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Towards standardizing basophil identification by flow cytometry

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Background: Basophils normally make up <2% of the white blood cells (WBC). There is no clear consensus for basophil identification by flow cytometry. The increased demand for basophil activation test (BAT) to identifying and monitoring allergic patients highlights the need for a standardized approach to identify basophils.

Methods: Using flow cytometry we analyzed whole blood stained with antibodies against: IgE, CD123, CD193, CD203c, CD3, HLADR, FcεRI, CRTH2 and CD45. We examined unstimulated blood as well as blood stimulated with Anti-IgE and fMLP. Finally, we compared the results to a complete blood count (CBC) from an FDA approved hematological analyzer.

Results: Basophil identification relying on just one surface marker performed worse than approaches utilizing two identification markers. The percentage of basophils from WBC determined by flow cytometry results had a good correlation with the CBC results even though the CBC results were generally higher. Stimulating whole blood with the basophil activators did not interfere with the basophil identification markers.

Conclusion: In flow cytometry assays, two surface markers should be used for identifying basophils and if a very pure basophil fraction is desired a third marker can be considered. In our hands the approaches that included CD123 in combination with either CD193, HLADR^{negative} or FcεRI performed the best.

KEYWORDS

basophil activating test (BAT), basophil identification markers, basophil activation test, CBC (complete blood count), flow cytometry

Introduction

The basophils make up <2% of the white blood cells (WBC) in healthy individuals. Historically basophils have not been given much attention. The rapid development in flow cytometry combined with the discovery that CD63 is translocated to the basophil surface after activation lead to the development of the flow cytometry-based basophil activation test (BAT) and has revitalized the interest in basophils (1–5). The BAT is increasing in popularity because it offers a safe alternative to the oral food challenge when diagnosing food allergy (6). Measuring CD63 by flow cytometry has been established as the best marker for basophil activation in the BAT assay. Basophil identification is done with a variety of markers and no clear standard has been established. It is possible to identify basophils using only one surface marker combined with FSC/SSC but these methods do not always give the best result (7–13). Many clinical and research laboratories use a combination of markers to identify basophils including but not limited to: CD123⁺/CD193⁺, CD123⁺/HLADR⁻, CD3⁻/CD193⁺, CD3⁻/CRTH2⁺, CD193⁺/CD203c⁺ (3, 6, 8, 12, 14–16). In the BAT, basophils go through various stimuli such as anti-IgE and N-Formylmethionyl-leucyl-phenylalanine (fMLP) which are also considered as positive controls for this test, as well as different allergens that are being tested. These stimulations have been reported to affect the expression of several basophil identification markers including CD203c (increased expression), CD123 and CD193 (reduced

expression) (16–20). Furthermore, it might not always be possible to process a sample immediately which makes it critical that the identification markers that are chosen to be used in this test are stable *in vitro*. In this study we stain whole blood with an antibody cocktail containing all the above-mentioned antibodies and analyze them by flow cytometry. The aim of this study is to compare the different methods of identifying basophils by flow cytometry. We compare the impact of different gating methods. Furthermore, we compare the expression of the markers after activation with anti-IgE antibody and fMLP. We assessed the post collection stability of the identification markers. Finally, we compare the result for % basophils of WBC with the result of a CBC test obtained using an FDA approved hematology analyzer.

Methods

Donors

All clinical investigations were conducted according to Declaration of Helsinki principles. All human studies were approved by the Western Institutional Review Board (IRB 1285028). A total of 79 donors, age range 5 to 87, were utilized for the study. The donors were recruited from patients and employees at Amerimmune LLC, CBC was done by Quest Diagnostics using the FDA approved Sysmex XN11 automated hematology analyzer (Kobe, Japan).

Basophil phenotyping

Whole blood was collected in a heparin and an EDTA tube. The EDTA tube was used for external CBC analysis. The heparin tube was kept at 18–25°C. Basophil identification was done using unstimulated blood (PBS) as well as blood stimulated with either Anti-IgE-FITC (Thermo Fisher, Waltham, MA) or fMLP (Sigma, St. Louis, MO). The samples were incubated for 20 min at 37°C followed by 10 min at 4°C (4, 14, 21–24). Each sample was stained with the following antibodies anti-IgE-FITC (Clone Ige21), anti-CD193-PE (Clone 5E8), anti-CD123-PerCPCy5.5 (Clone 6H6), anti-CD203c-PECY7 (Clone NP4D6), anti-CRTH2-APC (Clone BM16), anti-CD3-AF700 (Clone UCHT1), anti-CD45-EF506 (Clone HI30) anti-FcεRI-SB600 (Clone AER-37) (all Thermo Fisher, Waltham, MA) and anti-HLADR-Pacific blue (L243) (Biolegend, San Diego, CA) for 30 min at 4°C. Each antibody was titrated to obtain the best separation (25). The red blood cells were lysed using BD FACS lysis solutions (BD Bioscience, San Jose, CA) and resuspended in 400 μl PBS before acquisition.

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.

All samples were acquired for 5 min at the highest acquisition speed setting.

Data analysis

Data analysis was performed using FCS Express software (De Novo software, Glendale, CA). The gating strategy is to gate on singlets using FSC-A/FSC-H plot. A CD45/SSC plot is used to identify WBC. This is followed by an FSC/SSC gate to gate out eosinophils and majority of neutrophils. The basophils are subsequently identified in 13 different plots: CD123/CD193, IgE/FcεRI, HLADR/CD123, CD203c/FcεRI, FcεRI/CD193, CD3/CD294, CD3/CD193, FcεRI/CD193, CD123/FcεRI, IgE/SSC, FcεRI/SSC, CD193/SSC and CD203c/SSC (Figure 1 and Table 1). The % basophil result is the gated population in each of these plots as percentage of the WBC population.

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 (32, 33).

Results

Basophil marker stability

Recent published papers by us and other groups show that the BAT is stable up to 28 h post collection (14, 22). The efficacy of this assays as well as other flow cytometry assays involving basophil identification is dependent on stable expression of the chosen markers on the basophils. Table 1 summarizes the different markers and parameters utilized in the study, and the gating strategy is shown in Figure 1.

We started by testing the stability of the different gating strategies to see if the expression of the markers that help identify basophils would change over time. Whole blood was collected in heparin tubes and the expression of the markers were measured by flow cytometry 0–4 h post collection and again after 22–26 h. The blood was stored at room temperature (18–25°C).

The results show a slight reduction in the % basophils to 89%–92% at Day 1 compared to Day 0. The absolute number of basophils collected dropped to 83%–87% of the value at Day 0. The results are very similar for all the tested gating combination with no method detecting a significant different percentages or absolute number of basophils (Figure 2). Based on this experiment we conclude that we can accurately evaluate the percentages of basophils within 22–26 h post collection.

Frequency of problematic gating

We examined if it was possible to gate on a distinct basophil population with all gating approaches in all the samples. We recorded the instances where the basophil population does not

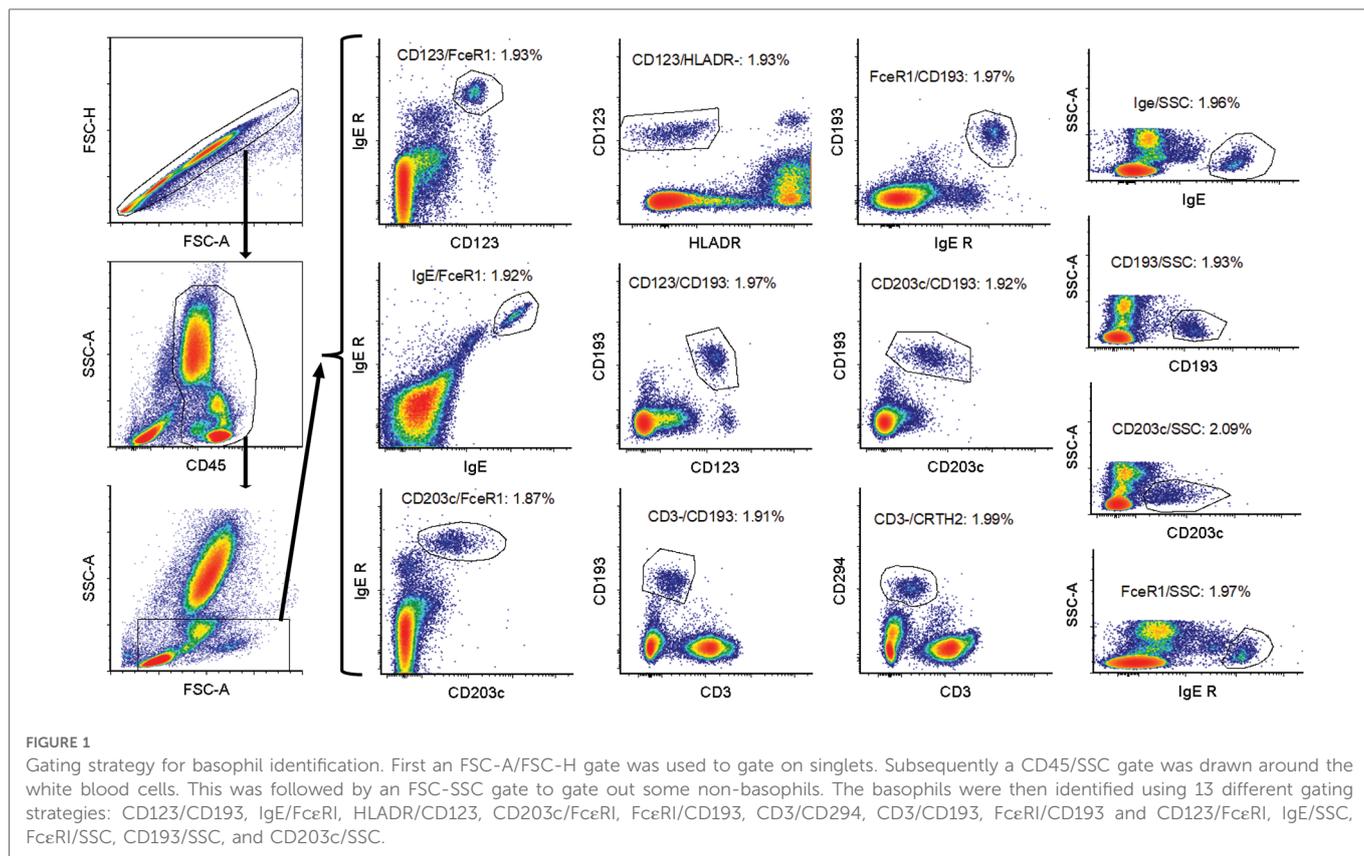


FIGURE 1
Gating strategy for basophil identification. First an FSC-A/FSC-H gate was used to gate on singlets. Subsequently a CD45/SSC gate was drawn around the white blood cells. This was followed by an FSC-SSC gate to gate out some non-basophils. The basophils were then identified using 13 different gating strategies: CD123/CD193, IgE/FcεRI, HLADR/CD123, CD203c/FcεRI, FcεRI/CD193, CD3/CD294, CD3/CD193, FcεRI/CD193 and CD123/FcεRI, IgE/SSC, FcεRI/SSC, CD193/SSC, and CD203c/SSC.

TABLE 1 Basophil identification markers and flow cytometry parameters used to identify basophils in whole blood samples.

	Marker	Cell expression	Description and gating strategy	References
Basophil identification markers expressed on the cell surface	CD123	Basophils, eosinophils, dendritic cells,	Highly expressed on basophils. Is normally used in combination with another basophil marker or HLADR ^{negative} .	(8, 17, 26)
	CD193	Basophils, eosinophils, mast cells, Th2 lymphocytes	Solid marker for basophil identification. Have been used alone, in combination with other basophil markers or CD3 ^{negative} .	(8, 10, 12, 27)
	CD294 (CRT2)	Basophils, eosinophils, T-lymphocytes	Basophils can be differentiated from T-lymphocytes by CD3 and from eosinophils by side scatter	(28, 29)
	IgE	Basophils, monocytes, dendritic cells	Expressed as both a soluble molecule and bound to the FcεRI on basophils.	(9, 13, 30)
	FcεRI	Basophils, mast cells, dendritic cells, monocytes in patients with allergic disorder	Bound to IgE. Crosslinking receptors with a relevant allergen of anti-IgE activates the basophil.	(7, 26)
	CD203c	Basophils, CD34+ progenitor cells, mast cells	Used as both an identification and an activation marker for basophils. Is expressed at low levels on resting basophils.	(31)

form a separate population and the instances where it was impossible to identify a basophil population at all. Our results show that CD123/CD193 and CD123/HLADR⁻ gave a clear separate population in all analysis. CD203c/FcεRI, CD193/FcεRI and CD123/FcεRI worked for all but one donor. In the other end of the spectrum are IgE/SSC, FcεRI/SSC and CD3⁻/CDTH2 which frequently could not detect any basophils or often did not give a clear separate population (Table 2). In the patients where it was impossible to identify basophils at all with a given gating combination then the specific

combination from that patient was removed from the subsequent analysis.

Correlation between methods the different flow-based methods

Next, we used linear correlation to see if the % basophils of WBC results are similar using the different gating approaches. The R

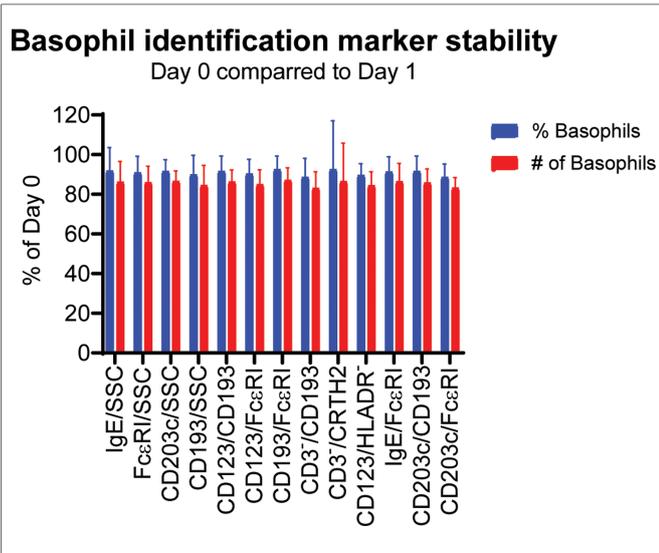


FIGURE 2
Stability of the different basophil identification markers. Whole blood from 9 donors was collected in heparin tubes and the expression of the markers were measured by flow cytometry after 0–4 h (Day 0) and again after 22–26 h (Day 1) using the gating strategy shown in Figure 1. The blood was stored at room temperature (18–25°C). The % basophils of WBC as well as the total number of basophils identified were measured. The results for Day 0 are set to 100% and the results for Day 1 are normalized accordingly. Student’s *t*-test paired, *n* = 9.

TABLE 2 Problematic basophil identification.

Issue	Impossible to gate on basophils			Basophils form a shoulder rather than a detached population		
	PBS	Anti-IgE	fMLP	PBS	Anti-IgE	fMLP
IgE/SSC	10	10	10	0	0	0
FcεRI/SSC	7	14	6	7	8	6
CD203c/SSC	1	1	1	3	2	1
CD193/SSC	1	0	1	3	5	8
CD123/CD193	0	0	0	0	0	0
CD123/FcεRI	0	0	0	0	1	0
CD193/FcεRI	1	0	0	2	7	4
CD3 ⁻ /CD193	2	0	0	5	8	9
CD3 ⁻ /CRTH2	3	3	2	10	11	14
CD123/HLADR ⁻	0	0	0	0	0	0
IgE/FcεRI	1	1	1	5	3	3
CD203c/CD193	1	1	1	2	1	3
CD203c/FcεRI	0	0	0	1	0	1

The table show the numbers of samples where it was not possible to identify a basophil population as well as the number of samples where the identification can be prone to error because the basophils does not form a separate population but rather a shoulder on the negative population.

square value ranges from 0.69 (FcεRI/SSC vs. FcεRI/IgE) to 0.99 (IgE/SSC vs. CD123/CD193; Ige/SSC vs. CD123/HLADR⁻; IgE/SSC vs. IgE/FcεRI; and CD123/CD193 vs. CD123/HLADR⁻). To easier appreciate the performance of each gating strategy we calculated

the average for the R square values for each gating strategy, The average R square ranking are (high to low): IgE/SSC, CD123/CD193, CD123/FcεRI, CD123/HLADR⁻ and CD203c/CD193 > CD203c/FcεRI > IgE/FcεRI > CD203c/SSC, CD193/FcεRI, CD3⁻/CRTH2 > CD193/SSC > CD3⁻/CD193 > FcεRI/SSC. The graphs are all shown in **Supplementary Figure S1**. The values for the R square and the slope are summarized in **Table 3**.

Specificity and inclusiveness of the different gating strategies

An essential question is if the basophils identified by one gating strategy will also be identified as basophils by the other gating strategies. We addressed this by displaying the basophils obtained by one gating strategy in each of the 12 other strategies and recording the percentages of the cells that were then identified as basophils. The individual box plots are shown in **Supplementary Figure S2** and the mean values are shown in **Table 4**. The highest specificity defined as the gating method where most of the identified basophils were also identified as basophils in the 12 other approaches is CD123/HLADR⁻ followed by (high to low, as shown in the bottom row in **Table 4**): CD123/CD193 > IgE/SSC > CD203c/CD193 > CD123/FcεRI > CD193/FcεRI > IgE/FcεRI > CD203c/FcεRI > CD193/SSC > CD3⁻/CD193 > CD3⁻/CRTH2 > CD203c/SSC > FcεRI/SSC. This ranking shows how pure the initial captured basophil population is. The summary column on the far right of the table shown the inclusiveness, defined as how well the different gating options are at capturing all the basophil events with the best being IgE/SSC followed by (high to low): CD123/HLADR⁻ > CD193/FcεRI > CD123/CD193 > CD123/FcεRI > CD3⁻/CD193 > CD193/SSC > CD203c/CD193 > IgE/FcεRI > FcεRI/SSC > CD3⁻/CRTH2 > CD203c/FcεRI > CD203c/SSC.

Comparing flow cytometry results to CBC

A CBC with differential test is a well-established method that among other results provides the percent basophils of whole blood. For each donor we collected a tube for CBC analysis at an external reference laboratory. The CBC were all run within 24 h of sample collection on an FDA approved hematology analyzer. The linear correlation analysis between the CBC and each of the flow methods show some degree of correlation with an R square value between 0.59 to 0.8. The highest being IgE/SSC, CD193/SSC, CD123/CD193 followed by CD123/FcεRI, CD123/HLADR⁻ > IgE/FcεRI, CD203c/FcεRI > CD203c/CD193 > CD193/FcεRI > CD203c/SSC > CD3⁻/CRTH2 > FcεRI/SSC. The sloop is between 0.74–0.84 indicating that the flow values generally are lower than the CBC values. To better understand the difference between the methods we visualized the data in two different Bland-Altman plots, Difference vs. Average and Ratio vs. Average (32, 33). The results for all flow methods except FcεRI/SSC show that the CBC systematically are higher than the flow cytometry results. The most pronounced difference is for CBC results between 0.3%–1.3% basophils but even for the higher values the CBC result is higher. The Ratio vs. Average plot show that the CBC values are generally higher by 40%–50% percentages rather than fixed value. The

TABLE 3 Linear correlation between different flow methods.

		R square													
		IgE/SSC	FcεRI/SSC	CD203c/SSC	CD193/SSC	CD123/CD193	CD123/FcεRI	CD193/FcεRI	CD3 ⁻ /CD193	CD3 ⁻ /CRTH2	CD123/HLADR ⁻	IgE/FcεRI	CD203c/CD193	CD203c/FcεRI	Average R square
Slope	IgE/SSC	0.73		0.94	0.92	0.99	0.98	0.91	0.95	0.92	0.99	0.99	0.96	0.97	0.94
	FcεRI/SSC		0.72	0.72	0.75	0.74	0.79	0.81	0.79	0.78	0.74	0.69	0.79	0.74	0.75
	CD203c/SSC			0.72	0.88	0.94	0.93	0.88	0.91	0.88	0.94	0.90	0.93	0.94	0.90
	CD193/SSC				0.72	0.95	0.93	0.86	0.90	0.85	0.93	0.89	0.92	0.88	0.89
	CD123/CD193					0.98	0.98	0.91	0.95	0.92	0.99	0.95	0.97	0.96	0.94
	CD123/FcεRI					1.01		0.96	0.95	0.94	0.98	0.93	0.97	0.95	0.94
	CD193/FcεRI						0.99		0.91	0.91	0.91	0.89	0.93	0.91	0.90
	CD3 ⁻ /CD193						0.98	0.96	0.96	0.94	0.94	0.95	0.98	0.94	0.85
	CD3 ⁻ /CRTH2						0.95	0.94	0.90	0.95	0.93	0.89	0.93	0.91	0.90
	CD123/HLADR ⁻						0.98	0.94	0.96	0.96	0.98	0.96	0.97	0.96	0.94
	IgE/FcεRI						0.99	0.93	0.93	0.94	0.96	0.96	0.93	0.94	0.91
	CD203c/CD193						0.96	0.96	0.93	0.96	0.96	0.94	0.96	0.96	0.94
CD203c/FcεRI						0.91	0.87	0.89	0.90	0.91	0.91	0.94	0.91	0.92	

A summary of the linear correlation analysis between the different flow-based basophil identification methods shown in Supplementary Figure S1. Light red background are the R square values, and the light blue background is the slope. The average R square values are the average of the 12 R square values where the gating combination on the right is involved.

TABLE 4 Linear correlation between different flow methods.

	Initial_gate												Average (Inclusiveness)				
	IgE/SSC	FcεRI/SSC	CD203c/SSC	CD193/SSC	CD123/CD193	CD123/FcεRI	CD193/FcεRI	CD3 ⁺ /CD193	CD3 ⁺ /CRTH2	CD123/HLADR ⁻	IgE/FcεRI	CD203c/CD193		CD203c/FcεRI			
% Back-gated																	
	88	74	81	88	94	94	93	86	80	96	98	91	91	91	91	91	88.8
	88		75	80	83	86	84	77	72	84	88	81	86	86	86	86	82.0
	82	65		78	81	81	79	74	70	83	79	83	87	87	87	87	78.5
	90	70	78		93	88	91	88	79	91	86	91	85	85	85	85	85.8
	92	71	80	90		92	92	87	81	95	89	92	88	88	88	88	87.4
	94	73	80	86	93		91	84	79	95	92	89	91	91	91	91	87.3
	92	73	78	89	94	92		88	81	92	90	92	90	90	90	90	87.6
	88	69	76	91	93	88	91		83	90	86	94	85	85	85	85	86.2
	81	65	71	80	84	82	82	82		84	80	84	79	79	79	79	79.5
	94	72	81	88	95	94	92	86	81		91	91	90	90	90	90	87.9
	92	73	75	81	86	88	87	80	76	88		84	87	87	87	87	83.1
	86	67	78	87	90	85	88	86	79	88	84		84	84	84	84	83.5
	84	68	78	77	82	84	82	75	71	83	85	84		84	84	84	79.4
Average (Specificity)	88.6	70.0	77.6	84.6	89.0	87.8	87.7	82.8	77.7	89.1	87.3	88.0	86.9	86.9	86.9	86.9	86.9

A summary of the linear correlation analysis between the different flow-based basophil identification methods shown in Supplementary Figure S2. Last row with light red background is the average of the 12 values in the column and is a measurement of how specific the gate is for basophils when compared to the 12 other gating methods. The last blue column is the inclusiveness which represents how big percentages of the basophils fall within the given gate.

TABLE 5 Correlation between the different flow cytometry methods of identifying basophils and the results of a CBC obtained using the XN11 automated hematology analyzer at an accredited reference laboratory.

	Correlation analysis		Bland-Altman analysis (Difference vs. Average)		Bland-Altman analysis (Ratio vs. Average)	
	R square	Sloop	Bias	SD	Bias	SD
IgE/SSC	0.80	0.82	0.14	0.19	1.5	0.8
FcεRI/SSC	0.59	0.84	0.02	0.29	1.2	0.6
CD203c/SSC	0.71	0.74	0.14	0.23	1.4	0.6
CD193/SSC	0.80	0.83	0.13	0.18	1.4	0.6
CD123/CD193	0.80	0.81	0.14	0.18	1.5	0.8
CD123/FcεRI	0.79	0.81	0.13	0.19	1.5	0.8
CD193/FcεRI	0.74	0.80	0.13	0.21	1.5	0.8
CD3 ⁻ /CD193	0.73	0.79	0.11	0.22	1.4	0.8
CD3 ⁻ /CRTH2	0.67	0.74	0.13	0.24	1.4	0.6
CD123/HLADR ⁻	0.79	0.81	0.14	0.19	1.5	0.8
IgE/FcεRI	0.78	0.81	0.15	0.19	1.6	0.9
CD203c/CD193	0.77	0.78	0.15	0.20	1.5	0.9
CD203c/FcεRI	0.78	0.76	0.18	0.19	1.6	0.8

The graphs are shown in [Supplementary Figure S3](#).

results for the FcεRI/SSC gating method the diversion between flow cytometry and CBC seems more of a random nature ([Table 5](#) and [Supplementary Figure S3](#)).

Identification of activated basophils

The BAT involves stimulating whole blood with positive controls that activates the basophils in both a IgE/FcεRI dependent and independent manner. The activation of basophils might affect the expression of the identification markers.

We stimulated whole blood with Anti-IgE-FITC or fMLP before staining for basophil identification and compared it to unstimulated blood (PBS control) to see if stimulation affects our ability to identify basophils.

We observed that fMLP activation decreases CRTH2 expression slightly. Anti-IgE activation reduce the FcεRI signal. The antibody used for stimulation was the same used for detection which result in an increase in IgE signal after Anti-IgE stimulation. Both anti-IgE and fMLP activation increases CD203c expression. The rest of the markers were unaffected by stimulation ([Supplementary Figure S4](#)).

Next, we performed a linear correlation to determine if the percentages of basophil are similar before and after stimulation. The results show an R square above 0.95 for Anti-IgE and fMLP for IgE/SSC, CD123/CD193, CD123/FcεRI, CD193/FcεRI, and CD123/HLADR⁻. The lowest correlation with an R square below 0.9 was seen for FcεRI/SSC, CD193/SSC, CD193/CD3⁻, CD294/CD3⁻ and CD203c/CD193 ([Figure 3A](#)).

Finally, we compared the absolute number of basophils identified to show whether basophil events are gained or lost by stimulating in any of our basophil identification approaches. The results are similar in stimulated and unstimulated samples ([Figure 3B](#)).

Discussion

In this study we examined 13 different gating strategies for basophil identification in a whole blood by flow cytometry. For each gating strategy we looked at (1) Reliability/easiness of gating, (2) Specificity vs. inclusiveness, (3) Correlation between the different approaches, (4) Correlation with CBC results, (5) Stability of the markers, (6) Effect of stimulation.

All the strategies used in this paper to identify basophils utilizing published markers and marker combinations. We started by examining if we get similar results between the different methods. The linear correlation analysis showed the highest correlation between gating strategies utilizing two surface markers unless one of the markers is CD3^{negative} in which case the correlations were low. The single surface markers approaches did not perform well except for IgE/SSC that was among the best. It is worth noting that for 10 of the samples IgE/SSC could not identify any basophils at all. All these patients have elevated levels of circulating IgE that will compete with the surface bound IgE for anti-IgE antibody binding. This approach does not provide us information on the extent the gates captures all the basophils or how basophil specific they are. To understand which strategies are best at capturing the highest percentages of basophils with the least contamination we investigated to what extend a population identified by a given gate would also be identified as basophils using the other gating strategies. The results of this approach are very similar to what we saw in our initial correlation analysis, and it confirms that best gating strategies utilizing two surface markers unless one of the markers is CD3^{negative}. It is important to remember that this form of comparison has a bias towards higher specificity and inclusiveness when the same marker is present in both strategies such as comparing CD123/HLADR^{negative} vs. CD123/CD193 or IgE/SSC vs. IgE/FcεRI.

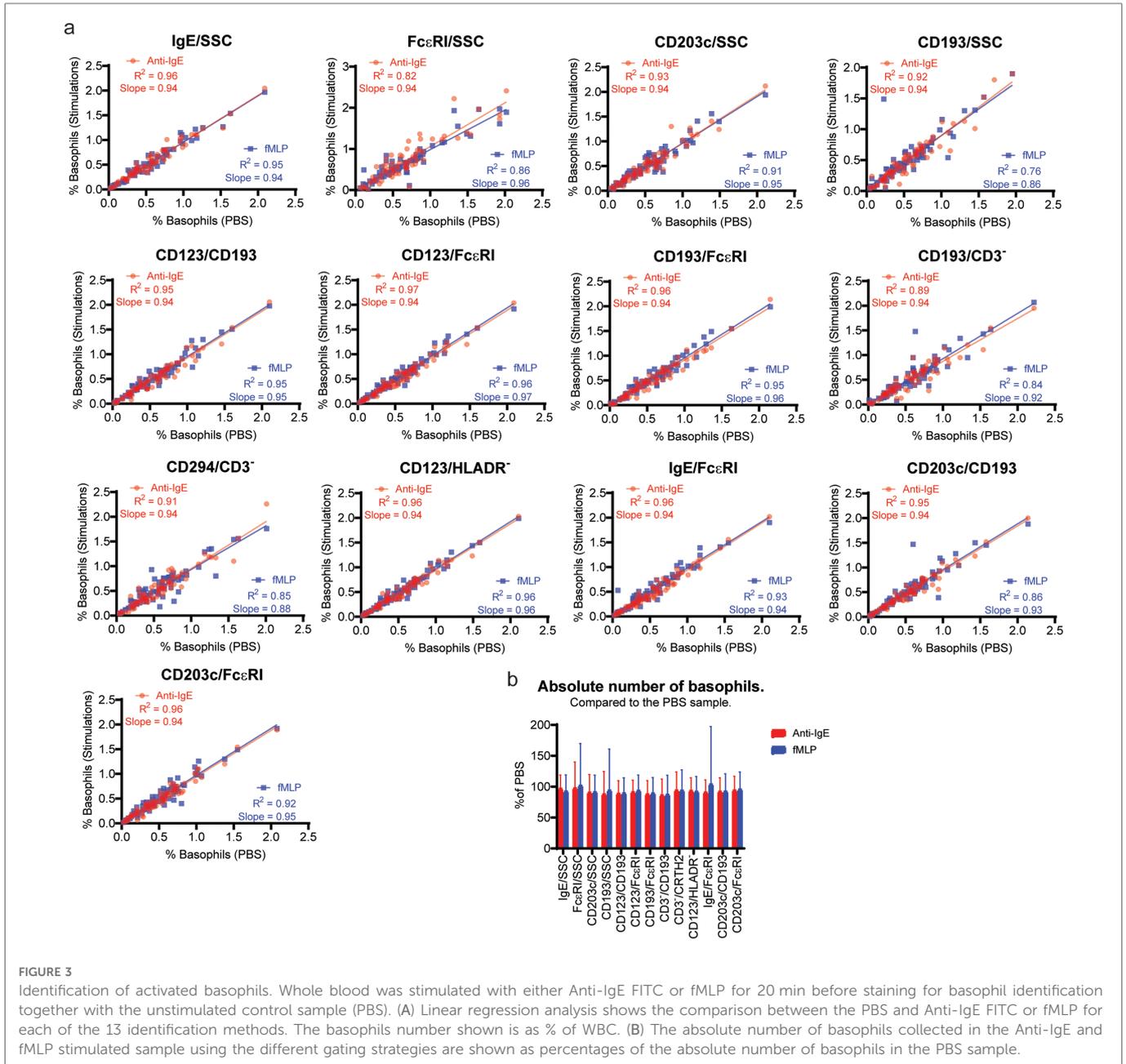


FIGURE 3

Identification of activated basophils. Whole blood was stimulated with either Anti-IgE FITC or fMLP for 20 min before staining for basophil identification together with the unstimulated control sample (PBS). (A) Linear regression analysis shows the comparison between the PBS and Anti-IgE FITC or fMLP for each of the 13 identification methods. The basophils number shown is as % of WBC. (B) The absolute number of basophils collected in the Anti-IgE and fMLP stimulated sample using the different gating strategies are shown as percentages of the absolute number of basophils in the PBS sample.

A selection of hemolytic analyzers is approved by FDA and used at almost all reference laboratories. No flow method for identifying basophils has been FDA approved and we tested to see how well the two approaches compare. Several other studies have compared % basophils from a CBC with flow cytometry and each time the correlation is mediocre with the best R square value at 0.68 (8, 27, 34). The correlation in our experiments has an R square ranging from 0.59 to 0.80 with seven of our gating strategies having an R square of at least 0.75. The slope ranged from 0.74 to 0.84 showing that the flow result was generally lower than the CBC result. This was confirmed by the Bland Altman plot that showed the CBC to be 40%–50% higher than the flow results with the difference being lower as the basophil percentages increase. The fact that basophil count in a CBC can be overestimated rather than underestimated especially for when the

basophil percentages is in or below the normal range has been described previously (34–36).

It has, for the longest time, been a dogma that basophils were unstable cells and that the BAT should be run within 4 h of collecting the sample. Recently studies have shown that the BAT results are stable up to 1 day (20–28 h) post collection (14, 22). Our results show that no matter which gating strategy is used the percentage and absolute number of basophils identified in a 22–26 h old sample is very similar to what can be identified within 4 h post collection. An essential part of the BAT is the stimulation. We did not see any systematic drop in the identified basophils for any of the gating strategies after stimulation with either fMLP or anti-IgE showing that the markers are not shredded or internalized after stimulation to an extent that makes utilization impossible. The gating strategies with the lowest correlation between

stimulated and control samples are also the strategies where there were most instances of difficult to identify basophils due to the population being an attached rather than a separate population (Table 2). The strategies with few or no instances of shoulder population had both R square and sloop values very close to 1.

A recurring dilemma in designing a BAT flow panel is on its simplicity and cost-effectiveness. We included CD45 because it is essential for a good WBC gate. We would recommend including it rather than relying solely on FSC and SSC to narrow in on the basophil population.

We do not recommend relying on just one marker for identifying basophils as those strategies did not perform very well in our study. Three of them were not very precise or accurate. IgE/SSC works very well when it does not completely fail which it did more than 13% of the time. Among the gating strategies utilizing two surface markers CD3⁻/CRTH2 and CD3⁻/CD193 performed the worst with respect to inclusiveness and specificity. The IgE/FcεRI gate has too many instances difficult/impossible to gate. This is typically observed in individuals where the IgE staining does not work. The CD193/FcεRI approach also have some instances of difficult to gate issues, especially after anti-IgE stimulation, even though the specificity and inclusiveness is among the best. Not having a well-defined population can cause variation between technologists analyzing the samples and problems if using automatic gating. CD203c expression is low in resting cells and increase after basophil stimulation. This can cause the gate used for identification to shift between samples. Our identification was mainly done on resting cells and for both CD203c/CD193 and CD203c/FcεRI we saw a high specificity but the inclusiveness was low reflecting that is a problem capturing all the basophils. The remaining three approaches, CD123/CD193; CD123/HLADR^{negative}; and CD123/FcεRI all performs as the best in all our tests, and we recommend choosing one of these. If the flow panel allows for one more parameter, it is possible to combine two methods such as CD123/CD193 and CD123/HLADR^{negative}. All three methods include CD123 as one of the parameters and CD123 expression has been reported to be reduced after stimulation (12, 17). This statement has been rebuked by others (37–39). We did not see any reduction in CD123 after stimulation in our experiments. CD123 gave a clear separation in all our experiments but there might be instances where it is not the case, and it will make gating that includes it impossible. Another approach could be to combine one of the CD123 strategies with IgE/SSC. This gate has a very high inclusiveness and specificity but should be excluded from the analysis when a basophil population cannot be identified. If the flow panel allows for four markers to identify basophils it is possible to combine several of the approaches shown here. The advance of using more than one approach is that the gates can be more inclusive, and the specificity can at the same time be increased.

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Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Western Institutional Review Board (IRB 1285028). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

SUS and OA contributed to project conceptualization. DL and OA coordinated the sample collection and project administration. SS and MP contributed to formal analysis. SU did the experimental work and wrote the original draft. All authors contributed to the article and approved the submitted version.

Conflict of interest

Author SUS, MP and DL are employed by Amerimmune. OA is the owner of Amerimmune. This study was funded by resources internally at Amerimmune. The funder (OA) had the following involvement with the study: Approved study design and the final manuscript. All authors declare no other competing interests.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/falgy.2023.1133378/full#supplementary-material>.

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