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ORIGINAL RESEARCH

Standardizing a Four-Marker Basophil Activation Test: Influence of Storage Time, Temperature, and IgE Levels

WeiZe Li[1,2,*], XuanYue Qu[1,*], LiHui Lin[1], Juan Wang[1], Jia Li[1], Yue Yin[1], YiQin Ge[1,3], JinYan Zhao[1], Yi Liu[4], Li Li[1], Xia Peng[1]

[1]Department of Laboratory Medicine, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, People's Republic of China; [2]Department of Laboratory Medicine, Zhongshan Hospital, Fudan University, Shanghai, People's Republic of China; [3]Department of Laboratory Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, People's Republic of China; [4]Department of Plastic Surgery, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, People's Republic of China

*These authors contributed equally to this work

Correspondence: Xia Peng, Department of Laboratory Medicine, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, No. 100 Haining Road, Hongkou District, Shanghai, 200080, People's Republic of China, Tel +8602163240090, Email pengxiajiao1@163.com; Li Li, Department of Laboratory Medicine, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, People's Republic of China, Email annylish@126.com

Background: Allergic diseases affect 25% of the global population, yet current diagnostics like specific IgE (sIgE) and skin prick tests lack specificity. The basophil activation test (BAT) offers high specificity but requires methodological standardization.

Objective: This study develops gating strategies and pre-analytical storage conditions to develop an analytically validated BAT protocol for *Dermatophagoides farinae* (Df) allergy.

Methods: Seventy-nine adults (Df-sensitized and controls) were enrolled. Basophil activation was assessed by CD63 expression following *Df* extract, anti-IgE, or fMLP stimulation. Evaluations included gating strategies, correlations with serological markers, and storage stability.

Results: The three-marker gating strategy ($CD123^+CD203c^+CD45^+SSC^{low}$) achieved analytical equivalence to a four-marker gating approach with 25% cost reduction, and improved phenotypic purity over a two-marker method (97.2% vs. 91.8% HLA-DR negativity). Df-induced BAT results correlated moderately with *Df* sIgE (r=0.4352) and membrane-bound IgE (mIgE) (r=0.6264–0.6659) (r^2<0.4), leaving >60% of variability unexplained. Quantitatively, CD63 MFI declined significantly with storage. Qualitatively, storage at 2–8°C better preserved *Df* responsiveness up to 48–52 h, while room temperature (RT) was superior for fMLP.

Conclusion: This study demonstrates that a three-marker gating strategy within a four-color BAT panel reduces costs while maintaining analytical equivalence to a four-marker gating reference. Stimulus-specific storage patterns support flexible processing for research applications, and BAT outcomes provide complementary functional information beyond IgE levels. Clinical utility requires prospective validation.

Keywords: Basophil activation test, BAT, allergy diagnosis, storage stability, gating strategy

Introduction

Allergic diseases affect approximately 25% of the population worldwide.[1–3] Current diagnostics, such as skin prick testing (SPT) and allergen-specific IgE (sIgE) detection, lack sufficient specificity and correlate poorly with clinical symptoms.[4] Provocation testing constitutes the gold standard for the definitive diagnosis of food and drug allergies. However, they are rarely performed in clinical practice due to being labor-intensive, costly, and posing potential safety risks to patients.[5,6]

Accumulating studies have demonstrated the important role of basophils in both immediate allergic reactions and chronic inflammation.[7] The basophil activation test (BAT), which measures IgE-mediated allergic reactions in vitro, has gained increasing attention in recent years. Following allergen-specific and non-specific stimulation, basophils rapidly

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degranulate, releasing histamine, leukotrienes, and cytokines. They also upregulate surface activation markers, most commonly CD63 and CD203c, which can be quantified by flow cytometry.[8] This method demonstrates higher specificity in allergy diagnosis.[9–12]

As an in vitro diagnostic method, BAT offers considerable safety advantages by eliminating direct allergen exposure while preserving diagnostic accuracy. BAT is recommended when routine clinical diagnostics (eg., SPT and specific IgE testing) yield ambiguous or clinically discordant results, or when in vivo testing poses unacceptable risks to patients, or when necessary reagents for conventional tests are unavailable.[9] However, its widespread clinical adoption is hindered by two unresolved methodological barriers: (1) the absence of quantitative, cost-benefit evaluated guidance for gating strategy selection, and (2) conflicting, stimulus-specific storage recommendations that remain to be reconciled.

Regarding the first barrier to gating strategy selection, prior studies have compared gating strategies descriptively, without quantifying the incremental specificity gain relative to cost increase. While multi-marker strategies (eg., incorporating CD123, CD203c, CCR3, and HLA-DR) enhance sensitivity and specificity, their high reagent cost and complexity limit clinical feasibility. Simplified approaches using fewer markers risk contaminating the target population with other cell types.[13–16] Evidence-based optimization is needed to identify the minimal marker panel achieving acceptable specificity without prohibitive cost.

Regarding the second barrier for storage conditions, existing studies report inconsistent time-temperature combinations without distinguishing allergen-specific from non-specific responses. Some studies advocate processing within 4 h of collection at RT,[15,17] while others demonstrate 24 h stability at 4°C.[18] Critically, no prior study has compared temperature effects across different stimuli within a unified framework, preventing evidence-based standardization for routine laboratory workflows involving specimen transportation and batch testing.

To address these methodological gaps, house dust mite (HDM) allergy was selected as a clinically relevant model. HDM was chosen because it is globally prevalent, often presents with discordant sIgE and clinical symptoms, and is commercially available in standardized formulations suitable for methodologically rigorous evaluation.[19]

This study addresses the lack of analytically validated, cost-efficient BAT workflows. Our primary objective is operational feasibility: to develop a minimal gating panel with defined cost-efficiency boundaries and unify stimulus-specific storage parameters within a single analytical framework. To achieve this, we conducted three evaluations: (1) quantitative comparison of two-, three-, and four-marker gating strategies to balance analytical specificity against reagent cost; (2) systematic assessment of storage time/temperature effects on basophil responses to allergen-specific (Df) and non-specific (anti-IgE, fMLP) stimuli; and (3) exploratory analysis of correlations between BAT outcomes and serological markers (such as sIgE, total IgE). These findings represent an analytically validated methodological framework for future diagnostic accuracy studies.

Methods

Study Population

Seventy-nine adult patients from the respiratory department were included. Participants were divided into two groups based on symptoms and serological testing. The *Dermatophagoides farinae* (Df) sensitized group: patients with symptoms of allergic rhinitis or asthma and serum Df specific IgE (Df sIgE) ≥0.35 KU/L.[20] Control group: respiratory patients without allergic disease (no allergic symptoms and Df sIgE <0.35 KU/L). Exclusion criteria included: anti-histamine use within 72 hours, systemic corticosteroid treatment within 2 weeks, acute infections, hematological malignancies, pregnancy, or lactation.

Establishment of Basophil Activation Test

BAT was performed according to the following procedure. Fresh venous blood was collected into heparin tubes. Immediately after collection, the tubes were gently inverted 8–10 times, and transported at RT to the laboratory within 30 minutes. Samples were maintained at RT and processed within 6 hours of collection.

For the assay, whole blood was incubated with Df extract *(G*lallergen Co., Ltd) at 37°C for 15–30 minutes. The optimal concentration of 10 μg/mL was selected based on preliminary titration experiments (serial dilutions from 0.01 to 10 μg/mL tested in 19 Df-sensitized patients) and previous literature on HDM-stimulated BAT.[21,22] Anti-IgE antibody (Sigma-

Aldrich, USA) and N-Formyl-Met-Leu-Phe (fMLP; Sigma-Aldrich, USA) stimulation were used as positive controls, while reaction buffer alone served as the negative control.

Following stimulation, samples were stained with fluorescently conjugated antibodies (CD63, CD203c, CD123, and CD45) (Beckman Coulter, USA), subjected to erythrocyte lysis, and acquired by flow cytometry (Navios, Beckman Coulter, USA). Data were analyzed with Kaluza software (Beckman Coulter, USA).

Basophils were identified using three different gating strategies: (1) four-marker strategy: $CD123^{+}CD203c^{+}HLA\text{-}DR^{-}CD45^{+}$side scatter $(SSC)^{low}$, (2) three-marker strategy: $CD123^{+}CD203c^{+}CD45^{+}SSC^{low}$, (3) two-marker strategy: $CD203c^{+}CD45^{+}SSC^{low}$. The four-marker gating strategy was employed as the reference for comparative evaluation within a five-color panel that also included the activation marker CD63. The three-marker and two-marker gating strategies were used within a four-color and a three-color panel, respectively, each also incorporating CD63 as the activation marker.

Basophil activation was quantified using two parameters: (1) the percentage of CD63 positive basophils ($CD63^{+}$%), corrected by subtracting the negative control value, and (2) the stimulation index of CD63 (CD63SI), calculated as the ratio of the mean fluorescence intensity (MFI) of CD63 in stimulated samples to that in unstimulated samples. A positive response is defined as both $CD63^{+}$% ≥5% and CD63 SI ≥2, based on previously established thresholds in BAT studies.[23,24]

Patients whose basophils did not respond to anti-IgE stimulation but exhibited a response to fMLP were classified as non-responders according to Pascal M and excluded from activation analysis.[25] For Df-specific analyses, only sensitized patients with anti-IgE-responsive basophils were included, unless otherwise specified.

Quantification of Membrane-Bound IgE, Df sIgE, Total IgE, Basophil Count, and Eosinophil Count

We performed a comprehensive correlation analysis between BAT outcomes and key immunological parameters, including basophil membrane-bound IgE (mIgE), Df sIgE, total IgE (tIgE), basophil count, and eosinophil count. All quantitative measurements were carried out using standardized methods: tIgE and Df sIgE levels were quantified using the ImmunoCAP™ 250 system (Thermo Fisher Scientific, Uppsala, Sweden), a fluorescent enzyme immunoassay (FEIA) platform. Basophil and eosinophil counts were determined using automated hematology analyzers (BC-6800 Plus, Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China). MIgE was assessed via flow cytometry using FITC-conjugated anti-human IgE antibodies.

The Effect of Storage Temperature and Duration on BAT Outcomes

To evaluate storage stability, a total of 21 Df-sensitized patient samples were used. The initial BAT analysis was performed within 6 hours after collection (designated as Day 0). Following this, aliquots from each sample were stored under two different conditions: 2–8 °C (low temperature, LT) and room temperature (RT, 18–25°C). Each of the 21 samples was tested at all subsequent time points (day 1: 24–28 h and day 2: 48–52 h) under both storage conditions, following a paired, repeated-measures design.

Statistical Analysis

Continuous data were presented as median and interquartile range (IQR). Categorical variables were expressed as frequencies and percentages. The normality of distribution was assessed using the Shapiro–Wilk test. As the data were not normally distributed, non-parametric tests were used for all group comparisons. Differences between two independent groups (eg., Df-sensitized vs. control) were analyzed using the Mann–Whitney *U*-test. Differences among paired samples (eg., across storage time points, cell counts) were analyzed using the Wilcoxon signed-rank test. Comparisons of categorical variables between groups were performed using the chi-square test or Fisher's exact test, as appropriate. Associations between variables were evaluated using Spearman's rank correlation coefficient (r), with coefficients of determination (r^2) to indicate the proportion of explained variance. For these exploratory correlation analyses between BAT outcomes and multiple immunological parameters, p values are reported without formal correction for multiple comparisons, and results should be interpreted as hypothesis-generating. A two-sided p-value < 0.05 was considered statistically significant. Analyses of basophil counts included all participants (including non-responders), whereas analyses of basophil reactivity restricted to participants with

responsive basophils. For Df-specific analyses, this subset was further limited to Df-sensitized patients. All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Agreement between gating strategies for qualitative BAT outcomes was assessed using Cohen's kappa coefficient (κ).

Results

Characteristics of the Study Population

A total of 79 patients were included in this study, and 7/79 (8.86%) failed to respond to anti-IgE. Finally, 32 Df-sensitized patients and 39 non-allergic controls were analyzed. The baseline characteristics of the study population are summarized in Table 1. TIgE and Df sIgE levels were markedly higher in the sensitized group compared to the control group. Age and gender distribution were similar between the two groups.

Establishment of the Basophil Activation Test

Gating Strategies and Identification of Basophils

Flow cytometric identification of basophils is a prerequisite for measuring their activation in response to IgE-dependent or IgE-independent stimuli. Basophils were identified and gated as $CD123^{+}CD203c^{+}CD45^{+}SSC^{low}$ cells (Figure 1A and B). A distinct cell population was evident within the $CD123^{+}CD203c^{+}$ region (Figure 1A). To confirm basophil identity, we evaluated the absence of HLA-DR. Nearly all gated cells were negative for HLA-DR (Figure 1C), consistent with a basophil phenotype.

To validate the accuracy of our three-marker gating panel ($CD123^{+}CD203c^{+}CD45^{+}SS^{low}$), we compared it with a more extensive four-marker gating strategy ($CD123^{+}CD203c^{+}HLA\text{-}DR^{-}CD45^{+}SSC^{low}$) reported.[26] Basophil counts did not differ significantly between the two strategies under any of the stimulation conditions tested (Figure 2).

Given the high specificity of CD203c for basophils, we investigated a simplified, cost-effective gating strategy utilizing $CD203c^{+}CD45^{+}SSC^{low}$ without CD123. This approach consistently identified a well-defined cellular population (Figure 1G–H), approximately 92% of which exhibited $HLA\text{-}DR^{-}$ phenotype (Figure 1I). However, the basophil count obtained with $CD123^{+}CD203c^{+}CD45^{+}SSC^{low}$ strategy was significantly lower than that obtained with $CD203c^{+}CD45^{+}SSC^{low}$ (two marker gating approach) (Table 2), confirming that the inclusion of CD123 helps exclude non-basophil populations.

Changes of Molecular Expression Levels

The expression of these membrane markers was analyzed in basophils from all enrolled patients, excluding non-responders. Among markers evaluated, CD45, CD63 and CD203c (expressed as MFI), as well as side scatter, all increased significantly after anti-IgE stimulation compared to unstimulated conditions, whereas CD123 remained unchanged (Figure 3). CD63 exhibited the most pronounced response (32.5-fold increase), consistent with its established role as a principal activation indicator in BAT.[17] Consequently, CD63 was selected as the activation marker for subsequent analyses. Flow cytometric gating resolved two discrete subpopulations upon stimulation: CD63-negative (resting) and CD63-positive (activated) (Figure 1D–F and J-L). Activation was quantified as CD63+% or CD63SI.

Table 1 Characteristics of Df-Sensitized and Non-Sensitized Patients

Characteristic	Df-sensitized Patients (n=32)	Control Subjects (n=39)	*p*
Age, years (median [IQR])	62 (41.75,71.5)	68 (60.5, 70.25)	0.0564
Male (%)	62.5%	66.67%	0.8047
Total IgE for kU/L, Mdn (IQR)	447.5 (186.5, 920)	35.1 (17.1,74)	<0.0001
Df sIgE (kU/L) Mdn (IQR)	2.895 (1.0075, 8.5225)	0.0 (0.0,0.055)	<0.0001

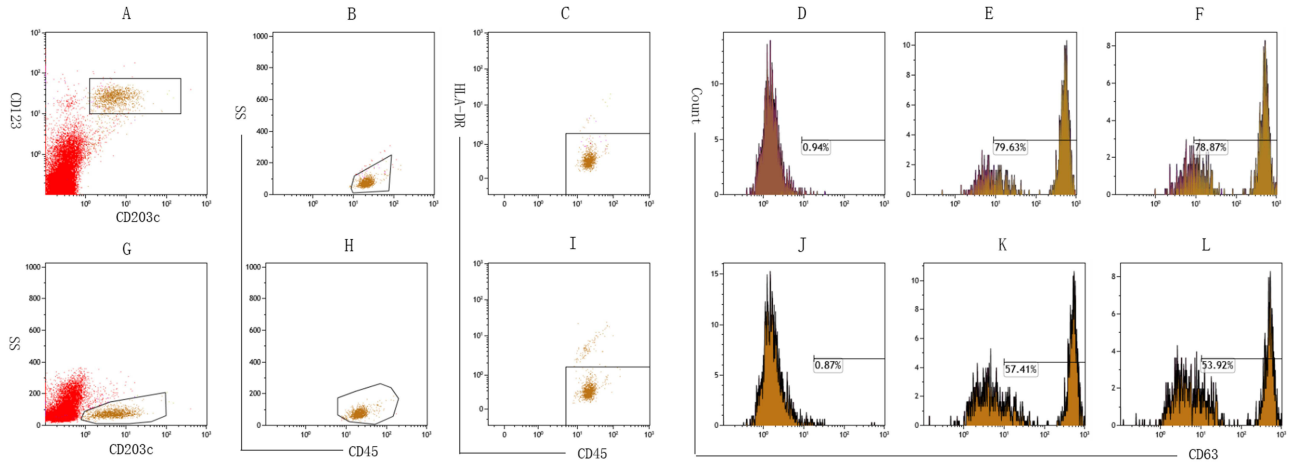

Figure 1 Representative gating strategy and immunophenotypic verification of human basophils. Basophils were identified using two distinct gating strategies: $CD123^{+}CD203c^{+}CD45^{+}SS^{low}$ (**A** and **B**) and $CD203c^{+}CD45^{+}SS^{low}$ (**G**–**H**). Basophil identity was confirmed by absence of HLA-DR (**C** and **I**). Representative CD63 expression on basophils under negative control, anti-IgE, and Df stimulation is shown for the three-marker gate (**D**–**F**) and the two-marker gate (**J**–**L**), respectively.

Number of basophils defined by the two phenotypes

	n	CD123+CD203c +CD45+SSlow	CD123+203c+HLA-DR- CD45+SSClow	*P*
Pre-stimulation	19	1362(1028, 1892)	1390(976.0, 1850)	0.9612
Post-stimulation-aIgE	19	1290(922.0, 1818)	1266(928.0, 1820)	0.2619
Post-stimulation-Df	19	1406(1064, 1620)	1404(1030, 1646)	0.5579
Post-stimulation-fMLP	19	1092(680.0, 1496)	1116 (684.0, 1548)	0.6471

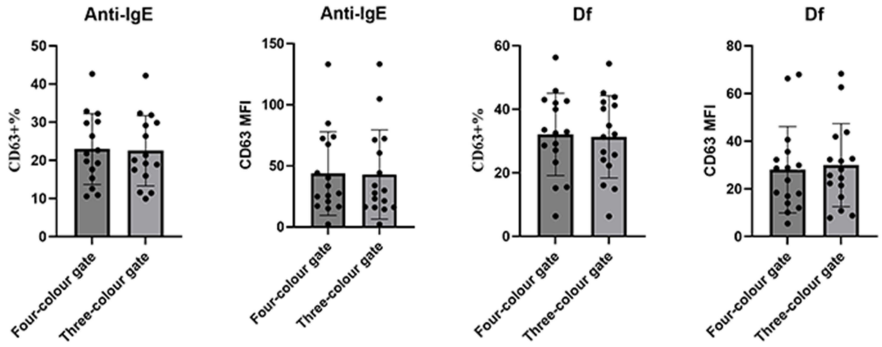

Figure 2 Comparison of basophil counts and CD63 expression between three-marker ($CD123^{+}CD203c^{+}CD45^{+}SS^{low}$) and four-marker ($CD123^{+}CD203c^{+}HLA^{-}DR^{-}CD45+SS^{low}$) gating strategies across stimulations.

No differences in CD63+% and CD63 MFI were observed between the three- and the four-marker gating strategies, whereas the two marker-gating approach yielded significantly lower CD63+% values than the three-marker gating strategy (Figures 2 and 3).

Taken together, these findings indicate that the three-marker gating panel identifies basophils with purity comparable to the four-marker gating reference and higher than the two-marker gating approach, while maintaining equivalent activation readouts-representing a balance between analytical specificity and cost-effectiveness.

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Table 2 Comparison of Basophil Counts Obtained by $CD123^+CD203c^+CD45^+SSC^{low}$ and $CD203c^+CD45^+SSC^{low}$ Gating Strategies

	n	$CD123^+CD203c^+CD45^+SSC^{low}$	$CD203c^+CD45^+SSC^{low}$	*P*
Pre-stimulation	79	1326 (1017, 2025)	1581 (1191, 2062)	<0.0001
Post-stimulation-aIgE	79	1197 (918, 2578)	1310.5 (722.5, 2075)	<0.0001
Post-stimulation-Df	58	1352 (818, 1991)	1584 (1113.25, 2206)	<0.0001
Post-stimulation-fMLP	42	1328 (755.5, 2152)	1730 (996, 2560)	<0.0001

Agreement Between Gating Strategies for Qualitative BAT Classification

We evaluated agreement between the three-marker and four-marker gating strategies for qualitative classification (positive/negative) using the predefined threshold. Among the 19 Df-sensitized patients with responsive basophils, the two gating strategies showed perfect concordance (κ = 1.00, 95% CI: 1.00–1.00), with 16/19 positive and 3/19 negative by both methods.

For comparison between the three- and two-marker gating strategies (n = 72), concordance was 97.2% (κ = 0.917, 95% CI: 0.832–1.000), with two samples classified as positive by three-marker but negative by two-marker, consistent with lower analytical sensitivity of the two-marker gating approach in this cohort.

These findings support analytical equivalence of the three-marker and four-marker gating strategies, with the three-marker gating strategy showing better concordance with the reference standard than the two-marker gating approach.

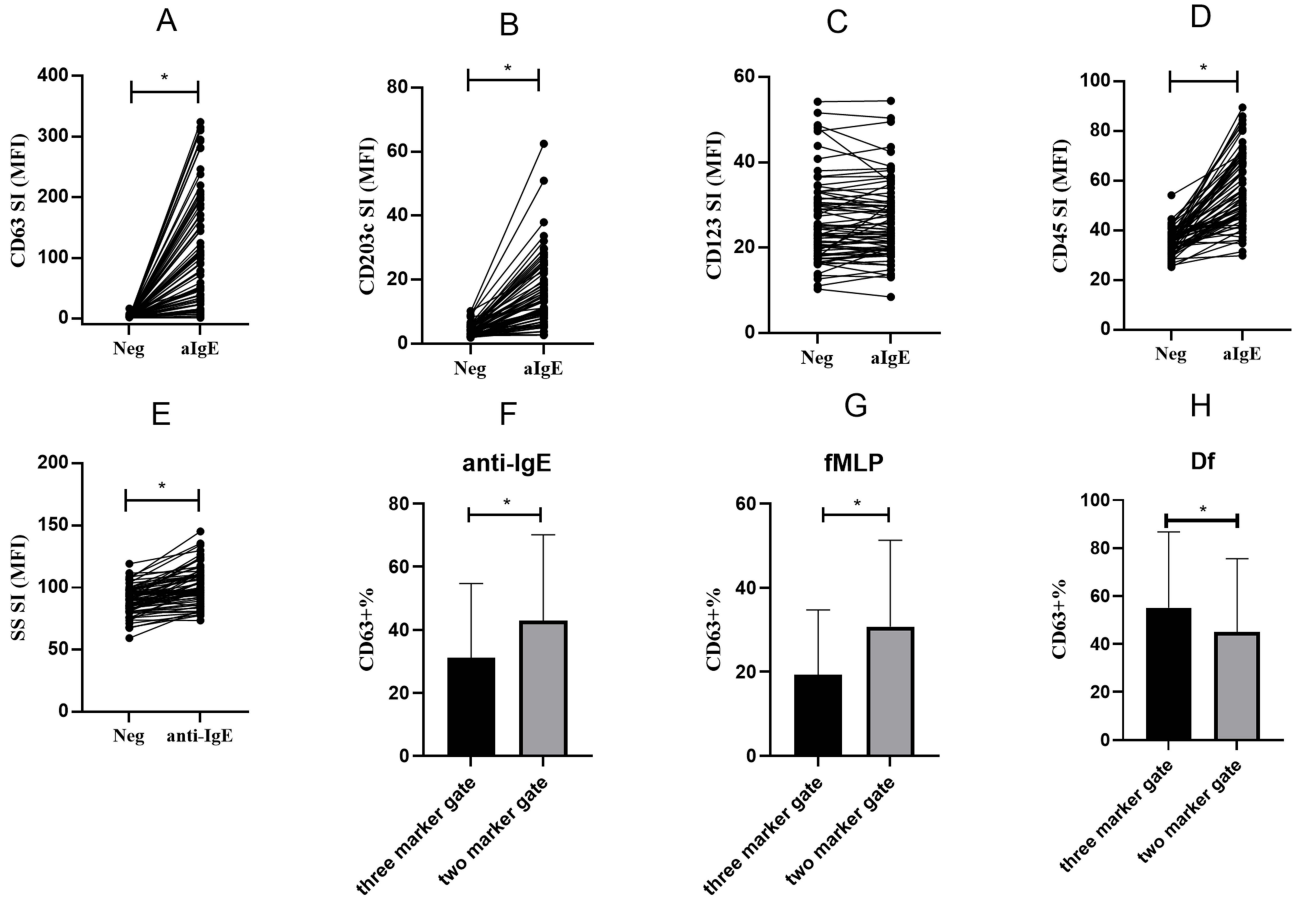

Figure 3 Basophil activation characteristics and evaluation of gating strategies. (**A**–**E**) Changes in CD63, CD203c, CD123, and CD45 on basophils and side scatter characteristics following anti-IgE stimulation compared with negative control assessed using the three-marker gating ($CD123^+CD203c^+CD45^+SS^{low}$). (**F**–**H**) Comparison of CD63+% between three- and two-marker ($CD203c^+CD45^+SS^{low}$) gating strategies for anti-IgE, fMLP and Df stimulation. * $p<0.05$.

The Association Between Basophil CD63 Expression and Biomarkers of Allergic Inflammation

IgE levels and eosinophil counts are commonly used laboratory parameters for allergy diagnosis. We therefore examined correlations between these markers and BAT outcomes in Df-sensitized patients (Table 3). Df-induced basophil CD63 expression exhibited moderate correlations with Df sIgE levels (r=0.4352 for CD63 MFI, 95% CI: 0.1020–0.6805) and mIgE levels (r=0.6659 for CD63+%, 95% CI: 0.1925–0.8879; and r=0.6264 for CD63 MFI, 95% CI: 0.1263 to 0.8726), though confidence intervals were wide indicating substantial uncertainty. But a negative correlation was observed with tIgE (r=−0.3798 for CD63 MFI, 95% CI:-0.6496 to −0.02511). In contrast, basophil activation did not associate with basophil counts, eosinophil counts, or fMLP-stimulated CD63 expression.

We further assessed the correlation between anti-IgE-induced CD63 expression and the aforementioned parameters in the entire study population, excluding non-responders. A weak positive correlation was observed between CD63+% and mIgE levels (r=0.363, 95% CI:0.0161 to 0.6600), whereas a weak inverse correlation was found between CD63SI and tIgE levels (r=−0.2854, 95% CI:-0.4945 to −0.0451). No significant associations were detected between anti-IgE-induced CD63 expression and other variables, including Df sIgE levels, basophil counts, or eosinophil counts (Table 4).

Additionally, we analyzed the correlation between CD63 expression under different stimulation conditions. The correlation between Df- and anti-IgE induced basophil CD63 expression was also moderate (r=0.4483 for CD63+%, 95% CI: 0.1075 to 0.6949; r=0.5117 for CD63 MFI, 95% CI: 0.1881 to 0.7351), though confidence intervals were wide (Table 3).

Table 3 Association of IgE Levels, Eosinophil and Basophil Counts with Df-Induced CD63 Expression on Basophils

	Basophil CD63% (Df)		**Basophil CD63 MFI (Df)**	
	r	***p***	***r***	***p***
Df sIgE	0.3284	0.0665	0.4352	0.0128(*)
tIgE	−0.2924	0.1044	−0.3798	0.032(*)
mIgE	0.6659	0.0113(*)	0.6264	0.0191(*)
Eosinophil number	0.1142	0.5406	0.0981	0.5996
Eosinophil ratio	0.0817	0.6622	0.0847	0.6505
Basophil number	0.0788	0.6734	−0.0244	0.8962
Basophil ratio	0.0836	0.6547	0.0546	0.7702
Anti-IgE stimulated CD63 expression	0.4483	0.0101(*)	0.5117	0.0028(*)
fMLP stimulated CD63 expression	0.0181	0.9673	0.1545	0.6538

Notes: total N = 32 (sensitized group), with 14 for mIgE analysis. * Indicates statistically significant differences.

Table 4 Association Between IgE Levels, Eosinophil and Basophil Counts, and Anti-IgE Induced CD63 Expression on Basophils

	Basophil CD63% (anti-IgE)		**Basophil CD63 MFI (anti-IgE)**	
	r	***p***	***r***	***p***
Df sIgE	0.0141	0.9801	−0.0076	0.9505
tIgE	−0.1479	0.2253	−0.2854	0.0174(*)
mIgE	0.3630	0.0321(*)	0.1914	0.2706
Eosinophil number	−0.0643	0.6136	−0.0061	0.9617
Eosinophil ratio	−0.0941	0.4596	−0.0075	0.9527
Basophil number	−0.0923	0.4679	−0.0794	0.5327
Basophil ratio	0.03646	0.7749	0.0699	0.5828

Notes: All 72 samples (including sensitized group) were analyzed, beyond mIgE (n=35). *Indicates statistically significant differences.

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The coefficient of determination (r^2) indicated that tested markers explained only 8.15% to 44.34% of the total variability in BAT outcomes, leaving 55.66% to 91.85% unexplained.

Influence of Storage Temperature and Duration on BAT Outcomes

To assess the impact of storage time and temperature on basophil reactivity, we assessed responses to anti-IgE, fMLP and serially diluted Df extract under two storage conditions: RT (18–25°C) and LT (2–8°C), at three points post-collection: 0–6 hours (day 0), 24–28 hours (day 1), and 48–52 hours (day 2).

Quantitative Changes in CD63 Expression

We first analyzed the basophil activation magnitude by measuring CD63 MFI. Overall, quantitative analysis revealed significant temporal- and temperature-dependent declines in activation (Figure 4).

For Df stimulation, CD63 MFI decreased significantly after 1 or 2 days of storage at LT relative to baseline (day 0). A similar but less pronounced trend was observed at RT. For anti-IgE stimulation, CD63 MFI declined significantly by day 2 under both storage conditions, with no significant decreases on day 1. When comparing the two storage conditions directly, both Df and anti-IgE stimulation exhibited a trend toward greater decline in CD63 expression from baseline under LT relative to RT, though this difference was not statistically significant. In contrast, for fMLP stimulation, CD63 MFI was significantly lower in LT-stored samples at both days 1 and 2 relative to day 0. Moreover, at each time point, LT storage led to significantly greater suppression than RT, indicating that fMLP-induced basophil activation is uniquely sensitive to LT storage.

Impact on Qualitative (Positive/Negative) Classification

We next evaluated whether the quantitative declines described above translated into qualitative classification using the predefined positivity threshold (CD63+% ≥5% and SI ≥2). Qualitative outcomes are detailed in Table 5.

Anti-IgE stimulation showed high stability. Only 3 of 20 (15%) samples stored at RT and 3 of 21 (14.28%) samples stored at LT yielded negative on Day 1, with no further change on day 2. In contrast, fMLP-induced activation exhibited marked temperature sensitivity. 1 of 20 (5%) RT-stored samples turned negative on day 1, compared to 7 of 21 (33%) LT-stored samples, indicating that RT is required for fMLP control preservation. Df stimulation (10 μg/mL) showed initial stability with only one sample per group converting to negative on Day 1. By day 2, temperature-dependent divergence emerged. 5 of 21 (24%) RT-stored samples, but in only 2 of 21 (9.5%) LT-stored samples lost reactivity. This pattern was amplified under gradient-dilution Df challenge (1–0.01 ug/mL), with 8 of 21 (38%) RT-stored versus 5 of 21 (23.8%) LT-stored assays became negative on day 1.

These data indicated stimulus-specific storage patterns. Quantitatively, CD63 MFI declined significantly with storage. Qualitatively, LT better preserved Df positivity beyond 24 h, whereas fMLP required RT storage, and anti-IgE remained stable under either condition for 24–28 hours.

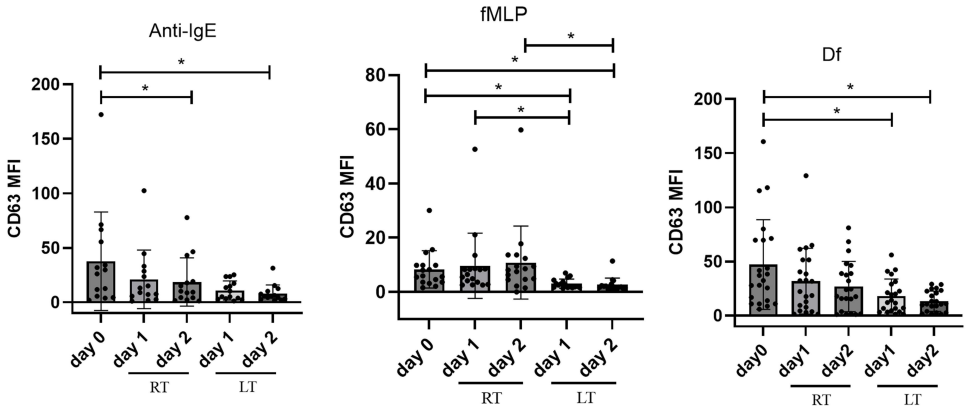

Figure 4 The effect of storage time and temperature on basophil CD63 expression stimulated by Df, anti-IgE and fMLP. * *p<0.05*.

Table 5 IgE and CD63 Values at Different Storage Time and Temperature for Df-Sensitized Patients

Patient	Sex/Age	IgE (KU/L)				Day0/1/2 (for RT), Day 1/2 (for 4°C),					
		Total	Derf	Derp		aIgE	fMLP	10 ug/mL	1ug/mL	0.1ug/mL	0.01ug/mL
P1	M/67	460	2.46	2.2	RT	+/+/+	-/-/-	+/+/+			
					4°C	+/+	-/-	+/+			
P2	M/53	61.7	8.98	14.5	RT	+/+/+	+/+/+	+/+/+			
					4°C	+/+	+/+	+/+			
P3	F/22	597	66	65.2	RT	+/+/+	+/+/+	+/+/+			
					4°C	+/+	+/+	+/+			
P4	F/39	>5000	1.36	2.23	RT	-/-/-	+/+/+	+/±	±/-	-/-/-	-/-/-
					4°C	-/-	±	±	±	-/-	-/-/-
P5	F/61	250	21.5	24.4	RT	+/?/-	+/+/?	+/±	±/-	+/±	+/±
					4°C	+/+	-/-	+/+	+/+	+/+	+/+
P6	F/61	>5000	4.48	4.42	RT	±/-	+/+/+	+/±	±/-	-/-/-	-/-/-
					4°C	-/-	-/-	+/+	-/-	-/-	-/-
P7	M/18	252	8.43	13	RT	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
					4°C	+/+	+/+	+/+	+/+	+/+	+/+
P8	M/67	727	5.43	8.92	RT	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	-/-/-
					4°C	+/+	+/+	+/+	+/+	+/+	-/-
P9	F/38	93.1	15.5	19	RT	+/+/+	-/-/-	+/+/+	+/+/+	+/+/+	+/+/+
					4°C	+/+	-/-	+/+	+/+	+/+	+/+
P10	F/42	88.1	4.49	7.15	RT	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
					4°C	+/+	-/-	+/+	+/+	+/+	+/+
P11	F/55	185	4.58	6.42	RT	+/+/+	±/-	+/+/+	+/+/+	+/+/+	+/+/+
					4°C	+/+	-/-	+/+	+/+	+/+	+/+
P12	F/64	389	9.98	7.3	RT	+/+/+	+/+/+	+/+/+	-/-/-	-/-/-	-/-/-
					4°C	+/+	+/+	+/+	-/-	-/-	-/-
P13	M/66	676	6.11	1.65	RT	±/-	+/+/+	±/-	-/-/-	-/-/-	-/-/-
					4°C	-/-	-/-	-/-	-/-	-/-	-/-
P14	M/32	68.1	13.6	21.8	RT	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/±
					4°C	+/+	-/-	+/+	+/+	+/+	+/+
P15	F/33	126	0.81	0.81	RT	+/+/+	+/+/+	+/+/+	+/+/+	-/-/-	-/-/-
					4°C	+/+	+/+	+/+	+/+	-/-	-/-
P16	F/22	229	43.5	75.4	RT	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
					4°C	+/+	+/+	+/+	+/+	+/+	+/+
P17	M/66	62.3	1.5	2.72	RT	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	±/-
					4°C	+/+	+/+	+/+	+/+	+/+	±
P18	F/33	—	2.74	0.63	RT	±/-	+/+/+	+/±	±/-	±/-	-/-/-
					4°C	-/-	+/+	+/+	+/+	-/-	-/-
P19	F/49	11.1	2.61	5.14	RT	+/+/+	+/+/+	+/+/+	+/+/+	±/-	±/-
					4°C	+/+	±	+/+	+/+	-/-	-/-
P20	F/62	—	26.6	65.8	RT	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/±
					4°C	+/+	±	+/+	+/+	+/+	±
P21	M/26	—	0.49	0.34	RT	+/+/+	-/±	+/+/+	+/+/+	+/+/+	+/+/+
					4°C	+/+	-/-	+/+	+/+	+/+	-/-

Notes: The results marked with "?" are blank results due to instrument malfunction. +: indicates positive result (BTA result-CD63+% ≥5% and SI ≥2) -: indicates negative result.

Discussion

This study describes a cost-efficient BAT workflow with demonstrated analytical validity through systematic comparison of gating strategy and definition of pre-analytical storage conditions. We caution that this establishes a methodological framework rather than diagnostic accuracy.

To date, no consensus gating protocol for basophil identification in BAT has been established, resulting in substantial inter-laboratory and inter-kit variability.[27–30] The analytical equivalence of three- and four-marker gating strategies aligns with prior work demonstrating that $CD203c^+CD123^+HLA\text{-}DR^-$ gating improves specificity compared to $CD123^+HLA\text{-}DR^-$ alone.[31] We extend these findings by showing that explicit HLA-DR staining is redundant when CD203c is combined with CD123, as >97% of $CD123^+CD203c^+$ cells are inherently $HLA\text{-}DR^-$. Whether this analytical equivalence translates to diagnostic equivalence requires prospective validation (NCT06559319, currently underway).

Compared with the two-marker gating strategy ($CD203c^+CD45^+SSC^{low}$), the three-marker gating panel demonstrated higher phenotypic purity (97.2% vs. 91.8%) and detection agreement (κ=0.917). The 8.2% contamination rate in the two-marker gating approach suggests potential inclusion of non-basophil populations, supporting the mechanistic rationale for three-marker gating adoption.

Df-stimulated BAT outcomes correlated moderately with Df sIgE and mIgE (r^2=0.19–0.44), indicating that the IgE parameters account for less than half of the variability in basophil reactivity. This substantial unexplained variance suggests that discordant profiles—such as elevated sIgE with low basophil reactivity—may represent asymptomatic sensitization rather than true allergy. Evidence indicates that basophil reactivity is governed by factors beyond IgE availability, including FcεRI surface density, SYK signaling pathway activity, and basophil priming status.[32–34] Thus, BAT provides functional information complementary to static IgE measurements.

Our observed Df sIgE correlation (r=0.44) is lower than reports in mite-allergic children (r=0.53–0.60)[35] and venom allergy (r=0.60–0.68).[36] These discrepancies likely reflect multiple factors: (1) age-related differences, as our elderly cohort contrasts with pediatric populations; (2) absence of IL-3 priming in our protocol, which upregulates FcεRI expression and enhances basophil responsiveness;[37] (3) allergen-specific variables, including extract composition differences between mite and venom allergy. Notably, tIgE showed weak negative correlations with Df- and anti-IgE stimulated CD63+%. This unexpected finding may reflect high polyclonal IgE occupancy of FcεRI limiting anti-IgE accessibility, though this mechanism requires direct experimental validation.[38]

In this cohort, we observed that storage stability depended on both analytical purpose (quantitative/qualitative) and stimulus type. Quantitatively, CD63 MFI declined significantly with storage: fMLP responses were most sensitive to LT, followed by Df and anti-IgE. These differences may reflect stimulus-specific signaling lability: fMLP signals through G-protein coupled formyl peptide receptors (FPRs) that undergo irreversible or slowly reversible transitions between low- and high-affinity states,[39] whereas IgE-mediated FcεRI activation appears relatively preserved during short-term storage (24–28 h), though the molecular basis for this stability remains unclear. Df extract, with its complex composition, shows LT sensitivity distinct from anti-IgE.

Qualitatively, positive/negative classification based on the pre-defined positivity threshold proved more robust than quantitative measures. We observed differential storage stability: (1) Df: 2–8°C up to 48–52 hours (9.5% negative-conversion vs. 24% at RT); (2) fMLP: RT within 24–28 hours (5% negative-conversion vs. 33% at 2–8°C); (3) anti-IgE: stable under either condition for 24–28 h. These stimulus-specific requirements enable flexible batch processing.

This study systematically evaluates gating strategies, separates quantitative vs. qualitative storage outcomes across different stimuli, and integrates cost-effectiveness analysis. However, limitations must be acknowledged. First, our single-center clinic cohort, predominantly elderly, lacks healthy controls and detailed atopic histories, limiting generalizability. The absence of a clinical gold standard precludes diagnostic accuracy assessment. Second, the four-marker gating reference standard, though literature-supported, is not an absolute gold standard, and any systematic bias in this reference would affect both strategies. Third, no prospective power calculation was performed, and statistical power was limited by sample size. Post-hoc power analyses indicated 80% power to detect $|r|\geq 0.48$ (Df-specific) and ≥ 0.33 (anti-IgE). Weaker associations may have been missed, and findings should be interpreted as hypothesis-generating. Fourth, formal intra-/inter-assay variability was not assessed, though indirect indicators (perfect gating concordance, consistent storage trends) suggest acceptable reproducibility.

These methodological refinements offer several features relevant to clinical utility: cost reduction, stimulus-specific storage flexibility, and functional complementarity to IgE measurements. However, these features currently represent analytical and pre-analytical feasibility. Prospective diagnostic validation (NCT06559319) is required to establish clinical utility.

Conclusions

This study describes an analytically validated BAT protocol using a three-marker gating strategy ($CD123^{+}CD203c^{+}CD45^{+}SSC^{low}$) within a four-color panel that achieves 25% cost reduction while maintaining equivalence to four-marker gating reference. BAT outcomes showed only moderate correlations with IgE parameters, indicating its role as a complementary functional assay. For storage, quantitative analysis required processing within 4–6 h, whereas qualitative outcomes demonstrated stimulus-specific stability. These methodological refinements enhance feasibility for research applications, with prospective diagnostic validation underway.

Abbreviations

BAT, Basophil Activation Test; sIgE, specific IgE; tIgE, total IgE; HDM, house dust mite; AR, allergic rhinitis; SPT, skin prick testing; fMLP, N-Formyl-Met-Leu-Phe; SI, stimulation index; MFI, mean fluorescence intensity; FcεRI, high-affinity IgE receptor; SSC, side scatter; Df, Dermatophagoides farinae.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Declaration of Helsinki. And the study was approved by the Ethics Committee of the Shanghai General Hospital affiliated with Shanghai Jiao Tong University School of Medicine (reference number: 2021KY003). The requirement for individual informed consent was waived by the Ethics Committee because the research utilized anonymized leftover blood samples obtained from routine clinical diagnostics, posed no more than minimal risk to participants, and all patient data were kept strictly confidential.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest in this work.

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