

**Optimizing of the basophil activation test:  
comparison of different basophil identification markers**

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## Abstract

### Background

Flowcytometric identification of basophils is a prerequisite for measuring activation of basophils with IgE-dependent or IgE-independent stimuli. Aim of this study was to compare different marker combinations in a simultaneous multicolor flowcytometric measurement.

### Methods

Ten patients with a grass pollen allergy and 3 controls were included in the study. Basophilic cells were gated by using anti-CCR3<sup>+</sup>, anti-IgE, anti-CRTH2, anti-CD203c und anti-CD3. Cells were activated by a monoclonal anti-FcεRI antibody, N-formyl-methionyl-leucyl-phenylalanine (fMLP) and the allergen extract Phleum pratense. The activation marker anti-CD63 was used.

### Results

The highest relative number of basophils was found with anti-CCR3<sup>+</sup> cells, anti-IgE<sup>+</sup> and anti-IgE<sup>+</sup>/anti-CD203c<sup>+</sup> cells, the lowest with CRTH2<sup>+</sup>/CD203c<sup>+</sup>/CD3<sup>-</sup> cells. A very good and good concordance of CCR3<sup>+</sup> cells was seen with CCR3<sup>+</sup>/CD3<sup>-</sup> cells and CRTH2<sup>+</sup>/CD203c<sup>+</sup>/CD3<sup>-</sup> cells in all experiments. The contamination of the CCR3<sup>+</sup> population with CD3<sup>+</sup> cells and the contamination of the IgE<sup>+</sup>-population with CCR3<sup>-</sup> cells and CD203<sup>-</sup> cells were the lowest compared to all other marker combinations .

### Conclusions

As the highest relative number of basophils was identified by anti-CD193 (CCR3) followed by the anti-IgE and anti-IgE/antiCD203c positive population in most cases, these markers can generally be recommended for identification of basophils. If a basophil population with very high purity is needed, anti-IgE should be chosen. Key words: basophil identification markers, basophil activation test, multicolor flowcytometry

## Introduction

The basophil activation test (BAT) allows the in vitro analysis and quantification of activated basophils by flow cytometry. It was developed for type-I-allergy diagnosis by Sainte-Laudy et al. in the mid-1990s after discovery of CD63 as activation marker of basophils by Knol et al. (1,2). As CD63 was also detected on other cells like macrophages and mast cells as well as on platelets, basophils have to be selected before flowcytometric analysis. Until the year 2008 mainly anti-IgE protocols were used , but also other basophil identification strategies using anti-CD123<sup>high</sup>/HLA-DR<sup>neg</sup> and anti-CRTH2<sup>high</sup>/anti-CD3<sup>neg</sup> were published . Anti-CD203c can be used as identification as well as activation marker . Anti-CCR3 is another marker, which seems be more robust than anti-CD123<sup>high</sup>/anti-HLA-DR<sup>neg</sup> and anti-IgE (3-9) It was the aim of this study to compare different marker combinations in a simultaneous multicolor flowcytometric measurement in order to find the optimal marker combination for the basophil activation test using CD63 as activation marker. Furthermore “contamination” of nonbasophils within the basophil gates were investigated.

## Material and Methods

Blood donors

Ten patients (4 men, 6 women) at the age of 24 to 51 years (mean age: 31,4 years) with a grass pollen allergy (positive history of rhinoconjunctivitis, positive prick test to grass pollen, positive results for sIgE to grass pollen ranging from 0.91 kU/L [ImmunoCAP class 2] to > 100 kUA/L [ImmunoCAP class 6] and total IgE ranging from 71.2 kU/L to 1146 kU/L [mean: 263 kU/L]) and 3 controls (1 man, 2 women; negative history of rhinoconjunctivitis, negative prick test to grass pollen and negative sIgE to a panel of standard allergens including grass pollen) were included in the study. Tests were performed before the grass pollen season in Munich (November 2012 to February 2013). The study was approved by the local ethics committee.

#### Basophil activation test

Venous blood was collected in 10 mL EDTA tubes. The anticoagulated blood samples were gently homogenized by inverting several times. Basophil activation test was performed by using reagents of FlowCAST (Bühlmann, Schönenbuch, Switzerland) except for the staining reagents, the lyses/fix buffer 5X (BD Biosciences, San Jose, CA, USA) and the 0.1% NaN<sub>3</sub> (in PBS). For each patient 4 polystyrene tubes were prepared: One with 50 µL Phleum pratense extract (G6; Bühlmann, Schönenbuch, Switzerland) diluted in stimulation buffer at an end concentration of 4.8 ng/mL. The other tubes contained positive controls either a monoclonal anti-FcεRI antibody (IgE mediated positive control 1) or N-formyl-methionyl-leucyl-phenylalanine (2 mM) (non-IgE mediated positive control 2). In order to evaluate basal values without stimulation, 50 µL of pre-warmed stimulation buffer was applied to a separate tube. To each tube 100 µL of pre-warmed stimulation buffer (containing calcium, heparin and 3 ng/mL IL-3), 50 µL of patients' blood and simultaneously 10 µL V450 Rat Anti-Human CD294 (CRTH2) (BM16),

PE-Cy<sup>TM</sup>7 Mouse Anti-Human CD63 (H5C6; diluted 1:40), Alexa Fluor® 647 Mouse Anti-Human CD193 (CCR3) (5E8), PerCP-Cy<sup>TM</sup> 5.5 Mouse Anti-Human CD3 (SP34-2) (all from BD Biosciences GmbH, San Jose, CA, USA), monoclonal Anti-IGE-FITC (4H10) (Sigma-Aldrich, Saint Louis, MO, USA) and Anti-CD203c-PE (97A6) (Beckmann Coulter Company, Marseille, France) were added.

After gently mixing the tubes were covered with an adhesive plastic sheet and incubated at 37°C in a water bath for 20 minutes. The stimulation was stopped by adding 1 mL of prewarmed lysing/fix buffer (Lyse/Fix Buffer 5X; BD Biosciences GmbH, San Jose, CA, USA), and the tubes were incubated again at 37°C for 20 minutes. After centrifugation for 10 minutes at 4°C at 2400 rpm, the supernatant was decanted and 2 mL of PBS was added to each tube. After centrifugation at 2400 rpm for 10 min the cells were resuspended with 600 µl 0,1% sodium acid solution. Flow cytometric analysis of the cells was performed on a FACS BD LSRFortessa<sup>TM</sup> cytometer (BD Biosciences GmbH, Heidelberg, Germany) equipped with four lasers (Violet Laser (405nm), Blue Laser (488nm), Red Laser (630nm) und Ye-Gr Laser (561nm)) within 2 hours.

For the compensation of the fluorochroms BD<sup>TM</sup>CompBeads Anti-Rat Ig, κ (G16-510E3) as well as Anti-Mouse Ig, κ/Negative Control (FBS) Compensation Particles Set (BD Biosciences, San Jose, CA, USA) were used. When performing the bead compensation according to the manufacturer's protocol, autofluorescence was adjusted to basophils. As basophils cannot be identified in FSC/SSC plots due to co-occurrence with T and B cells, we first stained with CCR3 AF647 and adjusted the autofluorescence of the gated cells in all other channels (FITC, PE, PeCy7, PerCP5.5). Afterwards basophils were identified with algE FITC staining and the autofluorescence for the AF647 channel was

determined. These autofluorescence settings were used to perform the bead compensation and to define the cut-off for positive fluorescence.

### Statistical analysis

Statistical analysis was performed using the programme IBM SPSS 20 as well as Microsoft Office Excel 2007. Differences between the cellular markers were calculated by the paired t-test. Values of  $P < 0.05$  were considered significant. Furthermore the Kendall's coefficient of concordance was used. Kendall W ranges from 0 (no concordance) to 1 (complete concordance). Data in figure 3 and 4 are presented as boxplots showing the median in the middle of the box (line), the area between the 1<sup>st</sup> (Q1) and 3<sup>rd</sup> (Q3) quartile (box), Q1-1,5 IQR (interquartile range) and Q3+1.5 IQR (whiskers) and outliers (o).

## Results

### Gating strategies and identification of basophils

Basophils were identified in two discrete cell populations of whole blood – an overall leukocyte and a lymphocyte population. Both populations were separated on a Forward Scatter (FSC)/Side Scatter (SSC) histogram (Fig. 1 (a)). Basophilic cells were identified by using anti-CCR3-Alexa Fluor 647, anti-IgE-FITC, anti-CRTH2-V450 and anti-CD203c-PE antibodies for flow cytometry. In the leukocyte population, basophils can be identified by single stainings (Fig. 1 (b)), however, contamination of the basophils with other leukocytes cannot be excluded. Therefore, basophils were further identified in the lymphocyte population with combinatorial stainings of CCR3 with CD203c, IgE and CRTH2 and CD203c with IgE (Fig. 1 (c)). To understand the potential cross contamination of the identified basophils with T cells, basophil markers

were plotted against CD3 (Fig. 1 (d)) revealing a contamination risk only for basophil identification with CRTH2.

To evaluate the impact of different marker combinations on the basophil activation test, basophils were activated in erythrocyte-lysed whole blood. Figure 2 gives an example of one of the used gating strategies using CCR3<sup>+</sup> CD203c<sup>+</sup> double positive basophils that have been identified in the lymphocyte population. Basophils were activated with antibodies against Fc $\epsilon$  receptor (positive 1), N-formyl-methionyl-leucyl-phenylalanine (fMLP) (positive 2) and Phleum pratense (allergen) or were left unstimulated as control (negative). Up-regulation of CD63 was used to calculate the percentage of activated basophils (Fig. 2).

Comparison of relative basophil numbers Cells labelled with the following single markers or marker combinations were used: CCR3<sup>+</sup>, IgE<sup>+</sup>, CD203c<sup>+</sup>, CCR3<sup>+</sup>/IgE<sup>+</sup>, CCR3<sup>+</sup>/CD203c<sup>+</sup>, CCR3<sup>+</sup>/CD3<sup>-</sup>, CRTH2<sup>+</sup>/CD3<sup>-</sup>, CRTH2<sup>+</sup>/CD203c<sup>+</sup>/CD3<sup>-</sup> and IgE<sup>+</sup>/CD203c<sup>+</sup> cells. The relative basophil number was expressed as percentage of total cell counts. Values of patients and controls were taken together. The highest medians of cell numbers (except for stimulation with monoclonal anti-Fc $\epsilon$ RI antibody) were found with CCR3<sup>+</sup> (negative control: 46.8% x 10<sup>-3</sup> of total cells, stimulation with fMLP: 56.5% x 10<sup>-3</sup> of total cells, stimulation with allergen: 52.0% x 10<sup>-3</sup> of total cells) the lowest with CRTH2<sup>+</sup>/CD203c<sup>+</sup>/CD3<sup>-</sup> cells (negative control: 16.1% x 10<sup>-3</sup> of total cells, stimulation with monoclonal anti-Fc $\epsilon$ RI antibody: 9.4% x 10<sup>-3</sup> of total cells, stimulation with fMLP: 26.8% x 10<sup>-3</sup> of total cells, stimulation with allergen: 13.9% x 10<sup>-3</sup> of total cells). Details are shown in Fig. 3. Statistical comparison of CCR3<sup>+</sup> cells with cells that were labelled with all other markers and marker combinations revealed no significant difference to IgE<sup>+</sup> cells, CCR3<sup>+</sup>/IgE<sup>+</sup> cells (except for stimulation with

monoclonal anti-FcεRI antibody and allergen), IgE<sup>+</sup>/CD203c<sup>+</sup> (except for stimulation with monoclonal anti-FcεRI antibody) and significant differences to CD203c<sup>+</sup> cells, CRTH2<sup>+</sup>/CD3<sup>-</sup> cells (except negative control), CCR3<sup>+</sup>/CD3<sup>-</sup> cells, CCR3<sup>+</sup>/CD203c<sup>+</sup> cells and CRTH2<sup>+</sup>/CD203c<sup>+</sup>/CD3<sup>-</sup> cells. In the concordance analysis after Kendall very good and good concordance of CCR3<sup>+</sup> cells were seen with CCR3<sup>+</sup>/CD3<sup>-</sup> cells and CRTH2<sup>+</sup>/CD203c<sup>+</sup>/CD3<sup>-</sup> cells in all experiments.

For details see table 1.

It is known, that IL-3 which was used in our stimulation buffer, activates CD203c in basophils (10), but with regard to the results this seems not to be relevant for identification of basophils.

#### Contamination with „non-basophils“

Non-basophils were defined as CD3<sup>+</sup>, CCR3<sup>-</sup> and CD203<sup>-</sup> cells. The contamination of the CCR3<sup>+</sup> population with CD3<sup>+</sup> cells (negative control: 7.6%, stimulation with monoclonal anti-FcεRI antibody: 14.3%, stimulation with fMLP: 9.8%, stimulation with allergen: 9.0%) and the contamination of the IgE<sup>+</sup>-population with CCR3<sup>-</sup> cells (negative control: 8.9%, stimulation with monoclonal anti-FcεRI antibody: 9.9%, stimulation with fMLP: 8.7%, stimulation with allergen: 7.0%) and CD203<sup>-</sup> cells (negative control: 9.9%, stimulation with monoclonal anti-FcεRI antibody: 18.7%, stimulation with fMLP: 10.6%, stimulation with allergen: 10.4%) were the lowest in all experiments. The highest contamination was seen for the CRTH2<sup>+</sup>/CD3<sup>-</sup> or CCR3<sup>+</sup> population within the CD203<sup>-</sup> cells (negative control: 49.6%/30.7%, stimulation with monoclonal anti-FcεRI antibody: 43.5%/50.7%, stimulation with fMLP: 32.5%/35.2%, stimulation with allergen: 43.0%/36.0%). For details see Fig. 4. For statistical analysis see table 2.

## Discussion

Since the introduction of the basophil activation test different basophil identification markers were used, but comparisons between the different markers were analyzed to a limited extent. Sensitivity of a BAT using anti-IgE was higher than in the BAT using anti-CD123/anti-HLA-DR (85% vs. 72%) in patients sensitized to grass pollen (11). It could be shown that fMLP-induced up-regulation of CD63 was higher ( $31.2\% \pm 4.9$ ) in the CCR3 protocol than in the anti-IgE protocol ( $14.5\% \pm 3.4$ ) (8). Comparison of the identification marker anti-IgE-FITC and anti-CCR3-PE in patients with betalactam allergy showed comparable results with a slightly higher sensitivity (not significant) with anti-CCR3-PE (12). Comparison of the two different staining strategies anti-CD123-PE/anti-HLA-DR-PerCP/anti-lin1-FITC and anti-IgE-FITC/antiCD3-PerCP/anti-CCR3-PE revealed that CCR3 is a stable and highly expressed basophil selection marker independent of the atopic background or basophil activation state. In contrast the basophil markers anti-CD123/anti-HLA-DR and anti-IgE showed higher inter-individual variability (7). For this reason we decided to not include CD123 and HLA-DR in this study.

Activated Th2-lymphocytes can also express CCR3 and it was suggested to include anti-CD3 to exclude CCR3-positive T-cells (8). Measurements showed, that the number of CCR3-positive T-cells were negligible (13,14). This was also observed in our study (Fig. 5). Our results also showed that the Kendall's coefficient of concordance comparing CCR3<sup>+</sup>/CD3<sup>-</sup> cells and CCR3<sup>+</sup> cells was good or very good (Tab. 1). Similar good coefficients were also obtained with marker combinations using CD203c-PE as marker (CRTH2<sup>+</sup>/CD203c<sup>+</sup>/CD3<sup>-</sup>, CD203c<sup>+</sup> and CCR3<sup>+</sup>/CD203<sup>+</sup> cells), but not with marker combinations

using anti-IgE-FITC as marker (CCR3<sup>+</sup>/IgE<sup>+</sup> and IgE<sup>+</sup>/CD203c<sup>+</sup>). This may be due to the known inter-individual variability.

Non-basophils defined as CD203<sup>-</sup> cells were significantly higher in CCR3<sup>+</sup> and CRTH2<sup>+</sup>/CD3<sup>-</sup> cells than in IgE<sup>+</sup> cells (Tab. 2) suggesting that this marker revealed the purest basophil cell population in our comparisons.. If a basophil population with very high purity is needed, anti-IgE should be chosen. For other purposes a combination of SSC/anti-IgE/anti-CD203c or anti-CCR3 is recommended.

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<http://www.buhlmannlabs.ch/core/allergy/flow2-cast/>

## Legends

Fig. 1: Identification of basophils in whole blood

After erythrocyte lysis, leukocytes (black gate) and lymphocytes (red gate) were gated in a FSC/SSC plot (a). In the total leukocyte

population, basophils were identified using the markers CCR3, IgE, CRTH2 and CD203c (b). As basophils show the same size and granularity as lymphocytes, basophils were further identified in the lymphocyte population by co-staining of CCR3 with CD203c, IgE and CRTH2 and the combination of CD203c and IgE (c). To identify cross-contaminant T cells in the basophil population, all basophil markers were plotted against CD3 (d). Shown is one representative staining out of 13.

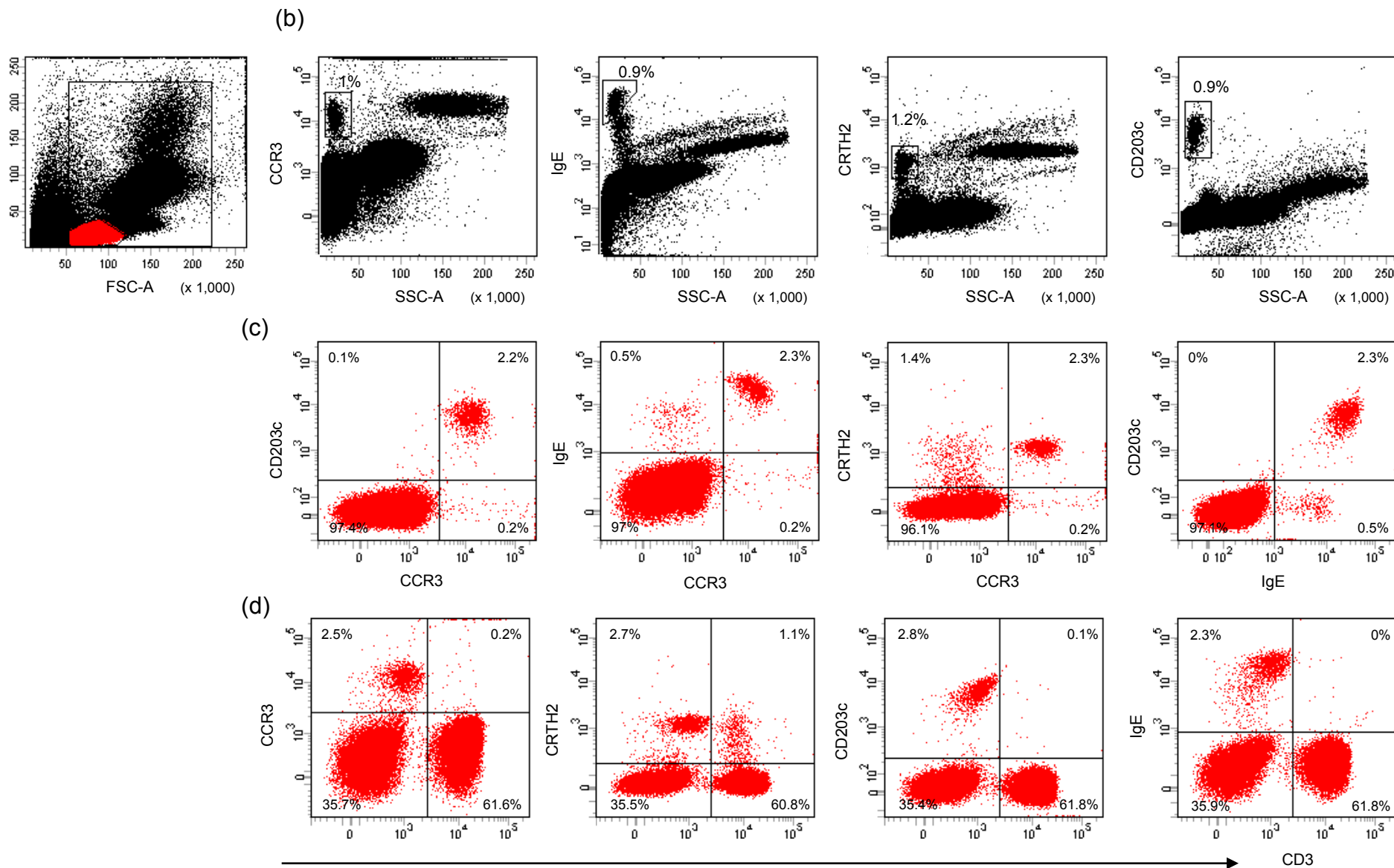
Fig. 2: Activation of basophils

Activation of basophils was determined according to induction of CD63 expression after stimulation. CCR3<sup>+</sup> and CD203<sup>+</sup> basophils were identified in the lymphocyte population and either left unstimulated (a) or were stimulated with antibodies against the FcεRI (positive 1) (b), N-formyl-methionyl-leucyl-phenylalanine (positive 2) (c) or Phleum pratense (allergen) (d). Shown is one representative experiment of an allergic donor out of ten.

Fig. 3: Relative cell counts (% x 10<sup>-3</sup> of total cells) with different labelled basophils in the negative control (a), positive control 1 using anti-FcεRI antibody (b), positive control 2 using N-formyl-methionyl-leucyl-phenylalanine (c), with allergen Phleum pratense (d)

Fig. 4: Contamination of basophils (defined as CCR3<sup>+</sup>, IgE<sup>+</sup> or CRTH<sup>+</sup>/CD3<sup>-</sup> cells) with non-basophils (defined as CD3<sup>+</sup>, CCR3<sup>-</sup> or CD203c<sup>-</sup> cells) in % of the cells labelled with the main marker: negative control (a), positive control 1 using anti-FcεRI antibody (b), positive

control 2 using N-formyl-methionyl-leucyl-phenylalanine (c), with allergen  
Phleum pratense (d)



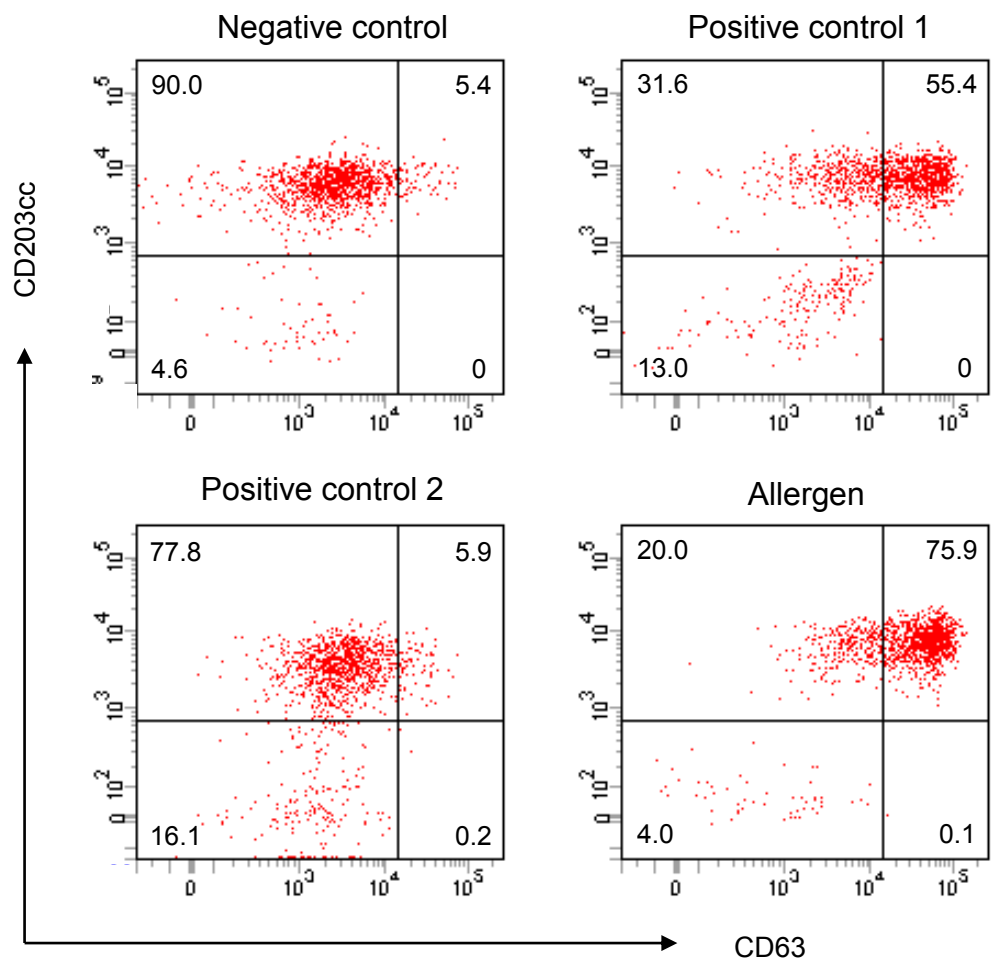
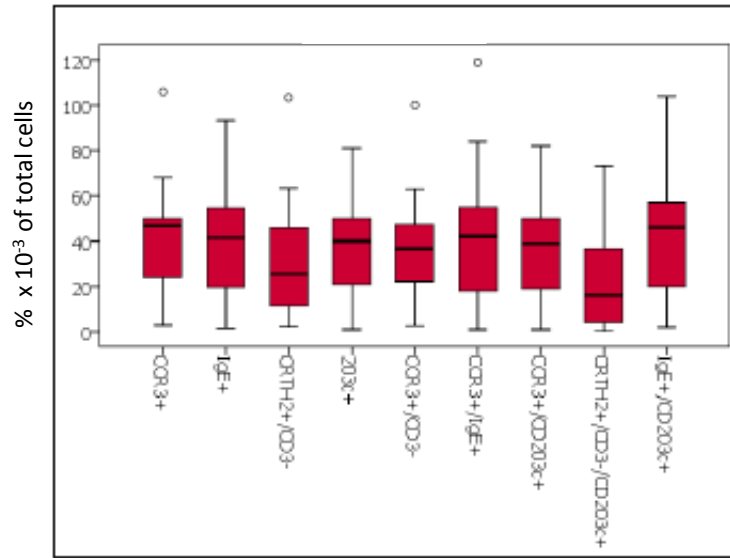
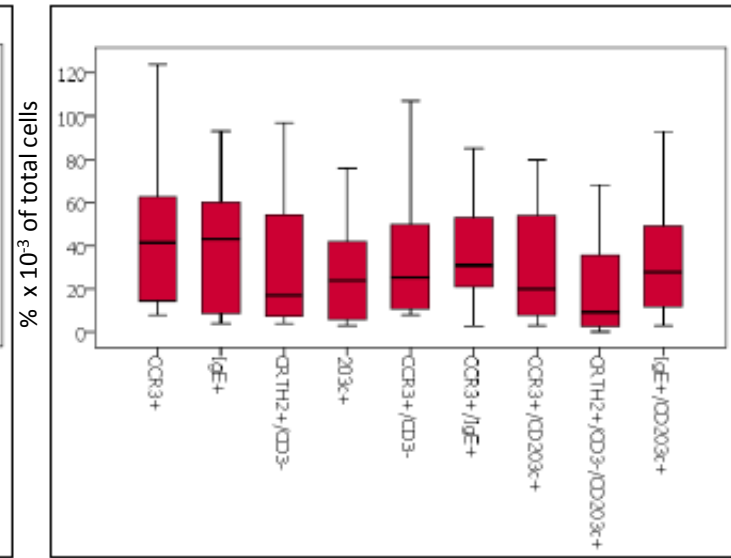


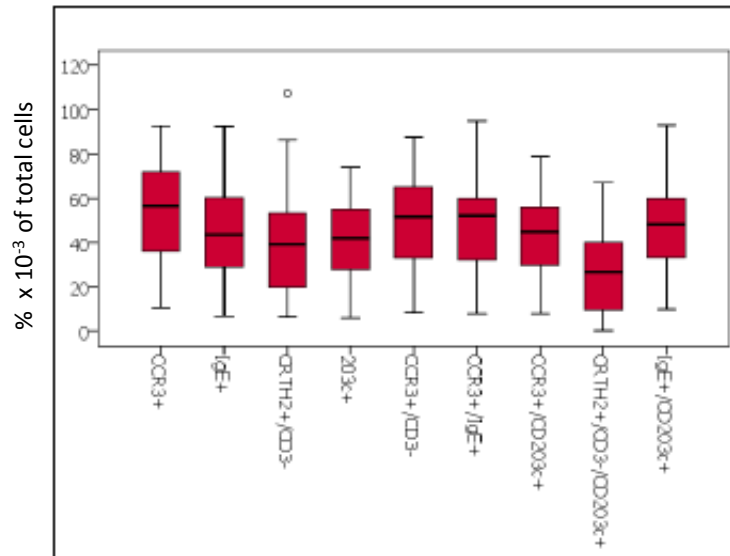
Fig. 3



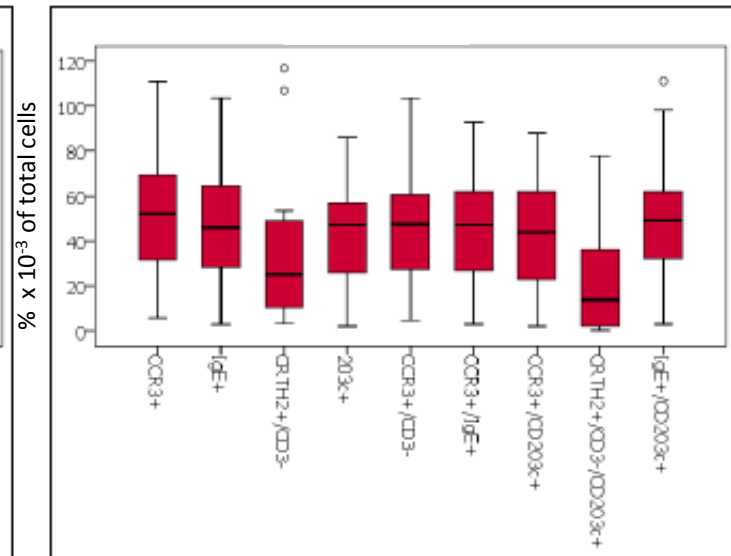
(a)



(b)

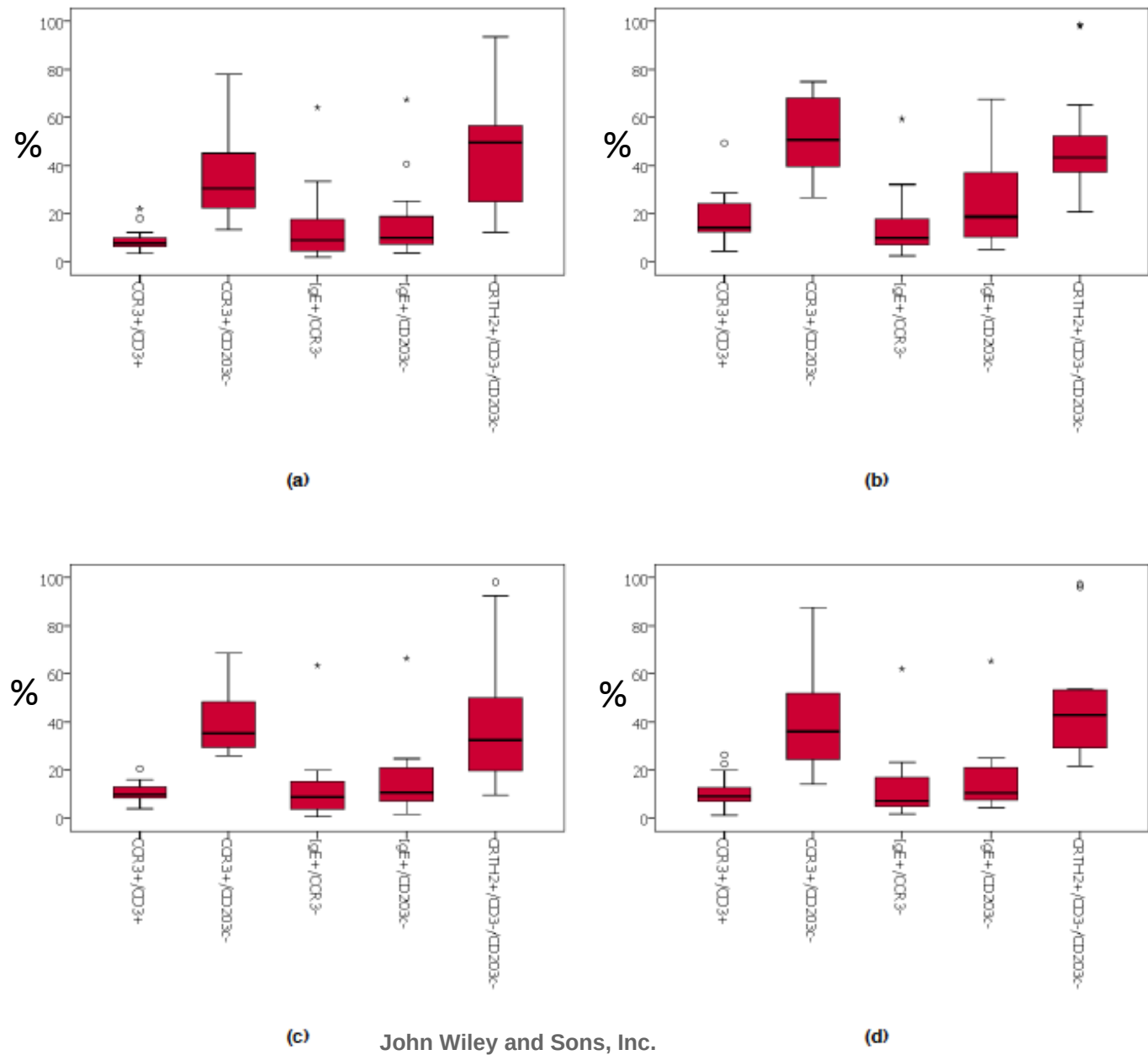


(c)



(d)

Fig. 4



Tab. 1 Comparison of the number of CCR3<sup>+</sup> cells (% x 10<sup>-3</sup> of total cells; median, range) with the number of cells labeled with the other markers and marker combinations (see also Fig. 3). Results of the paired t-test and the concordance analysis of Kendall.

| Cells                                                     | Negative control    | Kendall W    | p-value t-test  | Positive control 1  | Kendall W    | p-value t-test  | Positive control 2  | Kendall W    | p-value t-test  | Allergen            | Kendall W    | p-value t-test  |
|-----------------------------------------------------------|---------------------|--------------|-----------------|---------------------|--------------|-----------------|---------------------|--------------|-----------------|---------------------|--------------|-----------------|
| CCR3 <sup>+</sup>                                         | 46.8<br>(3.0-106.1) |              |                 | 41.5<br>(8.0-123.9) |              |                 | 56.5<br>(10.7-92.6) |              |                 | 52<br>(5.8-111.1)   |              |                 |
| IgE <sup>+</sup>                                          | 41.3<br>(1.6-93.1)  | 0.479        | n.s.            | 43.3<br>(4.0-93.2)  | 0.479        | n.s.            | 43.7<br>(6.8-92.5)  | 0.29         | n.s.            | 46<br>(2.9-103.5)   | <b>0.716</b> | n.s.            |
| CRTH2 <sup>+</sup> /CD3 <sup>-</sup>                      | 25.3<br>(2.2-103.5) | 0.053        | n.s.            | 16.9<br>(3.9-97.1)  | <b>0.716</b> | <b>&lt;0.01</b> | 39.3<br>(6.5-107.4) | 0.479        | <b>&lt;0.05</b> | 25.3<br>(3.3-116.6) | <b>0.716</b> | <b>&lt;0.05</b> |
| CD203c <sup>+</sup>                                       | 40<br>(1.0-81.0)    | <b>0.923</b> | <b>&lt;0.01</b> | 24<br>(3.0-76.0)    | <b>1.0</b>   | <b>&lt;0.01</b> | 42<br>(6.0-74.0)    | <b>0.716</b> | <b>&lt;0.01</b> | 47<br>(2.0-86.0)    | 0.479        | <b>&lt;0.01</b> |
| CCR3 <sup>+</sup> /CD3 <sup>-</sup>                       | 36.7<br>(2.7-100.1) | <b>1.0</b>   | <b>&lt;0.01</b> | 25.5<br>(8.1-107.1) | <b>0.716</b> | <b>&lt;0.01</b> | 51.5<br>(8.5-87.5)  | <b>1.0</b>   | <b>&lt;0.01</b> | 47.3<br>(4.7-103.4) | <b>1.0</b>   | <b>&lt;0.01</b> |
| CCR3 <sup>+</sup> /IgE <sup>+</sup>                       | 42<br>(1.0-119.0)   | 0.29         | n.s.            | 31.0<br>(2.8-85.0)  | <b>0.716</b> | <b>&lt;0.05</b> | 52<br>(8.0-95.0)    | 0.148        | n.s.            | 47<br>(3.0-93.0)    | 0.479        | <b>&lt;0.05</b> |
| CCR3 <sup>+</sup> /CD203c <sup>+</sup>                    | 39<br>(1.0-82.0)    | <b>0.719</b> | <b>&lt;0.01</b> | 20<br>(3.0-80.0)    | <b>1.0</b>   | <b>&lt;0.01</b> | 45<br>(8.0-79.0)    | <b>1.0</b>   | <b>&lt;0.01</b> | 44<br>(2.0-88.0)    | 0.410        | <b>&lt;0.05</b> |
| CRTH2 <sup>+</sup> /CD3 <sup>-</sup> /CD203c <sup>+</sup> | 16.1<br>(0.2-73.4)  | <b>1.0</b>   | <b>&lt;0.01</b> | 9.4<br>(0.1-68.1)   | <b>1.0</b>   | <b>&lt;0.01</b> | 26.8<br>(0.1-66.9)  | <b>1.0</b>   | <b>&lt;0.01</b> | 13.9<br>(0.1-78.0)  | <b>1.0</b>   | <b>&lt;0.01</b> |
| IgE <sup>+</sup> /CD203c <sup>+</sup>                     | 46<br>(2.0-104.0)   | 0.29         | n.s.            | 28<br>(3.0-93.0)    | <b>1.0</b>   | <b>&lt;0.01</b> | 48.0<br>(10.0-93.0) | 0.006        | n.s.            | 49.0<br>(3.0-111.0) | 0.29         | n.s.            |

n.s. = not significant

Good and very good concordance (Kendall W > 0.6) is highlighted

Tab. 2 Comparison of the number of non-basophils (% x 10<sup>-3</sup> of total cells; median, range) defined as CD3<sup>+</sup> or CD203c<sup>-</sup> cells within the CCR3<sup>+</sup> or IgE<sup>+</sup> labeled basophils (see also Fig. 4)

|                    | Cell populations                                          |                     | CCR3 <sup>+</sup> /CD203c <sup>-</sup> | IgE <sup>+</sup> /CD203c <sup>-</sup> |
|--------------------|-----------------------------------------------------------|---------------------|----------------------------------------|---------------------------------------|
| Negative control   |                                                           |                     | 30.7<br>(13.4-78.0)                    | 9.9<br>(3.8-67.5)                     |
|                    | CCR3 <sup>+</sup> /CD203c <sup>-</sup>                    | 30.7<br>(13.4-78.0) | -                                      | <b>p &lt; 0.01</b>                    |
|                    | CRTH2 <sup>+</sup> /CD3 <sup>-</sup> /CD203c <sup>-</sup> | 49.6<br>(12.3-93.7) | n.s.                                   | <b>p &lt; 0.01</b>                    |
|                    | CCR3 <sup>+</sup> /CD3 <sup>+</sup>                       | 7.6<br>(3.6-22.0)   | -                                      | n.s.                                  |
| Positive control 1 |                                                           |                     | 50.7<br>(26.8-74.8)                    | 18.7<br>(5.3-67.7)                    |
|                    | CCR3 <sup>+</sup> /CD203c <sup>-</sup>                    | 50.7<br>(26.8-74.8) | -                                      | <b>p &lt; 0.01</b>                    |
|                    | CRTH2 <sup>+</sup> /CD3 <sup>-</sup> /CD203c <sup>-</sup> | 43.5<br>(20.7-98.6) | n.s.                                   | <b>p &lt; 0.05</b>                    |
|                    | CCR3 <sup>+</sup> /CD3 <sup>+</sup>                       | 14.3<br>(4.6-49.3)  | -                                      | n.s.                                  |
| Positive control 2 |                                                           |                     | 35.2<br>(26.0-68.8)                    | 10.6<br>(1.7-66.5)                    |
|                    | CCR3 <sup>+</sup> /CD203c <sup>-</sup>                    | 35.2<br>(26.0-68.8) | -                                      | <b>p &lt; 0.01</b>                    |
|                    | CRTH2 <sup>+</sup> /CD3 <sup>-</sup> /CD203c <sup>-</sup> | 32.5<br>(9.5-98.2)  | n.s.                                   | <b>p &lt; 0.05</b>                    |
|                    | CCR3 <sup>+</sup> /CD3 <sup>+</sup>                       | 9.8<br>(4.1-20.5)   | -                                      | n.s.                                  |
| Allergen           |                                                           |                     | 36.0<br>(14.2-87.5)                    | 10.4<br>(4.6-65.3)                    |
|                    | CCR3 <sup>+</sup> /CD203c <sup>-</sup>                    | 36.0<br>(14.2-87.5) | -                                      | <b>p &lt; 0.01</b>                    |
|                    | CRTH2 <sup>+</sup> /CD3 <sup>-</sup> /CD203c <sup>-</sup> | 43.0<br>(21.5-97.3) | n.s.                                   | <b>p &lt; 0.01</b>                    |
|                    | CCR3 <sup>+</sup> /CD3 <sup>+</sup>                       | 9.0<br>(1.3-26.4)   | -                                      | n.s.                                  |

n.s. = not significant  
p < 0.05 is highlighted