

ORIGINAL ARTICLE

Basic Mechanisms in Allergic Disease

Updates on the surface antigens of basophils: CD16 on basophils of patients with respiratory or insect venom allergy and the rejection of CD203c and CD63 externalization decoupling by bisindolylmaleimides

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Summary

Background: CD16 was previously suggested to be a new marker of basophils that is subject to downregulation by FcεRI crosslinking. Certain compounds, including supraoptimal concentrations of the PKC inhibitors, bisindolylmaleimides, decouple the release of granules containing CD203c, CD63 and histamine, and may thus help to identify the mechanisms related to the CD16 externalization.

Objective: We hypothesized that CD16 is differentially expressed on the surface of basophils in patients with birch pollen or insect venom allergy and is subject to a regulation in response to allergens. We also employed CD203c and CD63 externalization decoupling by bisindolylmaleimides.

Methods: We performed a basophil activation test coupled with CD16 and histamine detection using cells isolated from patients with allergy to birch pollen or insect venom and negative controls. We employed two PKC inhibitors, bisindolylmaleimide II and Ro 31-8220 at their supraoptimal concentrations and, after difficulties reproducing previously published data, we analyzed the fluorescence of these inhibitors alone. We identified the CD16 isoforms by sequencing nested RT-PCR amplicons from flow cytometry sorted basophils and by cleaving the CD16b GPI anchor using a phospholipase C.

Results: We provide the first evidence that CD16a is expressed as a surface antigen on a small subpopulation of human basophils in patients with respiratory and insect venom allergy, and this antigen shows increased surface expression following allergen challenge or FcεRI crosslinking. We rejected the apparent decoupling of the surface expression of basophil activation markers following the administration of bisindolylmaleimides.

Conclusions & Clinical Relevance: The inclusion of αCD16 in negative selection cocktails selects against a subset of basophils that are CD16⁺ or CD16^{dim}. Using CD16^{dim} basophils and unstained leucocytes, we show that previous studies with supraoptimal concentrations of bisindolylmaleimides are likely flawed and are not associated with the differential expression of CD203c and CD63.

KEYWORDS

basophil activation test, bisindolylmaleimide, Fc γ RIIIA, flow cytometry artifact, IgG-mediated anaphylaxis

1 | INTRODUCTION

Monitoring the presence and activation of basophils by flow cytometry dates back to the 1990s when Edward F. Knol et al¹ developed a protocol involving the detection of CD63 surface expression. The CCR3 chemokine receptor was described as highly expressed on human blood basophils,² and CD203c was identified as another basophil-specific identification and activation marker.³ Further activation markers were suggested; see, for example, Heneberg⁴ and Chirumbolo⁵, for their detailed overview. Although mature human basophils are extensively studied and the basophil activation tests (BAT) are widely used, there is still surprisingly limited knowledge of the proteome associated with the external surface of these cells. Among the basophil surface markers with questionable status is CD16. Previous studies have shown that human basophils are CD16-negative.^{6–8} However, Yusuke Tsujimura et al⁹ demonstrated that mouse basophils play a key role in IgG-mediated, but not IgE-mediated, anaphylaxis. Subsequently, a report from the group headed by Marc Daëron provided the evidence of a substantial subpopulation of human basophils that express CD16b.¹⁰

The CD16a (Fc γ RIIIA) and CD16b (Fc γ RIIIB) surface antigens are low-affinity IgG receptors. CD16a, the activating IgG receptor, commonly serves as a marker of NK cells, NKT cells, monocytes and macrophages and is also present on eosinophils, reactive T cells, immature thymocytes and placental trophoblast cells. CD16b is the most abundant low-affinity IgG receptor on multiple types of leukocytes, but its functions remain unclear.^{4,11} In contrast to previous reports, Daëron and colleagues claimed that 24%–81% (median 44%) of basophils from healthy men express CD16b transcripts. Additionally, these authors showed the expression of CD16b transcripts in basophils from patients with various allergic diseases, with similar levels experienced in patients with respiratory allergy (median 57%) and chronic urticaria (median 34%) and significantly decreased levels in atopic dermatitis patients (22%), without any correlation with the sex or age of the patients or their serum IgE levels. Their report was based on the analysis of CD16a and CD16b transcripts instead of the proteins because the currently used α CD16 antibodies, such as 3G8 or LNK16, do not discriminate between the two isoforms.¹⁰ Using staining for the two above-mentioned antibody clones, Daëron and colleagues showed that a subset of human basophils expressed low but detectable levels of the CD16 protein. The expression levels were lower by one or two orders of magnitude when compared to those in monocytes, NK cells or neutrophils. By cleaving the GPI anchor of CD16 with phosphatidylinositol-specific phospholipase C (PI-PLC), these authors showed that the detected CD16 protein indeed represents glycosphosphatidylinositol (GPI)-anchored CD16b

and not transmembrane CD16a.¹⁰ Under most of the experimental setups, cell activation decreases the (surface) expression of CD16b,¹² which was also reported from human basophils stimulated with α IgE.¹⁰ Importantly, antibodies against CD16 were often included in the negative selection cocktails used for the enrichment of basophils; thus, the CD16-positive basophils may be absent from many previous analyses of their functions.^{7,13}

According to Salvatore Chirumbolo,⁵ it is paradoxical that there are no published BAT that would corroborate and further expand the initial observation of CD16b downregulation by the Fc ϵ RI-IgE-mediated activation of basophils, despite reports showing a basophil desensitization following omalizumab immunotherapy.¹⁴ The omalizumab therapy of patients with allergy blocks the increase in CD203c following the stimulation of basophils.¹⁵ If the CD16b surface expression is coupled with the CD203c, but not CD63 surface expression, then it should respond to the application of inhibitors that specifically block CD203c surface expression but enable CD63 surface expression during antigen-induced basophil activation. These effects have been previously reported as side effects of the protein kinase C (PKC) inhibitors bisindolylmaleimide II and Ro 31-8220.¹⁵ CD203c is an ecto-nucleotide pyrophosphatase/phosphodiesterase with an unknown physiological role, the surface expression of which is rapidly upregulated following the basophil activation in a manner more rapid than that of CD63. The surface expression of CD203c and CD63 in α IgE-stimulated cells was insensitive to the two above-mentioned PKC inhibitors when applied at the half-maximal inhibitory concentration (IC₅₀) of the PKC itself (~100 nM). In contrast, the 3 μ M concentration of the same inhibitors led to a differential increase in the surface expression of CD203c (15-fold) when compared to that of CD63 (2-fold) and the release of histamine (1.5-fold) when stimulated by Ro-81-3220 with α IgE; with slightly less pronounced differences also obtained when applying bisindolylmaleimide II with α IgE.^{16,17} The mechanisms of this regulation are unclear, independent from the activation and/or translocation of PKC. However, this regulation of CD63 surface expression suggests the existence of multiple forms of granules in peripheral blood basophils as previously suggested by electron microscopy experiments. Thus, decoupling CD63 and CD203c externalization enables an examination of the coupling of CD16b and CD203c surface expression suggested above.

Here, we hypothesized that (a) a fraction of human basophils express CD16, (b) the expression of CD16 differs in healthy controls and patients with birch pollen or insect venom allergy, (c) CD16 surface expression may be regulated during the activation of basophils by an allergen or α IgE, and (d) the decoupling of CD63 and CD203c externalization mediated by supraoptimal concentrations of Ro-81-3220 or bisindolylmaleimide II affects the surface expression of CD16 on human

basophils. The present study is the first to corroborate the initial findings of Marc Daëron and colleagues,¹⁰ showing CD16 expression on the surface of human basophils and the first to implement the concept of differential CD203c and CD63 regulation by supraoptimal concentrations of the PKC inhibitors initially suggested by Donald W. MacGlashan Jr.¹⁶ We provide the first evidence of the response of CD16 surface expression to naturally occurring stimuli, such as birch pollen allergen, and bee and wasp venom extracts, and show the first analysis of the expression of this marker in patients with venom or pollen allergy.

2 | MATERIALS AND METHODS

2.1 | Study population

We recruited three independent cohorts of adult (23–77 years) human subjects of Czech nationality, who provided written informed consent for BAT. The informed consent form and experiments were approved by the Ethics Committee of the Third Faculty of Medicine, Charles University; the approval was not numbered and was issued on April 4, 2018. The study conforms to the standards of the Declaration of Helsinki. The absolute exclusion criteria included the use of antihistamines or oral steroids within 4 days prior to the challenge. We screened the following cohorts:

- (1) Patients with the history of anaphylaxis to Hymenoptera (bee or wasp) venom of grade II to IV as classified according to the H. Mueller schedule, positive skin test results and/or elevated specific IgE levels ($n = 16$, of these 12 patients with reactions to wasp venom and 4 patients with reactions to bee venom).
- (2) Patients reporting subjective birch pollen allergy symptoms, objective allergy signs on conjunctival and nasal mucosa, and with records of positive skin prick test (≥ 3 mm) for birch pollen allergen or elevated IgE specific to birch pollen allergen ($n = 5$).
- (3) Control subjects without any known history of asthma, allergy and anaphylaxis ($n = 10$).

We did not apply any relative exclusion criteria based on the ratios of cells positive for basophil activation markers. There were no blood specimens with negative results in α IgE stimulations; thus, no patients were excluded from the presentation and statistical analyses. For the α IgE positive control, we determined the cut-off values by adding the double standard deviation value of CD63⁺ basophils observed in patients' background tubes to the mean value.

2.2 | The application of PKC inhibitors

We applied two PKC inhibitors, bisindolylmaleimide II (Santa Cruz Biotechnology, Santa Cruz, CA) and Ro-31-8220 (Merck, Darmstadt, Germany), which were previously reported to alter the ratio of CD193 and CD203c at the surface of basophils when used at low micromolar concentrations,¹⁶ and which thus were thought to enable the differential surface exposure of a specific type of

basophil granules. We observed the previously reported effects on the change of CD193 and CD203c ratios when using 3.5 μ M bisindolylmaleimide II or 3.0 μ M Ro 31-8220 but only when we did not correct the experiments for the fluorescence of the aromatic ring-containing study compounds themselves (see Results). Thus, we modified the previously suggested study protocol to monitor FL1, FL2 and FL3 changes in unstained white blood cells exposed to multiple dilutions of the two PKC inhibitors. We used bisindolylmaleimide II at 0.0, 1.75, 3.5 and 7.0 μ M and Ro 31-8220 at 0.0, 1.5, 3.0 and 6.0 μ M.

2.3 | BAT

We defined basophils as SSC^{low}CD193⁺CD203c⁺ cells. We quantified the total numbers of SSC^{low}CD193⁺, SSC^{low}CD203c⁺, SSC^{low}CD193⁺CD203c⁺, SSC^{low}CD16⁺, SSC^{low}CD193⁺CD16⁺, SSC^{low}CD203c⁺CD16⁺ and SSC^{low}CD193⁺CD203c⁺CD16⁺ cells. We quantified the median fluorescence signals of CD193 within the SSC^{low}CD193⁺ gate, CD203c within the SSC^{low}CD203c⁺ gate, CD16 within the SSC^{low}CD193⁺ gate and CD16 within the SSC^{low}CD193⁺CD203c⁺ gate. Further, we quantified the peak fluorescence signal (modus) of CD16 within the SSC^{low}CD193⁺CD203c⁺ gate; the relative share [in %] of CD63⁺ cells among SSC^{low}CD193⁺, SSC^{low}CD193⁺CD203c⁺ and SSC^{low}CD193⁺CD203c⁺CD16⁺ cells; and the relative share [in %] of CD16⁺ among SSC^{low}CD193⁺ and SSC^{low}CD193⁺CD203c⁺ cells. The specificity of the markers used was summarized, for example, by Heneberg et al.¹⁸ Because the CD16 surface expression on basophils was typically dim but high on other cell types (and can also be high at the surface of basophils under the conditions specified in the Results), in some analyses, we split the CD16^{+/dim} cells into CD16^{dim} and CD16⁺ cells, with the threshold arbitrarily set at 100 AU. We defined the CD63⁺ cells as those with the CD63 fluorescence above a threshold arbitrarily set at 100 AU.

We estimated the basophil activation using flow cytometry by measuring increases in CD63 on the cell surface prior to and after the ex vivo stimulation of cells with recombinant birch (*Betula verrucosa*) pollen allergen 1, isoform a (Bet v 1a) (Biomay AG, Vienna, Austria) at 100 ng/mL, the lyophilized venom extracts of yellow jacket *Pharmalgen Vesputa* spp. or honey bee *Pharmalgen Apis mellifera* (both ALK Abelló, Hørsholm, Denmark) at 1 μ g/mL, and a positive control (monoclonal mouse α IgE, clone E124.2.8; Immunotech, Marseille, France) at 2 μ g/mL. To obtain the negative control, we incubated the cells in an equal volume of the vehicle only. We first optimized the length of incubation with respect to the effects on CD16 expression by measuring CD16, CD203, CD193 and CD63 on cells isolated from 11 patients. We activated these cells with α IgE for 5 minutes, 25 minutes and 4 hours or with vehicle only for the same time intervals.

We initiated the experimental protocol for a single analysis with 80 μ L of peripheral blood with lithium heparin as the anticoagulant. We incubated the whole blood with IL-3 (PeproTech, Rocky Hill, NJ; final concentration 2 ng/mL) for 30 minutes at 37°C. Subsequently, we added the PKC inhibitors at the indicated concentrations; for

experiments with basophils, we used bisindolylmaleimide II at 3.5 μM and Ro-31-8220 at 3.0 μM . We incubated the inhibitors for 2 minutes at room temperature. In the next step, we added antigen or allergen and incubated for 20 minutes at 37°C. We then transferred tubes to ice and incubated for 5 minutes to stop the degranulation. Next, we incubated the cells with a panel of antibodies for 20 minutes on ice in the dark. We used the following panel of monoclonal antibody conjugates: $\alpha\text{CD63-FITC}$ (clone MEM-259; EXBIO Praha, Prague, Czech Republic), $\alpha\text{CD193-PE}$ (clone 5E8, BD Biosciences, San Jose, CA), $\alpha\text{CD203c-APC}$ (clone NP4D6; EXBIO Praha, Prague, Czech Republic) and $\alpha\text{CD16-PerCP}$ (clone 3G8; EXBIO Praha, Prague, Czech Republic). After incubation, we subjected the cells to erythrocytes lysis and subsequent washing with ice-cold wash buffer. We stored the cells on ice and immediately measured them at FACSCalibur. As the red blood cell lysis buffer, we used 155 mM NH_4Cl , 10 mM KHCO_3 and 0.1 mM EDTA (all from Carl Roth, Karlsruhe, Germany), pH 7.3. For tubes that did not contain any added compounds, we added the vehicle, followed by sham incubation for the indicated time intervals. As a vehicle and wash buffer, we used modified phosphate-buffered saline (PBS) containing Dulbecco's PBS, modified, without calcium chloride and magnesium chloride (D5652, Sigma-Aldrich, St. Louis, MO) with 0.02% (w/v) EDTA and 0.1% (w/v) CA 24 (ACE Trade, Ústí nad Orlicí, Czech Republic). We calibrated the flow cytometer using BD Calibrite 3 Beads and APC Beads (both BD Biosciences, San Jose, CA). The results of a representative experimental protocol are provided in Figure S1.

2.4 | BAT coupled with ex vivo histamine release ELISA assay

To corroborate the outcomes of BAT, we coupled BAT with the ex vivo histamine release ELISA assay. We tested six human volunteers in total, consisting of three patients with the history of allergy or anaphylaxis and three controls without any history of allergic or anaphylactic reactions. From each individual, we isolated and tested histamine content in: (a) untreated plasma from peripheral blood, (b) mixture of 40% plasma following the whole-blood incubation with IL-3 and the follow-up incubation with αIgE , (c) mixture of 40% plasma following the whole-blood incubation with IL-3 and the follow-up incubation with birch pollen allergen, (d) mixture of 40% plasma following the whole-blood incubation with IL-3 and the follow-up incubation with the Hymenoptera venom extract and (e) mixture of 40% plasma following the whole-blood incubation with IL-3 and sham incubation without any antigen/allergen. The protocol was as follows: We prepared four aliquots of the whole blood (800 μL each) and added IL-3 (in 200 μL of vehicle per tube) to a final concentration of 2 ng/mL and incubated for 30 minutes at 37°C. Subsequently, we added 1 mL of vehicle to each test tube, containing either αIgE (final concentration of 4 $\mu\text{g/mL}$), Bet v 1a allergen (final concentration of 100 ng/mL), lyophilized venom extract (final concentration of 1 $\mu\text{g/mL}$) or mock. We incubated the tubes for 20 minutes at 37°C, and then for 5 minutes on ice to stop degranulation. From each test tube, we collected 200 μL of the suspension, which

were later used for the flow cytometry measurements. We centrifuged the remaining 1800 μL of the suspension for 5 minutes at 250 $\times g$ and 4°C and frozen down the supernatant for further histamine content measurements. For the purpose of flow cytometry measurements, we incubated 200 μL of cell suspension with $\alpha\text{CD63-FITC}$, $\alpha\text{CD193-PE}$, $\alpha\text{CD203c-APC}$ and $\alpha\text{CD16-PerCP}$ for 20 minutes on ice in dark. In the next step, we lysed the erythrocytes by adding 1.5 mL of the red blood cell lysis buffer for approximately 11 minutes at room temperature. We centrifuged the cell suspension for 5 minutes at 250 $\times g$ and 4°C, washed with one volume of the ice-cold vehicle, centrifuged again for 5 minutes at 250 $\times g$ and 4°C, resuspended in 500 μL of the ice-cold vehicle and immediately measured by FACSCalibur.

We measured the histamine content using the ELISA assay (RE59221, IBL International, Hamburg, Germany) according to manufacturer's instructions. The assay was based on the competition principle, when the tested histamine was competing for binding sites on the $\alpha\text{histamine}$ antibody-coated wells with a fixed amount of enzyme-labelled antigen. We tested a standard curve of histamine in a range from 0.35 to 150 ng/mL. It revealed that the method was somewhat less sensitive compared to the manufacturer's instructions and we considered only histamine concentrations >0.5 ng/mL as reproducibly detectable. We measured all samples in duplicates; the intra-assay variability was 13.1%.

2.5 | CD16b cleavage

To differentiate between the CD16a and CD16b isoforms at the protein level, the GPI cleavage can be employed since only the CD16b isoform is GPI-attached, whereas the CD16a isoform is considered a transmembrane form. We cleaved the GPI anchor by incubating leucocytes with PI-PLC from *Bacillus cereus* (Molecular Probes, Eugene, OR). When setting-up the GPI cleavage experiment, we tested the PI-PLC activity by observing changes in the $\alpha\text{CD16-PerCP}$ fluorescence of neutrophils (based on the SSC/FSC gating). We treated the cells as follows: we incubated leucocytes from 80 μL erythrocytes-depleted peripheral blood with 0.1 U or 0.25 U of PI-PLC for 20 minutes at 4°C or 37°C or mock; alternatively, we incubated leucocytes from 0.8 μL of erythrocytes-depleted peripheral blood with 0.1 U of PI-PLC for 20 minutes at 37°C. Raw data from this test are available in Table S1; we chose 0.1 U of PI-PLC incubated for 20 minutes at 37°C for further experiments.

We based the protocol on the treatment of 80 μL of peripheral blood per test tube and carried out the lysis of erythrocytes by adding 120 μL of the vehicle and 1.5 mL of the erythrocyte lysis buffer with the subsequent incubation for approximately 11 minutes at room temperature. We centrifuged the cell suspension for 5 minutes at 250 $\times g$ and 4°C, washed with one volume of the ice-cold vehicle, centrifuged again for 5 minutes at 250 $\times g$ and 4°C and resuspended in 100 μL of the ice-cold Dulbecco's PBS with 1 mM CaCl_2 and 2 ng/mL IL-3. We incubated the suspension for 10 minutes at 37°C. Subsequently, we added or not 0.1 U of PI-PLC and incubated the test tubes for 20 minutes at 37°C. Following the addition of 200 μL of

the ice-cold vehicle, we centrifuged the test tubes for 5 minutes at $250 \times g$ and 4°C , washed the cells in another 2.5 mL of the vehicle and centrifuged again. Then, we added 200 μL of vehicle to each test tube, containing either αIgE (final concentration of 4 $\mu\text{g}/\text{mL}$), lyophilized venom extract (final concentration of 1 $\mu\text{g}/\text{mL}$) or mock. We incubated the tubes for 20 minutes at 37°C , and then transferred them on ice for 5 minutes to stop degranulation. Then, we added 200 μL of vehicle, containing the antibody cocktail $\alpha\text{CD63-FITC}$, $\alpha\text{CD193-PE}$, $\alpha\text{CD203c-APC}$ and $\alpha\text{CD16-PerCP}$ and incubated for 20 minutes on ice in dark. Following the incubation, we added 500 μL of vehicle, centrifuged the cell suspension for 5 minutes at $250 \times g$ 4°C , washed with 2.5 mL of the ice-cold vehicle, centrifuged again for 5 minutes at $250 \times g$ 4°C , resuspended in 500 μL of the ice-cold vehicle and immediately measured by FACSCalibur. The results of a representative experimental protocol are provided in Figure S2.

2.6 | Detection of mRNA for CD16a and CD16b in human basophils

To differentiate between the CD16a and CD16b isoforms at the protein level, we coupled the flow cytometry sorting of basophils with various CD16 surface expression levels with subsequent RT-PCR and sequencing of the resulting amplicons. Based on the findings of the present study that indicate increase of CD16 surface expression following basophil activation, we proportionally scaled-up the BAT protocol (see above) to the input of 40 mL of peripheral blood subjected the lysis of erythrocytes. The only two exceptions were in the use of antibody conjugates, which we used in the following combination $\alpha\text{CD63-PerCP}$, $\alpha\text{CD193-PE}$, $\alpha\text{CD203c-APC}$ and $\alpha\text{CD16-FITC}$ (all EXBIO Praha, Prague, Czech Republic) and in the calibration, for which we used CompBeads Set αmlg , κ (BD Biosciences, Franklin Lakes, NJ) single-stained with the above-mentioned four antibody conjugates. We sorted FSC-A-gated single $\text{SSC}^{\text{low}}\text{CD193}^+\text{CD203c}^+$ cells into three tubes based on the level of their CD16 surface expression (CD16^- , CD16^{dim} , CD16^+). In addition, we sorted CD16^+ neutrophils identified based on their position in the SSC/FSC scatterplot, and used them as a positive control for the CD16b detection. The ratios of the sorted populations were similar as those found in course of the BAT test, with $\text{SSC}^{\text{low}}\text{CD193}^+\text{CD203c}^+\text{CD16}^+$ cells being particularly difficult to obtain (approximately 0.5% of the sorted population of basophils).

To amplify mRNA for CD16, we isolated total RNA from sorted cells using PicoPure RNA isolation kit (ThermoFisher Scientific, Waltham, MA) and immediately prepared cDNA using SuperScript VILO cDNA Synthesis kit (Invitrogen, Carlsbad, CA). We subsequently amplified the desired sequences using two series of primers. The first set of primers had a nested design and aimed to detect a short DNA locus that contains several single-nucleotide polymorphisms when comparing mRNAs for CD16a and CD16b. The second set of primers had a semi-nested design and aimed to detect specifically mRNA for CD16a isoform only. The third set of primers had a nested design and served as a positive control by means of detecting

the glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The list of primers and their positions in mRNA sequence for CD16a is provided in Table S2. We amplified the fragments using Q5 Hot Start DNA Polymerase (New England Biolabs, Ipswich, MA); with negative controls prepared for all reactions. We subsequently sequenced the CD16 amplicons bidirectionally using an ABI 3130 DNA Analyzer (Applied Biosystems, Foster City, CA) and searched for matching CD16a and CD16b sequences using NCBI Blast.

2.7 | Data analyses

We analyzed the flow data using CellQuest (BD Biosciences, San Jose, CA). The data are presented as the means $\pm$ standard errors (SE) and ranges, unless otherwise stated. We tested the significance of observed differences by Kruskal-Wallis ANOVA on ranks (as the data did not pass Shapiro-Wilk normality test) followed by Dunnett's post-tests or by Mann-Whitney rank sum tests in case when we compared only two groups. We examined the significance of activation-induced changes by using paired *t* tests. We calculated Pearson product moment correlation coefficients and Spearman rank order correlation coefficients in order to correlate the flow cytometry and histamine measurements. We prepared the graphs and performed statistical calculations in SigmaPlot v 12.0 except histamine concentrations, which we calculated in GraphPad Prism v 7.

3 | RESULTS

3.1 | Fluorescence changes in response to supraoptimal concentrations of PKC inhibitors

We first recapitulated the background conditions suggested for the use of supraoptimal ($>1 \mu\text{M}$) concentrations of PKC inhibitors based on the protocol previously published by D. MacGlashan Jr.¹⁶ The use of either bisindolylmaleimide II or Ro 31-8220 led to strong increases in the background fluorescence in FL2 and FL3 channels, a moderate increase in the FL1 channel fluorescence and no increase in FL4 channel fluorescence (Figures 1, S3). We quantified the decreases for two morphologically easy-to-distinguish populations of peripheral blood cells, monocytes and lymphocytes. Although the extent of the changes slightly differed between these two cell populations, the overall course of the changes observed was similar in both populations and was proportionally dependent on the concentrations of the compounds used. The changes were observable at all three supraoptimal concentrations of the study compounds used (1.5 μM through 6.0 μM for Ro 31-8220, and 1.75 μM through 7.00 μM for bisindolylmaleimide II). For example, when using 6.0 μM Ro 31-8220, we obtained the following net median relative fluorescence intensities ($\pm\text{SE}$) for lymphocytes compared to the negative control (100%): FL1 $129 \pm 5\%$, FL2 $573 \pm 84\%$, FL3 $3650 \pm 606\%$ and FL4: $83 \pm 1\%$ (Figure 2A,B). These differential changes in fluorescence intensities led to consistent and robust decreases in the FL1/FL2, FL1/FL3 and FL4/FL1 ratios (Figure 2C,D).

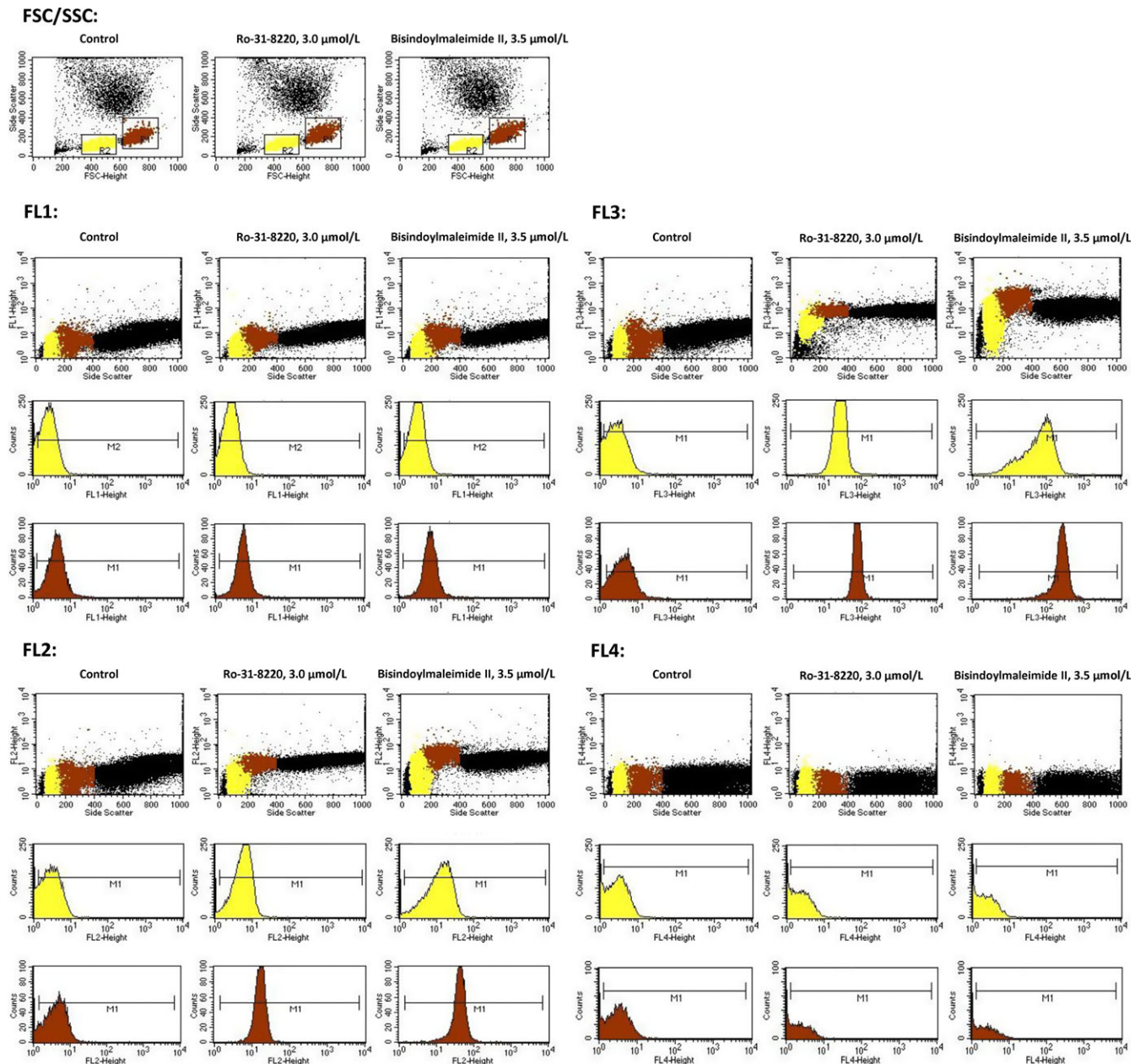


FIGURE 1 The fluorescence induced by supraoptimal concentrations of the PKC inhibitors bisindolylmaleimide II and Ro-31-8220. The figure shows the white blood cells that we did not stain with any antibodies. Based on the forward scatter and side scatter, we gated the populations of monocytes (brown, R1) and lymphocytes (yellow, R2) and tracked these cells in the four channels allowed by the flow cytometer used. For each fluorescence channel, we show the control (untreated) cells, cells treated with 3 μM Ro-31-8220 and cells treated with 3.5 μM bisindolylmaleimide II. Data from a representative patient are shown; raw data from all patients are provided in Figure S3

3.2 | Supraoptimal concentrations of PKC inhibitors in BAT

The above-reported differential changes in background fluorescence of the four channels closely resemble the previously reported apparent differences in the increased CD203c expression (twofold increase following the application of 3 μM Ro 31-8220) compared to CD63 expression (15-fold increase following the same treatment).¹⁶ Thus, we next examined the implementation of supraoptimal concentrations of the PKC inhibitors in BAT. To this end, we selected

BAT combined with the detection of changes in CD16 surface expression. Evidence for the expression of CD16 on basophils is controversial, suggesting the absence of dim expression. We activated the cells with αIgE , Hymenoptera venom extracts or the recombinant birch pollen allergen Bet v 1a, and with or without Ro-31-8220 (3.0 μM) or bisindolylmaleimide II (3.5 μM). An analysis of a series of patients (an example of the full protocol is provided in Figure S1) showed that the cells display standard activation patterns following stimulation with αIgE or allergens in case of the allergic patients. We also showed that the patients express certain

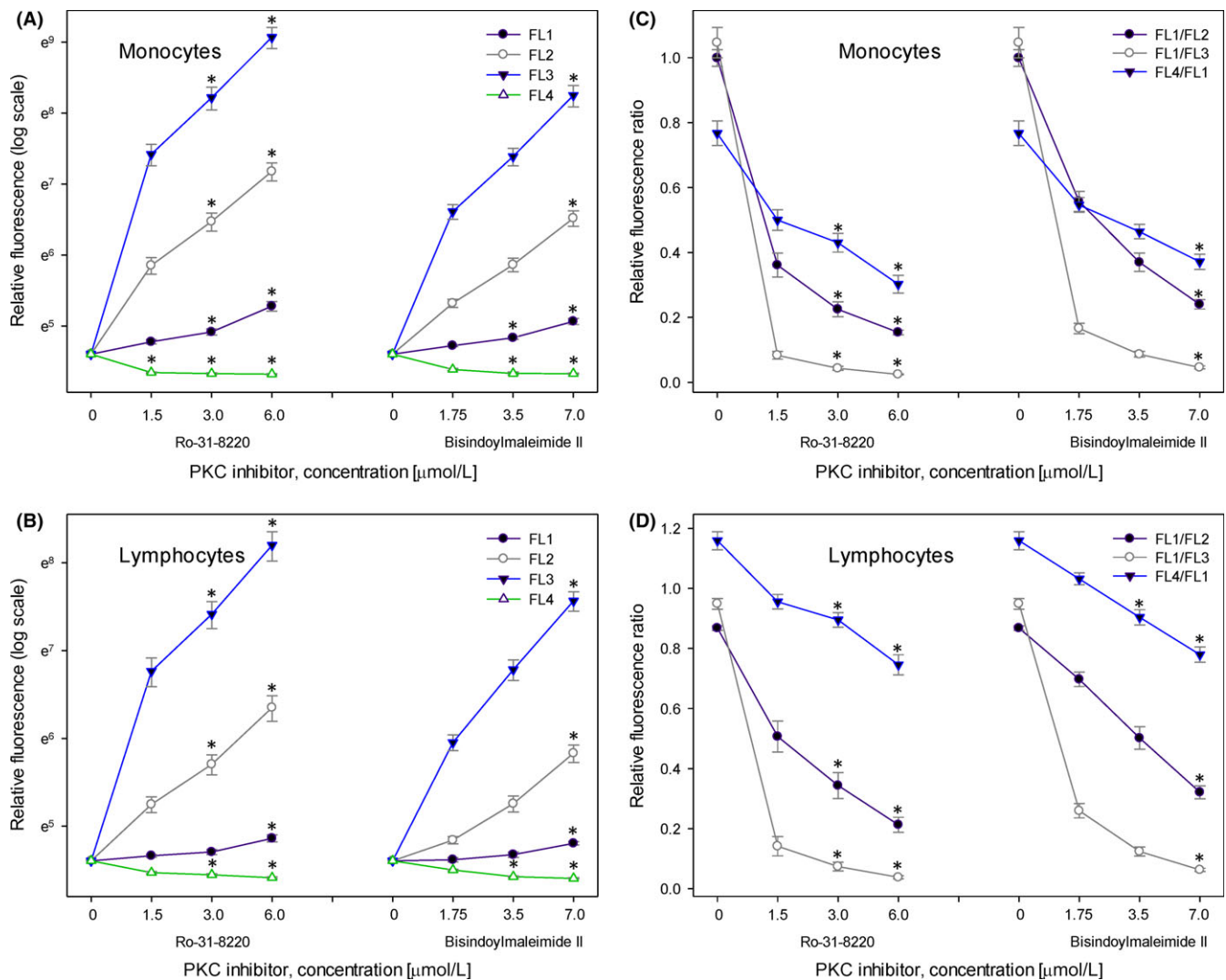
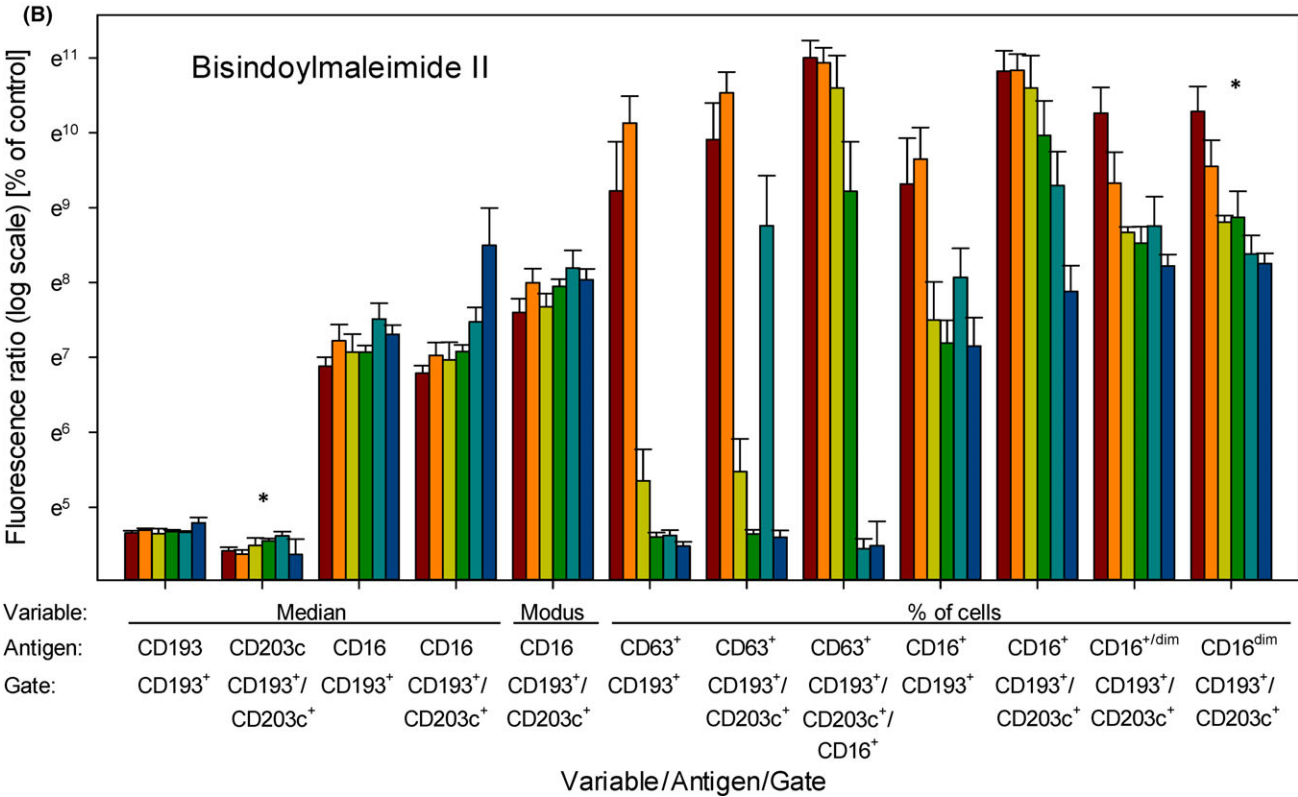
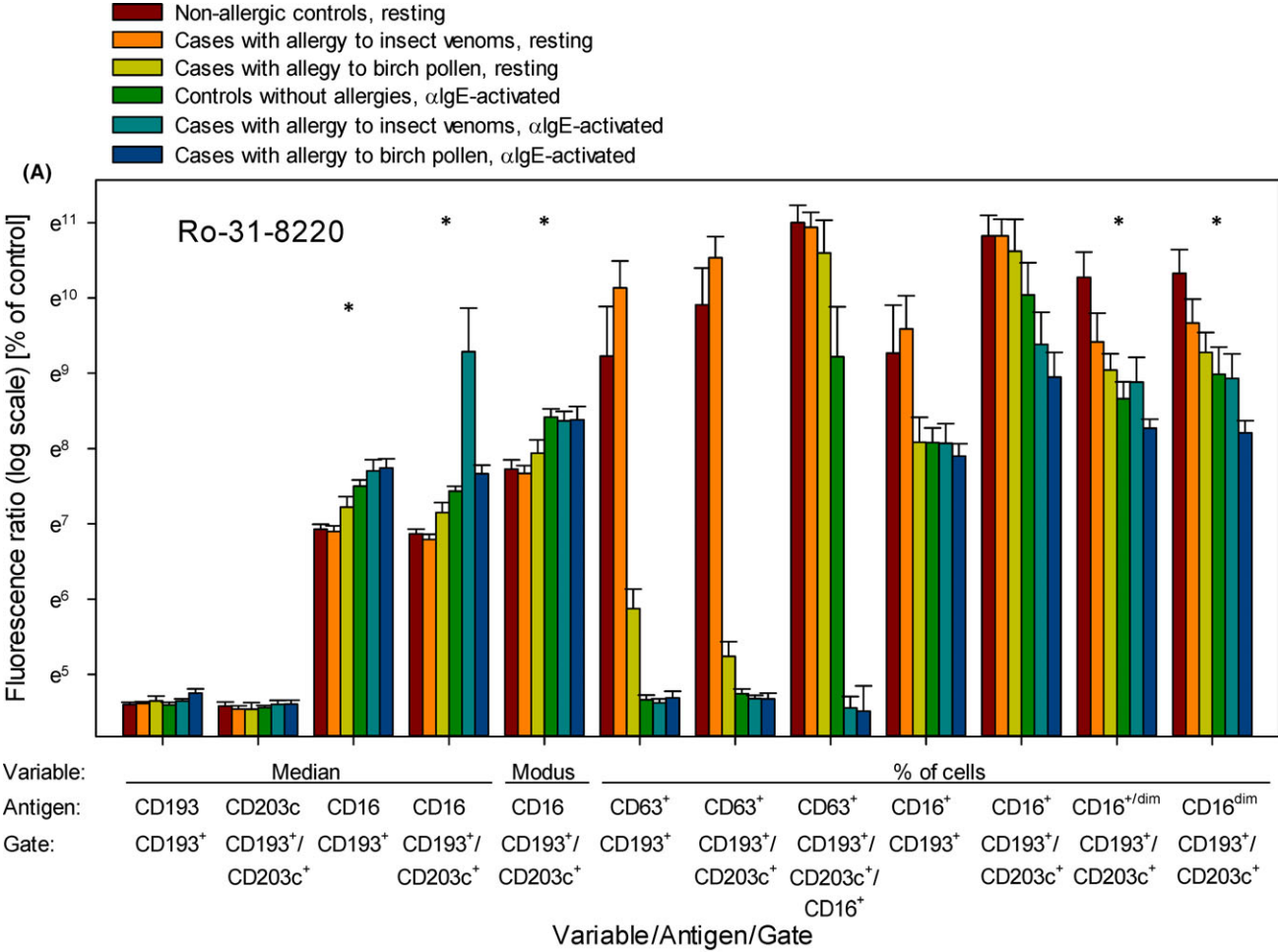


FIGURE 2 Net median fluorescence intensities of cells treated with supraoptimal concentrations of the PKC inhibitors bisindolylmaleimide II and Ro-31-8220. A,B, Changes in net median relative fluorescence intensities ($\pm$ SE) in monocytes/macrophages (A) and lymphocytes (B) calculated relative to the negative control (100%). C,D, Changes in fluorescence intensity ratios FL1/FL2, FL1/FL3 and FL4/FL1 in monocytes/macrophages (C) and lymphocytes (D). We did not stain the cells with any antibodies and identified the populations of monocytes/macrophages and lymphocytes based on their SSC and FSC fluorescence. We tested the significance of observed changes by Kruskal-Wallis ANOVA on ranks (as the data did not pass Shapiro-Wilk normality test) followed by Dunnett's post-tests that compared the respective values with the mock-treated control. Asterisks indicate significant differences at $\alpha = 0.05$

background levels of CD16 as specified in detail in the next chapter. Following the application of the two PKC inhibitors at supraoptimal concentrations, the median values of the relatively robust signals of CD193-PE and CD203c-APC did not change. In contrast, the weak signal of CD16-PerCP increased by one order of magnitude, and the numbers of CD63⁺ cells decreased in non-activated CD193⁺

CD203c⁺CD16⁺ basophils in response to the administration of supraoptimal concentrations of the PKC inhibitors (Figure 3). The apparent response of CD63-FITC and CD16-PerCP can be attributed to their low absolute fluorescence values in non-activated cells (CD63) and basophils (CD16). Thus, the contribution of the background fluorescence of the PKC inhibitors added at supraoptimal

FIGURE 3 Apparent changes in CD16 and CD63 fluorescence caused by the application of supraoptimal concentrations of the PKC inhibitors Ro-31-8220 (3 μM) (A) and bisindolylmaleimide II (3.5 μM) (B). We treated the cells isolated from controls without allergy, from cases with allergy to Hymenoptera venoms, and cases with allergy to birch pollen with the two inhibitors or mock and activated them with αIgE or mock. Median, modus and share of cells positive for the respective antigen are indicated together with the gating strategies used. We compared the fluorescence signals to those obtained when using cells that we did not expose to the PKC inhibitors. Asterisks indicate groups with significant differences at $\alpha = 0.05$ as revealed by Kruskal-Wallis ANOVA on ranks



concentrations was sufficient to induce changes in the apparent fluorescence of CD63-FITC and CD16-PerCP, whereas the robust signal of CD193 and CD203 was only marginally affected.

3.3 | CD16 is newly confirmed as a surface antigen on human basophils

Previously published data on CD16 expression on the surface of human basophils are conflicting and inconclusive. Here, we show that the CD16 signal was consistently present on the surface of a rare subpopulation of analyzed basophils. In healthy controls, the proportion of CD16^{+/dim} cells among resting SSC^{low}CD193⁺CD203c⁺ basophils was low, and we observed only a few such cells in isolates from each individual tested (CD16⁺: 0.18 ± 0.09%, range 0.00%-0.99%; CD16^{dim}: 0.64 ± 0.16%, range 0.00%-1.58%), with peak relative CD16 fluorescence at 1.5 ± 0.2 AU (range 1-3 AU). Consistent with their non-activated status, the resting SSC^{low}CD193⁺CD203c⁺ basophils were associated with only 0.72 ± 0.19% (range 0.00%-1.78%) positivity for the CD63 activation marker, while their CD16⁺ subpopulation displayed CD63 positivity in 17.8 ± 7.0% (range 0.0%-66.7%) of the cells.

Following αIgE-mediated FcεRI crosslinking, the proportion of CD16^{+/dim} cells among SSC^{low}CD193⁺CD203c⁺ basophils from healthy controls tripled, but remained low (CD16⁺: 0.64 ± 0.3%, range 0.00%-2.53%; CD16^{dim}: 2.75 ± 0.7%, range 0.20%-7.07%), with peak relative CD16 fluorescence unchanged, remaining at 1.2 ± 0.1 AU (range 1-2 AU). Consistent with their activated status, the CD63 positivity of resting SSC^{low}CD193⁺CD203c⁺ basophils increased to 39.5 ± 5.7% (range 13.6%-65.3%), while the CD16⁺ subpopulation was associated with a further increase to 61.0 ± 8.8% (range 0.0%-92.1%) of CD63⁺ cells.

In patients with Hymenoptera venom allergy, the proportion of CD16^{+/dim} cells among resting SSC^{low}CD193⁺CD203c⁺ basophils increased compared to the controls but was still low (CD16⁺: 0.45 ± 0.27%, range 0.00%-4.49%; CD16^{dim}: 2.15 ± 0.75%, range 0.00%-11.54%), with a peak relative CD16 fluorescence remaining at 1.6 ± 0.3 AU (range 1-6 AU). Consistent with their non-activated status, the resting SSC^{low}CD193⁺CD203c⁺ basophils from patients with allergy were slightly increased but still only 0.96 ± 0.33% (range 0.00%-5.13%) of them were CD63⁺, while their CD16⁺ subpopulation displayed CD63 positivity in 15.9 ± 4.9% (range 0.0%-60.0%) of the cells.

Following exposure to Hymenoptera venom extract, the proportion of CD16^{+/dim} cells among SSC^{low}CD193⁺CD203c⁺ basophils from patients with Hymenoptera venom allergy increased fivefold (CD16⁺: 2.34 ± 0.77%, range 0.00%-10.66%; CD16^{dim}: 5.28 ± 1.36%, range 0.40%-18.83%), with peak relative CD16 fluorescence slightly increased to 2.2 ± 0.5 AU (range 1-9 AU). Consistent with their activated status, the CD63 positivity of resting SSC^{low}CD193⁺CD203c⁺ basophils increased to 54.0 ± 4.6% (range 18.3%-94.4%), while the CD16⁺ subpopulation was associated with a further increase to 70.3 ± 6.4% (range 16.7%-100.0%) of CD63⁺ cells. After the induction of αIgE-mediated FcεRI crosslinking in the

basophils from patients with Hymenoptera venom allergy, the obtained values were similar or slightly lower than those obtained following allergen stimulation (Figure 4).

In patients with birch pollen allergy, the proportion of CD16^{+/dim} cells among resting SSC^{low}CD193⁺CD203c⁺ basophils was low, and the numbers of CD16^{+/dim} cells (CD16⁺: 0.18 ± 0.07%, range 0.00%-0.36%; CD16^{dim}: 0.99 ± 0.16%, range 0.36%-1.38%) were similar to those in the healthy controls, with similar peak relative CD16 fluorescence at 1.4 ± 0.2 AU (range 1-2 AU). Consistently, the resting SSC^{low}CD193⁺CD203c⁺ basophils were associated with 0.73 ± 0.15% (range 0.34%-1.11%) positivity for the CD63 activation marker, while the CD16⁺ subpopulation displayed 19.8 ± 9.4% (range 0.00%-57.14%) of CD63⁺ cells; both of these values were similar to those found in resting basophils from healthy controls.

Following exposure to the recombinant Bet v 1a allergen, the proportion of CD16^{+/dim} cells among SSC^{low}CD193⁺CD203c⁺ basophils from patients with birch pollen allergy increased (CD16⁺: 0.46 ± 0.14%, range 0.00%-0.92%; CD16^{dim}: 1.66 ± 0.62%, range 0.47%-4.13%) to level similar to those in healthy controls, with peak relative CD16 fluorescence unchanged, remaining at 1.6 ± 0.2 AU (range 1-2 AU). Consistent with their activated status, the CD63 positivity of resting SSC^{low}CD193⁺CD203c⁺ basophils increased to 48.0 ± 11.6% (range 15.2%-88.5%), while the CD16⁺ subpopulation was associated with further increase in the number of CD63⁺ cells to 68.2 ± 10.0% (range 50.0%-100.0%) of the cells; both of these values were similar to those found in activated basophils from healthy controls. The exposure of basophils from patients with Hymenoptera venom allergy to αIgE-mediated FcεRI crosslinking generated values similar to those obtained following allergen stimulation (Figure 4). The raw data for CD16 expression on the surface of the basophils from all tested patients are provided in Figure S4.

3.4 | Correlation of CD16 surface expression with histamine release

Reflecting the indirect nature of BAT with regard to the detection of the basophil degranulation, we repeated the above experiments under the settings for direct measurements of histamine concentrations. The histamine concentrations correlated with standard BAT outcomes, such as the numbers of CD63⁺ cells on the surface of basophils (defined as SSC^{low}CD193⁺CD203c⁺ cells) (Pearson 0.645, $P < 0.001$; Spearman 0.687, $P < 0.001$). Considering CD16 surface expression, the proportion of CD16^{+/dim} cells on SSC^{low}CD193⁺CD203c⁺ cells was only weakly correlated with histamine release (Pearson 0.398, $P = 0.009$; Spearman 0.379, $P = 0.014$). The medians of CD16 expression on SSC^{low}CD193⁺CD203c⁺ cells and peak fluorescence signal of CD16 on the SSC^{low}CD193⁺CD203c⁺ cells did not correlate with histamine release (Pearson -0.176, $P = 0.26$; Spearman -0.109, $P = 0.48$; and Pearson -0.18, $P = 0.25$; Spearman -0.002, $P = 0.99$, respectively). The raw data for histamine release matched with the detection of CD16 surface expression and BAT outcomes from all tested patients are provided in Table S3.

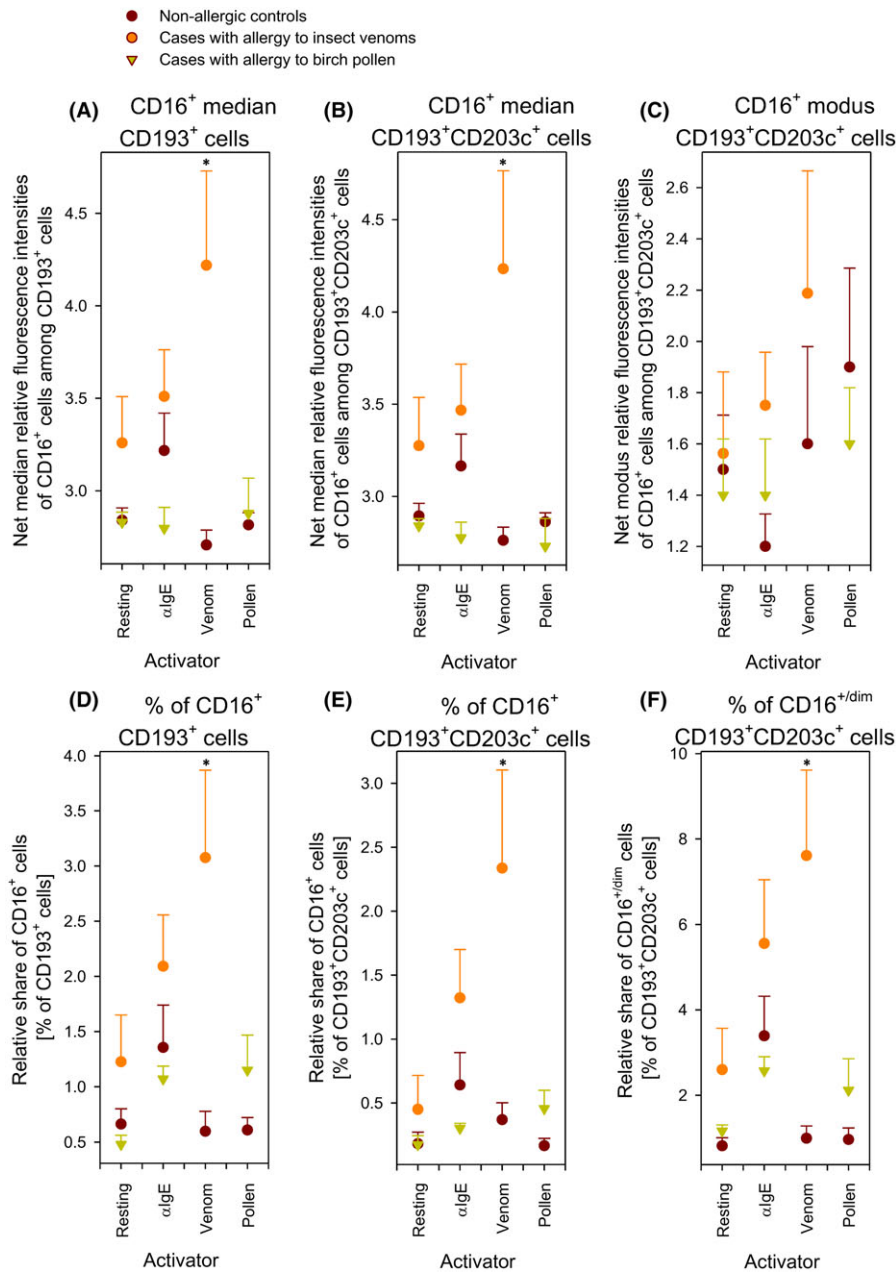


FIGURE 4 Surface expression of CD16 on human basophils and its changes following their exposure to antigen or allergens. A-C, Net median (A,B) and modus (C) relative fluorescence intensities of CD16 on the surface of $SSC^{\text{low}}CD193^+$ (A) and $SSC^{\text{low}}CD193^+CD203c^+$ (B,C) cells. (D-F) Relative share of CD16⁺ (D-E) or CD16^{+/dim} (F) cells expressed as a percentage of $SSC^{\text{low}}CD193^+$ (D) and $SSC^{\text{low}}CD193^+CD203c^+$ (E,F) cells. We analyzed separately cells from controls without allergy, patients with allergy to Hymenoptera venoms and patients with allergy to birch pollen. We analyzed the cells activated by the αIgE-mediated FcεRI crosslink, Hymenoptera venom extracts (except cells from patients with allergy to birch pollen) and the major birch pollen allergen Bet v 1a (except cells from patients with allergy to Hymenoptera venoms) or mock-treated. Asterisks indicate groups with significant differences at $\alpha = 0.05$ as revealed by Kruskal-Wallis ANOVA on ranks or Mann-Whitney rank sum tests in case when we compared only two groups

3.5 | Identification of the CD16 isoform expressed in basophils

To elucidate the status of CD16a and CD16b expression at the mRNA level, we isolated three populations of basophils by flow cytometry sorting, reflecting the level of CD16 surface expression (CD16⁺, CD16^{dim} and CD16⁻) and the control population of CD16⁺ neutrophils. We found that all the three populations of basophils,

including those that were sorted as CD16⁻ (i.e., with the CD16-FITC fluorescence $<10^1$) were positive for CD16 (Figure S5). Surprisingly, the isoform-specific RT-PCR suggested that CD16a is present in all the three populations of basophils (Figure S5B). We thus sequenced the obtained amplicons from basophils, as well as the control RT-PCR from CD16⁺ neutrophils. The NCBI Blast of the resulting sequences suggested that mRNA for CD16a represents a dominant CD16 isoform present in basophils regardless of CD16

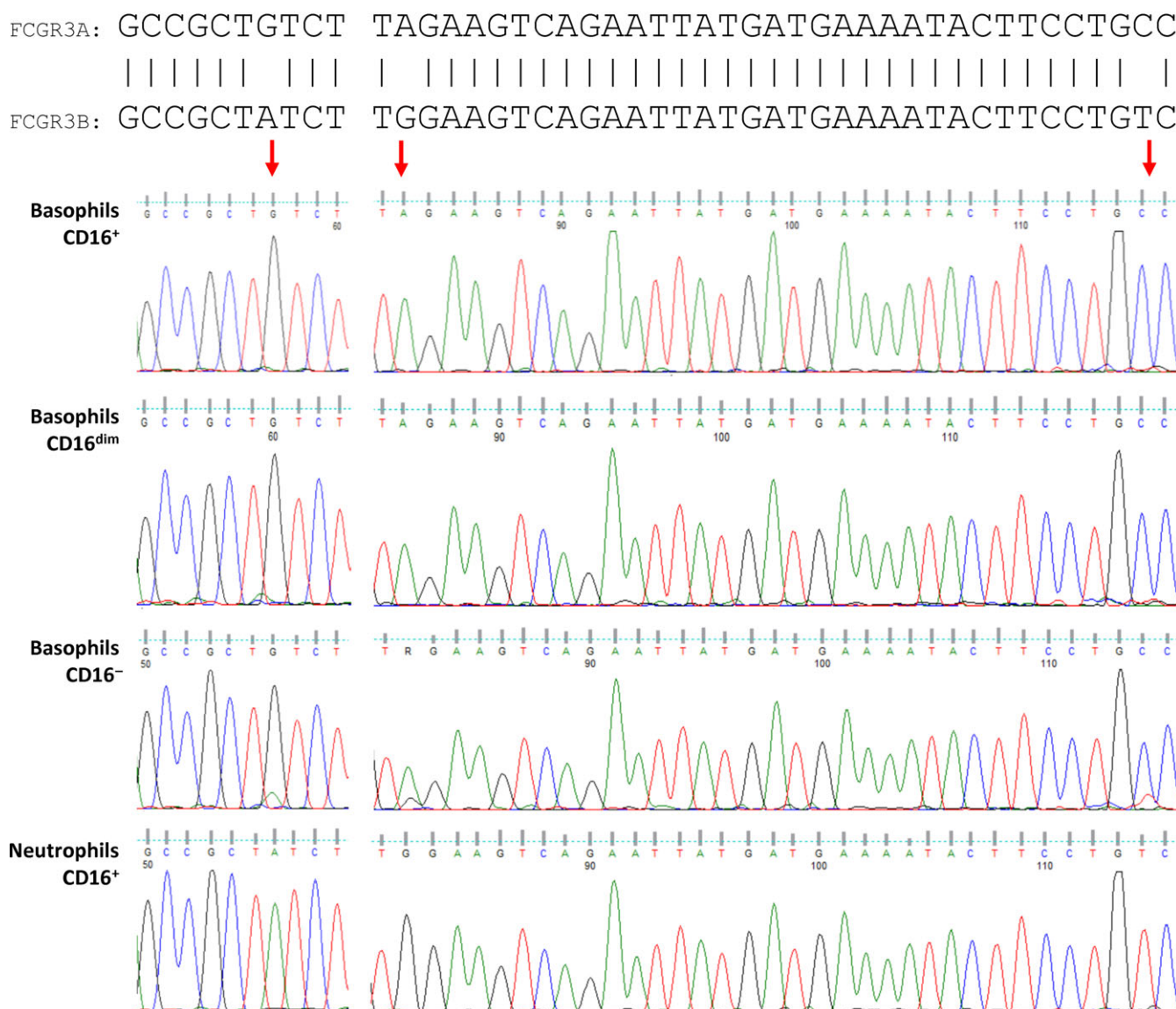


FIGURE 5 Chromatograms of sequences of mRNA for CD16 that we obtained from CD16⁺, CD16^{dim} and CD16⁻ basophils and CD16⁺ neutrophils (listed in order as they appear from the top to the bottom in the figure). We sorted all the cells at a flow cytometer based on levels of surface expression of the CD16 antigen. Nucleotides that differ between the FCGR3A (mRNA for CD16a) and FCGR3B (mRNA for CD16b) isoforms are indicated at the top of the figure

surface expression at the protein level. In contrast, we found that mRNA for CD16b isoform is also expressed by basophils but only at low level (CD16^{dim} basophils); we did not find mRNA for the CD16b isoform in CD16⁺ and CD16⁻ basophils despite the same nested RT-PCR approach amplified CD16b as the only detectable CD16 isoform in the CD16⁺ neutrophils (Figure 5).

We obtained similar results at the protein level. The PI-PLC-mediated cleavage of the GPI-anchored isoform CD16b revealed that this isoform is dominant on neutrophils that served as a positive control. Nevertheless, the PI-PLC treatment did not stimulate the decrease of CD16 signal on basophils from patients with the history of bee venom anaphylaxis as well as from control subjects when

treated with α lgE, bee venom extract or mock (Table S4). The experiment that involved PI-PLC-mediated cleavage suggested that CD16b may not be considered a dominant CD16 isoform on basophils of any of the tested patients and control subjects.

4 | DISCUSSION

The two tested PKC inhibitors, Ro-81-3220 (bisindolylmaleimide IX) (Figure 6A) and bisindolylmaleimide II (Figure 6B) are structurally related to the bis-indole alkaloid staurosporine. Bisindolylmaleimides in general possess multiple aromatic rings and many of these

compounds actually serve as red fluorescent materials due to their non-planar structure and D-A system.¹⁹ Various bisindolylmaleimides have fluorescence emission maxima wavelengths longer than 500 nm, with large Stokes shifts longer than 200 nm, and different fluorescence emission wavelengths at an excitation wavelength of 365 nm. The expansion of the π -conjugation, the π -bond electronic structure and the oxidation of the CC bond at the 2,3-position of the maleimide moiety are crucial for producing these fluorescence properties.²⁰ As shown in the present study, the two tested PKC inhibitors, Ro-81-3220 and bisindolylmaleimide II, cause negligible fluorescence when used at their IC₅₀ (with respect to PKC) but cause strong fluorescence when applied at low micromolar concentrations (Figure 1). This intrinsic property of the two PKC inhibitors caused the previously reported apparent differences in the CD63 and CD203c fluorescence¹⁶ and affected the measurements of weakly expressed surface antigens CD63 (in the FL1 channel) and CD16 (in the FL4 channel) in the present study (Figure 3). This issue likely affects any flow cytometry or fluorescence microscopy research that exploits bisindolylmaleimides in low micromolar ranges. For example, Haase et al²¹ published the analysis of the labile zinc content in cells by using a FluoZin-3 AM ester probe applied simultaneously or not with 2 μ M bisindolylmaleimide II and performed flow cytometry analysis in the FL1 channel. In another study, Edgar B. Cepeda et al²² suggested that the application of 10 μ M bisindolylmaleimide II did not affect the fluorescence of the APC-labelled CXCR4 antibody at the surface of human hepatocellular carcinoma cells without providing compensation for the fluorescence induced by the inhibitor itself. Many other examples exist. Nevertheless, Ann Fowler et al²³ correctly highlighted the orange colour of 2 μ M bisindolylmaleimide II and its potential interference in the performance fluorescence-based kinase activity screens, but this study was not considered among flow cytometry users and was only cited with respect to its high-throughput screening applications. Combined, the present study clearly rejected the hypothesis that supraoptimal concentrations of Ro-81-3220 or bisindolylmaleimide II mediate the significant decoupling of CD63 and CD203c externalization following the activation of human basophils and, thus, these compounds do not mediate any effects on the surface expression of CD16 measurable by flow cytometry.

The rejected role of supraoptimal concentrations of PKC inhibitors in decoupling CD63 and CD203c externalization has indirect applications beyond the scope of the present study. Particularly, the loss of validity of experiments that used these drugs eliminates arguments for the previously proposed hypothesis that the total amount of histamine released is the sum of piecemeal degranulation and anaphylactic degranulation. Particularly, the model of human basophil degranulation consisting of weak to modest IgE-mediated histamine release and CD203c expression together with a marked enhancement of CD63 expression¹⁶ is likely flawed. However, the support for differential CD63 and CD203c localization on the granules of human basophils is still supported by preferential externalization of CD203c over CD63 and histamine in response to interleukin-3,²⁴⁻²⁶ prostaglandin D₂²⁷ and phorbol myristate acetate.²⁸

We corroborated the surprising and previously unverified finding by Marc Daëron and colleagues, showing CD16 expression on the surface of human basophils.¹⁰ Consistent with these authors, there was primarily dim expression of this antigen on human basophils isolated from peripheral blood. However, a few cells with bright CD16 signals exist among the resting basophils of most of the patients (Figure 4). In contrast to Marc Daëron, we observed the increase in the CD16 surface expression following cell activation by FcεRI crosslinking. In addition, we provided the first evidence that CD16 surface expression on human basophils is increased in response to naturally occurring stimuli, such as birch pollen allergen, and bee and wasp venom extracts (Figure 4) in a correlation with basophil degranulation as measured by the histamine release (Table S3). A previous study suggested that the mRNA levels of CD16 in patients with various allergic diseases are disease-specific, with increases in patients with respiratory allergy and decreases in patients with atopic dermatitis. The data on the surface expression have never been shown. Thus, in the present study, we provided the first evidence of the surface expression of CD16 on basophils isolated from the peripheral blood of patients with birch pollen and Hymenoptera venom allergy. Consistent with previous findings at the mRNA level, the two groups of patients with allergy did not manifest identical CD16 levels at the surface of their basophils. Instead, the patients with Hymenoptera venom allergy expressed CD16 on the surface of

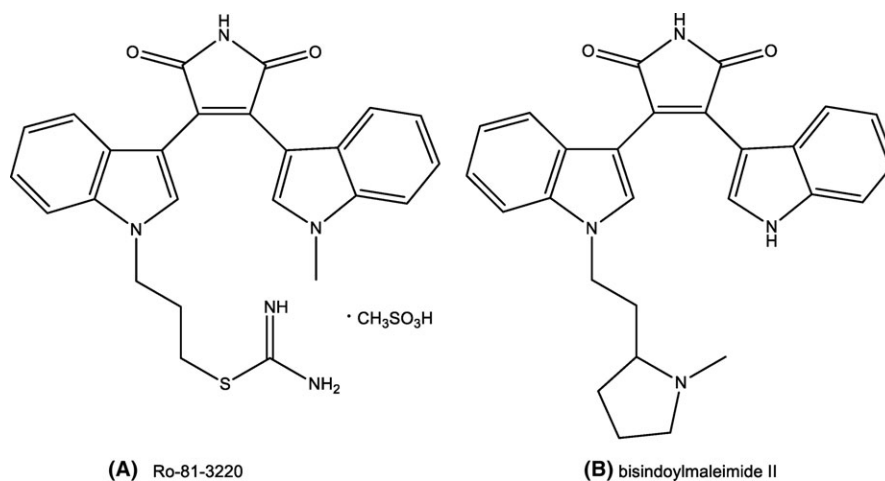


FIGURE 6 Structures of the study compounds, the PKC inhibitors Ro-31-8220 (A) and bisindolylmaleimide II (B)

a large fraction of basophils, and the number of CD16-expressing basophils was increased to a larger extent in response to the stimulation with allergen or by FcεRI crosslinking (Figure 4). In contrast, the patients with respiratory allergy to birch pollen had numbers of CD16-expressing basophils similar to those in healthy controls both before and after the activation of the basophils. Thus, the present study supports the assumption that multiple methods of basophil enrichment by using elutriation and/or sorting based on antigenic features, for which the basophils are considered as negative, may lead to the depletion of specific basophil populations expressing CD16. CD16⁺ cells are particularly abundant among activated basophils, and among those that express high CD63 levels even on resting cells (Figure 4).

Unexpectedly, we found that at the mRNA as well as at the protein level, the dominant CD16 form, which is expressed on human basophils, is CD16a. This contradicts the data by Marc Daëron and colleagues, who concluded that only CD16b is expressed in this cell type.¹⁰ Despite they amplified mRNA for CD16, they did not verify to which isoforms it belongs and their conclusion was based solely on the PI-PLC-mediated GPI cleavage of the CD16b anchor. We employed the same method, optimized it in a way that we reached more striking decreases of CD16b fluorescence on neutrophils (serving as a positive CD16b control) compared to those reported by Daëron and colleagues but still we did not see any major changes of the CD16 fluorescence on basophils. It is possible that there may be differences at the individual level as there are indices that the presence of certain CD16a polymorphisms may increase CD16a expression in NK cells.^{29,30} Nevertheless, among the six patients and control subjects tested in the present study, we did not see any that would express CD16b as a dominant form of surface-bound CD16 (Table S4). We reproduced these unexpected findings at the mRNA level, where we confirmed by Sanger sequencing of nested RT-PCR products that CD16a is the dominant form of CD16 in basophils although we also detected low expression of CD16b (Figure 5). Combined, these results unambiguously suggest that CD16a is a dominant CD16 isoform in human basophils.

Previously, CD16⁺ cells were reported to represent 24%–81% basophils from control subjects and 12%–100% basophils from patients with allergies.¹⁰ However, the same report, which claimed such a high positivity, showed only a single scatter plot that allows directly observing the CD16 gating strategy used.¹⁰ From the only figure shown, it can be speculated that any fluorescence >10¹ RFU was considered a positive signal when evaluating the fluorescence of αCD16 antibody conjugates. Despite that, their αCD16 signal on neutrophils was approximately at 10³ RFU and there were only a few basophils that reached this signal, if any (Figure 4A in Ref. [10]). Thus, to be able to compare directly the outcomes of the present study with those by Marc Daëron and colleagues,¹⁰ we would need to summarize the data reported in the present study as both CD16⁺ and CD16^{dim}. Such alternatively processed data do not differ from those reported previously. In the present study, we, for example, reached 13.4%–35.0% share of CD16^{+/dim} cells among basophils from the three control subjects tested in the experiment with

PI-PLC, and 8.8%–16.6% share of CD16^{+/dim} cells among basophils from the three patients with the history of bee venom anaphylaxis tested in the experiment with PI-PLC (Table S4). Combined, these results suggest that dim expression of CD16 is a common feature of human basophils but only a rare subpopulation of basophils expresses displays bright CD16 signal, which would be of similar intensity as that of CD16 on human neutrophils.

In conclusion, we rejected the apparent dissociation of the surface expression of human basophil activation markers following the administration of supraoptimal concentrations of PKC inhibitors, the bisindolylmaleimides. These compounds have fluorescent properties with potential to obscure the fluorescent signal of common fluorophores used in flow cytometry unless properly compensated for their spectrum. Particularly vulnerable are the analyses of antigens with dim expression, as shown for CD16. The present study confirmed CD16 as mostly weakly expressed surface antigen of a subpopulation of human basophils, identified the CD16a isoform as dominant in human basophils, and reported CD16 surface expression to increase following the allergen challenge or FcεRI crosslinking and in patients with certain types of allergy. Understanding the regulation of CD16 in human basophils allows further translational research, which would focus on whether CD16 expressed by human basophils may play similar role in IgG-mediated passive systemic anaphylaxis as its ortholog does in mice.⁹

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SUPPORTING INFORMATION

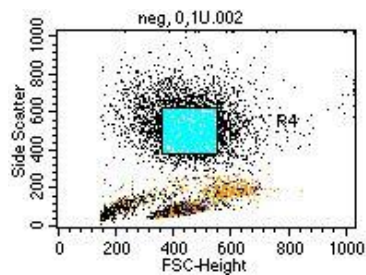
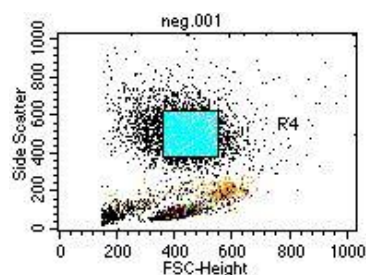
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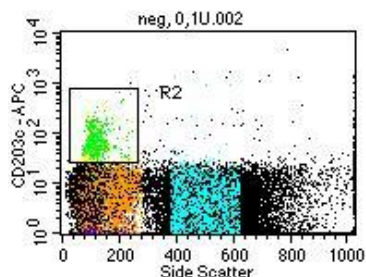
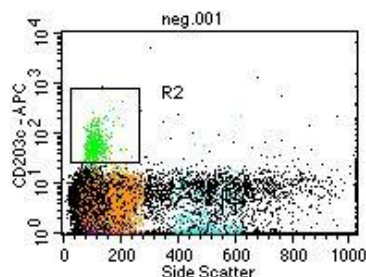
Mock

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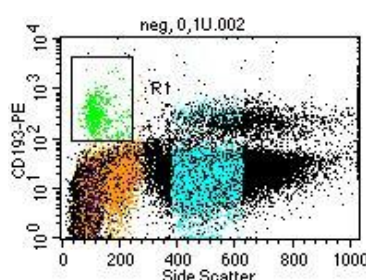
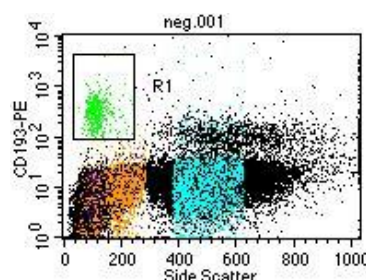
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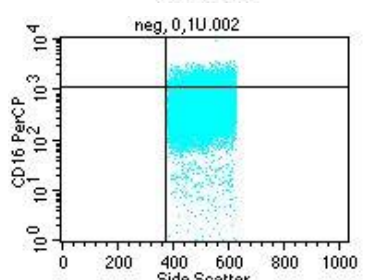
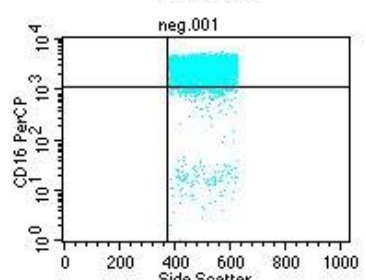
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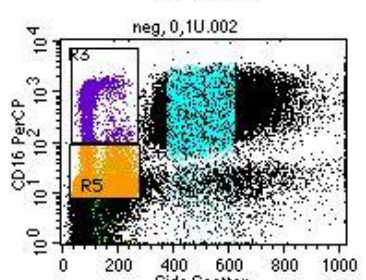
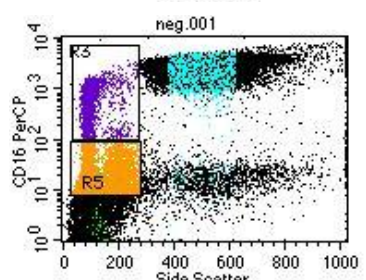
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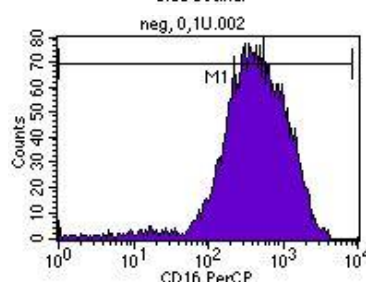
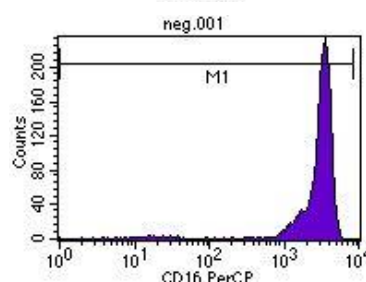
Neutrophils (R4)



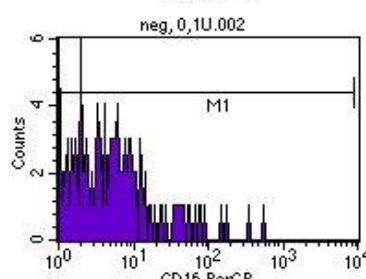
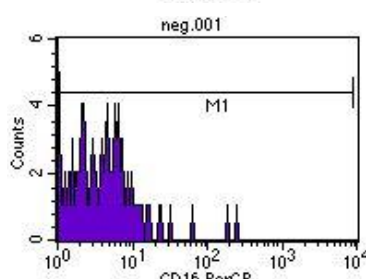
Total leukocytes



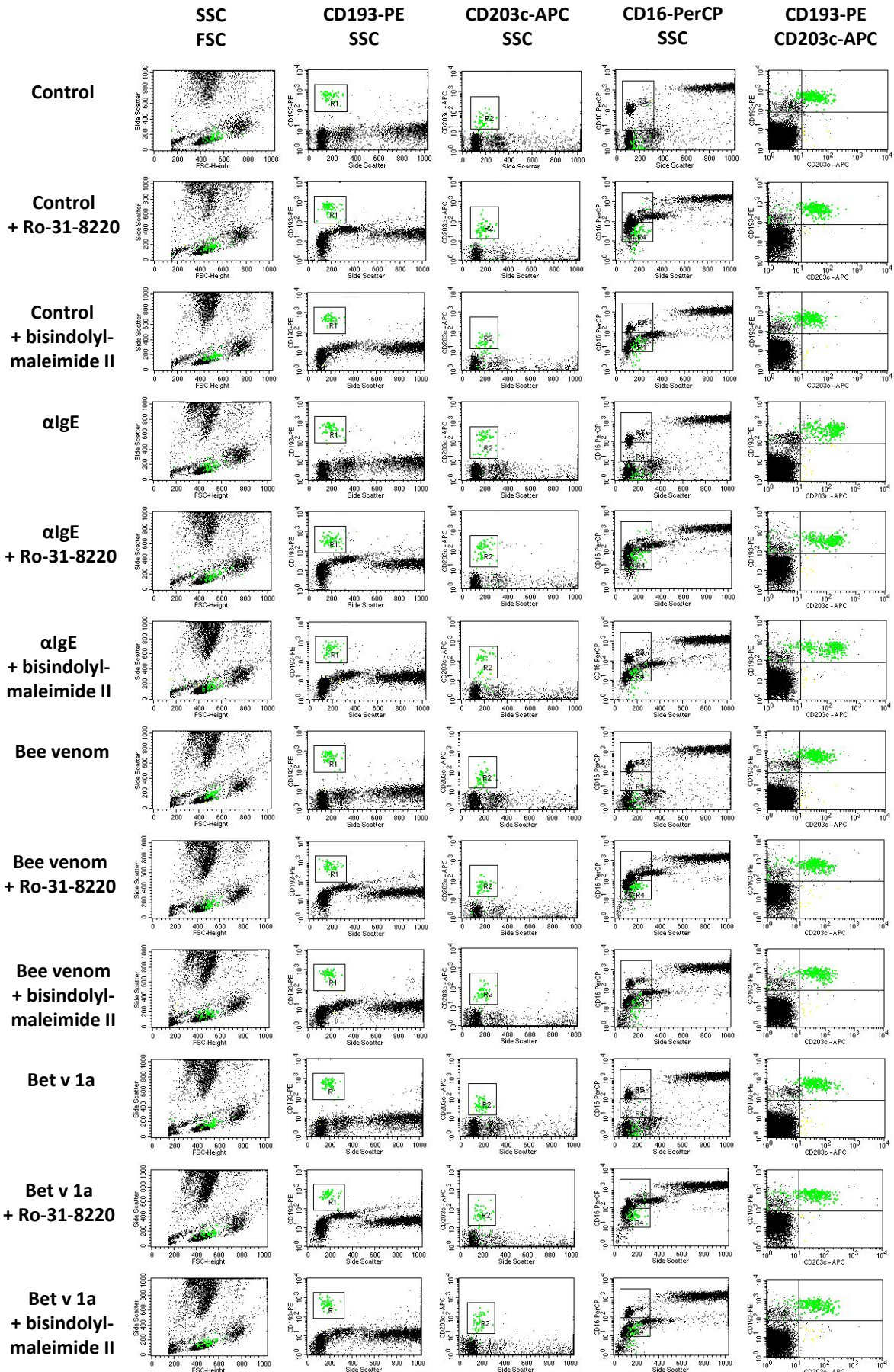
Neutrophils (R4)



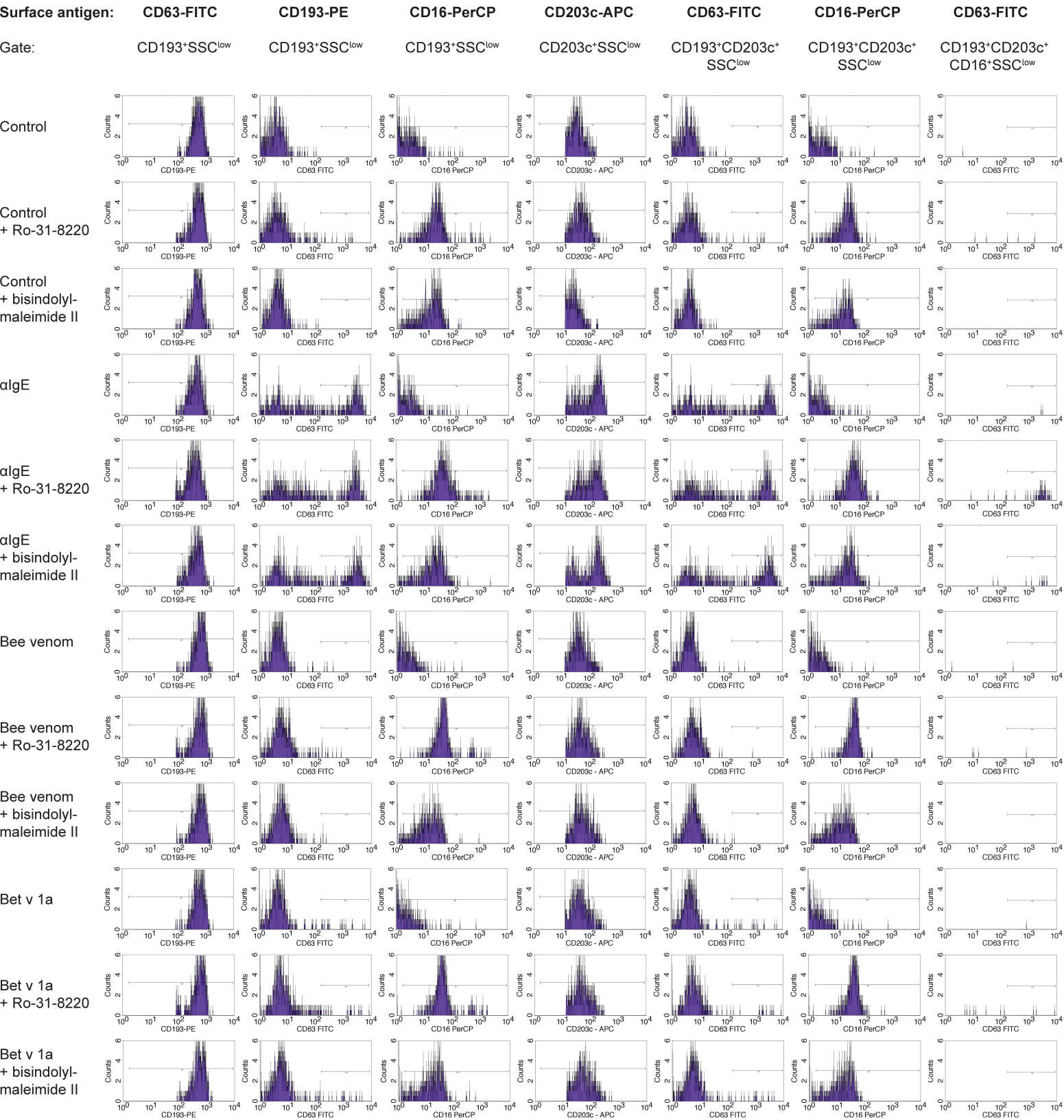
Basophils (R1 and R2)



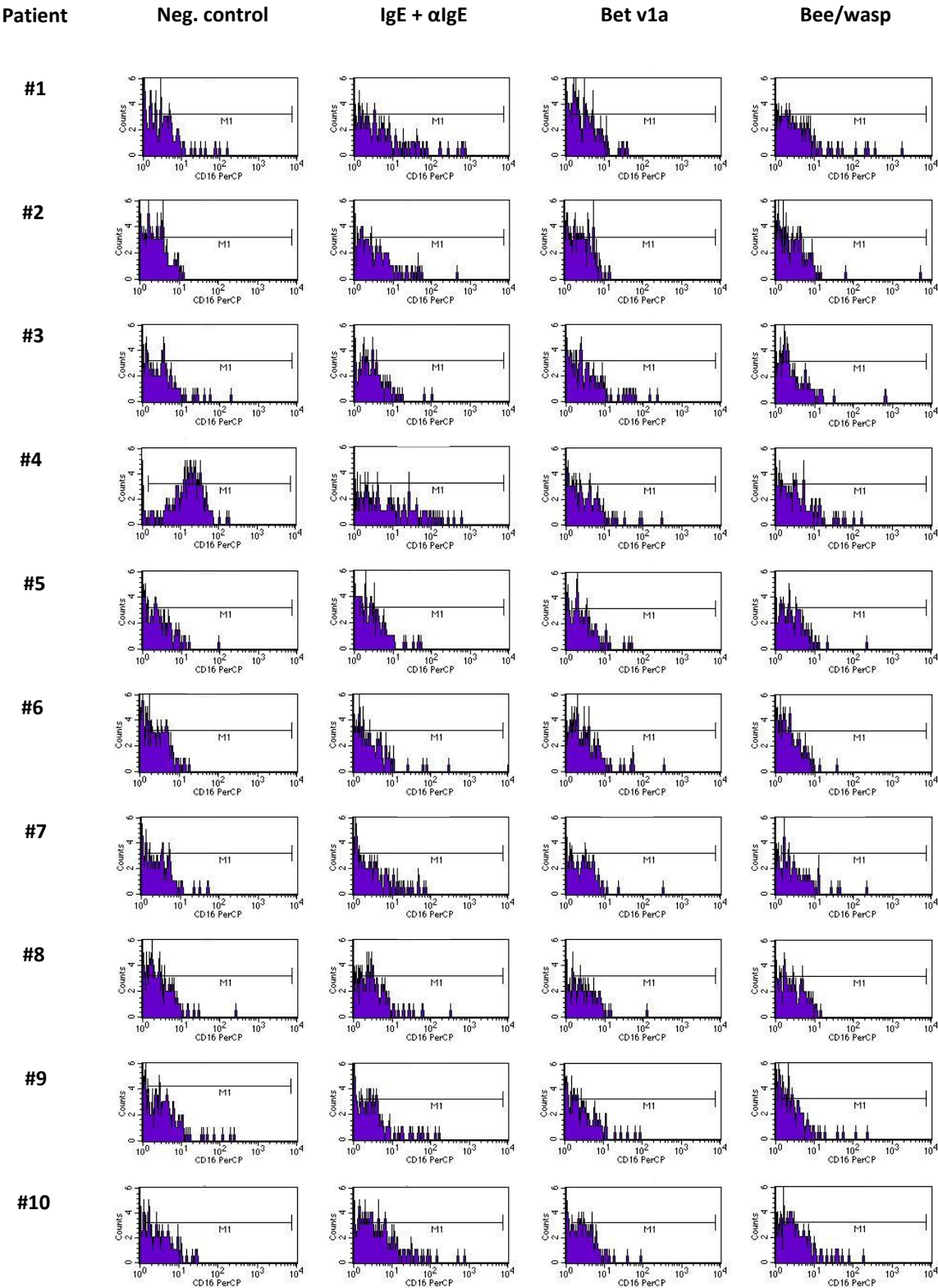
Example of the evaluation protocol (control case without allergy):



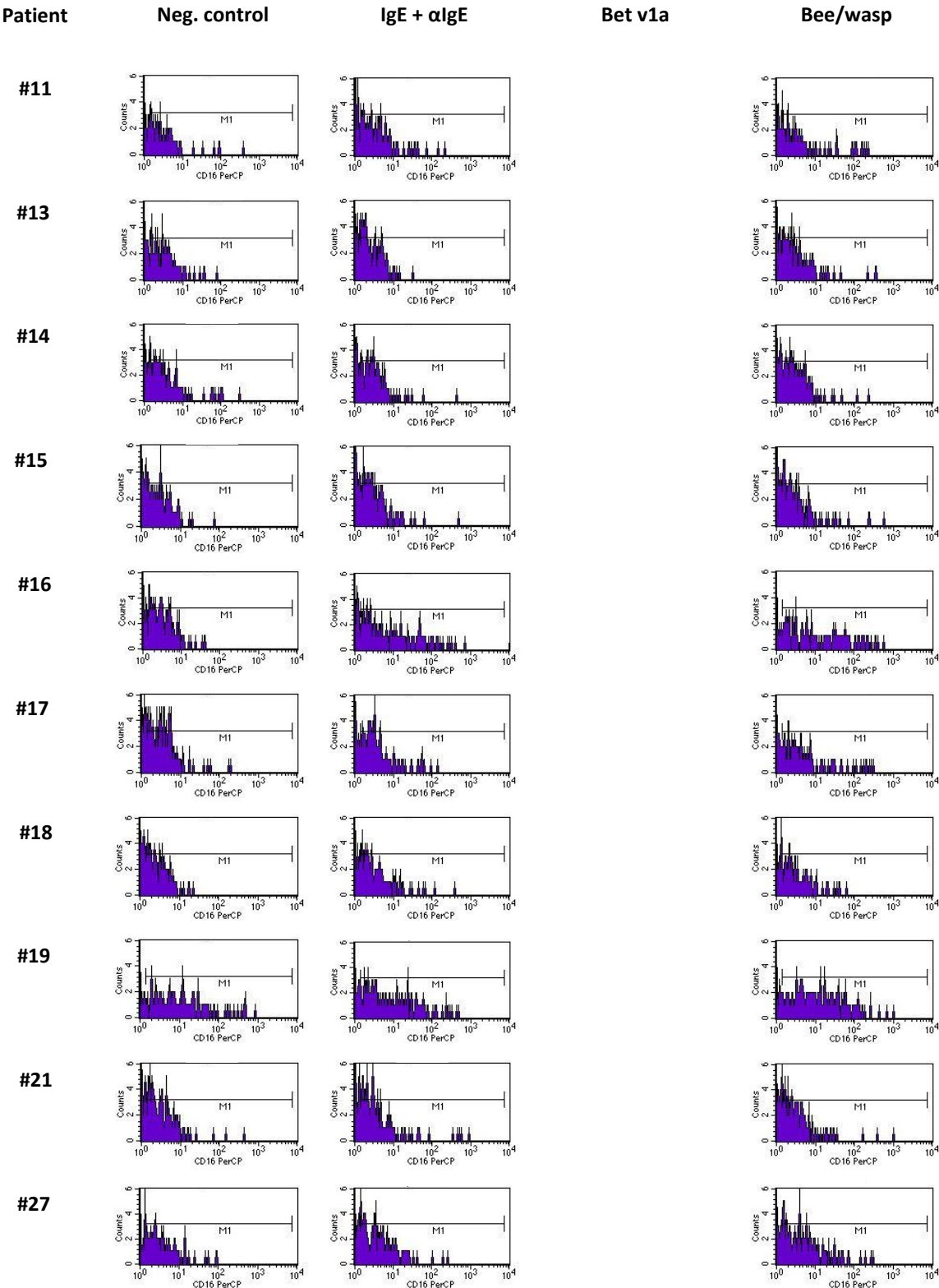
Example of the evaluation protocol (control case without allergy):



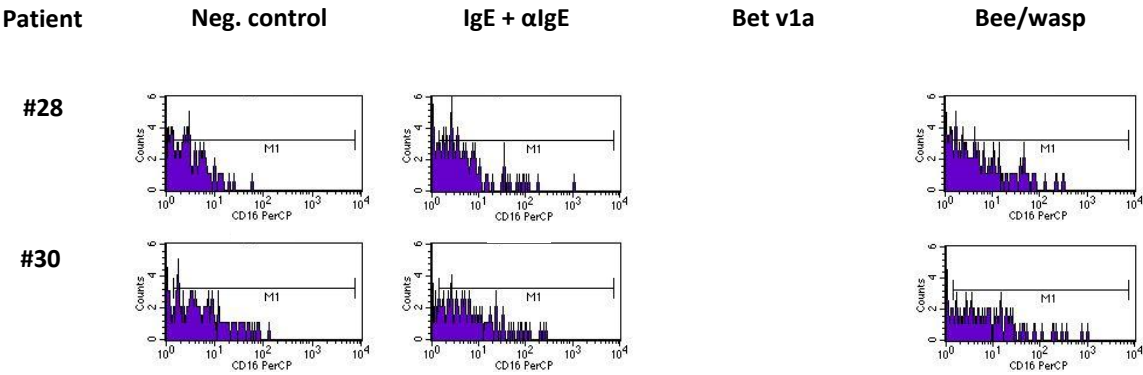
Patients without known allergic reactions:



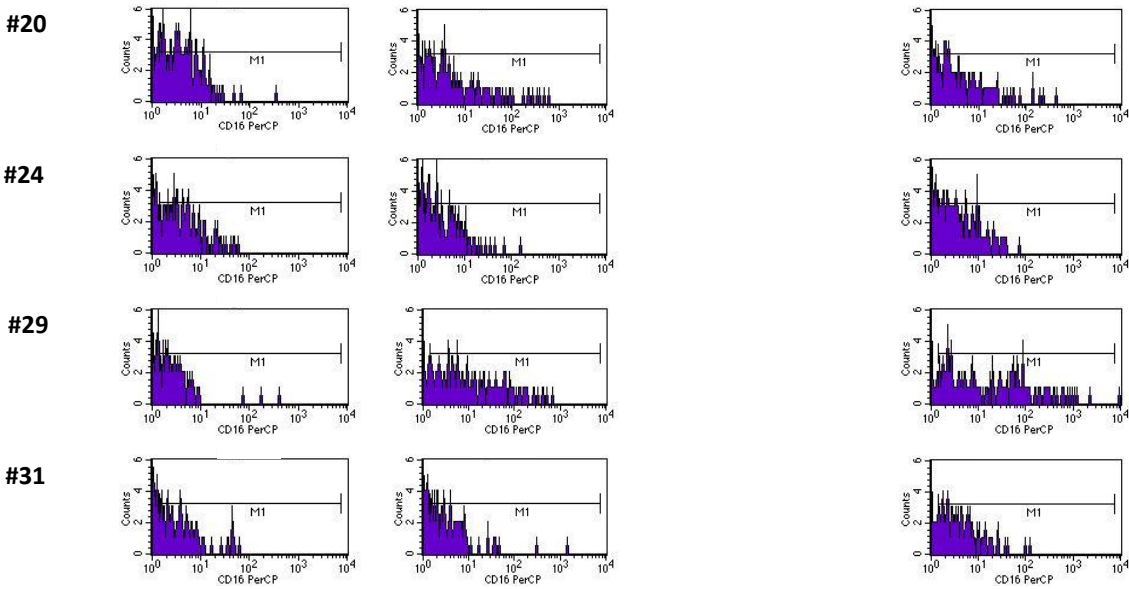
Patients with known wasp venom allergy:



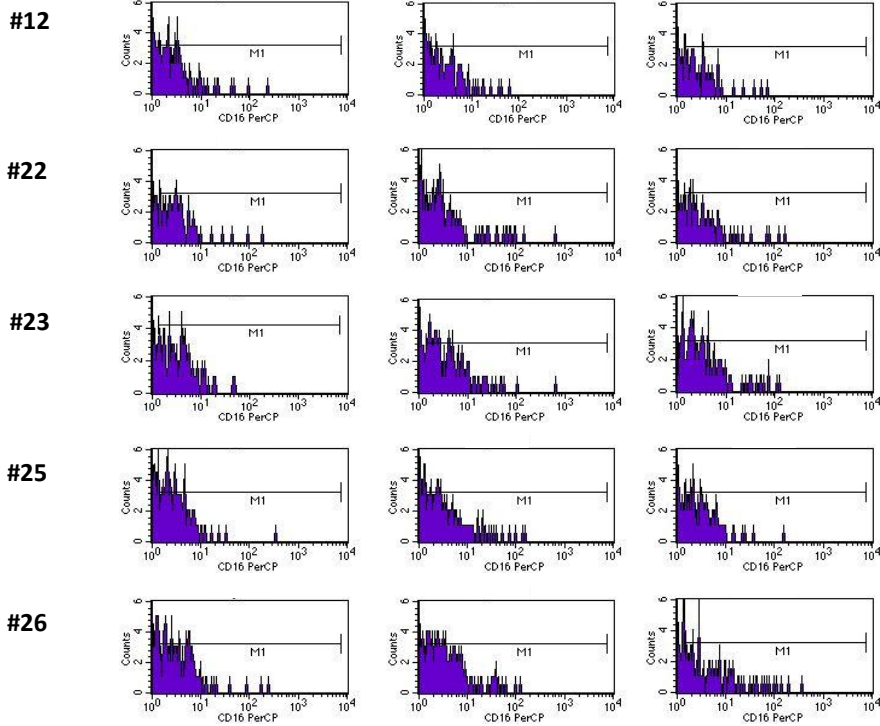
Patients with known wasp venom allergy:



Patients with known bee venom allergy:



Patients with known birch pollen allergy:



Basophils sorted according to their surface expression of CD16:

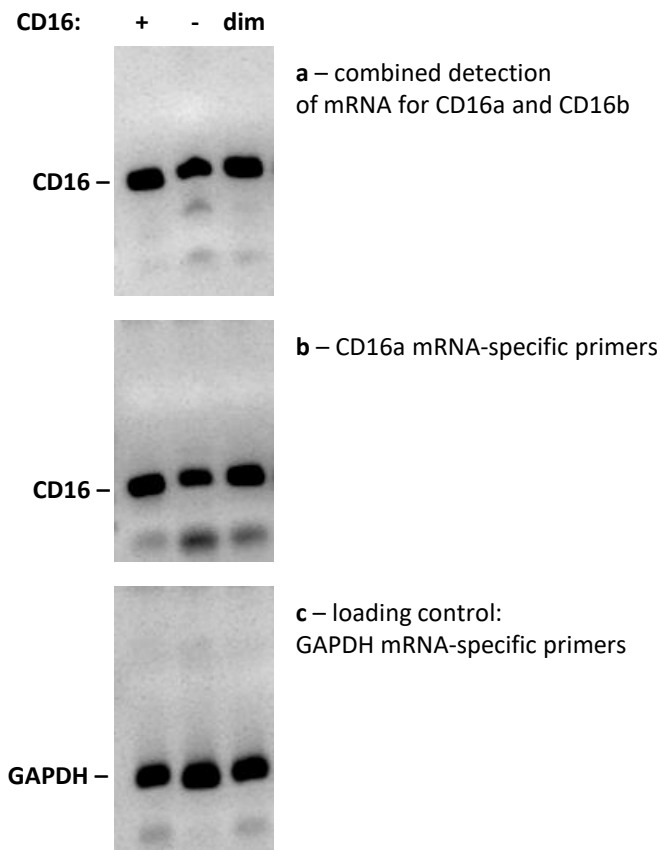


Table S1. Experimental set-up of the CD16b cleavage by PI-PLC as tested on the SSC/FSC-gated neutrophils from red blood cell-depleted peripheral blood.

Conditions	Median of CD16-PerCP fluorescence	Peak of CD16-PerCP fluorescence
Mock	3109.9	3248
80 µl of blood, 0.1 U of PI-PLC, 20 min, 4°C	429.4	406
80 µl of blood, 0.25 U of PI-PLC, 20 min, 4°C	361.9	324
80 µl of blood, 0.1 U of PI-PLC, 20 min, 37°C	385.4	324
0.8 µl of blood, 0.1 U of PI-PLC, 20 min, 37°C	385.4	196

Table S2. The list of primers used for amplification of mRNA for CD16a, CD16b and GAPDH.

Locus	Primer name	5'→3' sequence	Reference sequence	Position of 5'end
CD16a/b	CD16A/B-1 st -fw	GCTGCCACAGTCAACGACAGTG	NM_001244753.2	314
	CD16A/B-1 st -rv	CCATCACCAAGCAGAAAGAGACTT	NM_001244753.2	728
	CD16A/B-2 nd -fw	TAGAAGTCCATATCGGCTGGCTGTTG	NM_001244753.2	387
	CD16A/B-2 nd -rv	GCCAAACCTTGAGTGATGGTGATGTTG	NM_001244753.2	666
CD16a	CD16A-1 st -fw	TCAGCCACAGACCTTTGAGGGA	NM_000569.7	100
	CD16A-1 st &2 nd -rv	TCTCGAGCACCTGTACCATTGAG	NM_000569.7	485
	CD16A-2 nd -fw	CCTCCTGTCTAGTCGGTTTGGTCC	NM_000569.7	294
GAPDH	GAPDH-1 st -fw	CATCACCATCTTCCAGGAGCGAG	NM_002046.6	295
	GAPDH-1 st -fw	AGGGATGATGTTCTGGAGAGCCCC	NM_002046.6	700
	GAPDH-2 nd -fw	ATGGAGAAGGCTGGGGCTCATTG	NM_002046.6	389
	GAPDH-2 nd -rv	GCATTGCTGATGATCTTGAGGCT	NM_002046.6	525

Table S3. The raw data for histamine release matched with the detection of CD16 surface expression and BAT outcomes.

Subject	Sample	Treatment	Median of surface	Peak fluorescence of	%	%	Histamine release [ng/ml]
			expression of CD16	CD16	CD63 ⁺	CD16 ⁺	
			from SSC ^{low} CD193 ⁺ CD203c ⁺				
#1	2/1	Mock	2.57	1	0.27	0.27	0.0
history of anaphylaxis wasp venom	2/2	anti-IgE	2.94	1	24.25	1.50	13.4
	2/3	Wasp venom	2.84	1	52.86	1.18	140.2
#2	6/1	Mock	2.86	1	0.60	0.89	0.0
history of allergy birch pollen	6/2	anti-IgE	2.46	1	65.24	0.00	21.3
	6/3	Bet v 1a	2.35	2	85.15	0.82	24.9
#3	5/1	Mock	2.89	1	0.10	0.00	0.0
history of anaphylaxis bee venom	5/2	anti-IgE	2.97	2	33.51	0.68	15.5
	5/3	Bee venom	2.44	1	55.60	0.35	92.0
#4 control	3/1	Mock	2.97	2	0.00	0.00	1.5
	3/2	anti-IgE	2.61	1	48.62	0.59	28.2
	3/3	Bee venom	3.05	1	2.10	0.00	0.0
	3/4	Bet v 1a	2.84	5	0.22	0.00	0.0
#5 control	4/1	Mock	2.80	1	0.17	0.34	0.0
	4/2	anti-IgE	2.94	2	56.32	0.36	50.3
	4/3	Bee venom	2.69	3	0.83	0.67	0.0
	4/4	Bet v 1a	2.68	2	0.66	0.33	0.0
#6 control	1/1	Mock	2.83	1	0.31	0.40	0.0
	1/2	anti-IgE	2.89	2	15.81	0.00	6.9
	1/3	Bee venom	2.62	1	0.31	0.00	0.0
	1/4	Bet v 1a	2.79	1	1.33	0.30	1.3

Table S4. The raw data for CD16b cleavage by PI-PLC as tested on basophils and neutrophils from patients with the evidence of anaphylactic reactions to bee venom (n=3) and control subjects (n=3). We treated the red blood cell-depleted peripheral blood with PI-PLC or mock, and we activated the basophils with α IgE, bee venom extract or mock.

Subject	PI-PLC treatment	Activation of basophils	Number of basophils	Share of CD16 ⁺ basophils [%]	Share of CD16 ^{dim} basophils [%]	Median of CD16-PerCP fluorescence (basophils)	Peak of CD16-PerCP fluorescence (basophils)	Median of CD16-PerCP fluorescence (neutrophils)	Peak of CD16-PerCP fluorescence (neutrophils)
#1, bee venom anaphylaxis	Mock	Mock	368	0.3	14.4	2.01	1	2371.37	2392
	Mock	α IgE	535	0.4	12.7	2.57	1	2246.79	2246
	Mock	Bee venom	590	0.2	8.6	1.97	1	2226.67	2436
	PI-PLC	Mock	381	0.8	9.7	2.11	1	532.8	637
	PI-PLC	α IgE	364	0.0	11.5	2.33	1	577.72	500
	PI-PLC	Bee venom	405	0.0	9.6	1.93	1	491.37	798
#2, bee venom anaphylaxis	Mock	Mock	384	0.0	11.5	2.34	1	2246.79	2414
	Mock	α IgE	386	1.6	14.2	2.89	1	2109.69	2226
	Mock	Bee venom	364	0.3	12.4	2.4	1	2458.24	2502
	PI-PLC	Mock	398	0.8	18.6	3.15	1	237.14	266
	PI-PLC	α IgE	385	0.3	18.7	2.9	1	316.23	280
	PI-PLC	Bee venom	354	0.3	17.8	2.9	1	310.1	276
#3, bee venom anaphylaxis	Mock	Mock	339	0.3	14.5	2.53	1	2371.37	2392
	Mock	α IgE	517	0.2	16.4	2.79	1	2167.39	2350
	Mock	Bee venom	382	0.0	10.7	2.4	1	2297.88	2371
	PI-PLC	Mock	647	0.0	13.3	2.5	1	410.47	395
	PI-PLC	α IgE	351	0.6	15.4	3.28	1	395.96	417
	PI-PLC	Bee venom	470	0.6	13.8	2.79	1	410.47	395
#4, control	Mock	Mock	356	0.8	12.6	2.53	1	3023.21	3219
	Mock	α IgE	396	1.0	24.5	3.73	1	3133.96	3190
	Mock	Bee venom	383	3.7	20.9	3.16	1	3050.53	3190
	PI-PLC	Mock	382	1.6	17.5	3.12	1	410.47	500
	PI-PLC	α IgE	404	1.0	19.8	2.49	1	385.42	276
	PI-PLC	Bee venom	470	0.9	20.9	2.9	1	342.89	207
#5, control	Mock	Mock	470	0.4	18.1	3.13	1	3023.21	3078
	Mock	α IgE	483	0.0	18.6	3.11	1	2923.15	2916
	Mock	Bee venom	496	1.0	17.1	2.98	1	3023.21	3105
	PI-PLC	Mock	417	0.7	17.5	2.59	1	299.61	226
	PI-PLC	α IgE	495	1.2	27.9	3.82	1	333.76	257
	PI-PLC	Bee venom	472	0.8	23.5	3.68	1	261.8	232
#6, control	Mock	Mock	327	0.3	24.5	3.16	1	2436.23	2480
	Mock	α IgE	378	0.8	18.0	2.81	1	1963.22	1910
	Mock	Bee venom	351	3.4	31.6	4.63	1	2548.3	2548
	PI-PLC	Mock	372	0.5	25.5	3.08	1	352.27	243
	PI-PLC	α IgE	330	0.9	15.5	2.89	1	355.45	388
	PI-PLC	Bee venom	300	4.3	18.7	2.92	1	345.99	425

Patient #1

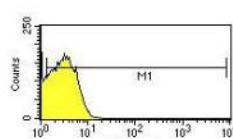
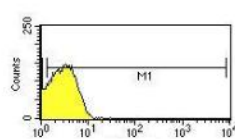
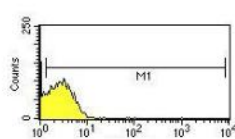
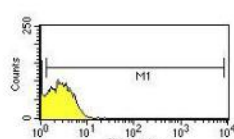
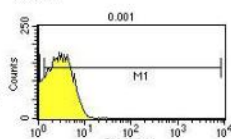
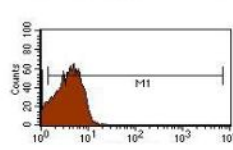
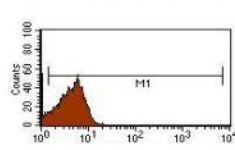
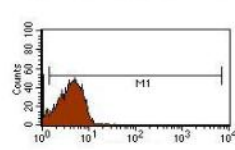
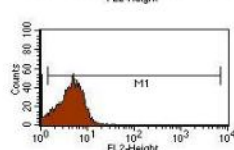
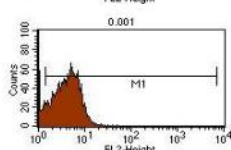
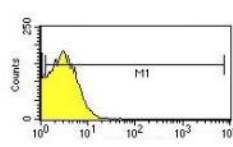
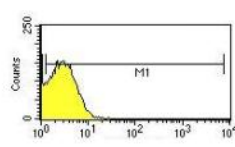
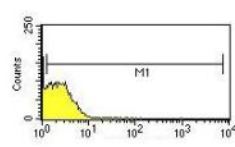
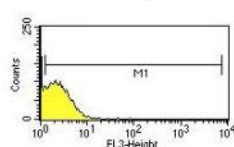
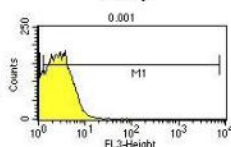
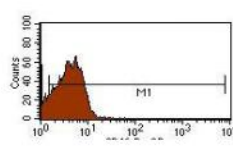
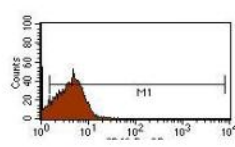
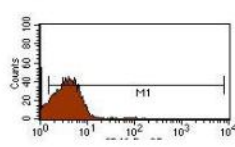
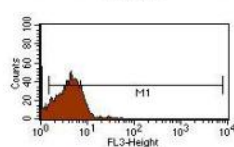
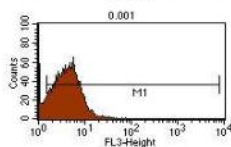
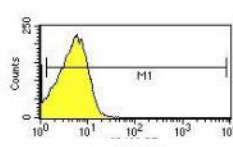
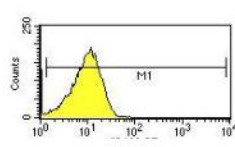
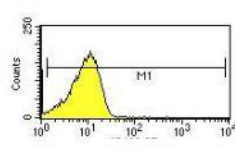
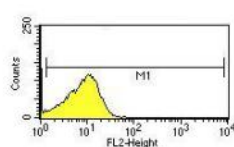
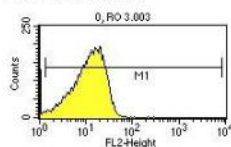
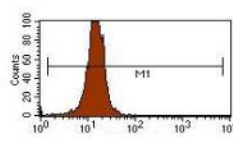
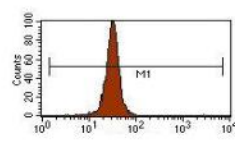
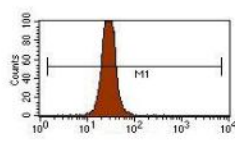
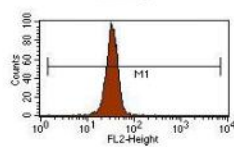
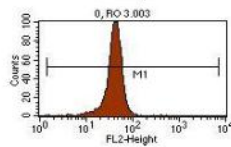
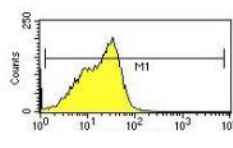
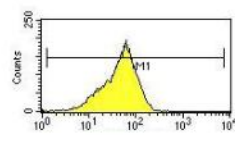
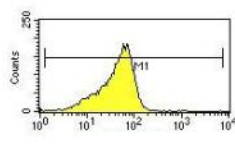
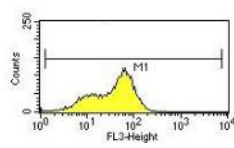
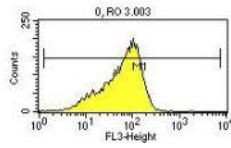
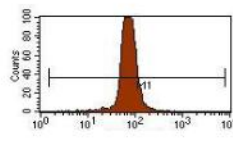
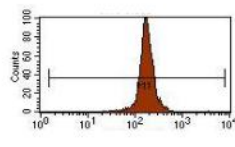
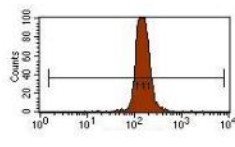
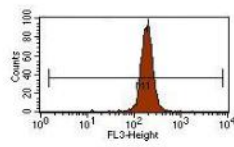
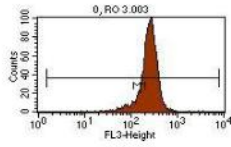
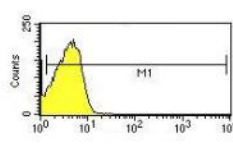
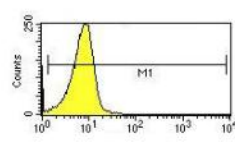
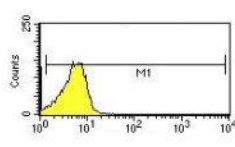
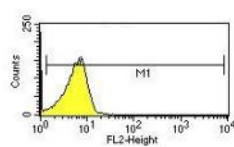
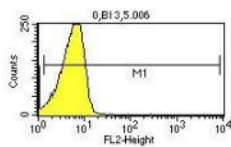
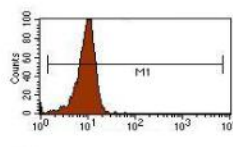
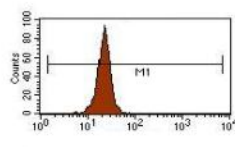
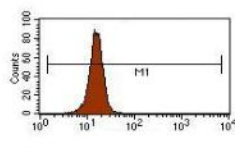
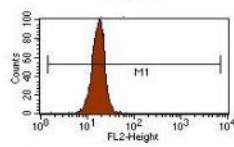
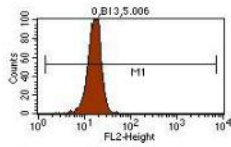
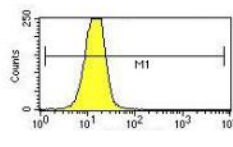
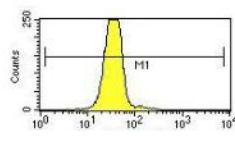
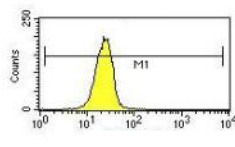
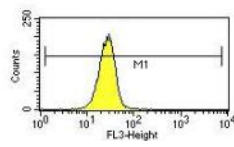
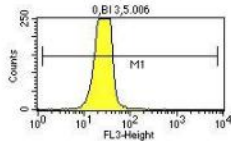
Patient #2

Patient #3

Patient #4

Patient #5

Control:

FL2
(lymphocytes)FL2
(monocytes)FL3
(lymphocytes)FL3
(monocytes)Ro-31-8220, 3.0 μ M:FL2
(lymphocytes)FL2
(monocytes)FL3
(lymphocytes)FL3
(monocytes)Bisindolylmaleimide II, 3.5 μ M:FL2
(lymphocytes)FL2
(monocytes)FL3
(lymphocytes)FL3
(monocytes)