VIA MEDICA

ORIGINAL RESEARCH

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Using a $CD45^{dim}/CD123^{bright}/HLA\text{-}DR^{neg}$ phenotyping protocol to gate basophils in FC for airway allergy. CD123 does not decrease

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Abstract

Physicians in the field of respiratory medicine are particularly concerned about the availability of a reliable diagnostic tool to investigate respiratory allergy. Usually, basophils are easy to obtain from peripheral blood and therefore they represent a reproducible model to assess allergy in individuals. Cell assays called basophil activation tests (BATs) are widespread tools for allergy diagnosis and are easily available in most of the medical labs. The strategy by which basophils are captured in a flow cytometry protocol has met many suggestions, recommendations and experimental novelties in recent years, yet the debate needs to be further expanded. Concerns still remain about the suitability of the many approaches to make the basophil activation test (BAT) an excellent and practical tool to diagnose allergy, while improving its analytical performance. This technical report describes the methodological aspects of the use of the protocol adopting the panel $CD45^{dim}/CD123^{bright}/HLA\text{-}DR^{neg}$ to gate basophils in flow cytometry, trying to highlight the main biases related to an incorrect use of this protocol.

Key words: basophil activation test, allergy, CD123, flow cytometry

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Introduction

Airway allergy is a major cause of exacerbation of respiratory pathologies and in this sense a reliable laboratory tool is fundamental to achieve a correct allergy diagnosis. In the very recent years, the basophil activation test (BAT) has become an outstanding tool to diagnose allergy, though still with some contradictory opinion [1]. The way by which basophil activity is investigated in a BAT involves the adoption of flow cytometry methods, which are generally suited to capture cells in a virtual electronic approach, not physically, and therefore this might generate bias and misinterpretation on the real nature of the events dotted in a gated channel. In few words, flow cytometry shows and counts cells (appearing as electronic "dots" in a software screen) on the basis of their marker-associated fluorescence, so this is an indirect approach to "see" physical cells" passing though a laser detection device. Events and dots in a dot plot are not necessarily physical single cells, therefore, but their nature depends on the features of the fluorochromes-associated markers, usually expressed on the membrane of the scattered cells, then depending on the complex biology of the same leukocytes. If we could express this concept with a simplistic example, markers are molecules usually up- or down-regulated upon activation, which are actually located on the cell membrane and are easily targeted by a fluorochromes-conjugated monoclonal antibody, in order to render the marker "visible" to the laser system of the flow cytometry devices and then to the operator. The same can be said for markers used to detect where basophils

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are (phenotyping), when a complex population of cells (whole blood) is examined in flow cytometry. This approach would mean that the "amount" of markers detected depends on the antibody and the fluorochromes performance and also on the expression levels of the markers during the assay. This issue represents the main technical concern of a BAT. A first reason is because researchers are endeavoured in finding stable and highly specific markers in order to phenotype basophils and separate them from other cells in a complex milieu such as the whole blood, second because in a BAT basophils perfectly distinguished from other cells have to be followed up by some highly specific markers that should show better than other ones the cell dynamics of the allergy activation [2]. In few words, to "see" (phenotype) basophils in flow cytometry, researchers needs stable membrane markers, which do not change their expression during the cell activation. Different markers should be used to "measure" this activation. Due to the very complex matter addressed in this field, the debate is particularly crowded of different and often controversial comments, often prompted to expand the discussion in the scientific community.

In a recent paper, for example, some authors addressed the reliability of a flow cytometry protocol used to phenotype basophils from peripheral blood samples. This protocol included a) a phenotype-model made by fluorochrome-conjugated monoclonal antibodies against CD123 (the IL-3 receptor alpha-subunit) and HLA-DR (a MHC class II molecule) arranged to gate basophils and b) the simple activation marker CD203c to assay basophil activation. The authors sustained the opinion that the use of CD123 as a single, specific marker to capture basophils as $CD123^{pos}$ cells, should lead to an underestimation of basophils upon cell activation [3, 4]. They concluded that, taking into account gating approaches that are based solely on CD123, the performance should lead to a loss-to-analysis of basophils, particularly of the ones that highly express CD63 and CD203c, i.e. a cellular loss in flow cytometry upon cell activation, resulting in a false-negative outcome for the test[4]. In particular, CD123 is down-regulated following basophil activation [4].

We believe that the gating strategy using CD123 merits some further insight. Previous reports have tried to elucidate the role of this marker in the phenotyping of basophils for a BAT. Therefore, after having come through these interesting observations and comments [4], we decided to report some technical notes on the $CD123^{bright}$/$HLA\text{-}DR^{neg}$ protocol, which we have adopted in our research in association with the CD45, in order to give a constructive contribution on the issue[5, 6]. The new protocol, which allows to gate basophils as $CD45^{dim}$/$CD123^{bright}$/$HLA\text{-}DR^{neg}$ cells, is discussed in this article.

Material and methods

Blood collection

Basophils were collected from peripheral whole blood of healthy blood donors in sodium citrate anti-coagulated specimens from the routinely running course of the blood transfusion unit . When specified, peripheral whole blood samples were collected in TruCount™ tubes (Becton Dickinson, CA, USA), according to the indicated procedures and all vials processed within 4 hrs.

MoAbs, fluorochromes and staining procedures

The following monoclonal antibodies conjugated with fluorochromes, i.e. CD8-ECD; CD4-PC7; CD45-APC750; CD3-APC700; CD16-Pacific Blue; CD19-KRO; CD38-PC5, CD203c-PE. were from Beckman Coulter, Pasadena, CA, USA. The following ones, CD123-PeCy5; HLA-DR-APC, CD9-FITC; CD63-PE, from BD-Pharmigen Becton Dickinson, San Josè, CA, USA. According to the Mo-Abs producer's specifications, an aliquot of 100 μl was incubated for total 30 minutes and when requested activation was carried out according to the procedures described in the text. About 10 minutes before ending the activation step (stopping reaction), the indicated MoAbs panels were added, following separate protocol patterns. Subsequently reaction was stopped by erythrocyte lysing and sample fixation by using the Coulter ImmunoPrep® Reagent used with the Coulter TQ-Prep Workstation.

Equipment, FC devices and technical performance

Analyses were performed with a Beckman Coulter Navios™. FL1-FL10 channels, with three lasers, i.e. a) Blue Solid State Diode: 488 nm, 22 mW laser output; b). Red Solid State Diode: 638 nm, 25 mW laser output; c) Violet Solid State Diode: 405 nm, 40 mW laser output. In the scatter analysis the device allowed the resolution process of 0.404 μm diameter particles from background noise using forward scatter with maximum detection up to 40 μm diameter particles. Its flow rate specification (high yield 25,000 events/sec) was ensured by the continuous pressure applied to

the sample tube based on user selected flow rates: Standard: 780 ml/hour with a carryover: < 0.1%.

Software and data elaboration

Compensation followed cytometer manufacturer's instruction according an off-line procedure by applying automated electronics algorithms and preset templates, by using bi-parametric logarithmic dot plots, gate-specific tubes and single-tube data analysis, and optimizing FSC threshold and fluorochrome voltage as set up parameters. Beckman Coulter Navios Tetra System's fully automated instrument set-up. Flow analysis was performed using the laser indicated system with not less than 10,000 events/sec capability, where the throughput of 10 k normal lymphocytes is 80 tubes/hour. Mean of fluorescence intensity (MFI) for each fluorochrome-labeled monoclonal antibody was calculated automatically with the cytometer software by averaging the total fluorescence of the marker in the basophil gate. As well percentage of activated cells was calculated by the software considering the CD63-PE bright cells counted to the right of a threshold that was established including the main peak of fluorescence of a sample of resting cells. In order to reduce standard deviation due to brightly fluorescent cells respect to the dimly/negative ones, a logarithmic scale and the coefficient of variation to measure variability dispersion were used. When the test needs further controls, Bigos' formula to normalize brightness over background was also applied [7].

Controls

In order to evaluate fluorochrome unspecific staining, isotype controls for anti-IgG_1 and anti-IgG_{2a} were systematically introduced in the preliminary procedure to set up photomultiplier and instrument technical parameters; when necessary. A control using a staining procedure carried out without introducing in the assay system the fluorochrome of interest was also performed

Statistics

When described, mean ± standard deviation (SD) were reported. Parametric distributions, when assessed following the Shapiro-Wilk test, were evaluated in an ANOVA test (Tukey post hoc analysis), with $p < 0.05$. Data were elaborated with an SPSS v. 22.0.0.0 software.

Results

Figure 1 shows the electronic capture of basophils and their purification in flow cytometry, according to this approach. Events in the SSC^{low} region of the SSC/CD45 plot were selected from the $CD45^{dim}$ area (Fig. 1A) and this region was subsequently gated for the expression of CD123 and HLA-DR (Fig. 1B). A distinct events area, expressing CD123 at least ten times higher than other cells and negative for HLA-DR expression (indicated as basophils in Fig. 1B), is also $CD4^{neg}$, $CD8^{neg}$, $CD9^{pos}$, $CD16^{neg}$, $CD3^{neg}$, $CD19^{neg}$, $CD38^{bright}$, $CD203c^{pos}$ (Fig. 1, panels C-H). Therefore, the $CD123^{bright}$/$HLA\text{-}DR^{neg}$ gated events from the $CD45^{dim}$ area are purified basophils[6,8]. Although basophils were gated as $CD123^{pos}$/$HLA\text{-}DR^{neg}$ cells also in a paper by other authors published elsewhere [4], showing a perfect distinct group of $CD123^{pos}$/$HLA\text{-}DR^{neg}$ events (see Fig. 1 in ref 4), it is still possible that the gate contains $CD45^{pos}$ or $CD45^{bright}$ cells expressing CD123, as they are within the SSC^{low} region. We therefore approached this issue.

Figure 2 shows that when selecting basophils from the SSC^{low} region of the SSC/FSC gate, one might potentially exclude activated (and degranulated) cells while this bias is prevented by selecting a $CD45^{dim}$ area for the CD123 gate. When cells are selected only through a SSC/ /CD123 panel, some basophils might be excluded from the analysis. In particular, panel E of Figure 2 shows that in the SSC/FSC selected area, only the $CD123^{pos}$ gating (red dots) was included in the area, while many $CD123^{bright}$ events (blue dots), probably being activated or degranulated basophils, were excluded (Fig. 2 panel E).

Furthermore, we observed that upon stimulation with 10^{-7} M formyl-L-Met-L-Leu-L-Phe (fMLP) the correct count of cells in the sample, gated as $CD123^{bright}$/$HLADR^{neg}$ cells, according to the previously described method of ours using the $CD45^{dim}$ population, did not significantly change respect to the resting, non activated basophils, as calculated using BD-TruCount™ tubes (Becton Dickinson, San Jose, CA, ref. 340334, lot 15064). Figure 3 shows an exemplificative result of six different triplicate samples (1461.5 ± 53.9 SD resting cells, 1430.7 ± 65.1 SD activated, $p = 0.65$). This result should confirm previously reported evidence about the stability of the CD123 marker upon basophil activation.

Discussion

A first, major concern about the suitability of the protocol using the only CD123, as previously reported [4], deals with the kind of strategy actually used to perform the electronic capture and purification of basophils in flow cytometry,

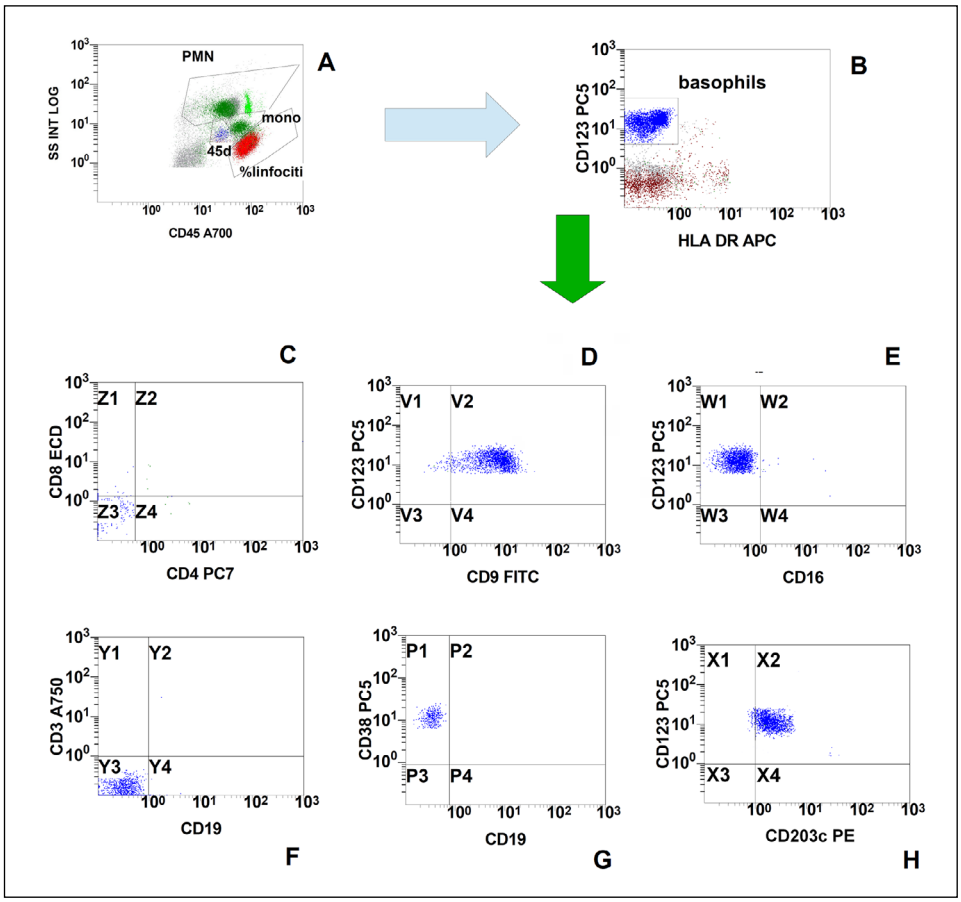

Figure 1. Gating strategy and electronic purification of basophils starting from a $CD45^{dim}$ selected area, exemplificative experiment of five on sodium citrate anticoagulated whole blood samples. The region indicated as 45d (**A**) was gated for the expression of membrane CD123 and HLA-DR and a separated area of events (indicated as basophils in the Figure) was recovered (**B**). This area was $CD4^{neg}$, $CD8^{neg}$ (**C**), $CD9^{pos}$ (**D**), $CD16^{meg}$ (**E**), $CD3^{neg}$, $CD19^{neg}$ (**F**), $CD38^{bright}$ (**G**), $CD203c^{pos}$ (**H**).

Samples were treated with N-formyl-L-Met-L-Leu-L-Phe (fMLP) 10^{-7} M when requested and incubated for a total of 30 minutes, where 10 minutes before the end of the incubation fluorochromes-conjugated MoAbs were added according to the producer's recommendation and standardized lab protocols. Subsequently reaction was stopped by erythrocyte lysing and sample fixation procedure by the use of the Coulter ImmunoPrep® Reagent used with the Coulter TQ-Prep Workstation for FC run according to the Beckman Coulter Navios™ flow cytometer, with FL1-FL10 detectors. Internal controls and compensation process were accomplished according the instrumental specifications (see text for details)

i.e. to separate and identify (purify) basophils in the gate. The simple use of CD123 as a gating marker [3, 4], without introducing in the panel further phenotyping markers such as HLA-DR, is usually performed following the steps below, in the gating strategy: a) selection of a region in the SSC^{low} events area (lymphocyte-monocyte region) in the SSC/FSC plot; b) gating the $CD123^{expressing}$ region (i.e. $CD123^{pos}$) of the selected SSC^{low} area; c) purify cells by selecting events expressing CD123 [3, 4]. This might be considered quite sufficient to purify basophils as cells expressing CD123, which later should show also the expression of CD203c, which is exclusively expressed by these cells [3, 4]. HLA-DR might be used, therefore, simply as a confirmatory marker of the gating process, as basophils are always negative for the expression of HLA-DR [8]. Therefore, some authors are used to select an area in the SSC^{low} region of the side scatter/forward scatter (SSC/FSC) plot and then gate this area simply for the expression of CD123. To assess that events are representative of basophils, events within the $SSC^{low}/CD123^{pos}$ gate are then examined for the expression of HLA-DR and a conclusion that the $CD123^{pos}$ and $HLADR^{neg}$ area includes only basophils should emerge [3, 4].

However, any of these approaches should contain some possible bias, in our opinion.

Fundamentally, basophils are $CD45^{dim}$ cells [6, 8], an issue which has never been previously addressed [3]. The SSC/CD45 plot is preferable respect to the SSC/FSC one in generating the first selection area, because it reduces technical bias. In our opinion, any selected area in the SSC^{low}

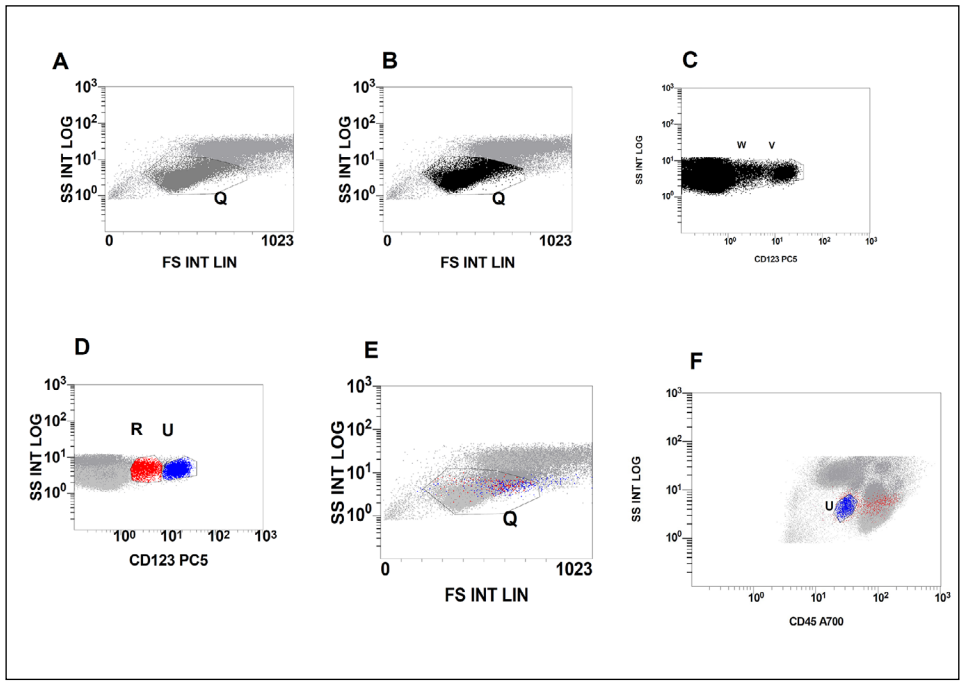

Figure 2. Simulation of the gating strategy with only CD123^{pos} method compared with the CD45^{dim} approach, as described in the text on healthy donor blood samples. (**A**). A region in the SSC/FSC gate, encompassing a wide FSC area may presumably e included in a SSC/CD123 gating strategy. This region is therefore selected (**B**) and read for the expression of CD123 (**C**). A selection of the CD123^{pos} region can be gated (see ref 4) but this gated region may include two separate areas (W and V), identified in our simulation as R (CD123^{pos}) and U (CD123^{bright}) (**D**). Panel E shows that in the SSC/FSC selected area, only the CD123^{pos} gating was included in the area, while many CD123^{bright} events, probably associated with activated or degranulated basophils, were excluded (**E**). If the same process is performed by starting with a CD45^{dim} selection, only CD123^{bright} cells are included in the area (**F**). These CD123^{bright} events represents basophils, inasmuch both resting and degranulated cells are captured if considering a CD45^{dim}-CD123^{bright}-HLADR^{neg} protocol. A percentage of degranulated cells usually occurs due to aspecific activation related to handling and pre-analytical procedures; SSC — side scatter; FSC — forward scatter

region might contain many ungated CD45^{pos} cells expressing CD123 and HLA-DR at various levels, such as T and mature B lymphocytes, monocytes, pre-B cells, CD56^{pos}-NK cells, myeloblasts, plasmacytoid dendritic cells and basophils [4, 8]. Without the introduction of a Pan-Leu CD45 marker, most probably many of these cells are captured when selecting the SSC^{low} region, as indicated by our results, causing bias in the basophil capture [4]. With the exception of basophils, which are CD123^{bright}, the expression of CD123 in any of these cells is not completely negative [8]. Therefore, we believe that basophils need to be initially captured from the CD45 dimmy population in the SSC^{low} area of a SSC/CD45 plot, in order to reduce this cross-contamination and improve their electronic purification [8], as reported also in a past paper of ours [6].

Furthermore, basophils express markers such as CD9, CD13, CD22, CD33, CD36, so they are CD25^{dim} and CD38^{bright}, besides to be CD45^{dim}. In addition, they do not express CD19, CD16, CD4, CD8, CD3 and are actually CD45-dimmer than lymphocytes and CD45-brighter than myeloblasts, which would mean than in a SSC^{low}/CD45 gate, basophils are at the left side respect to lymphocytes and right to CD34 and CD117 positive cells (myeloblasts), which are CD45dimmer than basophils, i.e. the CD45 expression order in the SSC/CD45 plot is myeloblasts-basophils-lymphocytes. All these cells express CD123 and HLA-DR at different levels on the membrane, from negative to positive (see also Figure 1A red area). Notoriously, T-cells and mature B-cells are negative or very low expressing CD123 and myeloblasts are positive for CD123 but dimmy (i.e.CD123^{dim}), so they could affect basophil count as simply CD123 positive cells, although they are also HLA-DR positive. Any gate performed by using initially the SSC/FSC plot, most probably introduced cells with high SSC, such as lymphocytes and monocytes, which are CD123 weak positive cells but are not CD45^{dim} and do not express CD203c. As detailed in the Results section, Figures 1 and 2 would contribute in describing this issue. Therefore, in this report, we assessed that CD123^{bright}/HLA-DR^{neg} gated events from the CD45^{dim} area are quite presumably purified basophils [5, 6].

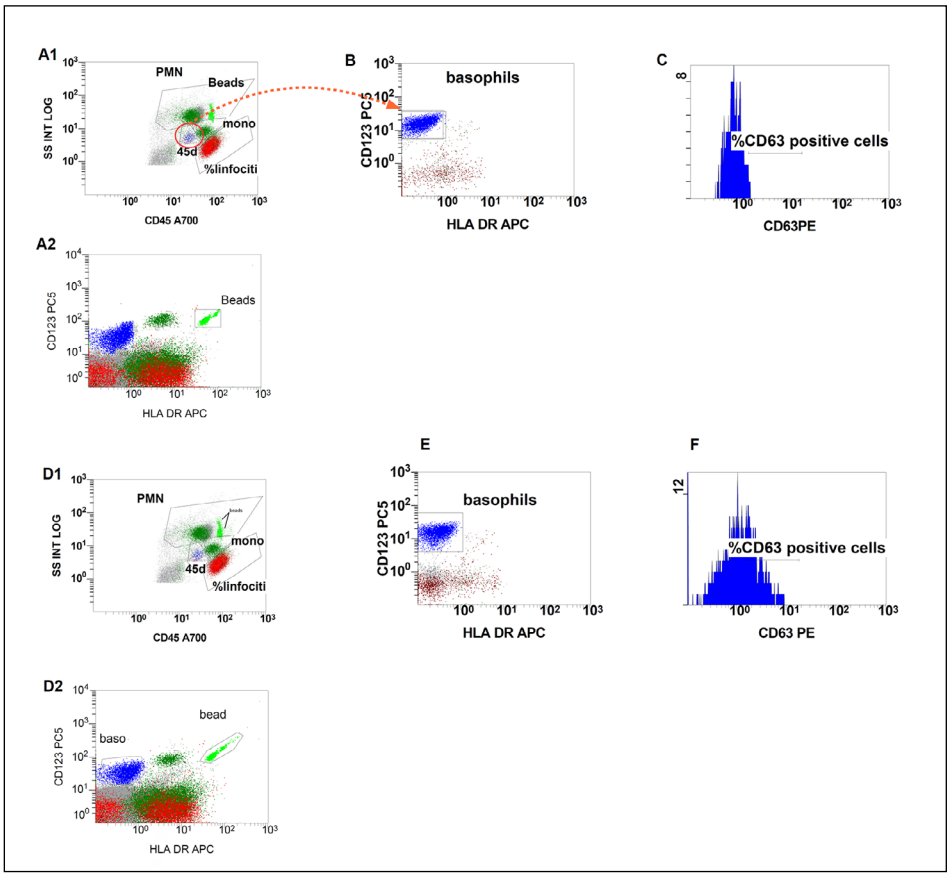

Figure 3. Exemplificative experiments showing the absolute cell count in the CD123^{bright}/HLA-DR^{neg} gate using BD TruCount™ tubes. Initially, a CD45^{dim} area was selected from TruCount™ tubes containing blood samples from healthy volunteers (**A1**). Events from this CD45^{dim} area were gated for the expression of CD123 and HLA-DR (**A2**) and values of beads considered for the calculation of the absolute count of basophil in the gate. Basophils as CD123^{bright}/HLADR^{neg} events (**C**), were evaluated for the expression of CD63% (**D**). Cells treated with 10^{-7} M fMLP for 30 min at 37°C, then stained and blocked as explained in the text, were introduced in the same test, using BD TruCount™ tubes (**D1, D2, E, F**). Calculations made according to the BD TruCount™ methods (beads = 48700), gave results discussed within the text. FC performance with Beckman Coulter Navios™ flow cytometer, with FL1-FL10 detectors. Internal controls and compensation process were accomplished according the instrumental specifications (see text)

Furthermore, the use of the simple CD123, particularly when not associated with the more specific marker CD203c, has been reported to cause some further concern, namely the presumptive reduction of CD123 during cell activation. Fundamentally, an up- or down-regulation of a marker may not result in an over- or underestimation of the count of the population. A possible reduction may be induced by experimental conditions, such as an incorrect erythrocyte lysis. However, the effect of some lysis reagent, such as Pharmlyse, was recently compared with other lysis solutions and does not seem to cause bias in the leukocyte count in FC [9], although previous reports claimed a possible impact on immuno-phenotyping caused by different lysis protocols and reagents [4, 10].

As demonstrated by results in Figure 3, we observed that upon stimulation with 10^{-7} M formyl-L-Met-L-Leu-L-Phe (fMLP), a bacterial-derived product, the correct count of cells in the sample, gated as CD123^{bright}/HLADR^{neg} cells, according to the previously described method using the CD45^{dim} population, did not significantly change respect to the resting, non activated basophils, as calculated using BD-TruCount™ tubes (Becton Dickinson, San Jose, CA, ref. 340334, lot 15064). This result should confirm previously reported evidence about the stability of the CD123 marker upon basophil activation [5, 6]. Besides the technical process, lysis reagents, pre-analytical bias, also the imaging approach might lead to bias and the interpretation of a CD123 downloading upon activation, if cells are not correctly gated as CD45^{dim} leukocytes [4]. For example, the use of density-weighted diagrams may generate some misinterpretation in the reading of CD123^{pos} cells, causing an apparent loss of events. Yet, a reliable normalized functional measure of reagent brightness is the so-called

"stain index", which is defined as D/W, where D is the difference between positive and negative cell populations, and W is equal to 2 SD of the negative population. Therefore, the way by which plots are shown might create bias in the selection process. A further bias is that events in the plot cannot be directly considered cells, at least when counted in a gate. As events depend on fluorescence performance, the methodological approach of flow cytometry, miight play a major role also in this debate. Furthermore, the different choice of fluorochromes may affect test performance, particularly if used as conjugates of phenotyping markers. It is probably a good idea to use a fluorochrome-combination which does not interfere with the signal of the activation markers used, for example: CD123-APC, HLA-DR (and lineage markers)-PacBlue and CD203c-PE.

In this respect, when the same antibody is conjugated to various dyes, their stain indexes may be compared to reach an idea of the relative brightness of the dyes on a particular FC instrument. For example, on BD instruments, FITC has a stain index of about 50, PerCp-Cy5.5 ≈ 80 while PE ≈ 250 but FITC has a relevant spillover on PE, which is usually conjugated with the activation marker CD203c [1, 11, 12]. moreover, it is widely known that, in order to optimize gating, bias from fluorescence spillover must be considered if the compensation process has not been properly done. This should not be a fundamental concern in a CD123-based gating strategy, as previously reported [4], but it may have an effect if the gating marker is widely shared with non-basophil cells. Levels and brightness of fluorochromes in different companies might also cause differences in the observed effects by different research groups and in conclusion expression of basophil markers and BAT performance depend on a good gating approach [2].

According to some author, activation may even up-regulate HLA-DR and down-regulates CD123 in basophils, yet these cells are notoriously negative for HLA-DR membrane expression [4, 5, 13]. If CD123 is really down-regulated by activation, it would be very interesting to assess if a gating strategy using CCR3 (CD193), the eotaxin receptor, might bear the same concern reported by others elsewhere [3, 4], as CCR3 appears to be down-regulated upon basophil activation [2, 14, 15]. Interestingly, some authors reported a paper where a phenotyping protocol using a combination of CD123 and CCR3 markers, improved significantly the performance of BAT, respect to the only CD123 [16].

This approach should affect also the way by which any activation marker is interpreted to evaluate activation in the *in vitro* basophil test. Which is therefore the bet way to gate and investigate basophils? This may depend also on those markers used to test activated basophils.

Some authors gated events from the lowest region (SSC^{low}) of the SSC/FSC plot for the CD203c expression, which is notoriously an activation marker, and realized that these events included only $CD123^{bight}$/$HLA\text{-}DR^{neg}$ cells, i.e. basophils [4]. Then, by comparing the activation behaviour with CD63 and CD203c of either cells gated as $CD123^{pos}$/$HLA\text{-}DR^{neg}$ or as $CD203c^{pos}$, no significant difference was reported [4]. Someone therefore concluded that the introduction of the CD203c marker to gate basophils, should improve the diagnostic accuracy of BAT from 91% to 97% [3, 4, 17]. Therefore, according to the different authors, the reliability of the CD123/HLA-DR protocol seems to need the inclusion of CD203c in the phenotyping process, in order to better ensure the electronic capture of basophils during the activation step and reduce the apparent cell loss in the gate due to CD123 down-regulation [3, 4].

However, we have assessed with BD TruCount™ tubes that CD123 does not change its expression during basophil activation, yet evidence that CD203c moves its expression pattern upon activation was already reported [6]. This trend confirmed previous data from our lab, performed with a BD-FACScanto flow cytometer [5, 6]. While basophils remained $HLA\text{-}DR^{neg}$, despite the different kind of stimuli adopted, CD123-PECy5 showed always a bright appearance, with MFI values in the range 10^4– 2×10^4, i.e. at least ten (10^1) times the basal positive expression of CD123 and with the absence of significant regression in a linear regression test from the CD123-PECy5 expression in resting, non activated control. This trend was confirmed also for the basophil events dots in the gate [6]. On the contrary, if events are gated with a CD123-FITC from the SSC^{low} region of the SSC/FSC plot, instead from a $CD45^{dim}$ area, all CD123-expressing cells cannot be considered only basophils and during the evaluation of CD63 or CD203c, an apparent loss can be observed due to the inability of the non basophilic CD-123-expressing cells to express and up-regulate CD63 or CD203c.

Finally, a loss of degranulated basophils was also demonstrated (see Fig. 2). These non basophilic cells, previously included in the gate, should show a low or dimmy expression of CD123, leading to a possible misinterpretation (compa-

re Fig. 2E, F). Apparently, the introduction of CD203c to gate events from an SSC/FSC plot and then purifying these cells as $CD123^{bright}$/$HLA\text{-}DR^{neg}$, might appear pleonastic, as BAT diagnostic approaches based on CD203c as the only phenotyping marker are reported in the literature [18]. CD203c is up-regulated during either an IgE-mediated or a non-IgE mediated activation [6]. Using CD203c as a phenotyping marker should prevent its use as an activation marker, although the authors showed its use both in gating and activation [4]. During the evaluation of the expression of CD63 in CD203c gated cells, the fluorescence intensity distribution of the CD203c used as an activation marker, may be completely shifted to higher values in activated cells respect to non activated ones [5] and this may prevent a correct evaluation of the percentage of $CD63^{pos}$ cells (calculated on the total gated basophils) [5, 19].

Basophils gated with CD203c-PE and then purified as $CD123^{bright}$/$HLA\text{-}DR^{neg}$, when evaluated for the expression of CD63 and CD203c upon activation with formyl-L-Met-L-Leu-L-Phe (fMLP), show an increase in the number of basophils expressing CD63 [4, 6]. This circumstance, shown by using the only CD123, is perfectly comparable to the one previously published where the simple gating protocol $CD45^{dim}$/$CD123^{bright}$/$HLA\text{-}DR^{neg}$ was adopted, practically suggesting that the introduction of CD203c is quite useless [4, 6].

Conclusions

These results should confirm that CD123 is expressed by basophil in a bright, constitutive form and probably changes in a very negligible way upon either an IgE- or non IgE-mediated activation.

According to our opinion, the best performance process should encompass the following bullet points:

1) Basophils should be gated from an initially selected $CD45^{dim}$ region in a SSC/CD45 plot, that includes also activated ("degranulated") basophils, contrarily to a SSC/FSC plot;
2) The expression of CD123 does not change the absolute basophil count in the gate upon activation, as assessed with TruCount™ tubes;
3) The use of CD203c within the CD123/HLA-DR phenotyping protocol upon activation with fMLP resulted in a CD63/CD203c dot plot perfectly similar to the one obtained by gating basophils only with a CD123/HLA-DR approach (compare Fig. 5b ref 4 with Fig. 4 ref. 6).
4) CD203c used as a gating marker may cause bias in the apparent basophil count in the gate upon activation and cannot be used at the same time as an activation marker.

Our final opinion, confirmed by experimental tests, is that CD123 does not significantly change upon basophil activation and that bias in the cell count are mainly generated by gating basophils from a SSC/FSC and by gating cells with activation markers.

The reported decrease in cell count in the gate might be an artefact due to the gating process and it is not confirmed by our studies [3, 4].

BAT is a cellular assay used to ameliorate our ability to diagnose allergy and hypersensitivity reactions and therefore the debate about its reliability and suitability for clinics still represents a fundamental activity of current research in allergy and immunology. Further research should improve our knowledge about basophil biology and its role in the immune system.

Conflict of interest

The authors declare no conflict of interest.

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