/-	-/-/-
					CD203c	+/+/-	+/+/-	-/-/-	-/-/-

F32	No				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	+/+/+	+/+/+	+/+/+	-/-/-
F3	No	309	1.25	1.17	CD63	+/+/+	+/+/-	+/+/+	+/-/-
					CD203c	+/+/+	+/+/+	+/+/+	+/-/-
F9	Yes	723	12.8	13.7	CD63	+/+/+	+/+/+	-/-/-	-/-/-
					CD203c	+/+/+	+/+/+	-/-/-	-/-/-
M8	No	956	>100	>100	CD63	+/+/+	+/+/+	+/+/+	+/+/+
					CD203c	+/+/+	+/+/+	+/+/+	+/+/+
M5	No	48	0	0	CD63	+/+/+	+/+/-	-/-/-	-/-/-
					CD203c	+/+/+	+/+/-	-/-/-	-/-/-

Table 1 shows the age, sex, OIT information and IgE levels (overall IgE, peanut specific IgE and Arah2 IgE) together with the individual patient's positive and negative to the different concentrations of peanut allergen based on CD63 and CD230c.

Supplemental Figure 1:

Initially a generous FCS/SSC, singlet (FCA-A/ FCS-H) (not shown) and CD45/SSC are applied. This is followed by gating on CD123/CD193/IgE positive basophils. In patients with high level of circulating IgE only CD123 and CD193 are used to identify basophils. Basophil activation is determined by measuring percentages of CD63 positive cells as well as the fold change in CD203c MFI compared to the negative control.

Supplemental data 2

Sex, age	M10			M20			F5			F14			M6			M5			M7		
Days post collection	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
% CD63																					
negative	0.1	0.6	0.8	0.4	0.4	0.8	0.1	0.3	0.7	0.0	0.3	0.5	0.1	0.4	0.4	0.1	0.3	1.4	0.1	0.2	0.7
positive	46.4	45.1	24.8	42.9	49.2	38.3	11.0	8.8	4.4	11.9	20.2	4.0	32.8	58.6	72.5	9.3	5.9	3.5	15.2	10.8	5.0
peanut 1	83.0	70.9	42.4	10.6	8.7	6.5	8.0	6.5	2.9	21.2	25.4	2.3	28.1	50.7	65.8	6.1	5.0	5.1	7.4	5.8	3.4

peanut 2	42.0	10.2	6.4	0.7	1.9	1.9	13.5	19.1	7.1	32.8	53.5	4.8	21.0	62.8	64.5	8.0	6.6	2.9	19.2	13.1	4.3
peanut 3	23.1	3.6	3.4	0.1	1.4	0.9	20.7	17.3	1.6	26.5	46.0	1.1	9.3	35.2	4.0	13.1	7.8	3.6	17.8	10.3	2.9
peanut 4	0.7	0.3	0.6	0.1	0.3	1.4	0.7	0.5	0.3	0.9	1.2	0.2	0.1	0.3	0.4	0.3	0.8	0.3	0.3	0.5	0.2
Fold CD203c change																					
positive	3.7	3.5	2.0	11.6	5.9	4.7	3.3	2.8	1.9	3.0	2.7	2.4	3.6	3.5	4.9	3.3	3.5	1.6	4.0	3.9	2.3
peanut 1	5.4	4.0	3.0	2.8	1.7	1.1	2.1	2.3	1.4	2.9	2.4	1.4	3.6	3.4	5.1	3.5	3.4	1.6	4.1	4.1	2.4
peanut 2	3.1	2.0	1.6	1.5	1.2	0.9	3.1	2.7	2.0	3.5	2.8	2.0	3.4	3.9	5.3	3.3	3.9	1.9	5.0	4.7	2.8
peanut 3	2.7	1.4	1.2	1.0	1.0	0.9	4.4	3.1	1.8	3.4	2.6	1.8	2.4	2.6	1.1	4.4	4.1	2.0	5.9	4.4	3.2
peanut 4	1.5	1.0	1.0	0.9	0.9	0.9	1.1	1.0	1.0	1.3	1.4	1.1	0.9	1.1	1.1	1.0	1.1	0.7	1.2	1.3	0.9

OIT	No	No	Yes - on 20.5mg of PP	Yes- on 6mg of PP	Yes - on 8.2mg of PP	Yes - on 8.2mg of PP	Yes - on 8.2mg of PP
Total IgE kU/L	N/A	45	N/A	1561	8096	314	1290
Peanut specific IgE level kU/L	5.5	N/A	67	>100	>100	52.3	>100
Peanut component IgE level	N/A	N/A	Arah2 =65	Arah2 >100	Arah2 >100	Arah2 41.6	Arah2 99.1
Clinical history (reaction type: vomit, hives ect)			Hives, itchy eyes, wheezing, nasal congestion	Hives, itchy tongue, runny nose, facial angioedema, vomiting,	History of AD. Pn positive by test. Never has Peanuts	Never had peanuts. Positive by test. Sibling ith PN allergy	Hives only

Supplemental Data 3: Two different times the same day.

CD63 and CD203c expression on the surface of basophils.
Day 0 whole blood was stimulated with peanut allergen concentrations as indicated in the figure

at two different timepoints. The expression of CD63, measured as percentages positive basophils, and CD203c, measured as fold change in MFI of the basophil population, was determined by flow cytometry.

Supplemental Figure 4:

Whole blood collected and stored in the laboratory were compared with whole blood collected at various locations and shipped overnight. The blood was stimulated with peanut allergen concentrations as indicated in the figure at Day 0, Day 1 and Day 2 after blood collection. The expression of CD63, measured as percentages positive basophils, and CD203c, measured as fold change in MFI of the basophil population, was determined by flow cytometry.

Supplemental methods

Basophil phenotyping
Whole blood collected in heparine tubes were mixed with the relevant concentrations of peanut allergen (Stallergenes Greer, Cambridge, MA). Anti-IgE (BD Bioscience, San Jose, CA) stimulation was used as positive control whereas unstimulated blood functioned as negative control. The samples were incubated for 20 min at 37c followed by 10 min at 4c. Each sample were stained with the following antibodies anti-CD63-PE, anti-CD203c-PECY7, anti-CD45-AF700, anti-IgE-FITC, anti-CD123-PerCPCy5.5 and anti-CD193-APC (Thermo Fisher, Waltham, MA) for 30 minutes at 4c. The red blood cells were lysed using BD FACS lysis solutions (BD Bioscience, San Jose, CA).

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 instrument has been CAP and CLIA validated for clinical diagnostic studies.

Data analysis: Data analysis was performed using FCS Express software (De Novo software, Glendale, CA). Graphs were generated as scatter plots, and statistical analysis was performed using GraphPad Prism showing mean ± SD. 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