Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

CCR3 as a Single Selection Marker Compared to CD123/HLADR to Isolate Basophils in Flow Cytometry: Some Comments

Introduction

Flow cytometry of activated basophils is extensively used to assess cell response to allergens or to soluble agonists and has immediately proved a successful approach for studying the function of basophils *in vitro* (1). The mechanism of expression of various membrane proteins is the basis of basophil activation tests (BATs). Several questions and influencing factors on the analytical feasibility and reliability of BATs still remain and generally the potentials and pitfall of these diagnostic tools are a point at issue (2,3). Activated basophils up-regulate several membrane proteins, such as the tetraspan CD63 and the ectoenzyme CD203c but also express many other molecules on the surface that can be used to characterize these cells allowing their separation from other leukocytes (4,5). A large number of gating strategies to phenotype basophils have been proposed in the past and most of them are currently in use: IgE^{pos}, $HLA\text{-}DR^{neg}/CD123^{pos}$, $CD45^{dim}/CCR3^{pos}$, $CD3^{neg}/CRTH2^{pos}$ (3). An approach for best basophil electronic capture is the use of a single selection marker, with a bright fluorochrome, as basophils gated with the brighter fluorochrome PE resulted in a better separation of the target basophilic cells compared to FITC (2). One candidate to isolate basophils in whole blood or in leukocyte-enriched buffy coats might be CD203c, as this molecule is expressed only on basophilic cells (3,6); however this marker changes with activation, it is constitutively present on resting basophils with a wide interindividual variability in cell surface expression and it is influenced by preanalytical process and by autocrine interleukin 3 (2). Another well-known selection approach using the highly basophil specific marker anti-IgE has raised serious objections, because anti-IgE is a potential triggering agent, because other leucocytes express IgE-receptor (Fc RII) and because the density of IgE and Fc RI may vary widely among subjects and even among basophils in the same individual (3). So, recently published papers have suggested the use of eotaxin receptor CCR3, also known as CD193, as a single selection marker to separate basophils from other leukocytes in flow cytometry, though with some controversial issue (7,8). This marker has the indisputable advantage of being highly expressed in cells at the basal level with a comparable extent in different individuals but it changes its expression upon basophil stimulation (7). Phenotyping markers, which up- or down-regulate membrane expression following cell stimulation, make it very difficult to perform a gating process without introducing cellular contamination into the gate and/or to attain a loss of target cells when activated. Choosing the best gating strategy often has to face all of these topics, to evaluate basophil function the best possible way. This work trying to address these controversial issues raises some comments and suggestions. A total of 32 volunteer randomized blood donors (mean age 44.61 $\pm$ 4.57 SD, female/male ratio = 1.28) enrolled and evaluated over a period of 2 years were considered in this study. All the subjects were non allergic and non atopic, they did not suffer from any immunological disorder and had never reported any previous history or genetic diathesis of chronic allergy. Moreover, none underwent either drug therapy or antihistamine therapy during the 48 hrs before withdrawal. All participants signed an informed consent. Basophils were collected and treated as leukocyte-enriched buffy coats from venous K_2-ethylendiaminetetraacetic acid (EDTA) anticoagulated peripheral blood from four screened healthy donors in each experiment performed, according to previously described methods (1). When indicated, activation was performed with 100 nmol/L of fMLP or 2 μg/ml of goat anti-human IgE, according to published methods (1). Cells were blocked in ice-cold buffer with 2.8 mmol/L Na_3-EDTA, then stained with monoclonal antibodies (20 minutes at +4°C) and erythrolysed (4 min) with an ammonium-buffered solution, centrifuged and pellets recovered and resuspended in a PBS-buffered saline solution (pH 7.4) for

Received 18 October 2010; Accepted 17 November 2010

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Published online 30 December 2010 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.21008

flow cytometry reading. Basophils were gated as low side-scattered cells (SSC) in the $CD45^{dim}$ lymphocyte area (here indicated as "baso region"): the use of CD45 contributed to discriminate basophil area from other leukocytes better than FSC/SSC light-scattering, allowing to exclude cellular debris and to account for a further selective marker to phenotype basophilic cells. The alignment of the instrument was checked daily using fluorescent microbeads; also the accuracy of volumetric apparatus was addressed by using check commercial fluorescent beads having predetermined acceptable ranges on three different levels during the study. Compensation followed cytometer manufacturer's instructions granting an off-line procedure by applying automated electronics algorithms and preset templates, by using biparametric logarithmic dot plots, gate-specific tubes and single-tube data analysis, and by optimizing FSC threshold and fluorochrome voltage as set up parameters.

Figure 1 shows dot plots of different gating approaches. Basophils appeared very well separated when protocol A was used (panel 1). When cells were activated, the events cloud changed its dot area in the case of protocol B and of protocol C (panels 2 and 3) but this was not evident in the case of protocol A (panel 1). To assess this evidence and to address best suitability in basophil gating, CD123, CD203c, and CCR3 were evaluated as phenotyping markers (Fig. 2A bottom x-axis) and their expression upon activation evaluated (Fig. 2A top x-axis) in different gating strategies. Box plots are good tools to show the extent of variability of mean of fluorescence values (MFI) for each marker. CD123 behaved as a marker changing very little with activation in any of the gating strategies employed: differences between CD123 expression and other markers used were highly significant ($r = 0.965$, $P < 0.001$ for CCR3 and $r = 0.687$, $P < 0.01$ for CD203c, Fig. 2A). CCR3 evaluation in protocol B appeared underestimated compared to protocol A (Fig. 2A, median of boxes 4 and 5); on the contrary, CD203c showed over-estimation in protocol C (Fig. 2A, median of boxes 6 and 7): this trend actually occurred also when CD123 was evaluated with each of the different gating strategies used (Fig. 2A, boxes 1-3). Focusing on CD123 and CCR3 as the main object of our investigation, we shifted this result for each stimulation condition: differences between $CD45^{dim}/CCR3^{pos}$ gating and $HLADR^{neg}/CD123^{pos}$ one were more clearly evident. CCR3 expression changed widely when basophils were gated with a CD45/CCR3 protocol (Fig. 2B1) showing down-regulation during activation (see medians in box plots Fig. 2B1) while the variability in CD123 expression was much more limited in a HLADR/CD123 gated protocol (Fig. 2B2): this means and confirms that CCR3 membrane expression changes with activation more than CD123 ($r = 0.679$, $P < 0.05$). Down-regulation of eotaxin marker in activated basophils actually influences the read out of other well-known moving markers related to activation, such as CD63, as the expression of this molecule might be underestimated in CD45/CCR3 gated basophils (Fig. 2C1) when compared to $HLADR^{neg}/CD123^{pos}$ (Fig. 2C2; $r = 0.936$, $P < 0.001$). CD123 appears quite stable only in the same individual throughout the activation steps of basophils. However CD123, which identifies the alpha-subunit of IL-3 receptor, appears to change dramatically within the population in the resting condition; interindividual variability of basophils in the population (27 replicates) was evidenced for CD123 in the resting (basal) condition. This variability was as wide as for a usual activation marker, such as CD63, in response to 2.0 μg/ml anti-IgE, reflecting basophil heterogeneity within the sample population (Fig. 2D).

What conclusion can be forwarded? A good protocol to capture basophils in an electronic gate might be suggested by the use of a bright marker, for example a PE or a tandem PE-conjugated antibody against a highly upregulated molecule on cell membrane (which does not change its expression throughout the activation step) coupled with a clear nonexpressing (negative) marker. In any case, HLA-DR/CD123 seems to be an optimal approach to gate basophils in this context: basophils express high level of CD123 and do not express HLA-DR. In this report we have shown that CD123 expression within each sample analysis was very stable: it was close to the experimental variability related to manual procedures in the triplicate setting (CV = 12.9%, data not shown). So, CD123 behaves as a fixed marker in a cell population throughout an activation process, although its basal expression varies among different subjects. This feature allows to capture basophils in flow cytometry with a good reliability during a BAT. In the recently published paper by Hausmann et al. on the latest issue of Allergy, the eotaxin receptor CCR3 was addressed as a good single basophil selection marker, mainly because of its strong stability within the population (7). In this work some issue was also raised about CCR3 expression following basophil activation, namely that CCR3 changes (down-regulates) its expression upon activation. This would be an advantage for CCR3 as compared to CD123 while CD203c does not appear to be a good phenotyping marker due to its wide variability related to basophil function: CD203c is strictly related to basophil spontaneous activation and to interindividual variability and has a wide expression extent upon activation (2) (see also Fig. 2A). However the use of CCR3 as a gating marker actually underestimates the evaluation of basophil activation markers, such as CD63. Hausmann suggested that the observed $CD63^{pos}$-underestimation might be due to contamination of $CD63^{neg}$-lymphocytes into the gate (7). So, Monneret has proposed a $CCR3^{pos}/CD3^{neg}$ gating strategy to prevent this inconvenience (8). BATs may have many inconveniences due to several technical factors of which gating strategy may be only one (9). An inexpensive possibility to optimize basophil gating with CCR3 might be to relate this marker with an immunological gate, instead of a side scattered morphological plot, using a classical Pan-Leu CD45. Nevertheless this approach raises further criticism. Figure 1 panel 2 evidences, in a typical experiment, that activated basophils up-regulate CD45, as expected (10) (Mean values for 6 triplicates, CD45 MFI resting: 2883 ± 267 SD; CD45 MFI activated: 8269 ± 1001, CV respectively 9.26% and 12.11%, $P = 0.023$). This shifts basophils outside the gate and might result in a loss of $CD45^{pos}/CD63^{pos}$cells, unless starting gate does not include even $CD45^{bright}$ cells in the basal condition: of course, if so, this may result in a cell contamination, as only $CD45^{dim}$/

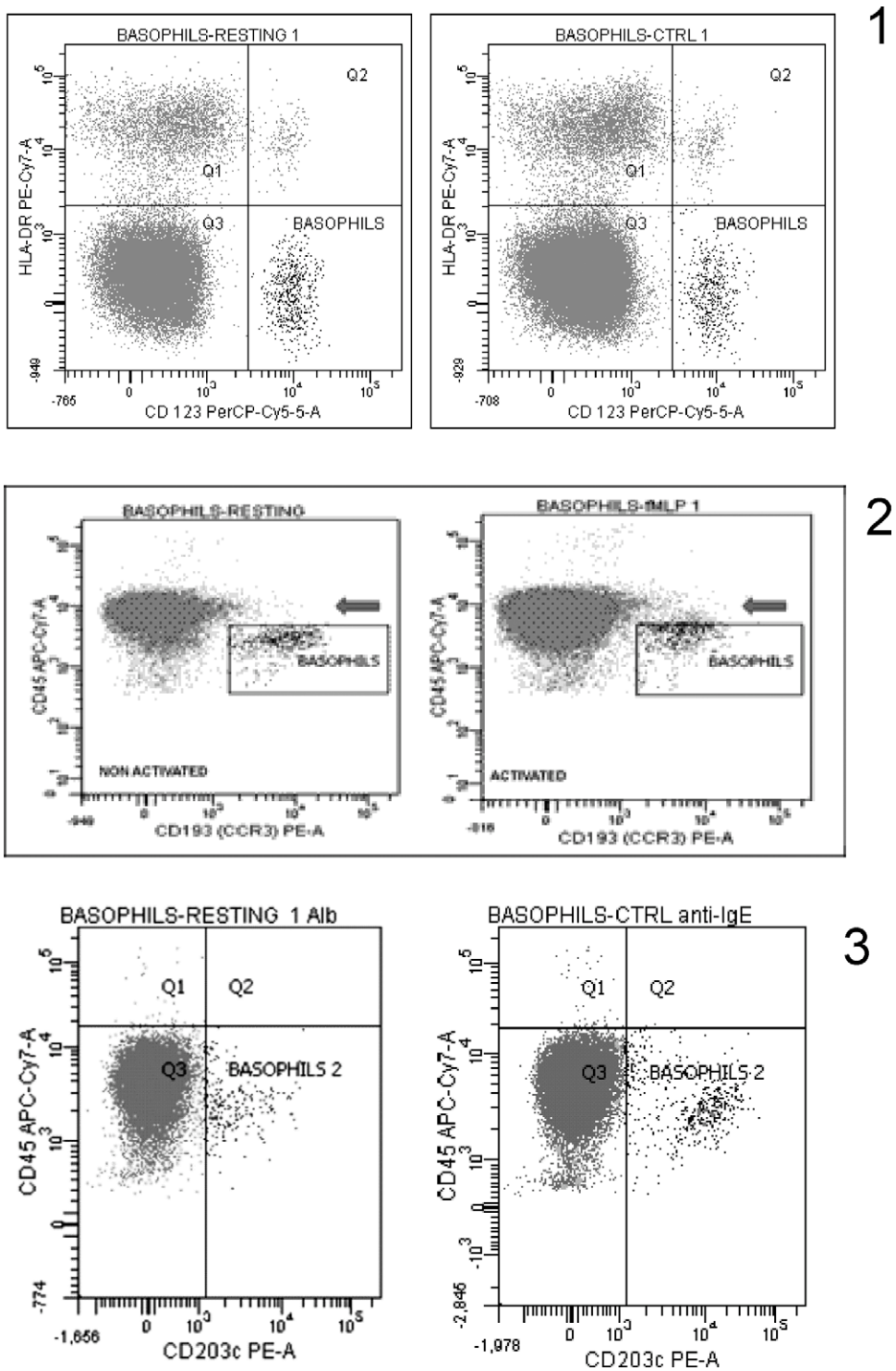

Figure 1. Panel 1: Basophil electronic capture with a HLA-DR/CD123 protocol (protocol A): basophils can be identified in this dot plot within the gate HLA-DR^{neg}/CD123^{bright} (bottom right). Events dots do not change their gating position in two functional states: resting (left) and activated (right). Q1: monocytes; Q2: dendritic cells; Q3: lymphocytes. Panel 2: Basophil electronic capture with a CD45/CCR3 protocol (protocol B), as described: basophils can be identified in the gate CD45^{dim}/CCR3^{bright}. 3. Panel 3: Basophil electronic capture with CD203c in the gate CD45^{dim}/CD203c^{expressing} (protocol C). In protocols B and C (panels 2, 3) events dots change their gating position in two different functional states: resting (left) and activated (right). Arrows show events that appear outside the gate area. For each sample a total of about 50,000 events was aquired in which, by applying this gating protocol, approximately 500–1,000 basophils were counted in the gate. To evaluate fluorochrome unspecific staining, isotype controls for anti-IgG$_1$ and anti-IgG$_{2\alpha}$ were introduced in the preliminary procedure to set up photomultiplier and instrument technical parameters; a control which used a staining procedure carried out without introducing in the assay system the fluorochrome of interest was also performed.

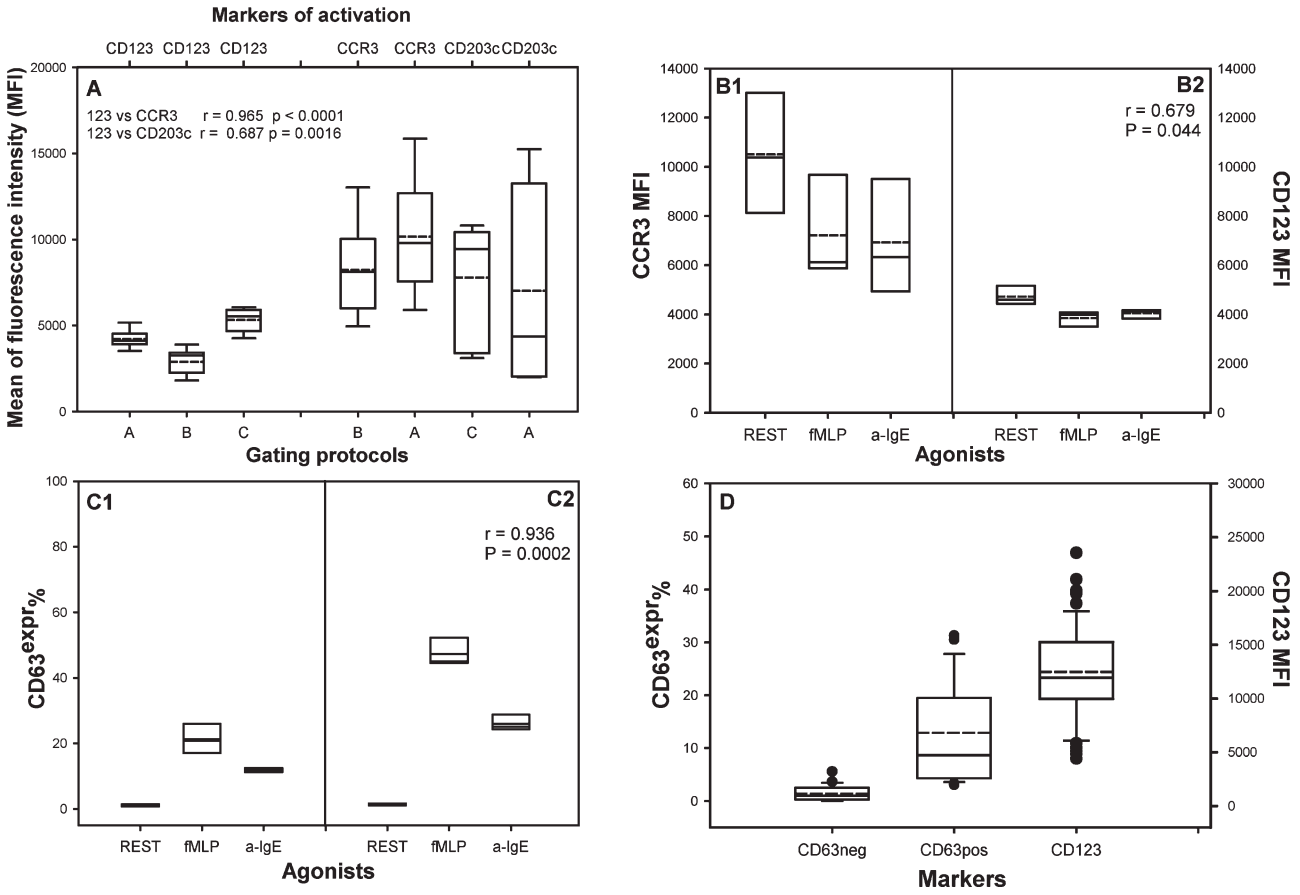

Figure 2. Panel A: Box plot distribution of CCR3, CD203c, and CD123 MFI from three separate experiments in which cells were gated by using the different protocols indicated in x-axis. In top x-axis those markers evaluated, as MFI, throughout the activation steps are indicated. Each box plot evaluates data from resting, fMLP-activated and anti-IgE-activated basophils (nine replicates/experiment). Panel B: A typical experiment/9 performed selected by a randomized approach in which CCR3 variability with activation (Fig. 1B1) compared to CD123 (Fig. 1B2) is shown. Basophils were gated with protocol B (B1) or with protocol A (B2). Panel C: Two typical experiments selected by a randomized approach in which box plots of $CD63^{expr}$% variability with activation are shown. Basophils were gated with different protocols (protocol B, Fig. 1C1; protocol A Fig. 1C2). Panel D: Variability of CD123 and of CD203c compared to a classical activation marker CD63. Each box plot reports data from nine separate triplicate experiments (27 replicates) and evaluating two activation conditions (resting and anti-IgE = total 54 replicates). Basophils were gated using the routine protocol A. Mean of fluorescence intensity (MFI) for each fluorochrome-labeled monoclonal antibody was calculated automatically by the cytometer software by averaging the total fluorescence of the marker in the basophil gate. Percentage of CD63 activated cells ($CD63^{expr}$%) was calculated by the software considering the CD63-FITC bright cells counted to the right of a threshold that was established including the main peak of fluorescence of a sample of resting cells. To reduce standard deviation due to brightly fluorescent cells compared to dimly fluorescent ones, a logarithmic scale and the coefficient of variation to measure variability dispersion were used. Data were described by plotting them as box plots using the Sigma plot 10 software. Box plots area was defined by 25–75% confidence limits, indicating median (solid line) or mean (dashed line) and represented one typical triplicate experiment/9 performed collecting data from three different conditions of cell activation. Statistics analysis was performed with the software SPSS, version 11 for Windows, Chicago, IL. Kolmogorov-Smirnov and Shapiro-Wilk goodness-of-fit tests were performed to determine whether the sample population followed a Gaussian distribution. Regression analysis and Durbin-Watson test were applied to evaluate effects related to different methods. Differences were analyzed by using a one-way analysis of variance (ANOVA) followed by Fisher LSD test. A value of $P < 0.05$ was considered statistically significant.

$CCR3^{pos}$cells are basophils (11). So, operator has to move the gate to capture basophils but, when doing so, he must face these technical issues. The evidence here reported was confirmed by other experiments performed in our laboratory aiming at assessing also the reliability of $CD45^{dim}$/ $CCR3^{pos}$ gating in BATs. The importance of a well suited flow cytometry (FC) protocol to investigate leukocytes is of paramount importance. Some evidence has stressed that FC was better than standard cytology in detection and quantification of circulating blast cells or immature granulocytes, with a first lineage orientation in the former case. All cases of lymphocytosis, with lineage assignment, were detected by FC (12).

In conclusion, these results lead to two main considerations. Taking into account the huge variability in phenotyping markers expression and in responsiveness performed by human basophils, a stable bright PE-conjugated marker, better if associated to a negative one to isolate basophils, is allowed; nevertheless evidence suggests that it is very difficult to gate basophils in blood using a single marker. Single markers, such as CCR3 or CD203c, have to be associated to an SSC plot and

this may raise issues concerning other cellular contaminants, mainly because basophils possess side scatter/forward scatter features within the lymphocyte area. The single marker should be highly specific and very stable, but at the same time it should allow a stability at least comparable to that observed here with CD123, to attain a reliable performing of the activation testing, which is decisive for allergy diagnosis.

So, which gating strategy would it be best to use? To date there are no valid protocols to capture basophils in flow cytometry using a single highly specific, highly expressed (bright) stable marker. Better purification of cells would advice to use CD45 to remove $CD63^{pos}$ platelets, cell debris and residual erythrocytes and the involvement of at least two phenotyping markers: CCR3/CD3, CRTH2/CD3, CD123/HLADR, CD14/CD13, etc. Faucher used a six markers/five colors to phenotype leukocyte with the CD36-FITC/CD2-PE+CRTH2-PE/CD19-ECD/CD16-Cy5/CD45-Cy7 combination: this protocol was able to detect 12 different circulating cell types and among them 11 were quantified. With this approach detection of quantitative abnormalities of whole leukocytes, neutrophils, eosinophils, basophils, monocytes, or lymphocytes was comparable by FC and by standard cytology in terms of sensitivity and specificity (12). Hence polychromatic flow cytometry is the way. Many protocols of basophil electronic capture include a minimal protocol made up of three fluorochrome-conjugated monoclonals, and CD45/CD123/HLADR might prove a good solution for gating basophils in BATs.

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Literature Cited

1. Chirumbolo S, Vella A, Ortolani R, De Gironcoli M, Solero P, Tridente G, Bellavite P. Differential response of human basophil activation markers: A multi-parameter flow cytometry approach. Clin Mol Allergy 2008;6:12–25.
2. Sturm EM, Kranzelbinder B, Heinemann A, Groselj-Strele A, Aberer W, Sturm GJ. CD203c-based basophil activation test in allergy diagnosis: Characteristics and differences to CD63 upregulation. Cytometry B Clin Cytom 2010;78B:308–318.
3. de Weck AL, Sanz ML, Gamboa PM, Aberer W, Bienvenu J, Bianca M, Demoly P, Ebo DG, Mayorga L, Monneret G, Sainte-Laudy J. Diagnostic tests based on human basophils: More potentials and perspectives than pitfalls: II Technical issues. J Invest Allergol Clin Immunol 2008;18:143–155.
4. Hennersdorf F, Florian S, Jakob A, Baumgartner K, Sonneck K, Nordheim A, Biedermann T, Valent P, Buhring HJ. Identification of CD13, CD107a, and CD164 as novel basophil-activation markers and dissection of two response patterns in time kinetics of IgE-dependent upregulation. Cell Res 2005;15:325–335.
5. Han X, Jorgensen JL, Brahmandam A, Schlette E, Huh YO, Shi Y, Awagu S, Chen W. Immunophenotypic study of basophils by multiparameter flow cytometry. Arch Pathol Lab Med 2008;132:813–819.
6. Buhring HJ, Streble A, Valent P. The basophil-specific ectoenzyme E-NPP3 (CD203c) as a marker for cell activation and allergy diagnosis. Int Arch Allergy Immunol 2004;133:317–329.
7. Hausmann OV, Gentinetta T, Fux M, Ducrest S, Pichler WJ, Dahinden CA. Robust expression of CCR3 as a single basophil selection marker in flow cytometry. Allergy 2011;66:85–91.
8. Monneret G. CCR3 for basophil activation test: a necessary but insufficient step. Clin Exp Allergy 2010;40:953.
9. Chirumbolo, S. The use of IL-3 in basophil activation test is the real pitfall. Cytometry B Clin Cytom (in press). Sept. 15, 2010; DOI: 10.1002/cyto.b.20570.
10. Gane P, Pecquet C, Crespeau H, Lambin P, Leynadier F, Rouger P. Flow cytometric monitoring of allergen induced basophil activation. Cytometry 1995;19:361–365.
11. Gane P, Pecquet C, Lambin P, Abuaf N, Leynadier F, Rouger P. Flow cytometric evaluation of human basophils. Cytometry 1993;14:344–348.
12. Faucher JL, Lacronique-Gazaille C, Frebet E, Trimoreau F, Donnard M, Bordessoule D, Lacombe F, Feuillard J. "6 markers/5 colors" extended white blood cell differential by flow cytometry. Cytometry Part A 2007;71A:934–944.