

Monitoring of CD63% in basophil activation test and suggested new parameters for allergy diagnosis

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To the Editor,

The basophil activation test (BAT) is the only cellular test used to investigate basophil function for allergy diagnosis [1–4]. Most basophil activation tests (BATs) evaluate the percentage of basophils expressing the CD63 molecule on activation, in order to address a possible hypersensitivity reaction to an allergen; non-activated resting basophils do not express this tetraspanin on the cell surface but, on the contrary, cells up-regulate the molecule upon stimulation [5]. CD63 up-regulation can be related to basophil degranulation and histamine release [6], two main activation steps of basophils, which have made this molecule a good activation marker for providing a reliable test of cellular function for allergy diagnosis. Although evidence of other mechanisms able to change CD63 up-regulation on the membrane by histamine release has been also reported [7, 8], its association with degranulation events enabled CD63 to be included in BATs preferentially to other activation markers having comparably good performance. How reliable a parameter is CD63% for evaluating basophil response to an allergen? This is an important issue in assessing how far BATs are reliable tools for diagnosing allergy and hypersensitivity reactions to different allergens. The relatively high frequency of involvement of this marker in allergy cellular tests necessitates a review of CD63 performance in BATs, and so a closer look at how this molecule is expressed as a result of immunoglobulin (Ig)

E-mediated activation has to be addressed. Normally, the extent to which CD63 is expressed is evaluated using the fluorescence pattern in a flow cytometry assay. In any case, very few papers seem to use the full potential of fluorescence information on expression of membrane markers, often limiting assessment to a fraction of cells which express the molecule and using only a percentage value to elaborate a possible diagnostic algorithm. However, the expression of CD63 related to basophil activation is a complex mechanism.

CD63 displacement onto the membrane occurs in the first few minutes following stimulation, after 5–10 min reaching a plateau lasting throughout the whole 30-minute period of incubation with the allergen or anti-IgE [9]. Most CD63 is stored within cell lysosome-associated granules; it is not synthesized very rapidly, it is not recycled, and the whole amount of CD63 does not appear to be externalized when cells undergo the strongest activation available [8]. Mean fluorescence intensity (MFI) should describe an activated population in which basophils express heterogeneously different amounts of CD63 on their surface, resulting in different parametric distributions in a fluorescence histogram. Flow cytometers are used regularly for the determination of the density of specific molecules on the surface of cells in the population analyzed. Obviously, these measurements may be relative, semiquantitative, or quantitative depending upon the question asked and the reagents and methods applied. In most cases, when fluorescence signals derived from monoclonal antibody (mAb) binding are measured, the data are log-transformed to provide sufficient resolution of the cells. If the fluorescence intensity of the population appears normally (i.e., Gaussian) distributed (not bimodal), this procedure means that the population is a “log-normal” distribution, in which the mean fluorescence intensity will be skewed to the right,

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that is the mean will overestimate the true average fluorescence intensity. In this circumstance, the best estimate of the average of log-arrayed fluorescence signals is the median or geometric mean, but median fluorescence intensity is a value that should be taken from a single log-normal distribution. Taking a single median value from a fluorescence signal that contains multiple log-normal distributions should be avoided [9]. The MFI of a population of cells labeled with a fluorochrome conjugated to a ligand or mAb is linearly related to the mean number of receptors/cell or mean number of mAb binding sites/cell. Calibrated bead populations that bind a quantifiable amount of antibody (antibody binding capacity: ABC) generate a calibrated standard curve (linear regression) that converts channels of MFI into molecular equivalents of soluble fluorochrome (MESF) [10]. In this respect, MFI appears to be the only parameter that can be reliably monitored by checking and managing flow cytometry performance. A CD63^{expressing} population is defined by the percentage of cells that express this molecule with a value higher than the threshold fixed in a non-activated, resting population. Another parameter, called cut-off, should set the minimal threshold below which CD63 up-regulation should not be related to an activated state. The parameter %CD63^{expressing} basophils proves to be a good approach because it does not appear to be influenced by the total number of basophils/gate. Nevertheless, basophils number and event count can affect CD63^{expressing} fluorescence distribution. The skewness of this distribution may significantly change histogram symmetry and MFI, and splitting of two quite distinct populations expressing this marker is not always possible. Because of the existence of these critical issues, CD63% expression might serve as a limiting parameter to achieve a reliable diagnostic outcome. While considering similar and comparable experimental materials and settings, what functional meaning could this difference have?

Expression of CD63 gives rise to a typical bimodal pattern [11]. A possibility for observing this distribution hallmark of CD63 is to phenotype basophils using flow cytometry and to investigate CD63 membrane expression of these cells. Basophils are low side-scatter/forward-scatter cells (Fig. 1a, circle), which express a low level of CD45 (CD45^{dimly} cells, Fig. 1b) and which can be gated as HLADR^{non expressing}/CD123^{bright} cells, regardless of whether they are non-activated (Fig. 1c) or activated (Fig. 1d) by whatever allergen or agonist. When cells are not activated, almost the total amount of CD63 is stored within the intracellular granules and appears to be not or very weakly expressed on the cell membrane (Fig. 1e). In this case, %CD63^{expressing} cells is very low (0.3%, Fig. 1e). Activation with the same batch and dose of goat polyclonal anti-human IgE, which mimics allergen-mediated stimulation, induces the up-regulation of CD63 but only in a small

fraction of the gated population (e.g., basophils identified by the gating protocol), thus leading to a bimodal distribution of fluorescence. This bimodal pattern is a typical clue that basophil populations are made of different levels of activatable cells and presumably have a different endowment of intracellular CD63; this would explain why, in an atopic subject in which almost all basophils are activated (93.7%), bimodal behaviour may still be reported (Fig. 1f). Why do most activated basophils express CD63 with a MFI of about 2,000–2,500 and about one-third of them CD63 ten times higher? This is the same situation as with many examples reported in the next figure (bimodal distribution of non-expressing and expressing cells) but with the substantial difference that, in this case, the majority of cells do express CD63. If we do not consider this aspect, the percentage of CD63 of more than 90% might be interpreted as a highly significant diagnostic index of hypersensitivity. The question is: did basophils massively up-regulate most of the intracellular CD63 because of a huge and strong response to an IgE-mediated stimulation or did they show an effect due to a quite “regular” response but involving an exhausted or limited pool of CD63? This means that if basophils activate a maximal membrane displacement of 20% CD63, this is a real 20% if the potentially up-regulating intracellular pool is 100 but it can reach 90% if this pool is reduced by more than 75%. A reduced availability of CD63 allows basophils to attain a higher expression of CD63 (higher %) but with a lower MFI and always complying with a bimodal pattern; MFI can discriminate, if used in association with CD63%, different basophil responsiveness power to the allergen. In the example, it is quite probable that most basophils are unable to express maximal concentration of CD63 on their membrane, because of down-regulation mechanisms and depletion of functional CD63 due to continuous allergen stimulation *in vivo*. In this circumstance, which kind of quantitative parameter might give us this important information?

Figure 2 should help to explain this issue. When basophils from atopic subjects are challenged with an optimal dose of anti-IgE, CD63 membrane up-regulation is described by the existence of two peaks in the fluorescence histogram and a good activation is characterized by the presence of fluorescence at about 20–35% over the resting threshold. Activation of basophils by up-regulation of CD63 should be described by the use of two parameters: percentage of CD63 fluorescent cells (otherwise known as %CD63^{expressing} cells) and MFI of these fluorescent cells. The new parameter, which could be called 63_{index}, is given by:

$$63_{\text{index}} = [\text{MFI}_{\text{EXPR}} \times (\% \text{CD63}_{\text{EXPR}}/100)]$$

The 63_{index} of Fig. 1a is 1634.99 but this value is significantly different if cells are fixed with formaldehyde,

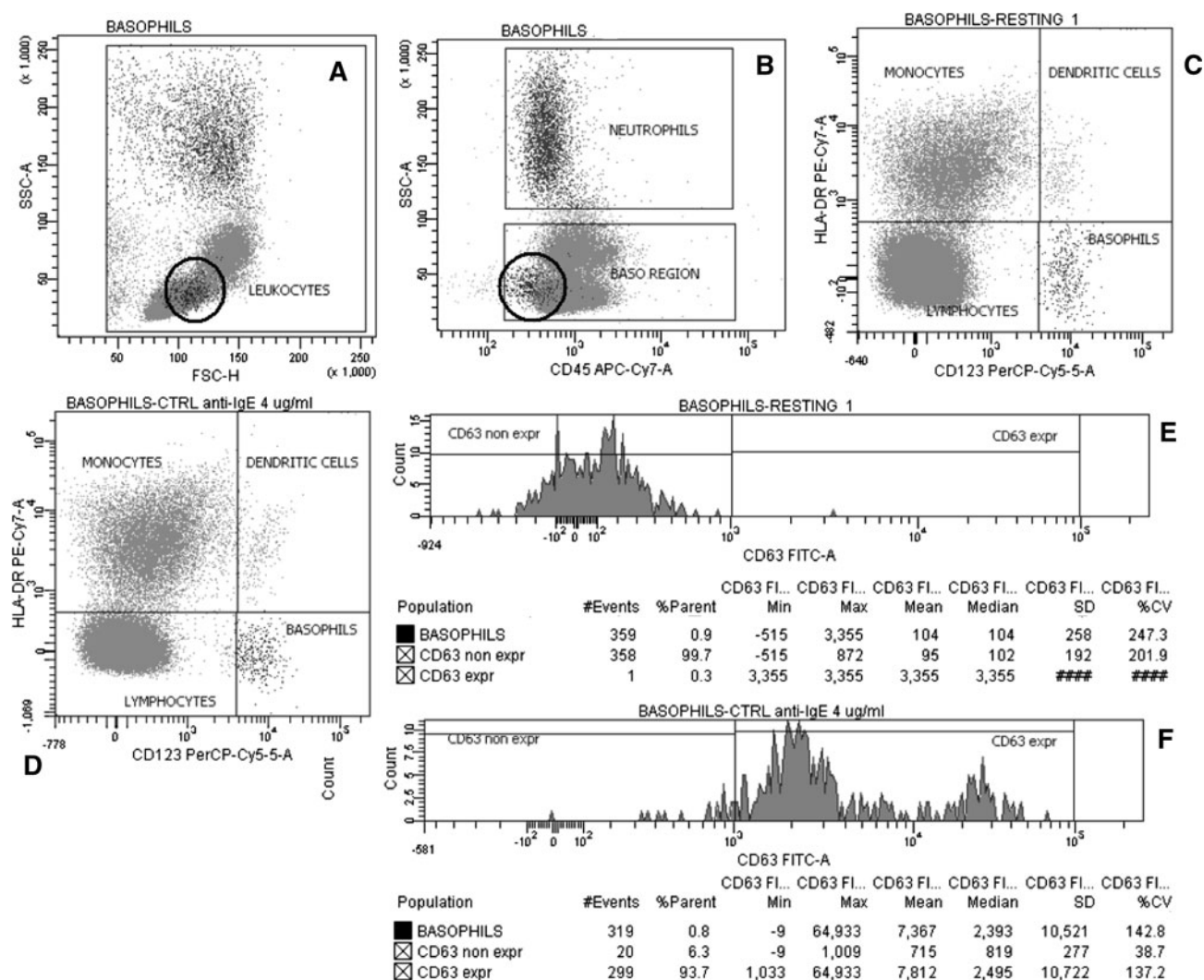


Fig. 1 Expression of CD63 in human basophils. In a flow cytometry dot plot, (morphological) basophils are located in the side scatter/forward scatter low region [(a), dots within the circle] and within the lymphocyte area in an immunological gate with CD45-APCCy7 (b). Basophils are electronically purified as HLA-DR^{non-expressing}/CD123^{bright} cells and their phenotyping does not change whether cells are not activated (c) or activated (d); they are included in the

same gate in both conditions. CD63 expression in resting, nonactivated cells (e) and in cells activated with 4.0 µg/ml mouse anti-human polyclonal IgE (Biolegend) in a birch pollen atopic symptomatic subject (f). In e and f the relative values of basophil count, percentages and fluorescence values are reported, as direct output from the flow cytometer software

kept at +4°C and read after 24 h ($63_{\text{index}} = 7,787.05$; Fig. 1b), thus demonstrating that different experimental conditions may significantly affect CD63% evaluation. If in a first attempt comparison is made by rating only %CD63 values, fixed cells are shown to express more than 2.8 times CD63 but if 63_{index} is used this ratio is over 4.7, i.e., about two-fold higher. This should mean that during storage, at least in the first hours, CD63 up-regulation on the membrane was sustained and at the same time CD63 down-regulation was inhibited or blocked, thus resulting in an increase both in percentage (number of cells expressing) and in fluorescence (number of molecules expressed). CD63 up-regulation on the surface of basophils is highly

susceptible to basophil manipulation. Of course, this is an hypothesis but, in any case, 63_{index} is proved to be a clear-cut parameter for performing suitable discrimination between different levels of activation, while MFI by itself is barely useful for clinical purposes. An IgE-mediated activation usually elicits about 20–30% of gated basophils to up-regulate CD63 on their membrane; in these circumstances 63_{index} may often be around 7,000–10,000 (Fig. 2d, $63_{\text{index}} = 7,171.27$). This can be considered an acceptable reaction to an allergen in an atopic individual. An activation of more than 90% CD63^{expressing} cells should imply that basophil responsiveness to the allergen must be at least three times higher. The following example shows

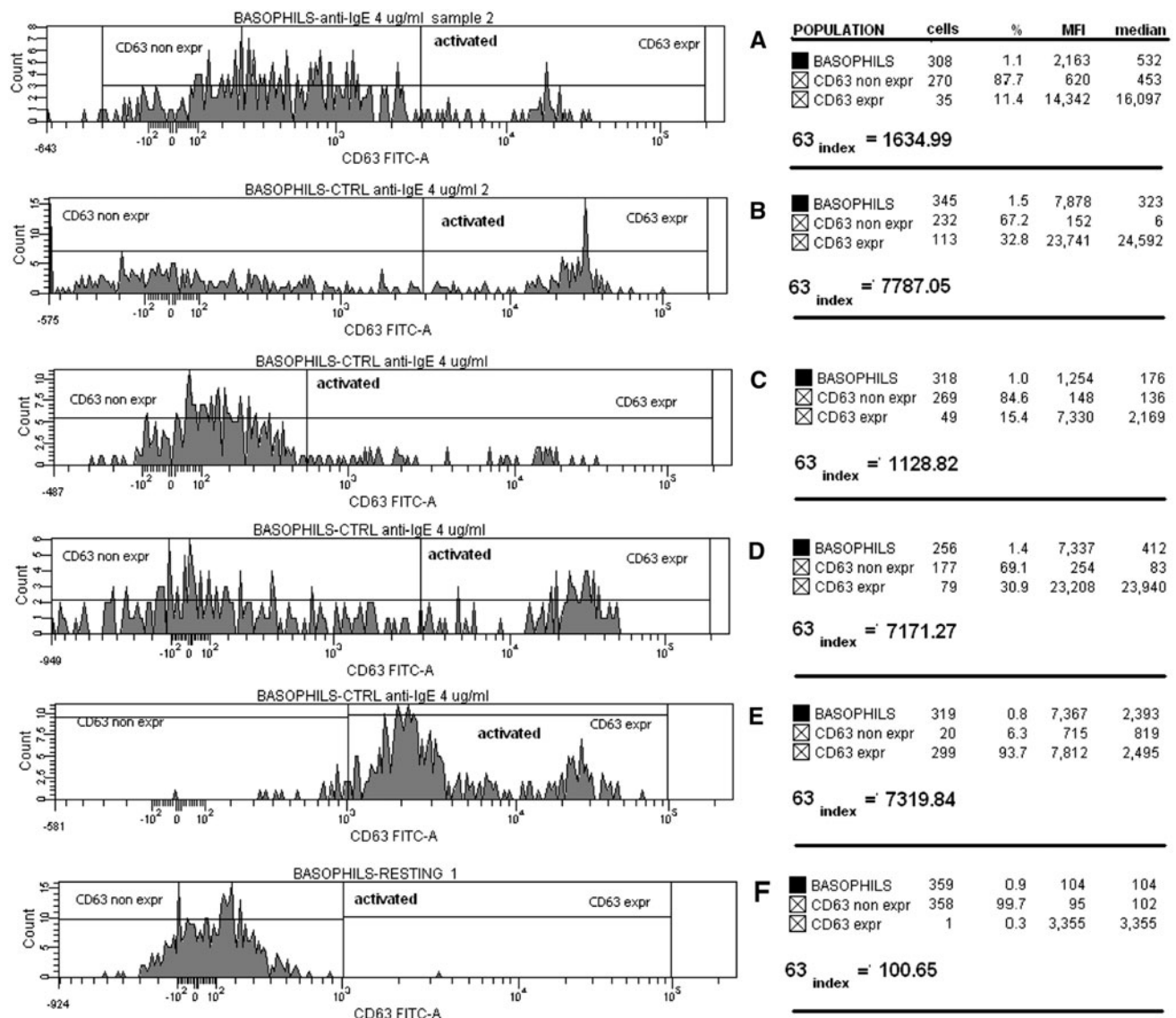


Fig. 2 Different expression of CD63 in different beta-lactam atopic individuals as shown by fluorescence histograms in a BDFACScanto II flow cytometer (*left*) and as counts, percentage, and fluorescence outputs from the instrument (*right*). For each sample, a total of about 50,000 events was acquired in which, by applying this gating protocol, approximately 500–1,000 basophils were counted in the gate. To evaluate fluorochrome non-specific staining, isotype controls for anti-IgG₁ and anti-IgG_{2x} 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 the fluorochrome of interest in the assay system was also performed. To estimate the number of antibodies bound per cell (APC), the Quantum™ Simply Cellular® kit distributed by Bangs Laboratories, Inc., designed for measuring immunofluorescence

directly and providing several different ranges of antibody-binding capacity (ABC) (1,000–80,000, 5,000–150,000, and 10,000–200,000) as well as MESF kits (Quantum-FITC 5 MESF) was used. 63_{index} was calculated according to the formula in the text. Typical experiments with six quadruplicate assays. **a** Basophils from whole blood activated with 4.0 $\mu\text{g}/\text{ml}$ anti-IgE and read 2 h after the treatment; **b** the same as **a** but reading performed 24 h later after formaldehyde fixation; **c**, **d**, **e** basophils from 3 different atopic subjects activated with 4.0 $\mu\text{g}/\text{ml}$ anti-IgE; **f** expression of the marker in non-activated basophils (resting cells). Methods according to reference [11]. The *right-hand part* of the fluorescence histograms indicated as *activated* shows the percentage of cells up-regulating CD63 on the membrane. See text for further explanation

that 63_{index} is able to suggest that basophils should work with the same activation strength, as 63_{index} shows similarity with the previous example described (Fig. 2e, $63_{\text{index}} = 7,319.84$). This case is not due to flow cytometry artefacts, as cells were viable and the resting non-activated

condition was perfectly comparable to other resting populations (Fig. 2f showing resting state of the same basophils as in Fig 2e). Otherwise, an anergic or non-releaser atopic subject [12, 13] may up-regulate CD63 in a small percentage of the gated population, even with an

optimal dose of activation (Fig. 2c), a pattern that is commonly frequent in healthy screened non-atopic blood donors (data not reported). Activation appears to induce different levels of CD63 expression on different basophils captured in the gate; in the routine condition of a basophil activation test, and keeping the same experimental flow cytometry setting and reagent batches, it is not possible to assess whether the resulting distribution is due to different expression or to different down-regulation (with subsequent internalization) of the marker. The use of simple %CD63^{expressing} basophils does not deal with this issue. For example, when the investigator assesses the fluorescence of a resting non-activated basophilic cell population, in order to evaluate a threshold, he does this procedure on a fluorescence distribution, with its own MFI. The non-activated population, evaluated as percent/total on the basis of the fluorescence expressed on the left of this threshold, is supposed to have the same basal MFI of the resting samples. This is theoretically true if the investigator uses the same basophil population and the same experimental setting and condition, but it cannot be proven in the CD63% by itself. If during the experimental procedure the whole basophil population changes their basal MFI before the allergen challenge, because of various pre-analytical factors, the investigator has the possibility to observe a shift of the fluorescence curves but not necessarily a sensitive difference in the percentage of cells expressing CD63-fluorescence on the right of the threshold. The final judgment may be the same, but the two samples, with the same CD63%, may have different diagnostic power if investigators pay attention to the surface expression of CD63 by introducing fluorescence measurements in their evaluations. However, this approach does not completely prevent the influence of many other critical issues, such as carbohydrate interference with BATs [14], and therefore its limitations encourage us to associate this parameter with others, such as serum IgEs and skin tests. Although these arguments appear rather speculative, while taking into account some experimental examples, the need for improving the diagnostic power of CD63% in BATs is becoming a major request in cellular allergy tests. Obviously, MFI cannot be used by itself in order to achieve a diagnostic suggestion, due to the many variables involved in its evaluation; however, modern instruments are quite sturdy in keeping to standardized pre-analytical conditions, namely laser voltage and fluorochrome fluorescence hallmarks, which directly influence MFI, in order to achieve a stable technical and analytical performance. Therefore, as set-up-dependent parameters are only lasers, filters, detectors, fluorochrome performance and antibody clones, variations in a standardized system are likely induced only by statistical errors and biological variability. In this context one author has subtracted a

possible cell autofluorescence, which might affect net MFI evaluation during analysis, but in our test condition this fluorescent noise was not present or negligible [15]. Diagnosis of allergy still represents a fundamental obstacle in the management of chronic pathologies, such as asthma or urticaria, and optimization of the current available analytical tools should also allow us to confront severe adverse effects to allergens such as anaphylaxis. The CD63-based test for investigating basophil function may be improved by the introduction of new evaluation parameters or new activation markers [16], by new gating strategies to capture basophils in blood samples, by collating all efforts from researchers to expand the debate and to build a consensus guideline of BAT standardization and application. Certainly, improving the use of this test for allergy diagnosis should ensure that medical intervention in hypersensitivity reactions may be increasingly successful in the near future.

Conflict of interest None.

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