

Basophil Activation Test: Implementation and Standardization Between Systems and Between Instruments

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• Abstract

The basophil activation test (BAT) is a good ex vivo alternative for measuring hypersensitivity to an allergen in sensitized patients but still lacks standardization. In this present study, we have implemented one of the systems and proposed inter-systems, inter-instrument standardization. Our method for basophil activation and labeling on whole blood: EDTA in one step using BasoflowEx[®] and FlowCast[®]. Setup on Navios and fluorescence targets converted to set up FACSCanto[™] instrument. Our results: 1) A CD203c/CD63 (BasoflowEx) method was adapted for EDTA samples and simplified. 2) Final washing and concentration and use of time parameter help acquiring as many basophils as possible, spare acquisition time and noise. 3) The modified method was validated according to ISO15189 with a precision at 5.1% RCV, linearity between 1 and 1/8 of anti-IgE stimulation. Results were very close with CCR3/CD63 system (Flow-Cast). 4) Standardization, between systems and even between instruments. Mean Fluorescence Intensity targets are proposed using standard beads (Cytocal[®]) middle peak: FITC = 19.4; PE = 28.8 on Navios[®] corresponding to FITC = 4,966; PE = 7,373 for FACSCanto. Data analyzed on common software (Kaluza[®]) were very closely correlated. 5) Co-labeling of B cells (CD20+) gives the possibility to monitor a significant drop of basophils under stimulation that could explain some underestimation in case of strong hypersensitivity. In conclusion, BAT would strongly benefit from easy implementation [EDTA, one step stimulation/labeling, wash, full sample analysis over time parameter, B cell relative basophil count] and standardization of instrument settings on MFI targets whatever system or instrument is used. © 2017 International Society for Advancement of Cytometry

• Key terms

flow cytometry; basophil activation test; standardization; degranulation; allergy

INTRODUCTION

Diagnosis of allergy is based on skin tests, but these tests are not always possible because of skin status or because the risks would be too high to challenge the patients who have had a previous severe reaction to allergen. The Basophil Activation Test (BAT) is a good ex vivo alternative for measuring IgE mediated hypersensitivity to allergens in sensitized patients, without exposing the patient to skin tests risks (1–5). The BAT is based on detection of phenotype changes of allergen induced basophil degranulation. Different protocols have been developed, using CCR3, CD123, CRTH2, CD203c, or anti-IgE to identify basophils (6–9). These markers are basically expressed on the basophil membrane but not always specifically and secondary markers are needed to exclude CRTH2+ T cells or CD123+ plasmacytoid dendritic cells. Among them CCR3 and CD203c are exclusively expressed on resting basophils and CD203c is up-regulated under activation (10). Degranulation is detected by surface expression of CD63 that is otherwise only expressed on the inner side of the granule membrane of

resting basophils (11). The external expression of CD63 is correlated with histamine release that gives most allergy symptoms, and allergen responses are correlated between BAT and the historical Histamine Releasing Test (HRT) (4). BAT is more precise, rapid, and less expensive than HRT.

Basophil activation is triggered by allergens in sensitized patients only. These patients have specific IgE, most of them being bound on basophil membrane Fcε receptors (12). Basophil activation is dose dependent with possible bell shape dose-response in case of very high sensitivity. In order to validate the *ex vivo* basophil reactivity, antibody anti-IgE or anti-Fcε receptors are used as positive controls. These polyclonal stimuli mimic the capture of allergens by membrane IgE, bridging membrane receptors required to trigger basophil degranulation. Basophil degranulation is almost complete within 20 min of exposure and is calcium and temperature dependent (8). As a matter of fact, calcium chelator completely inhibits the BAT while EDTA is preferably used as anticoagulant as it is supposed to better preserve and keep the sample in resting state prior to *ex vivo* activation. Basophils are fragile and BAT system manufacturers usually recommend doing the test within 8 hours of withdrawal but, as the test is performed in few labs, it is not always possible to receive the sample and perform the test in less than 24 hours (13). Nevertheless, BAT is closely correlated with the clinical response to an allergen (2,4,14–17).

Basophils are rare cells in the peripheral blood. This makes it difficult to acquire a sufficient number of cells for good test reliability, especially when the test is performed on whole blood as it is now proposed by most of the systems, for time and cost efficiencies. Sample washing (not usually recommended in the manufacturer instructions) before acquisition gives the possibility to concentrate cells and significantly shorten the acquisition time while increasing the signal/noise ratio. In order to acquire maximum of cells, acquisition can be planned long enough to analyze the entire sample. This exposes to sample shortage and air aspiration that produces extra noise and impairs data analysis unless they are excluded by following data acquisition using time parameter.

In each sample, basophils are activated variably according to their random distribution of the IgE specific for the allergens tested. In routine practice, there is always a part of basophils that is not activated. In case they respond, the degranulation is usually rapid and massive with very high and homogenous expression of CD63. It is usually believed that basophils survive to strong stimulation but this is not necessarily true. In any case, in BAT, degranulation is only measured on individual basophils which have maintained their shape and identity. This could explain some bell shape of the dose-response curves with progressive response to increasing doses but reduced response to the highest stimulation. It is difficult to measure cell survival as basophil absolute count is not possible in BAT because of their rarity and multiple steps in sample preparation. One possibility to measure basophil destruction is to compare their count to another cell lineage which passed the same process but is not sensitive to the stimulant. In order to compare cells easy to identify and in the same order of concentration, we propose to compare Basophil to B cell counts.

For the past few years, lots of clinical studies have validated the BAT but there is still some controversy on clinical significance of thresholds used by different studies (18). Rare studies have tried to check if basophils selections with the different strategies are equivalent (6). The great majority of clinical studies use CD63 as read out, but again, there is no attempt to standardize CD63 detection and the determination of the positive threshold (13). The main limitation of BAT to be routinely used in diagnosis is its lack of standardization between systems and between instruments while European authorities are asking for accreditation of all diagnosis methods (19). First, there is a need for standardization of sample preparation (allergen preparation, dilutions used, incubation time). Progressively, few points have already reached a consensus, such as the use of whole blood, Interleukin 3, short incubation time (15–30 min at 37°C). Most systems recommend a calcium chelator as anticoagulant and sample stabilizer such as ethylenediamine tetraacetic acid (EDTA) while few protocols still propose to use heparin. Second, in data analysis, the gating strategies and positivity thresholds are different among studies. Finally, instrument settings are rarely addressed while they represent a strong concern in hematology and immunology diagnosis (20). Working groups have proposed the standardization of instrument settings at least from the same company to get comparable data for immuno-phenotyping of acute leukemia and lympho-proliferative disorders (21,22). Very recently Solly et al. (23), have shown that it was possible to make FCM results comparable not only on cell counting, but also on graphic representations even between different manufacturers. But even in local practice, all diagnostic methods should progressively fit to Iso15189 recommendation according to EU rules (19).

The aim of this study was to 1) optimize and adapt to EDTA anticoagulant, one of the largely used systems that was developed on heparin; 2) to make proposals on a technical standardization, independent of the system or the instrument used; and 3) to implement the tests by taking into consideration the stimulation dependent loss of basophils that can impair the readout of basophil degranulation.

MATERIAL AND METHODS

Blood Samples and Staining

FlowCast[®] kit (Buhlmann, Schönenbuch, Switzerland): peripheral blood samples were collected from healthy volunteers on EDTA. Samples were diluted in stimulation buffer, stimulated or not with a monoclonal anti-IgE receptor (anti-FcεRI mAb), immediately stained with anti-CCR3-PE and anti-CD63-FITC and incubated for 20 min in the dark at 37°C, according to the manufacturer instructions. Erythrolysis was done, using the reagents of the kit. At the end, cells were washed with 4 mL PBS (Eurobio[®], France) and re-suspended in 150 µL PBS with 1% serum albumin (PBS-BSA).

BasoflowEx[®] kit (Exbio, Praha, Czech Republic): peripheral blood samples were collected with either heparin as recommended by the manufacturer or EDTA. EDTA samples were then diluted 1/1 with stimulation buffer complemented

with calcium chloride 20 $\mu\text{mol/mL}$ (Renaudin laboratory, France) and sodium heparinate 500 UI/mL (Choay; Sanofi-Aventis[®], France). Basophils were unstimulated or stimulated with anti-IgE antibody and stained with anti-CD203c-PE monoclonal antibody (clone NP4D6) and anti-CD63-FITC (clone MEM-259) for 20 min in the dark in a 37°C water bath. In later experiments, an anti-CD20 monoclonal antibody (clone 2H7, Exbio) was also added. Erythrolysis was performed using reagents included in the kit. At the end, the sample was washed and re-suspended in PBS-BSA. The changes of this method were validated for routine analysis according to iso15189 recommendations (7).

Standardization Between the Two Instruments

PMT settings were optimized on a Navios[®] using Navios[™] software (Beckman-Coulter; Fullerton, CA) and levels of fluorescence settings were measured using calibration beads (Cytocal beads, Distrilab[®] The Netherlands Multifluor Calibrator, Thermo Scientific, Carlsbad, CA) as targets to be transferred to a FACSCanto II, using FACS Diva 6.2 (BD Biosciences[®], San Jose, CA) according to Solly et al. (23). Compensations of spectral overlap were set up using single labeling and respective software application on each instrument.

Data from different systems were analyzed on Kaluza[®] software (Beckman-Coulter). Cell shape was checked on Forward/side scatter dot plots. Bubbles were excluded on basophil label versus time scatter. Cell doublets were excluded on Forward Area/high scatter. Basophils were then selected on their specific label that were either CCR3 (Buhlmann system) or CD203c (Exbio system) versus Side Scatter (SSC) and the fraction of activated basophils (CD63⁺) was measured among this population. The fraction of activated basophils as well as the labeling median fluorescence intensity (MdFI) were measured. Lately, B cells were measured on singlets, time restricted dot plot and expression of CD20 versus basophil label (Supporting Information Figure).

Method Validation

Precision was measured on 10 repeated tests on one fresh sample, on the same day, by the same operator. Dose response linearity of degranulation (percentage of CD63⁺ cells in CD203c⁺) was evaluated by serial 1/2 dilutions of stimulant anti-IgE on EDTA and heparin samples on one donor and was compared with theoretical values calculated by serial 1/2 divisions of results from the full activation. To check sample stability, BAT was analyzed on fresh samples (<8 hours) and repeated after one night storage at 4°C. This method validation was performed only in Navios flow cytometer (CE-IVD; 3 lasers, 10 colors) using Navios software.

Acquisition

Data acquisition was realized on both FACSCanto and Navios instruments after daily maintenance and quality controls according to the manufacturer instructions. Data were analyzed using the built-in softwares, respectively, Diva[®] (on

FACSCanto II) and Gallios-Navios (on Navios) or from both instruments using Kaluza (Beckman Coulter).

Statistical Analysis

Correlations were analyzed using linear regressions, with a Pearson coefficient and the Bland and Altman (B&A) method. Statistical analysis used either Excel (Microsoft Corporation, Redmond, WA) or Prism version 5 Software (GraphPad Prism, San Diego, CA, USA).

This technical study was performed on anonymous blood samples collected for diagnostic purposes in accordance to 2011-814 bioethical French law.

RESULTS

Protocol Implementation

In order to homogenize and simplify the procedure and because EDTA sampling is the most used in clinic, we adapted BasoflowEx[®] protocol developed for heparin anticoagulant by adding calcium chloride to the sample and reducing the stimulation/labeling process in one single step. Results on EDTA samples and modified protocol (Fig. 1a) were very similar to results on heparin samples following the manufacturer instructions (Fig. 1b) in terms of levels of Immunolabeling and degranulation efficiency as measured by up-expression of membrane CD63 (EDTA: 69.97% vs. Heparin 56.3% of CD203c basophils). The method validation on 10 activated samples has shown a precision of 5.1% as a related standard deviation (RSD) for inducing expression of CD63 (Fig. 1c), 3.4% for the levels of CD63 expression (MFI; Fig. 1d), and 1.5% for CD203c (Fig. 1e).

Activation with an anti-IgE gave similar responses in four volunteers, between EDTA (plain) and heparin (open labels; Fig. 1f, squares) although unstimulated controls (noise) have been frequently higher in heparin samples (Fig. 1f, lozenges). Serial 1/2 dilutions of anti-IgE stimulus has shown a linear response (EDTA sample $r^2 = 0.990$, slope 0.961; heparin sample $r^2 = 0.997$, slope 0.786; Fig. 1g). When four samples were stored at 4°C, stimulations were not altered after 24 hours for EDTA (Fig. 1h) and heparin samples as well (Fig. 1i).

Cross-Calibration of the BAT, Between Systems, Between Instruments

When the settings were optimized on a Navios, fluorescence targets were defined using the intermediate peak of standard beads (Cytocal) giving FITC = 19.4; PE = 28.8 (Fig. 2a) that were converted to the FACS Canto scale (FITC = 4,966; PE = 7,373; Fig. 2a) to get the same levels of detection on the FACSCanto. Comparative dot plots of one (EDTA, BasoflowEx, activated) representative sample acquired on Navios and on FACSCanto and analyzed using Kaluza software are shown in Figures 2c–2f. Samples prepared with FlowCast were also similar between the two instruments (not shown).

Overall, 16 samples were prepared using FlowCast and modified BasoflowEx protocols and analyzed on the two instruments. Induction of CD63 out-expression, prepared with FlowCast protocol was closely correlated when analyzed

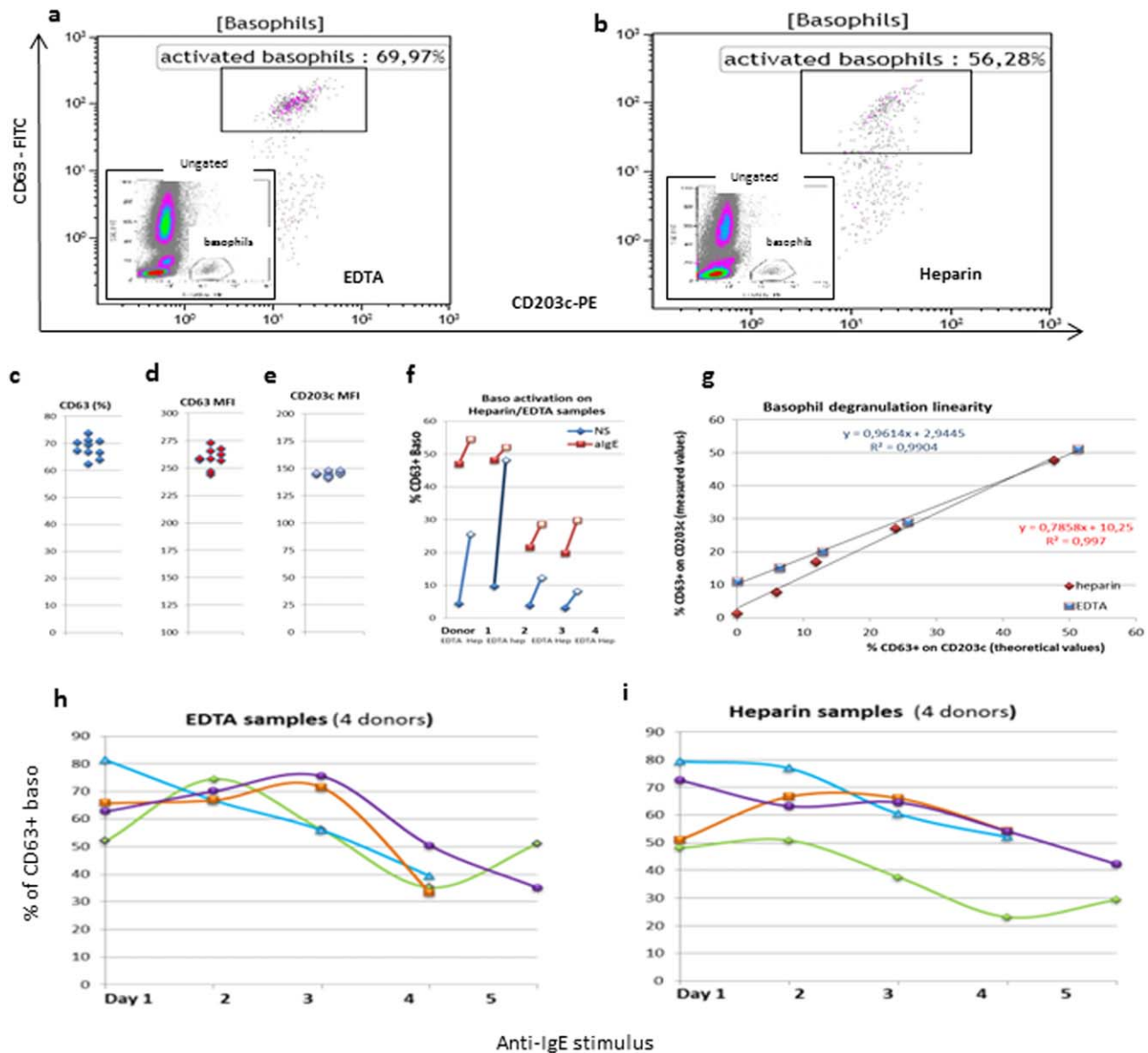


Figure 1. Validation of BasoflowEx[®] protocol implementation: Basophil identification and stimulation induced CD63 was similarly detected (EDTA, panel a) as compared with the preparation following the manufacturer's instructions (heparin, b). The method precision was measured on 10 repeated activations for CD63% (c), CD63MFI (d) and CD203c MFI (1e). The two methods were compared in four volunteers (f), between EDTA (plain) and heparin open under stimulation (□ squares) or not (◆ lozenges). The linearity of response according to the level anti-IgE activation was checked on four serial 1/2 dilutions on modified (EDTA, □) or not (heparin, ◆) methods. The effect of storage over 4 days at 4°C was tested in four samples, EDTA (h) and heparin (i). [Color figure can be viewed at wileyonlinelibrary.com]

on FACSCanto or Navios ($r^2 = 0.958$, slope 0.948; Fig. (2)g) without any bias as shown on Bland-Altman graph (Fig. 2i). Similarly, induction of CD63 expression prepared with the modified BasoflowEx protocol was closely correlated when analyzed on FACSCanto or Navios ($r^2 = 0.938$, slope 0.929; Fig. 2h) without any bias on Bland-Altman graph (Fig. 2j). The fluorescence intensity to detect basophils with CCR3 on FlowCast system ($r^2 = 0.990$; Fig. 2k) or CD203c on BasoflowEx ($r^2 = 0.887$; Fig. 2l) as well as the respective levels of CD63 MFI ($r^2 = 0.969$ Fig. 2m and $r^2 = 0.934$ Fig. 2n) were strongly correlated between the two instruments.

Protocol Implementation (that takes in account the basophil survival under stimulation)

In order to acquire the entire sample but exclude the noise due to air aspiration, we first gated events on signal over time dot plot (e.g., CD63 FITC; Fig. 3a). Then doublets were excluded and basophils were selected on their lineage label (CCR3, CD203c or others) versus low SSC for further analysis of eventual up regulation of membrane CD63 with minimal noise (Fig. 3b). By comparing unstimulated (Fig. 3b) and stimulated (Fig. 3c) samples, on whatever system (FlowCast or BasoflowEx) and instrument (FACSCanto and Navios), we

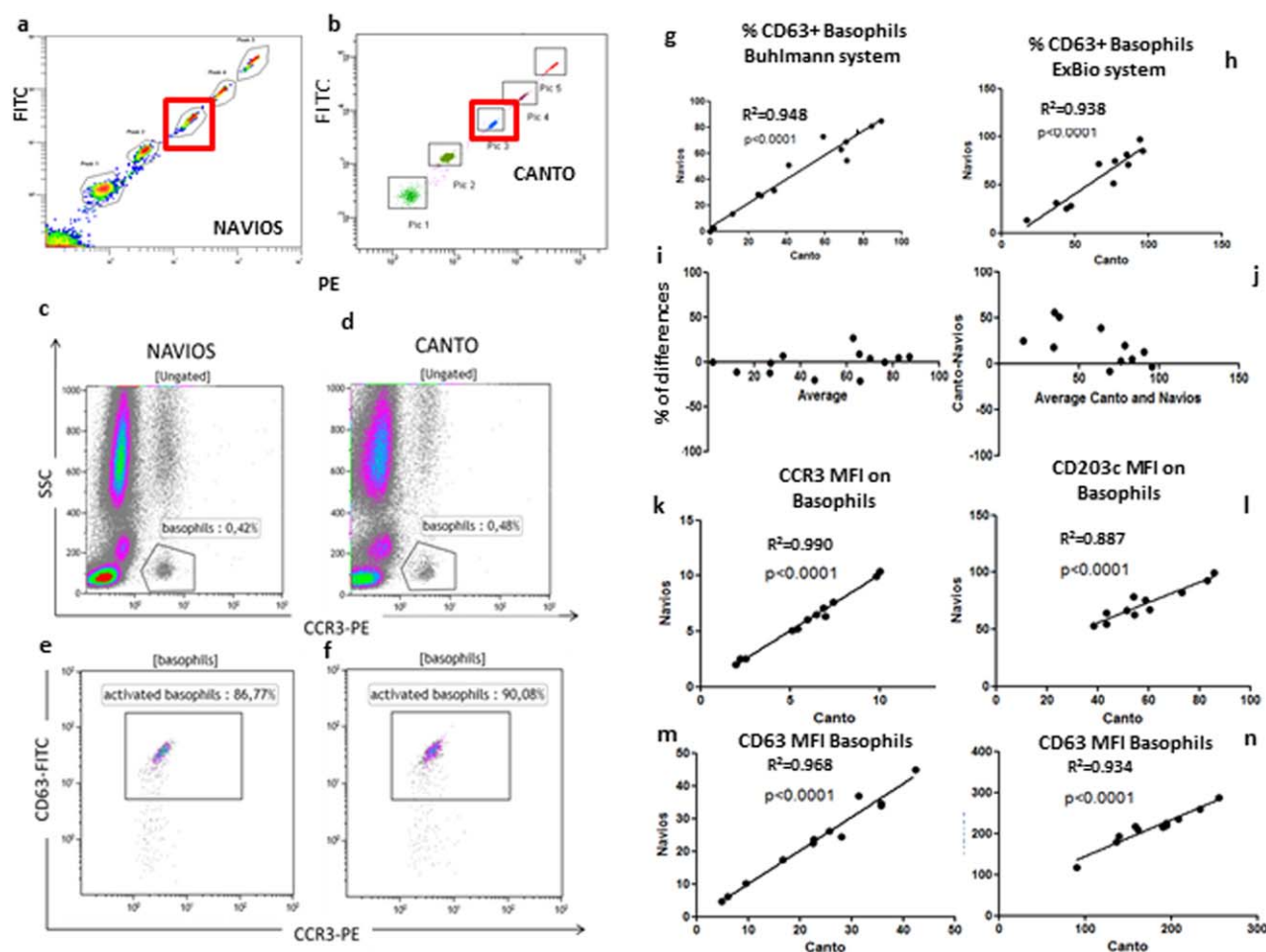


Figure 2. Method standardization: two methods (same stimulation) were compared on two instruments with Settings based on Fluorescence targets (Cytocal[®] peak 4) on Navios[®] (panel a) and FACSCanto[®] (b) giving similar dot plots for basophil identification (c and d) and degranulation (e, f) one representative sample, EDTA, BasoflowEx[®], activated) analyzed using Kaluza[®] software. More generally, induction of CD63 was compared on 16 samples were prepared using FlowCast[®] (g, i) and modified BasoflowEx (h, j) protocols and analyzed on the two instruments. The MFI of CCR3 (k) or CD203c (l) and CD63 MFI (m and n) were also compared. [Color figure can be viewed at wileyonlinelibrary.com]

could easily observe that the number of basophils that were identified (e.g., 1.2% in this representative sample, Fig. 3b) was lower after stimulation (0.85%, Fig. 3c). These numbers have little significance because the amount of debris and remaining red blood cells was changing and difficult to consider.

We thus monitored the number of basophils related to the number of CD20+ B cells in the same sample. In a representative sample, we acquired 1,432 basophils and 7,093 B cells meaning a *ratio* of 20.2 basophils for 100 B cells in the unstimulated sample (Fig. 3d) in which 0.37% of basophils expressed CD63 (Fig. 3e). Under stimulation, the *ratio* was 588/5092 meaning 11.6 basophils for 100 B cells (Fig. 3f) with 78% of basophils that expressed CD63+ (Fig. 3g) while the expression of CD203c raised from MFI of 10.6 to 47.8. Looking at 13 consecutive samples, we could observe a median *ratio* at 28.7 basophils for 100 B cells (range from 6.2 to 66.5; Fig. 3h, lozenges). After one night storage, the

median *ratio* of unstimulated sample was at 30.1 (from 11.3 to 62.6, $n = 11$; Fig. 3h, triangles). Under stimulation, the median *ratio* dropped to 5.1 (from 2.0 to 29.5; square labels) on 11 fresh samples and 5.6 (from 2.4 to 18.2; Fig. 3h, rounds) after one day of storage. Indeed, Baso/B cell *ratios* were significantly correlated between Day 1 and Day 2 (Fig. 3i) and we could easily see that all values of stimulated samples (squares) were severely lower than unstimulated samples (lozenges). Note that one case had a *ratio* at 218.3 unstimulated and a *ratio* at 128.9 after activation but was not repeated after one night.

Protocol Application on Allergens

This new protocol is now used in routine in our lab with positive controls (as anti IgE antibodies) and allergens at different doses. As an example: patient X, male, 57years old has an acute erythema's reaction under antibiotic amoxicillin-clavulanic acid. Skin test shows strong reaction (>8 mm) to

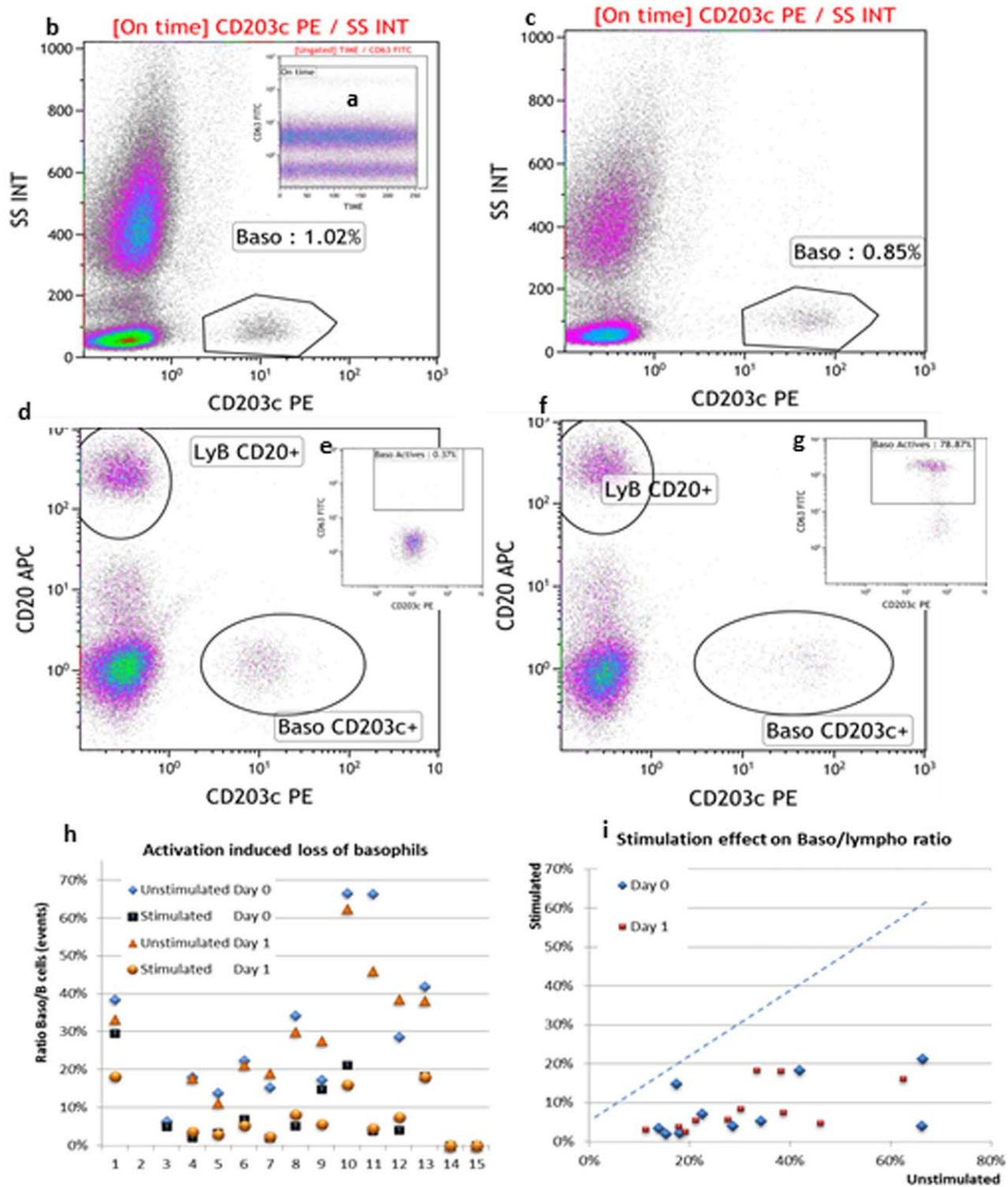


Figure 3. Acquisition protocol implementation: In order to get maximum basophils with little noise, we first selected cells on signal/time parameter (fig 3.a). As we could repeatedly observe a significant decrease of basophils on the sample without (b) or with stimulation (c) we considered Basophil (CCR3 or CD203c) versus B cells (CD20+) on unstimulated (d, e; one representative results, ratio = 20.2) or stimulated (f, g, ratio = 11.6). Results were confirmed on 13 consecutive donors (h) fresh unstimulated (◆) or stimulated (◻) and after one day storage (▽ triangles and • rounds respectively). Values were correlated between Day 0 (◆) and day +1 (◻). [Color figure can be viewed at wileyonlinelibrary.com]

amoxicillin-clavulanic acid and not to amoxicillin only. In basophil activation test with our new protocol, CD63 was expressed on 6.3% of non-activated basophils. Anti IgE induced CD63 expression on 91.2% of basophils. Amoxicillin at 50 mg/mL-clavulanic acid at 5 mg/mL induced CD63 expression on 53.9; 52.3% and 47.4% of basophils stimulated with antibiotic diluted at 1/100, 1/1000, 1/10,000, respectively, while Amoxicillin at 50 mg/mL alone induced CD63 out expression (degranulation) on 8, 12, and 10.2 of basophils. The test was repeated in the same Patient 1 month later with similar results.

DISCUSSION

In a first step, the aim of this study was to implement a CD203c/CD63 system (BasoflowEx; Exbio) from the manufacturer instructions which recommend heparin anticoagulant. EDTA anticoagulant is mostly used in routine practice for cell analysis, as it keeps a better morphological assessment of cells (24,25). Furthermore, as basophil degranulation is calcium dependent, using EDTA protects the samples against pre-analytical stress induced activation. So EDTA anticoagulant is recommended in all BAT kit available except BasoflowEx and we intended to adapt this test for EDTA samples. Calcium needs to be reconstituted for the basophils to be able to degranulate but calcium restitution will also induce coagulation. This is why we added Ca^{++} and heparin to the stimulation buffer, at optimal doses we have determined in a way it was preventing coagulation and making degranulation possible. We could show that a minimal implementation of the manufacturer protocol makes it possible to perform this test on EDTA samples similar to the test on heparin samples as recommended by the manufacturer.

Because we changed the manufacturer instructions, we had to validate this method. The performances of the test show a very good precision (RSD $\sim 5\%$) although the basophils are rare and fragile. This is in accordance with what the manufacturer claims to guarantee on his technical data sheets (between 2.4% and 3.4%). RSD between 5% and 10% is usually recommended by accreditation bodies in medical biology but there is no specific report on method validation of functional tests we could refer to yet. The linearity of the dose-response curve was also very good, using progressive doses of polyclonal stimulation. Of course, it would be better to test it with allergens, in sensitized patients. But it was difficult to get different types of fresh samples of highly sensitized patients at the time of the experiment. Furthermore, experience with one allergen in one patient does not necessarily represent other types of allergens or other patients (difference of epitopes, avidity...). On the other hand, anti-IgE stimulus strictly mimics basophil IgE-receptor bridging in the same way as the allergens induced basophil degranulation. As a matter of fact, anti-IgE or anti-IgE receptor are usually used as positive controls to check sample reactivity in BAT (6,26,27). The limit of detection of basophil degranulation depends on the type of allergen and on the patient sensitization profile. In our data, we still could get a significant positive response even at 1/

8 dilution of anti-IgE dose recommended by the manufacturer for a full basophil degranulation. We saw that 4 $\frac{1}{2}$ dilutions were not sufficient to get the lowest level of response above the unstimulating background. Because of the cost of the experiment in time, reagents and sample consuming, we propose to use 4–5 times $\frac{1}{4}$ dilutions instead of $\frac{1}{2}$ to better define the dynamic range of the method. In fact, the dynamic of the test is routinely checked. Indeed, it is highly recommended to test systematically three to six different doses of allergens in order to exclude the eventual hook effect of high doses in highly sensitized patients even if this increases the cost of the test (7,8).

We also propose to standardize fluorescence detection on time in one instrument and even between systems using CD63 (ex FlowCast and BasoflowEx) and between instruments (ex FACS Canto, FC500, Navios) in order to better compare positive thresholds between clinical studies. This is relatively easy to implement, at limited extra cost as previously described in hematology (23). We propose fluorescence target values on Cytocal beads as those which are available worldwide and guaranteed on fluorescence intensity over time (23). Standardization of identification markers (CCR3, CD203c, CD123, and CRTH2) is not so easy to achieve because of differences in levels of expression or performances of conjugated antibodies (6). Nevertheless, instruments settings can be standardized on this parameter with the same fluorescence targets. The levels of fluorescence then consider each antibody performance to get the same level of fluorescence on basophils whatever system is used. Our data show how good was the comparison we got between two different instruments was, not only on the major clinical outcome of BAT that is the level of degranulation (CD63+ basophils) but also on basophil identification (CCR3/CD302c).

We could show that our protocol adaptation is compatible with Iso15189 method validation rules (19) as much as it is possible for functional tests in allergy. Indeed, most Iso15189 recommended evaluations for quantitative cell analysis are not possible for BAT because of the lack of international standard, internal quality controls, External Quality Assessment program...). In any case, the quantitative value of basophil degranulation does not directly correlate with the risk or gravity of allergic crisis. The test should be interpreted in light of the clinical situation of the patient considering the type, severity, delay of the last reaction, allergen exposition, eventual treatment used at the time of sample collection, co-morbidity, and medical background such as mastocytosis, lung, or cardiovascular diseases (28–30). The type of allergen must also be considered. Drugs for example are frequently monovalent haptens that cannot bridge membrane IgE without the support of protein carriers. Response to proteins such as food, pollens, most antibiotics or venom components is usually good except in some patients who are refractory *ex vivo* and qualified as “non-responders” (15,29,31).

It is usually recommended to do the BAT on fresh samples, within 8 hours after collection, if stored at room temperature. This is part of the manufacturer's instruction. But this

is not always possible in real life practice. As an example, if samples are collected in the afternoon, it may be possible to start too late in the lab the test that takes a few hours. Conveniently, we could show that even 24 hour preservation gives good results, especially if the blood is collected in EDTA and kept in the fridge. This also gives the possibility to check with confidence the next day in case of unexpected results of the fresh sample.

Finally, one parameter has always been neglected until now that is the basophil survival under stimulation. It is frequently admitted that basophils do not die after degranulation. This is very difficult to demonstrate in clinical practice. So we propose to take this possibility into account by simply comparing their number to an internal control that should not be influenced by test conditions in such a short time. We considered that B cells are most probably not affected by the stimulation with anti-IgE in the 20 min of sample preparation. If an effect was possible, it would most probably reduce the number of B cells. Nevertheless, too rare circulating B cells express IgE among the total number of B cell to be significantly disturbed by the short challenge. We were surprised to show in our short study, how much the number of basophil decreased under anti-IgE stimulus as compared with B cells. Santos et al. (26) have previously reported a loss of CD123 basophils under activation, although the cell counting was not clearly detailed. This loss was then attributed to a specific down expression of the receptor as more basophils could be detected using a different label such as CD203c. In our test, we identified basophils using CD203c which expression is known to enhance and not reduce under anti IgE stimulus.

If the loss of basophils under strong activation is confirmed, it could explain some lack of sensitivity of the test occasionally observed in some patients despite strong clinical evidence of hypersensitivity. This could also give interesting input in understanding the mechanism of immunotherapy that is more and more used with great success in preventing anaphylaxis in patients who have had a severe allergic reaction. So, we believe this parameter should be taken in consideration in the routine BAT test and we have shown that it is quite easy to implement the tests with minimal extra cost as compared with its great biological advantages.

Just to mention that BAT is a functional test like skin tests. Functional tests consider all the mechanisms involved in allergy, including cell degranulation threshold while the detection of serum IgE specific for one allergen reflects previous sensitization and is necessary but not sufficient to explain allergic crisis. Furthermore, specific IgE immunodoseage is not available yet for some allergens (especially recent drugs) despite the very large portfolio made available by companies nowadays.

In conclusion, BasoflowEx can easily be adapted for EDTA samples as usually recommended in most if not all other systems. Sample wash improves data acquisition. The changes of manufacturer instructions were validated. We also propose to standardize the acquisition on whatever instrument that is used, and we propose fluorescence targets

as a start. Finally, the test would benefit from considering basophil loss during exposure to stimulation with simple implementation.

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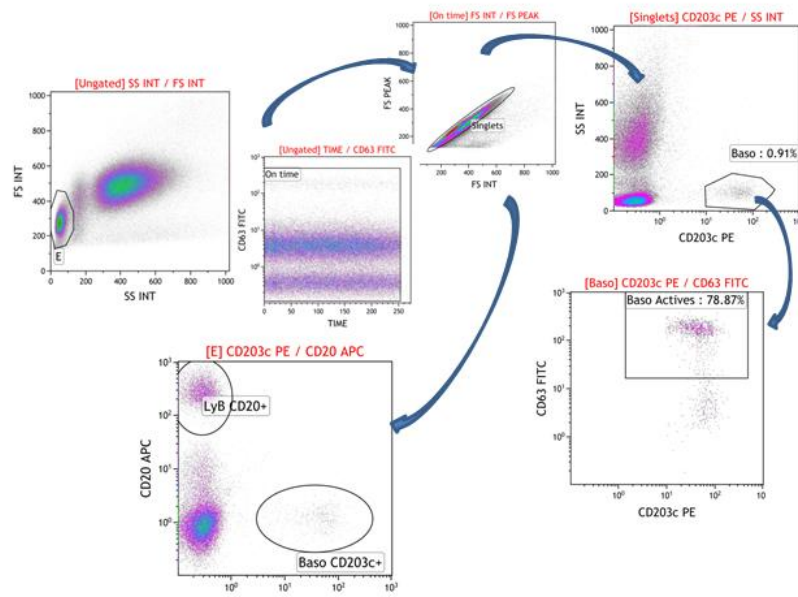
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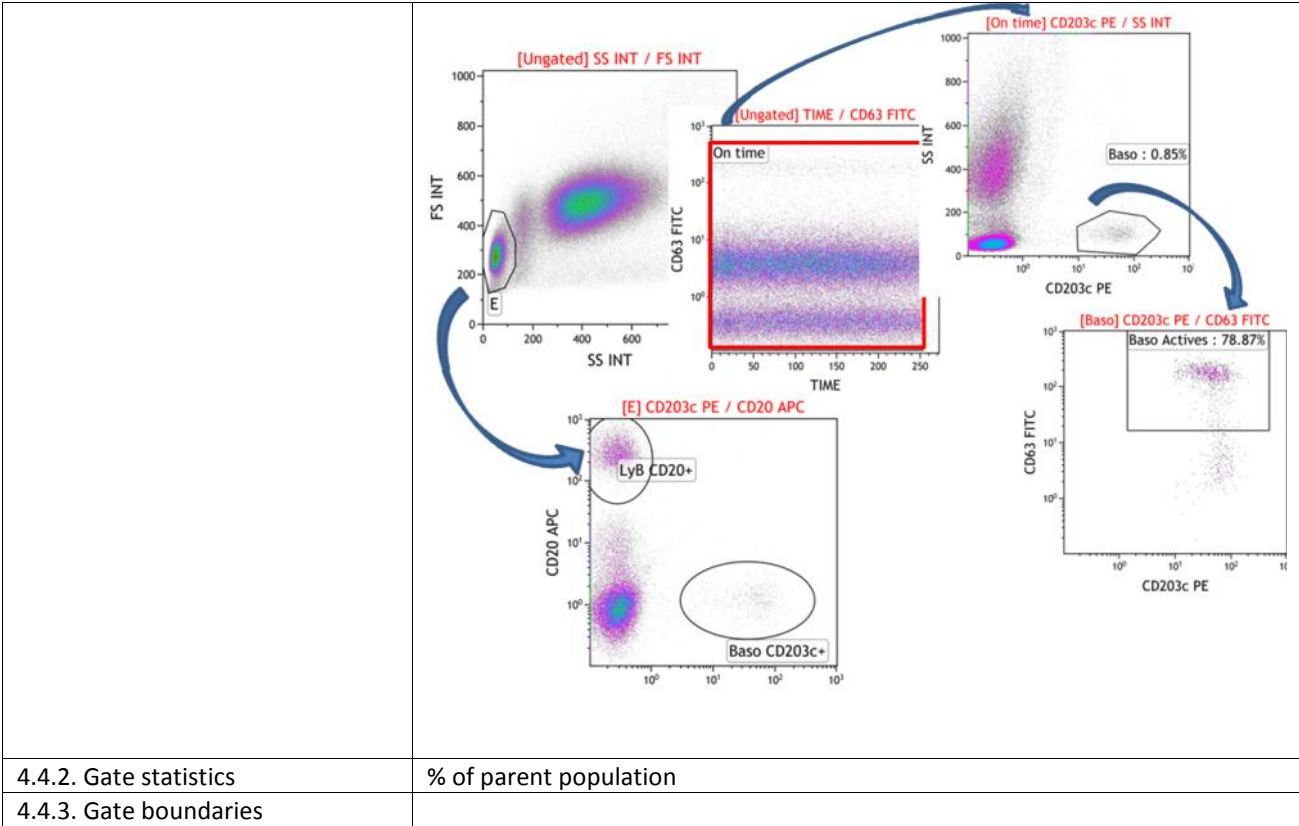
Supplementary Figure: Gating strategy



Supplementary figure: Representation of the gating strategy of basophils singlets, activated basophils and B cells.

Cytometry Part A
Author Checklist: MIFlowCyt-Compliant Items

Requirement	Please Include Requested Information
1.1. Purpose	Implementation/standardization Basophil activation test
1.2. Keywords	Basophil activation test, Allergy, Hypersensitivity,
1.3. Experiment variables	Basophil identification Basophil induced Degranulation (using anti IgE or Allergen)
1.4. Organization name and address	Immunology lab, University hospital Saint-ETIENNE FRANCE
1.5. Primary contact name and email address	Claude LAMBERT claudelambert@chu-st-etienne.fr
1.6. Date or time period of experiment	June 2015-July 2016
1.7. Conclusions	Need and faisibility for standardization of cytometric parameters Need for considering cell loss
1.8. Quality control measures	No internal / external quality control assessment Internal quality assurance based on Fluorescence ratio
2.1.1.1. (2.1.2.1., 2.1.3.1.) Sample description	basophils
2.1.1.2. Biological sample source description	Peripheral blood, possible Broncho Alveolar Lavages
2.1.1.3. Biological sample source organism description	Human, mammals
2.1.2.2. Environmental sample location	whole blood,
2.3. Sample treatment description	Stimulation/labelling (one step), Lysis, Wash
2.4. Fluorescence reagent(s) description	FITC, PE, APC
3.1. Instrument manufacturer	Beckman-Coulter, BD Biosciences
3.2. Instrument model	Navios; FACSCanto
3.3. Instrument configuration and settings	Navios 3 lasers, 10 col, ivd.eu; FACSCanto 3 lasers, 8 col ivd.eu
4.1. List-mode data files	http://flowrepository.org/id/FR-FCM-ZZTB
4.2. Compensation description	Single labelling, 3 colors; Comparison stimulated non stimulate (no need for FMO).
4.3. Data transformation details	Non required
4.4.1. Gate description	1 FSC/SSC 2 Time/SSC, 3 Baso identif (on time limitation : CCR3 or CD203c/SSC, 4 degranulation (CD63/baso)



Notes

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