

# Robust expression of CCR3 as a single basophil selection marker in flow cytometry

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## Abstract

**Background:** Basophil activation tests (BAT) rely on different combinations of basophil selection and activation markers. Whereas activation markers, especially CD63, are widely validated, the most suitable and robust marker for basophil selection is still a matter of debate.

**Aims:** Comparison of cell surface expression of two commonly used basophil selection markers (IgE, CD123/HLA-DR) with CCR3 in an unselected group of atopic and nonatopic donors in resting and activated basophils.

**Methods:** EDTA blood of 94 healthy adults, about half of them atopic by history, was analyzed using two different staining strategies: anti-CD123-PE/anti-HLA-DR-PerCP/anti-lin1-FITC and anti-IgE-FITC/anti-CD3-PerCP/anti-CCR3-PE. Additionally 40 pollen-allergic patients were recruited for the assessment of CCR3 expression after basophil activation.

**Results:** In resting basophils, cell surface expression of the three basophil selection markers was most constant for CCR3. IgE gating strategy showed the highest variation and up to 80% of nonbasophils in the selected gate in certain donors.

During basophil activation, a shift of the mean fluorescence intensity for CCR3 toward the lower third of the CCR3-positive population could be demonstrated, but neither were CCR3-positive cells significantly lost for further analysis nor was differentiation between CCR3-positive and CCR3-negative cell populations hampered by this shift.

**Conclusions:** CCR3 is a stable and highly expressed basophil selection marker, independent of the atopic background or basophil activation state and allows an accurate identification of basophils without need of a second marker. The basophil markers CD123/HLA-DR and IgE showed significantly higher interindividual variability in cell surface expression and are therefore less suited as selection markers.

Basophils are the least abundant granulocyte subpopulation (<1% of total leukocytes), which hampered their in depth analysis until the age of high-throughput flow cytometry. In the last decade, various centers developed flow cytometric basophil activation tests (BAT), mainly for diagnostic purposes in IgE-mediated allergies. The different BAT approaches in flow cytometry are based on varying combinations of basophil selection and activation markers [reviewed in (1)]. CD63 is currently the best-validated basophil activation marker directly linked to basophil degranulation. However, it is still unclear which of the basophil selection strategies is best suited

for an unbiased and robust identification of these cells (2, 3). In the present study, two commonly used basophil selection markers, IgE and CD123<sup>pos</sup>/HLA-DR<sup>neg</sup>, were compared to CCR3 (eotaxin receptor) concerning cell surface expression in an unselected cohort of atopic and nonatopic donors. Resulting basophil numbers and the potential 'contamination' of the selected cell population with 'nonbasophils' sharing cell surface markers with basophils (lymphocytes, monocytes, dendritic cells) were also investigated. The possible downregulation of CCR3 surface expression during basophil activation was another focus of our study.

## Methods

### Blood donors

After approval by the local ethics committee, we took blood samples (with EDTA as anticoagulant) from an unselected group of 94 healthy adult volunteers (62 women and 32 men, mean age 40 years, range 20–66 years) and analyzed it within the same day. About half of the donors (32 women and 13 men) were atopic by history, mainly mild form of seasonal rhinoconjunctivitis, taken by an independent physician unaware of the aim of this study. Additionally, 37 birch pollen-allergic patients (19 women and 18 men, mean age 33 years, range 18–61 years) and three grass pollen-allergic patients (men, age 34, 35, 61) were recruited for basophil activation experiments.

### Leukocyte staining

Hundred microliters of whole blood were incubated with fluorochrome-labelled antibodies (Abs) for 15 min at room temperature in the dark. After lysis of erythrocytes with LysingSolution<sup>®</sup>, cells were washed in CellWASH<sup>®</sup> and fixed with CellFIX<sup>®</sup> (all BD Bioscience, San Jose, CA, USA).

Two sets of Abs were used: (1) staining based on CD123: 5 µl anti-CD123-PE, 20 µl anti-lin1-FITC (lineage cocktail: CD3/14/16/19/20/56), 10 µl anti-HLA-DR-PerCP (BD, Franklin Lakes, NJ, USA). (2) Staining based on either CCR3 or IgE: 10 µl anti-IgE-FITC (Caltag, Burlingame, CA, USA); 10 µl anti-CD3-PerCP (BD); 10 µl anti-CCR3-PE (R&D, Minneapolis, MN, USA). The data of 50 000 events were acquired using a FACSCalibur<sup>®</sup>.

### Gating strategies

Basophils were identified using two different staining protocols:

CCR3/IgE staining: From the whole cell population, a primary gating region was set around the monocyte/lymphocyte overlap region, where basophils are located. Basophils were selected on the basis of low granularity (to exclude eosinophils) and either CCR3 positivity (side scatter (SSC)<sup>low</sup>/CCR3<sup>high</sup>) or IgE positivity (SSC<sup>low</sup>/IgE<sup>high</sup>).

CD123/HLA-DR staining: From the whole cell population, basophils were selected in the CD123<sup>high</sup>/HLA-DR<sup>neg</sup> cell gate.

### Basophil activation tests

Whole blood assay in 37 birch pollen-allergic patients: Basophil activation test was performed using reagents/protocols of Flow2CAST (Bühlmann, Schönenbuch, Switzerland) with slightly modified settings (stimulation time, individual antibodies). Briefly, 50 µl EDTA blood from birch pollen-allergic patients were stimulated with either 50 µl (0.5 µg/ml) anti-IgE (clone E124.2.8; mouse IgG1k; Beckman Coulter, Marseille, France), 50 µl fMLP or 50 µl (10 ng/ml) Bet v1 (Biomay AG, Vienna, Austria) in 100 µl (or 150 µl for unstimu-

lated control) stimulation buffer for 30 min at 37°C, 5% CO<sub>2</sub>.

Cells were stained simultaneously with anti-CCR3-APC and anti-CD203c-PE (BioLegend, San Diego, CA, USA). Basophils were selected similar as mentioned earlier and according to their CD203c expression (4). At least 600 basophils were acquired using FACSCanto<sup>®</sup>.

Assay with purified basophils from three grass pollen-allergic patients: Basophils have been purified to near homogeneity (>98%) as described previously (5), suspended in stimulation buffer, and stimulated and stained using reagents/protocols of Flow2CAST (Bühlmann, Schönenbuch, Switzerland). A range of 5000–10 000 events were acquired using FACSCalibur<sup>®</sup>.

### Statistical analysis

Analysis was performed using FLOWJO<sup>®</sup> Version 8.8.6 (Tree Star, Inc, Ashland, OR, USA) software. Data are expressed as percentage of total cell count or as mean fluorescence intensity (MFI). We used PRISM 5.0 (GRAPHPAD Software, La Jolla, CA, USA) for statistical analysis. Nonparametric Kruskal–Wallis or Mann–Whitney test was used to measure significance. Values of  $P < 0.05$  were considered significant.

## Results

### Variability in selection marker expression

Interindividual variability in cell surface expression of each basophil selection marker and the possible discrepancy between atopic and nonatopic donors were investigated. Figure 1 shows examples of dot plots for basophil selection in two donors. The selection of basophils with CCR3 and CD123/HLA-DR is comparable in both donors with a clear-cut separation between positive and negative cell population (Fig. 1 C/D and E/F). The situation is quite different when selecting basophils via IgE. Its density on basophil cell surface is dependent on the expression of its receptor (FcεRI), which is tightly regulated by IgE plasma levels (6, 7). Therefore, basophil gating is particularly difficult in donors with low IgE levels (as exemplified in Fig. 1 A/B, donor 2).

To visualize the differences in cell surface expression, we arranged the gated cell population of 16 randomly selected donors in successive histograms (Fig. 2 A–F). Statistical evaluation for the whole study population is depicted in Fig. 3. The expression of CCR3 was most constant in all donors, regardless of their atopic background, with a significant difference ( $P < 0.0001$ ) to IgE or CD123. As expected, the mean of MFI for IgE staining was higher in the atopic group, but with a similarly high variability.

### Basophil count in peripheral blood

The relative basophil number (expressed as percentage of total cell count) showed comparable results using the three different gating strategies in all donors with a tendency toward higher numbers in the IgE selection strategy (data

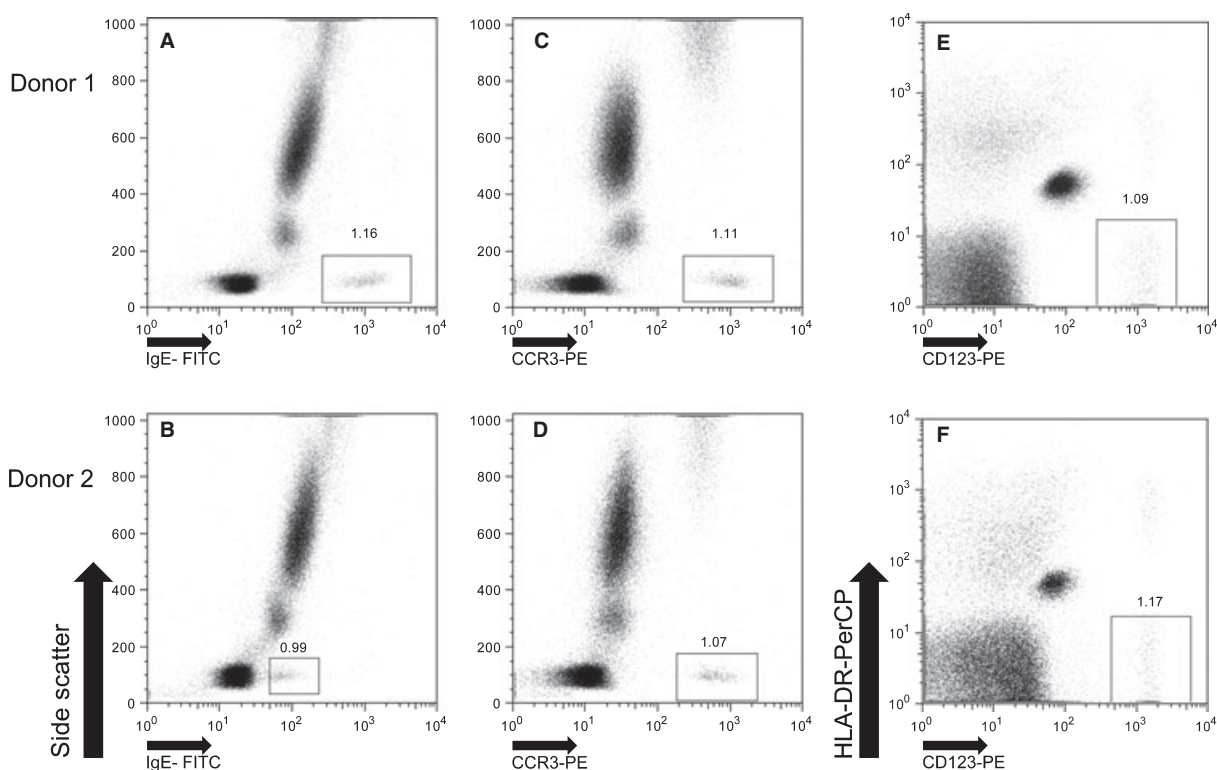


**Figure 1** Dot plot examples of basophil gating of two blood donors using three different selection markers (CCR3, IgE or CD123/HLA-DR). (A) and (B) Density plots of side scatter (SSC) and IgE-FITC. (C)

and (D) Density plots of SSC and CCR3-PE. (E) and (F) Density plots of CD123-PE and HLA-DR-PerCP. Numbers above the gate indicate the amount of positive cells (expressed in % of total cell count).

not shown). This holds true for the single donor as well as the group comparison between atopic and nonatopic donors.

#### 'Contamination' of nonbasophils within the basophil gate

An important issue is the possible 'contamination' of nonbasophils within the basophil gate, because lymphocytes, monocytes and dendritic cells cannot be safely excluded by size or granularity alone and share cell surface markers with basophils. We could substantiate the earlier observations (8) that basophil gating via IgE can result in up to 80% of nonbasophils in the selected gate in certain donors (Fig. 4C). In our study population, activated T<sub>H</sub>2-cells co-expressing CCR3 as potential 'contaminators' could not be detected in relevant amounts (Fig. 4A). The same holds true for the CD123/HLA-DR selection strategy, where other 'contaminating' cell populations (lin1<sup>pos</sup> cells) were below 4% (Fig. 4B).

#### Assessment of changes in CCR3-staining upon basophil activation

In eosinophils, CCR3 downregulation upon activation has been reported (9). Basophil stimulation of 37 whole blood samples of birch pollen-allergic patients resulted in a shift of the MFI for CCR3 toward the lower third of the CCR3-positive gate, especially with IgE-dependent stimuli, including Bet

v1, the major allergen of birch pollen. But none of these shifts led to a significant difference ( $P > 0.05$ , Kruskal-Wallis test) between unstimulated and stimulated samples (Fig. 5A). Furthermore, we observed a slight decrease in the relative number of acquired basophils upon stimulation, which did not reach statistical significance (Fig. 5B).

To further investigate possible changes in CCR3 density upon stimulation, we used purified basophils, allowing us to analyze the behavior of the entire cell population independently of staining/gating strategies that are needed to exclude contaminating leukocytes. Again, we observed a slight decrease in CCR3 staining upon activation, which was however statistically not significant (data not shown). Interestingly, the decrease in CCR3-staining was restricted to the degranulated, CD63-positive subpopulation (Fig. 6). This decrease, when compared to the CD63-negative cell fraction, was statistically significant ( $P < 0.005$ ). It should be noted, however, that based on the activation experiments in whole blood, CCR3 expression remained high still allowing selection of basophils based on this single marker.

#### Conclusions

Cell surface expression of the three basophil selection markers studied was most constant for CCR3 irrespective of the atopic background. This is in agreement with our previous

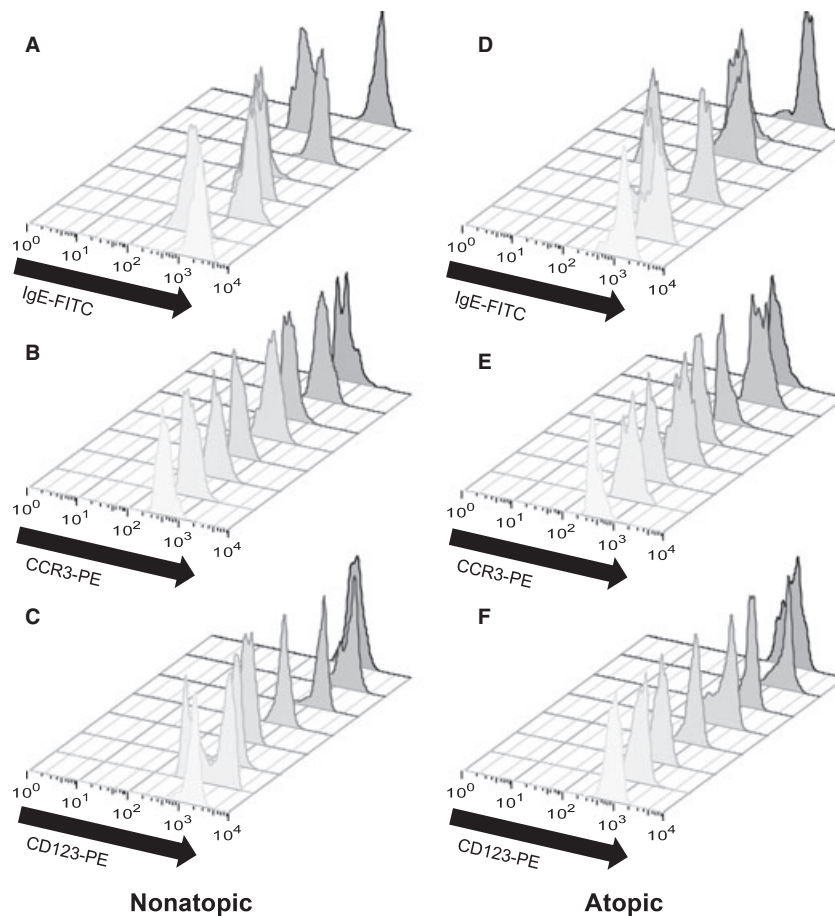


**Figure 2** Expression of the three different selection markers in 16 donors, visualized in 8 successively arranged histograms from nonatopic (A–C) or atopic donors (D–F).

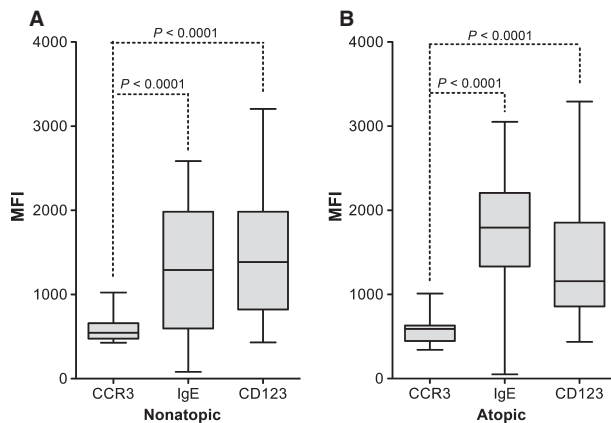


**Figure 3** Variations of the mean fluorescence intensity (MFI) in the expression of the three different selection markers. (A) MFI of CCR3-, IgE- and CD123-expression on basophils in nonatopic donors ( $n = 49$ ) or (B) atopic donors ( $n = 45$ ). (Nonparametric Kruskal–Wallis test).

work, which demonstrated a uniform density of CCR3 of around 20 000 receptors per cell on all the basophils in peripheral blood (10, 11). The basophil activation experi-

ments in birch pollen and grass pollen-allergic patients support the stable expression of CCR3 regardless of the basophils activation state. The present data are also consistent with our unpublished observation that CCR3 mRNA is constitutively expressed at high levels in basophils without regulation by different stimuli (e.g. IL-3, IL-33, IgE-receptor cross-linking). The other two basophil selection strategies showed significantly higher interindividual variations in cell surface expression, particularly the IgE-based approach. This could be expected as serum IgE levels influence IgE-receptor (FcεRI) density on basophils (6, 7) leading to weak staining at low IgE levels and difficulties in basophil gating (Figs 1–3). BAT is mainly used when routine serologic testing fails to define the relevant allergen, which is most often the case in patients with low IgE levels such as patients suffering from allergies to insect venoms and drugs or patients mono-sensitized to rare allergens. Thus, basophil selection via IgE is particularly inaccurate in those patients in which BAT is diagnostically most useful.

Selection based on CD123/HLA-DR-staining also provides an accurate method for basophil identification. Although some basophils are weakly HLA-DR positive, they can be easily separated from HLA-DR<sup>high</sup> plasmacytoid dendritic cells. However, CD123 expression is more variable than CCR3 (Fig. 3), and HLA-DR expression may also change

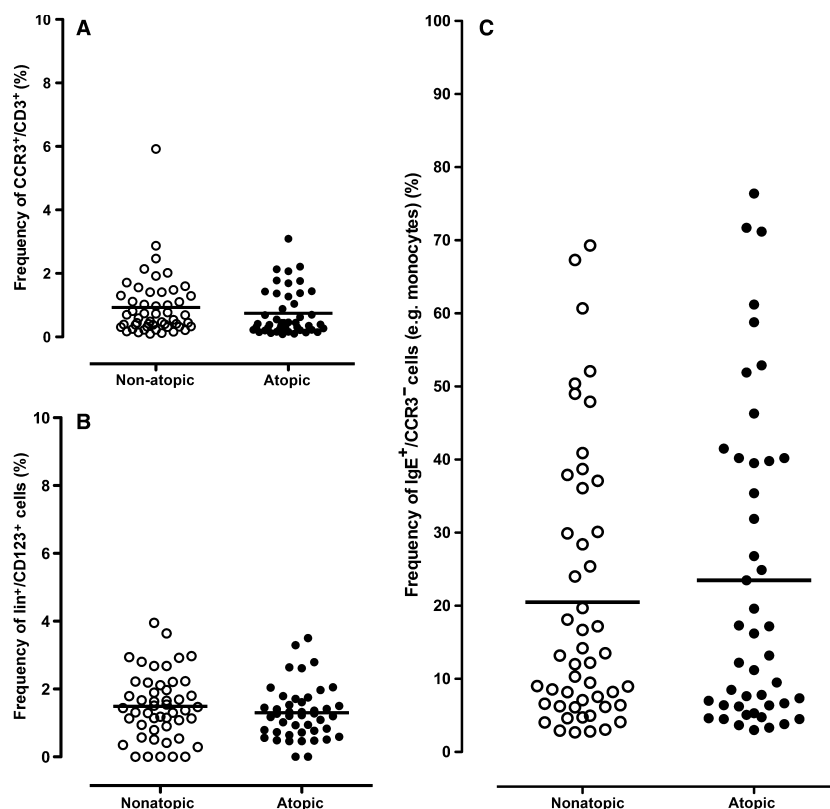


**Figure 4** 'Contamination' within the basophil gate by nonbasophils in the respective selection strategy. (A) Contamination of T cells, which co-express CCR3. (B) Contamination of other leukocytes

(lin1<sup>pos</sup> cells) within the CD123<sup>pos</sup>/HLA-DR<sup>neg</sup> population. (C) Contamination of CCR3-negative cells (nonbasophils) using selection with IgE. Results are expressed as percentage of total cell count.

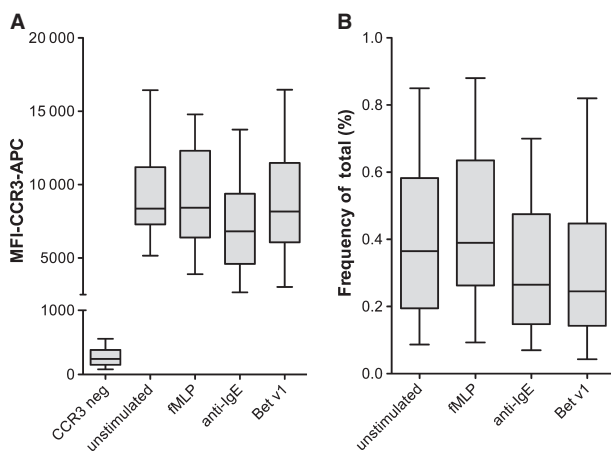


**Figure 5** CCR3 expression of basophil with and without activation in whole blood (A) MFI change of CCR3-positive cells using different stimuli (IgE-dependent and IgE-independent) and (B) frequency of basophils (CCR3<sup>pos</sup>) of total cell count. No significant difference between unstimulated and stimulated basophils regarding CCR3 expression (nonparametric Kruskal–Wallis test).

under inflammatory conditions (12). Finally, an additional color (HLA-DR) is needed in contrast to the CCR3-selection method.

Another chemotactic factor receptor expressed by basophils and eosinophils is CRTH2. However, in contrast to CCR3 that is only expressed by Th2-type lymphocytes in tissues but not in peripheral blood, CRTH2 is also expressed on blood memory Th2 cells (13, 14), and therefore a T-cell marker has to be included to gate out these cells.

Only the use of purified basophils made it possible to analyze all the cells without a possible bias of gating potentially leading to a 'loss' of basophils with low CCR3 staining. Additionally, it allowed us to compare the CCR3 staining intensity of stimulated basophils in the degranulated (CD63<sup>pos</sup>) and the CD63-negative cell fraction separately. We found that activation leads to a moderate decrease in CCR3 staining, which was restricted to CD63-positive (degranulated) basophils. Altogether, CCR3 staining intensity remains relatively stable upon stimulation, and these minor changes do not pose a significant problem in basophil selection: actually, CCR3 surface staining expression was not altered enough to lead to a significant 'loss' of basophils using this single selection marker. The reason for this slight decrease in CCR3 staining is unknown, but is unlikely because of chemokine release interacting with CCR3, as in this case the CD63-negative cell fraction would also be affected. The basis of this phenomenon requires further investigation. Also of note, IgE-receptor density is not only highly variable in basophils but also downregulated upon IgE-mediated activation (15).

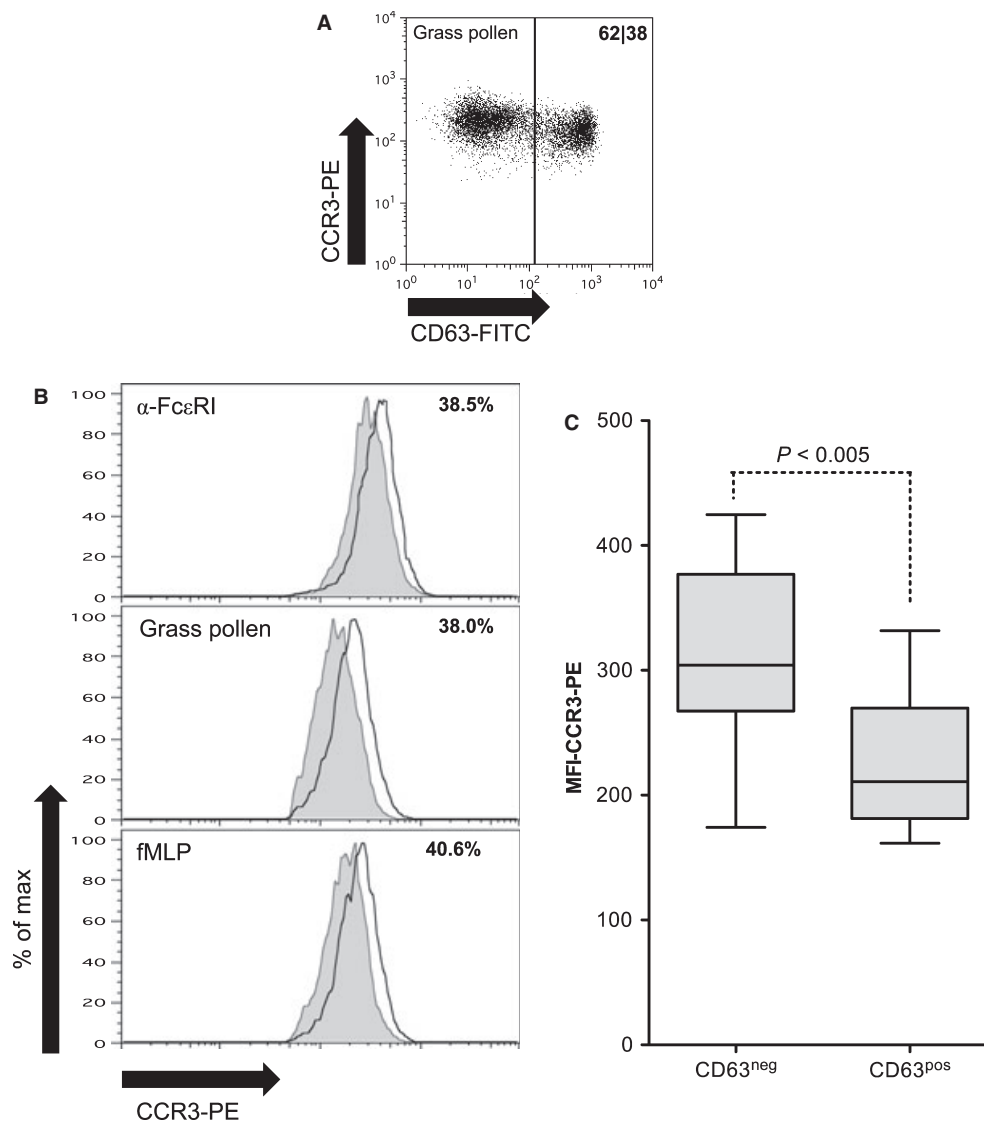


**Figure 6** Analysis of CCR3 expression in purified basophils (A) A representative dot plot (CCR3 vs CD63) of purified basophils from a pollen-allergic donor stimulated with the relevant allergen. (B) The histograms show a slight decrease in CCR3-staining in the CD63-positive population (black line: CD63-negative cell population. Shaded histogram: CD63-positive cell population, Numbers indicate CD63-positive basophils in percent). This was observed in response to all

degranulating stimuli (anti-Fc $\epsilon$ RI (100 ng/ml), grass pollen (30 ng/ml), fMLP (0.4 ng/ml)) as shown by a comparison of the histograms. (C) Statistical analysis of MFI of CCR3 in CD63-positive and CD63-negative cell populations of basophils from three donors stimulated with the 3 different stimuli mentioned above ( $n = 9$ ) (nonparametric Mann–Whitney test).

In conclusion, CCR3 is a stably expressed basophil marker that allows a robust and accurate selection of basophils without need of second marker for *in vitro* activation tests (i.e. CCR3/CD63) facilitating handling, automation and

analysis of BAT in whole blood. Inclusion of additional activation markers like CD203c, CD107a or CD13 is also possible without need of a sophisticated multichannel flow cytometer.

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