



Research paper

Flow cytometry for basophil activation markers: The measurement of CD203c expression is as reliable as CD63 up-regulation in the diagnosis of cat allergy

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Abstract

The flow cytometric basophil activation test (BAT), based on the detection of allergen-induced CD63 expression, has been proved effective in the diagnosis of various IgE-mediated allergies. However, there is not yet consensus about the suitability of CD203c expression as a specific basophil activation marker and its diagnostic reliability. The goal of the present study was to compare measurement of CD63 and CD203c expression using BAT in a model of cat allergy and to determine optimal experimental conditions for both markers. Heparinized whole blood samples from 20 cat allergic patients and 19 controls were incubated with Fel d1 (relevant allergen) or anti-FcεRI (positive control) either in IL-3 or IL-3-free conditions. An optimal gating of basophils was achieved in triple staining protocols: anti-IgE PE/anti-CD45 PerCP/anti-CD63 FITC or anti-IgE FITC/anti-CD45 PerCP/anti-CD203c PE. We demonstrated that IL-3 significantly enhanced CD63-induced expression by basophils obtained from cat allergic patients in response to Fel d1. Sensitivity was found to be 100%. The CD203c protocol, when performed under IL-3-free conditions, also demonstrated 100% sensitivity. Only one of the control subjects was positive in both tests. In conclusion, using well-defined experimental conditions, the measurement of CD203c up-regulation on basophils in response to specific allergens is as reliable as CD63-BAT for the in vitro diagnosis of patients with IgE-mediated allergy.

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Keywords: Allergy; Basophil activation tests; Flow cytometry; CD63; CD203c

1. Introduction

Challenging basophils with allergens leads to an activation status characterized by changes in the cell surface molecules and by subsequent release of several mediators such as histamine, sulfidoleukotrienes (LTs) and cytokines.

Abbreviations: MFI, mean fluorescence intensity; BAT, basophil activation test; SI, stimulation index; LTs, sulfidoleukotrienes.

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In the early 1990s, the discovery of the basophil activation marker CD63 resulted in the development of flow cytometry techniques which were useful to monitor allergen-specific activation of basophils. The flow-cytometric basophil activation test (BAT) based on the neo-expression of CD63 is now currently used and has been proved to be effective in the diagnosis of IgE-mediated allergy (Moneret-Vautrin et al., 1999; Abuaf et al., 1999; Siegmund et al., 2000; Sanz et al., 2001, 2002; Ebo et al., 2002; Erdmann et al., 2003).

More recently, the CD203c marker (ecto-nucleotide pyrophosphatase/phosphodiesterase 3) was proposed as a valuable tool for the detection of both non-activated and activated basophils by flow cytometry (Buhning et al., 1999). Indeed, this antigen, selectively expressed on basophils, mast cells and their progenitors, is rapidly up-regulated after activation of IgE-sensitized basophils with the relevant allergen. CD203c has already been used successfully for the diagnosis of allergy to hymenoptera venom (Platz et al., 2001) or grass pollens (Hauswirth et al., 2002; Kahlert et al., 2003). However, the few attempts to compare BAT using CD63 and/or CD203c expression for allergy diagnosis resulted in contradictory results (Boumiza et al., 2003; de Weck and Sanz, 2003; Ebo et al., 2003; Sudheer et al., 2005; Eberlein-Konig et al., 2006). Therefore, the main goal of this study was to compare BAT measuring either CD203c up-regulation or CD63 induction by basophils in patients with cat allergy. Furthermore, special emphasis was given to the experimental conditions, such as the basophil gating strategy, the need for an IL-3 priming stimulus and expression of basophil activation levels. Finally, comparison of BATs with the LTs released assessed by the CAST-2000 ELISA (de Weck and Sanz, 2004) was performed.

2. Materials and methods

2.1. Selection of patients

Twenty patients suffering from allergy to cat were included. All had a clinical history of rhinitis or asthma when exposed to cat and showed specific serum IgE (CAP-FEIA-Phadia, Uppsala, Sweden) and/or a positive skin prick test to cat extract (Stallergènes, Fresne, France). Nineteen healthy subjects with no history of allergy to cat and no sensitization to cat extracts (negative skin prick test and absence of specific IgE) served as controls.

The institutional scientific and ethic committees approved this study.

2.2. Allergens

The major purified cat dander allergen of *Felis domesticus* (Fel d1) and birch extract were purchased from ALK-Abello A/S (Horsholm, Denmark) and Stallergènes (Fresne, France), respectively.

2.3. Flow-cytometry-based basophil activation tests

All the tests were carried out within 4 h after blood sampling.

CD63 induced expression was evaluated using the Basotest® Kit (Orpegen Pharma, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, sodium heparin-anticoagulated peripheral blood aliquots (100 µl) primed or not with 20 µl of IL-3 containing buffer (10 ng/ml, 10 min, 37 °C) were stimulated (37 °C) for 20 min with 100 µl of optimal concentrations of Fel d1 (0.5 µg/ml) or birch allergen extract (0.01 µg/ml); antibody directed against the high affinity IgE receptor (FcεRI) (Bühlmann Laboratories AG, Allschwil, Switzerland) was used as positive control or washing solution as negative control. All dilutions were carried out in washing solution provided with the kit.

After incubation, samples were cooled on ice to stop basophil activation and degranulation and mixed with staining reagents including anti-CD63 FITC, anti-IgE PE (Basotest® Kit) and anti-CD45 PerCP (Becton Dickinson, San Diego, CA). After 20 min incubation on ice in the dark, erythrocyte lysis (Basotest kit), washing and centrifugation, leukocyte pellets were resuspended in washing solution and analyzed using a six-color flow cytometer (FACSCanto-BD Immunocytometry Systems). During acquisition, red fluorescence (FL2) and size scatter (SSC) were employed to gate on at least 1000 basophils that expressed high density surface of IgE per sample. For analysis, a first gate using FSC/SSC characteristics of lymphocytes was defined. Then, from these cells, basophils were selected as CD45^{low}/IgE^{high} on an FL3/FL2 dot plot (see Fig. 1). Within this gate, the percentage of activated basophils, i.e. those co-expressing CD63 (FL1) was measured. For this purpose, quadrant markers were set on the value obtained without stimulation. Percentages of activated basophils were corrected by subtracting spontaneous CD63 expression (negative control) from the values obtained after stimulation.

CD203c induced up-regulation was assessed using a similar experimental protocol. Briefly, whole blood, IL-3 primed or not, was stimulated (37 °C) for 15 min with the same dilutions of allergens or controls used in the CD63-BAT. The reaction was stopped on ice, followed

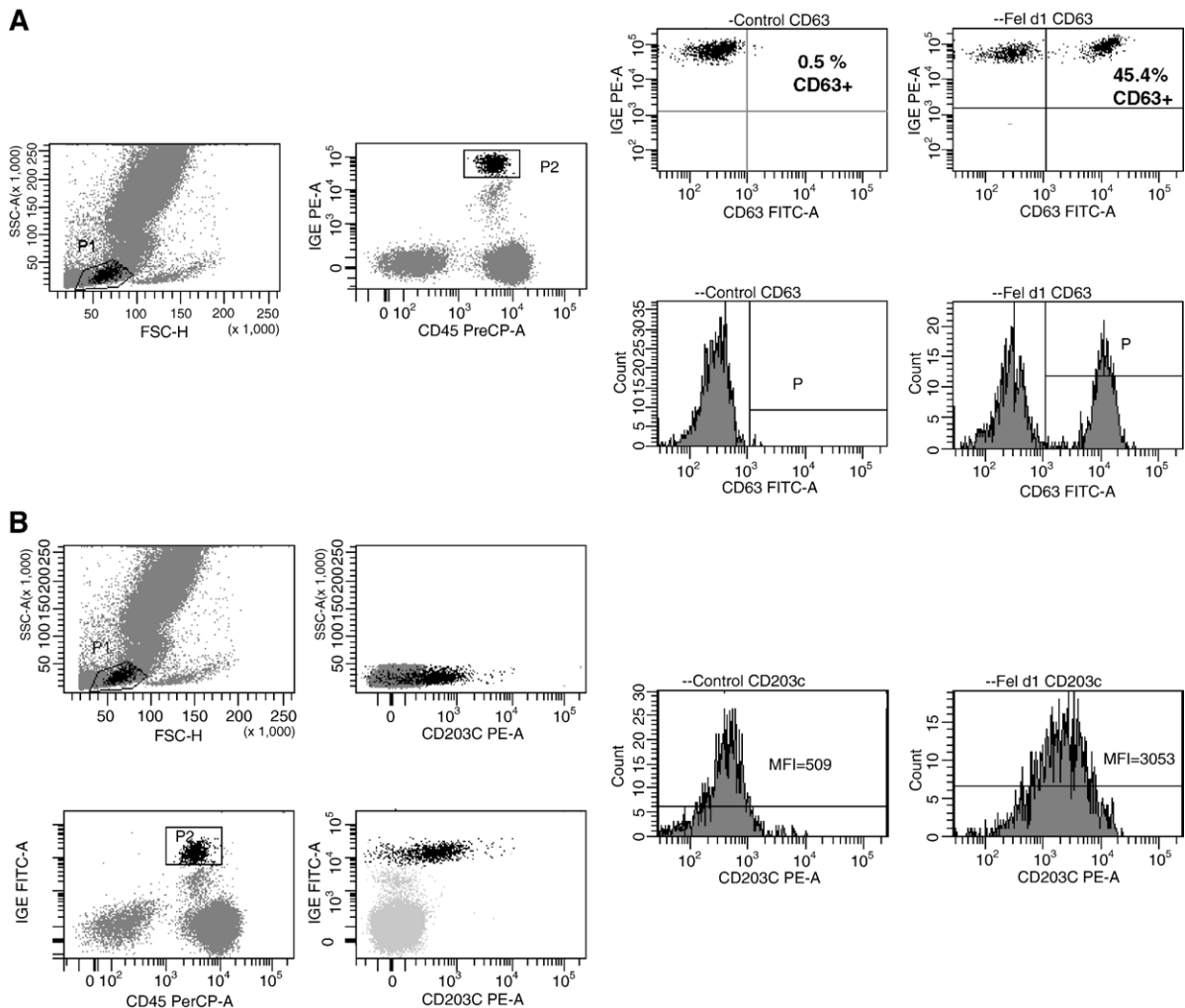


Fig. 1. Example of optimal basophil gating in the CD63 (A) or the CD203c (B) protocol. One hundred microliters of whole blood from one allergic patient were incubated with medium (control) or Fel d1 (allergen). After staining with anti-CD63 FITC/anti-CD45 Per-CP/anti-IgE PE or anti-CD203c PE/anti-CD45 Per-CP/anti-IgE FITC, the cells were analyzed on a FACS Canto flow cytometer. The FL-2/FL-3 or FL-1/FL-3 plots identify IgE+/CD45^{low} basophils (black boxes). The results are expressed in % CD63+ activated basophils or SIs (ratio MFI Fel d1/MFI control) for CD203c as described in Section 2.

by 20-min staining on ice with anti-IgE FITC (Caltag Laboratories, Burlingame, CA), anti-CD203c PE (Coulter Immunotech, Marseille, France), anti-CD45 PerCP (Becton Dickinson, San Diego, CA). One thousand basophils selected as IgE^{high} on an SSC/FL1 gate were counted. Basophils defined as CD45^{low}/IgE^{high} among lymphocytes were gated and analyzed for CD203c expression on an FL1/FL2 dot plot or on a simple histogram (count/FL2). Results were expressed as the stimulation index (SI), the ratio of the mean fluorescence intensities (MFI-FL2) related to the entire basophil population obtained with and without stimulation. For comparison purposes, the results were also

analyzed in net percentages of CD203c positive basophils (CD203^{high}), the threshold being defined by the negative control.

2.4. Allergen-specific sulfidoleukotriene release

The release of LTs by allergen stimulation was measured using the CAST 2000 ELISA (Bühlmann Laboratories AG, Allschwill, Switzerland). Within 4 h after blood sampling, whole blood taken on EDTA was sedimented in the presence of Dextran to obtain a leukocyte enriched suspension. Cells were suspended in a stimulation buffer containing IL 3 and stimulated for

40 min at 37 °C with optimal concentrations of Fel d1 allergen (0.2 µg/ml), anti FcεRI as positive control or stimulation buffer as negative control. All dilutions were carried out in stimulation buffer provided with the kit. LTs concentrations were measured by ELISA in the supernatants and the results were expressed in pg/ml.

2.5. Statistics

The non parametric Mann–Whitney and Wilcoxon tests were used for comparisons. The correlations were analyzed with Spearman's rank order correlation coefficient test.

3. Results

3.1. Multicolor flow cytometry strategy

In the present study, and as also reported by Boumiza et al. (2003), we used three-color flow cytometric BAT protocols, relying first on the same characteristics of basophils, i.e. low CD45 and high IgE expression, and second on the monitoring of the activation marker CD63 or CD203c. Therefore whole blood samples (100 µl) were incubated after stimulation with medium (negative control), anti-FcεRI (positive control) or relevant allergen (Fel d1) with the following association of antibodies: anti-CD45 PerCP/anti-IgE PE/anti-CD63 FITC or anti-CD45 PerCP/anti-IgE FITC/anti-CD203c PE. As shown in Fig. 1, triple staining readily identified the basophils (IgE^{high}) from other cells and localized them within the CD45^{low} region. This strategy also permitted a complete recovery of CD203c positive basophils. Indeed, in about half of the individuals tested (both patients and controls), resting basophils weakly expressed CD203c and can only be clearly distinguished from other cells when double-stained with an anti-IgE. Fig. 1 also demonstrates that CD63 and CD203c basophil activation markers are different in their up-regulation profile. Allergen stimulation generates a distinct sub-population of basophils that express CD63 with a high density and consequently can be expressed as percent activated CD63+ basophils. Up-regulation of CD203c by activated basophils is less prominent and often occurs on almost all basophils making the demarcation of negative (non-activated CD203c^{low}) and positive (activated CD203c^{high}) cells and the expression in percent activated CD203c+basophils not always appropriate. In view of these observations, the results were reported as the stimulation index (SI) for the CD203c marker. For comparison purposes, data were also analyzed as percentages.

3.2. Role of IL-3 priming in the CD63-BAT

The CD63-BAT Basotest® provided by Orpigen advocates a pre-incubation step of whole blood with a “stimulation buffer” containing IL-3 (final concentration 2 ng/ml). The need for such pre-priming of basophils with IL-3 in the CD63-BAT remains a matter of controversy (Ebo et al., 2006). With regard to this question, whole blood obtained from four cat allergic patients was incubated (or not) with IL-3 (10 min, 37 °C) and stimulated (20 min, 37 °C) with appropriate concentrations of anti-FcεRI as a positive control or Fel d1 as relevant allergen. Fig. 2 indicates that pre-incubation with IL-3 clearly improves the degree of allergen-specific induced expression of CD63 by basophils. The percentage of Fel d1 activated CD63+ basophils ranges from 1% to 28.6% in the absence of IL-3 and from 44% to 85% in the presence of IL-3. In three patients out of four, the CD63-BAT may even be considered negative when performed on whole blood in the absence of IL-3 priming. Basophil activation in response to the positive control anti-FcεRI is also clearly increased in IL-3 pre-activation conditions: 31.5–83.8% vs 74.4–90.8% in the absence or presence of IL-3, respectively. While IL-3 significantly potentiates specific allergen-induced activation of basophils, it has no effect on unstimulated (negative control) CD63 expression by basophils (data not shown). In view of these results, the CD63 tests in our patients with cat allergy were only evaluated in IL-3 priming conditions.

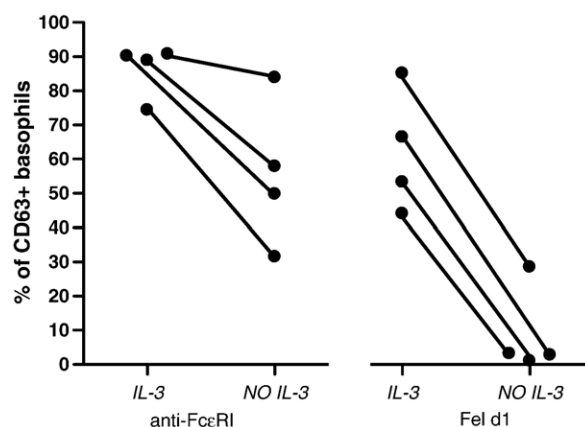


Fig. 2. Role of IL-3 priming in the CD63 BAT: whole blood obtained from four cat allergic patients was primed (or not) with IL-3 (final concentration 2 ng/ml, 10 min, 37 °C) and stimulated with anti-FcεRI as positive control or Fel d1 as relevant allergen. The results are expressed in % CD63+ activated basophils.

3.3. CD63-BAT

The individual percentages of CD63+basophils obtained after Fel d1 challenge of whole blood from healthy individuals and cat allergic patients are shown in Fig. 3.

With a cut-off of 8.1% (mean + 2 SD obtained after Fel d1 challenge in the control group) indicated by the dashed line, all patients allergic to cat (20/20) show a positive CD63-BAT (sensitivity 100%) while only one of the control subjects (specificity 95%) was positive. Comparison of the percentages of CD63+basophils shows significant differences between patients ($54.9 \pm 7.1\%$) and controls ($1.1 \pm 0.8\%$) ($p < 0.0001$). All allergic patients ($72.5 \pm 5.8\%$) and control subjects ($48.7 \pm 7.2\%$) ($p = 0.014$) responded to the anti-FcεRI challenge.

3.4. CD203c-BAT

In order to compare CD63 and CD203c BATs under the same analytical conditions, we first assessed the CD203c assay under IL-3 priming conditions. The individual results expressed as the SI obtained in non-allergic individuals and cat allergic patients are shown in Fig. 4. With a positive threshold value of 1.38 SI (mean + 2 SD after Fel d1 challenge in the control group), 19

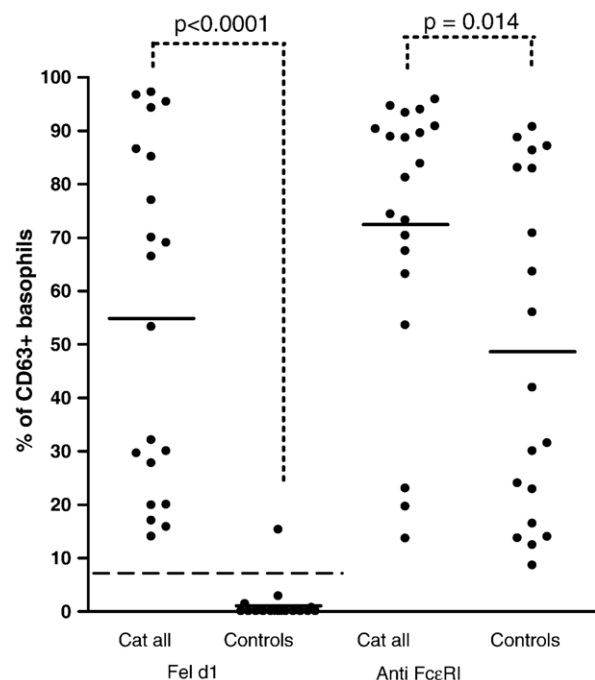


Fig. 3. CD63-induced expression by basophils in 20 cat allergic patients and 19 control individuals after Fel d1 or anti-FcεRI stimulation. Results are expressed in % CD63+ activated basophils.

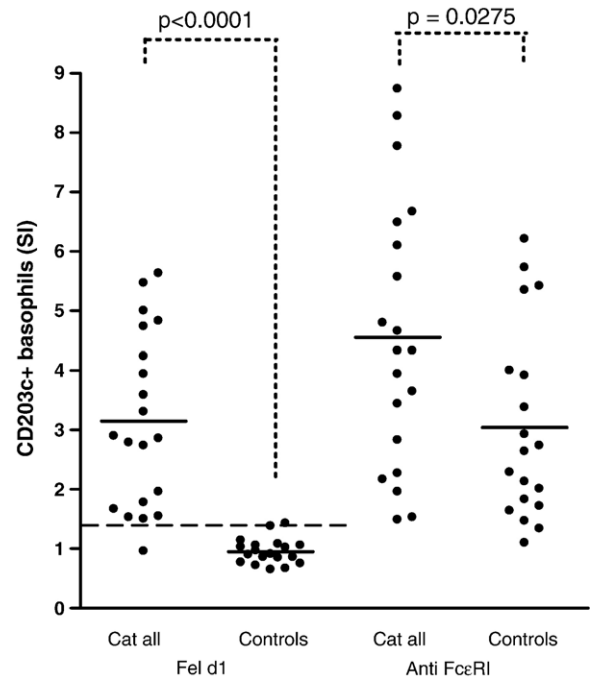


Fig. 4. CD203c up-regulation by basophils in 20 cat allergic patients and 19 control individuals after Fel d1 or anti-FcεRI stimulation under IL-3 priming conditions. Results are expressed in SIs.

patients out of 20 with allergy to cat were positive leading to a sensitivity of 95%. One out of 19 controls demonstrated a basophil activation test just above the cut-off value (specificity of 95%). Whole blood stimulation with anti-FcεRI resulted in activation of basophils from all allergic patients and control subjects, with levels of activation varying between 4.55 ± 0.5 SI and 3.05 ± 0.37 SI; $p = 0.0275$. When the results were analyzed as percentages instead of SI, the sensitivity of the CD203c BAT was reduced; e.g. two low responders (one control and one allergic) to anti-FcεRI (SI=1.53 and 1.72) became classified as non-responders (0.85% and 3.17%) and one cat allergic subject should have been considered non-responsive to the relevant allergen Fel d1 (SI=2.9 but only 2.51%). In this case, the activation of basophils did not induce enough positive CD203^{high} basophils to reach significant levels above the cut-off (=5.2%) defined as mean % + 2 SD obtained after Fel d1 challenge in the control group.

3.5. Role of IL-3 priming for CD203c

To our knowledge, the effect of IL-3 priming on the CD203c BAT has never been extensively analyzed. We compared Fel d1-mediated up-regulation of CD203c by basophils under IL-3 or no IL-3 priming conditions in

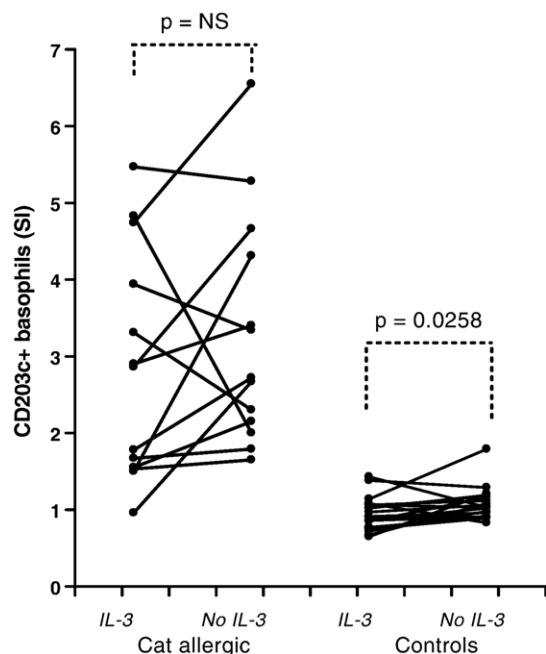


Fig. 5. CD203c up-regulation by basophils obtained in the presence and absence of IL-3 priming in response to Fel d1 in 13 cat allergic patients and 19 controls. Results are expressed in SIs.

13 individuals from the cat allergic group and 19 control subjects. First, we verified that the time course of basophil activation was not modified in the absence of IL-3 (data not shown). With a threshold of SI=1.51 for the CD203c BAT performed under IL-3-free conditions, the test was positive in 13/13 allergic subjects (sen-

sitivity 100%) and in 1/19 controls (specificity 95%). By comparison, when the IL-3-CD203c BAT was considered, the number of positive tests was 12/13 in the allergic group (Fig. 5). When data were analyzed as percentages instead of SI, similar results were obtained. These results indicate that the presence of IL-3 does not improve sensitivity and even may be detrimental. One reason for this may be that IL-3 by itself, in the absence of any other stimulation, up-regulates CD203c expression by basophils. In the present work, the only allergic patient who, in response to Fel d1, exhibited a negative IL-3-CD203c BAT (SI=0.96) but a positive IL-3-free-CD203c BAT (SI=2.67) showed a strong (five-fold) up-regulation of CD203c expression by its basophils exposed to IL-3 alone. Such a high level of cytokine activation seems to hamper the subsequent up-regulation of CD203c by the allergen, leading to an SI below the positivity threshold.

Considering all subjects (allergic and non-allergic) tested at the same time in IL-3 and in IL 3 free CD203c BATs, we found that resting basophils were more sensitive to the IL 3 effect than allergen stimulated basophils. Indeed, by comparison with cytokine free conditions, baseline MFIs of the CD203c marker were 3.38 times higher in the presence of IL 3. When combined with specific allergen stimulation, IL 3 similarly affected CD203c MFIs but to a lesser extent; following Fel d1 challenge, MFIs were 2.85 fold higher in the presence of IL 3 compared to IL 3 free conditions. Taken together, these observations make interpretation of the IL 3 CD203c BAT difficult.

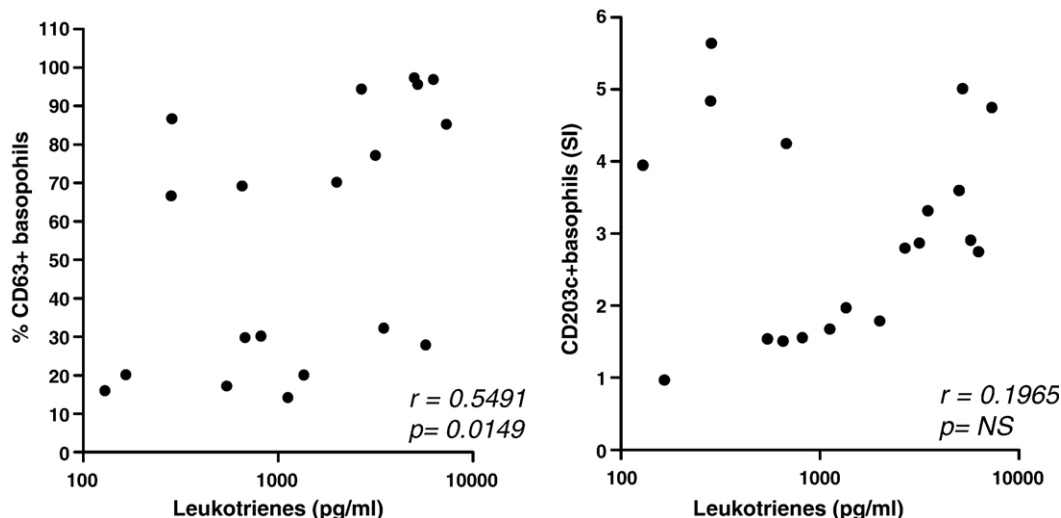


Fig. 6. Relationship between leukotriene levels and CD63 expression (left) or CD203c up-regulation (right) after Fel d1 challenge in 19 cat allergic patients.

3.6. Antigenic specificity of CD63 and CD203c responses

In order to assess the antigenic specificity of the CD63 and CD203c responses to Fel d1 in cat allergic patients, basophil activation was studied after birch challenge in five cat allergic patients not sensitized to birch (negative prick test and absence of specific IgE) compared to nine birch allergic individuals. Optimal conditions for measuring basophil activation in response to birch were determined previously in birch allergic patients (data not shown). None of the five cat allergic patients showed a positive basophil activation test in response to birch.

3.7. Activation of basophils measured by CAST

Following the manufacturer's instructions, LTs release or CAST test was performed in the presence of IL 3 on isolated leukocytes obtained from 19 cat allergic patients and 19 controls. The positivity threshold was set at 178 pg/ml (mean + 2 SD after Fel d1 challenge in the control group). All cat allergic and non allergic individuals responded to anti FcεRI (4157 ± 559 pg/ml vs 3082 ± 425 pg/ml). Statistically significant differences were found in the cat specific response between allergic and control individuals (2485 ± 546 pg/ml vs 34 ± 17 pg/ml; $p < 0.0001$); 18/19 patients and 1/19 controls showed a clear positive CAST test leading to a sensitivity and specificity of 95%.

Although no correlation was obtained between IL3 CD203c BAT and CAST, there was a significant positive correlation between the CAST test and the CD63 BAT (Spearman $r = 0.5491$ ($p = 0.0149$) (Fig. 6).

4. Discussion

Flow cytometry BATs based either on the detection of allergen induced CD63 expression or CD203c up regulation on basophil membrane have been proposed as promising alternative methods for *in vitro* diagnosis of IgE mediated reactions (for review see Ebo et al., 2006). Those two markers are quite different in their profile expression. CD63 is a basophil activation marker also present on other cells such as tissue mast cells, macrophages and platelets; CD203c is selectively expressed by the basophil/mastocytes lineage (Buhring et al., 2001). Resting basophils constitutively express plasma membrane CD203c, although sometimes weakly, whereas CD63, a secretory granule associated protein, is expressed by activated basophils concomitantly with cellular degranulation.

The present study shows that BATs measuring CD203c and CD63 are both reliable tests in the diagnosis of cat allergy. This confirms the results from

a recent study comparing the two markers in the diagnosis of venom allergy (Eberlein-König et al., 2006). Other previous studies were either in favor of CD203c (Boumiza et al., 2003) or in favor of CD63 (De Weck and Sanz, 2003; Sudheer et al., 2005). Such discrepant findings could result from different analytical approaches. Indeed, if the principle of the CD63 and CD203c BATs are quite similar, the optimal technical conditions for both tests might not be identical. Therefore, using a model of cat allergy, we investigated the crucial points of the flow cytometry protocols with respect to the combination of antibodies for optimal basophil gating and the role of IL 3 priming.

First, with regard to basophil gating strategy, the most common technique to select basophils by flow cytometry relies on identification of these cells by an anti IgE (high expression). In the present work, we performed a tricolor flow cytometry CD63 BAT combining an anti CD45 with an anti-IgE for characterization of basophils (IgE^{high}CD45^{low}) and an anti-CD63 for assessment of their activation. Gating around IgE^{high}/CD45^{low} cells was beneficial to exclude basophils from dendritic/monocytes (IgE^{low} cells) and platelets (CD45^{negative}, sometimes CD63+). Ideally, since CD203c is selectively and consecutively expressed by basophils, the CD203c BAT could allow a single color flow cytometry test (Hauswirth et al., 2002; Buhring et al., 2004). In this study, we demonstrated that staining with an anti-IgE was mandatory to yield optimal gating of basophils, especially when these cells express low constitutive levels of CD203c.

Second, IL-3, known to be an important growth factor for basophils, has been reported to improve basophil activation as measured by histamine and LTs release (Valent et al., 1989; MacDonald et al., 1989; Miura and MacGlashan, 2000; Sainte-Laudy et al., 2000) or CD63 expression (Cozon et al., 1999). This was confirmed by the present work in which IL-3 clearly improves the sensitivity of the CD63 assay. However, while IL-3 does not change spontaneous CD63 expression, it enhances that of CD203c by resting, non-activated basophils. Consequently, by reducing the difference between negative (non-stimulated basophils) CD203c^{low/MFI_{low}} and positive (allergen-stimulated basophils) CD203c^{high/MFI_{high}}, IL-3 may adversely affect CD203c BAT sensitivity. The differential influence of IL-3 on both markers of activation could explain the contradictory results observed when both tests are compared. For example, in latex allergy, Boumiza et al. (2003) reported 75% and 50% sensitivity for the CD203c BAT and CD63 BAT, respectively, when both protocols were performed in

the absence of IL-3. In contrast, when using IL-3 during the procedure, [de Weck and Sanz \(2003\)](#) found a marked improvement of the CD63-BAT in comparison with the CD203c BAT for the diagnosis of allergy to *Alternaria*, latex or peach. These results suggest that the usefulness of IL-3 priming depends on the marker selected to detect basophil activation. In this study, CD63 and CD203c BATs performed under adequate conditions, i.e. IL-3 priming for CD63 only, provide a similar degree of diagnostic accuracy. The sensitivity reaches 100% and the specificity is 95% for both markers. These optimized BAT techniques will further benefit from the recent introduction on the market of fluorochrome-labeled monoclonal antibodies directed against the CRTH2 antigen (CD294; chemoattractant receptor-homologous molecule expressed on T-helper 2 cells and basophils) ([Nagata et al., 1999](#); [Boumiza et al., 2005](#)) which, instead of an anti-IgE, will provide an optimal method for the detection of basophils in whole blood.

Distinct methods of regulation may explain the differential outcome of CD63 and CD203c expression by activated basophils. Indeed, CD203c and CD63 up-regulation appear to follow different kinetics and alternative activation pathways ([Hennersdorf et al., 2005](#); [Monneret et al., 2005](#)). Based on kinetic data and co-expression of CD63 with another marker of degranulation, CD107a, [Hennersdorf et al. \(2005\)](#) hypothesized that the CD63 pathway is closer to anaphylactic degranulation than the expression of CD203c. Our results support this dissection of the CD203c and the CD63 pathways. Indeed, first CD63 but not CD203c expression was correlated with the leukotrienes released. Second, Fel d1-specific allergen challenge rapidly up-regulated CD203c on almost all basophils leading to a shift towards higher MFIs of the entire population; in contrast, CD63 expression was induced with a delayed kinetic only in a sub-population of basophils. The question is whether CD203c BAT detects more activated basophils or a subset of activated basophils not tightly coupled to degranulation. Along these lines, it would be of interest to perform multicolor flow cytometry BAT combining CD203c with CD63 and/or CD107a markers for their expression analysis.

In conclusion, we showed that the CD203c BAT performed under optimal technical conditions, e.g. absence of the IL-3 priming step, triple-staining flow cytometric protocol, is as reliable as the CD63 BAT for the diagnosis of cat allergic patients. However, this enthusiasm for CD203c remains to be confirmed by additional studies dealing with different types of allergens and supported by inter-laboratory standardization.

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