



Research paper

Analyzing histamine release by flow cytometry (HistaFlow): A novel instrument to study the degranulation patterns of basophils ☆☆☆☆☆

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ABSTRACT

Background: Stimulated human basophils exhibit different degranulation patterns with release of mediators and appearance of activation markers such as CD63 and CD203c. Traditionally, released mediators are quantified in the supernatant of activated cells, whereas the expression of activation markers by individual cells is analyzed by flow cytometry. Alternatively, intracellular histamine and its release by basophils and mast cells have been repeatedly studied applying an enzyme–affinity–gold method based on the affinity of the histaminase diamine oxidase for its substrate histamine.

Objective: To develop a flow cytometric technique enabling to study histamine release by individual basophils in combination with the expression of activation markers. To elucidate the principles of basophil degranulation on a single cell level.

Methods: Intracellular histamine and its release is analyzed flow cytometrically by an enzyme–affinity method using diamine oxidase conjugated to laser-excitable fluorochromes. Phenotyping of cells implied flow cytometric quantification of CD63 and CD203c. Stimuli such as allergen, anti-IgE, N-formyl-met-leu-phe (fMLP), phorbol 12-myristate 13-acetate (PMA), ionomycin and interleukin (IL)-3 are applied to obtain different degranulation profiles.

Results: Stimulation with anti-IgE, allergen, fMLP and PMA ± ionomycin induces a rapid release of histamine that can be analyzed flow cytometrically. Analyses on a single cell level reveal that histamine release is not restricted to cells showing significant up-regulation of CD63. Alternatively, up-regulation of CD203c does not per se indicate histamine release. In some patients, priming of cells with IL-3 not only facilitates basophil responsiveness but also implies an increased ability of DAO to label the cells.

Conclusion: This study provides the proof-of-concept that histamine and its release can be studied by multicolor flow cytometry on a single cell level (HistaFlow). Coupling the data to simultaneous phenotyping of activated basophils confirms that histamine release principally results from anaphylactic degranulation and in a lesser extent from piecemeal degranulation.

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☆ Key message: this paper for the first time describes a flow cytometric technique that allows simultaneous studying of histamine release and expression of different surface activation markers on a single cell level. It is demonstrated that expression of activation markers can dissociate from histamine release and that total amount of histamine release results from piecemeal and anaphylactic degranulation.

☆☆ Conflict of interest: none.

☆☆☆ Statement of contribution: DGE and CHB have developed the technique and written the manuscript. CHM has performed the tests. MMH, WJS and LSD helped to interpret the data and write the paper.

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1. Introduction

Upon encountering specific allergen that cross-links FcεRI-bound specific IgE (sIgE) antibodies, basophils release various mediators, such as histamine (MacGlashan, 2003; Jutel et al., 2009). Traditionally, histamine release is quantified using assays measuring its extracellular content in the supernatant of degranulated basophils. However, in these assays results do not give information on individual cells or cell subpopulations with heterogeneous responses.

In the early 1990s, the discovery of the basophil activation marker CD63 induced the development of a flow cytometric technique to analyze *in vitro* basophil activation (Knol et al., 1991). The potentials and limitations of this basophil activation technique (BAT) are reviewed elsewhere (Ebo et al., 2006, 2008). At present, CD63 and CD203c are the most commonly applied readouts for BAT (Ebo et al., 2007a; Buhning et al., 1999, 2001, 2004; Hennersdorf et al., 2005). However, no technique capable of simultaneously analyzing histamine release and the appearance of activation markers on a single cell level has been described.

Dvorak et al. (1993) successfully localized intracellular histamine in mast cells by applying an enzyme-affinity-gold method based on the affinity of diamine oxidase (DAO) for its substrate histamine. In 1994, this DAO-colloidal gold based technique was applied to localize histamine in activated basophils (Dvorak et al., 1994). Furthermore the technique allowed to demonstrate histamine secretion by classical granule exocytosis by human mast cells *in vivo* (Dvorak et al., 1997). Finally, the technique has permitted the study of histamine transport and the ultrastructural alterations of piecemeal degranulation and anaphylactic degranulation (Dvorak, 1998, 2005a, 2005b, 2005c).

At present, appearance of CD63 is considered to reflect anaphylactic degranulation with significant release of mediators, whereas, an isolated up-regulation of CD203c can clearly dissociate from mediator release (Crivellato et al., 2003, 2010; MacGlashan, 2010).

The aim of this study was to develop a flow cytometric technique to analyze intracellular histamine and its release by coupling the histaminase DAO to fluorochromes. In addition, we investigated whether this technique could be integrated in multiple parameter flow cytometric cell analysis enabling further disentanglement of the principles of piecemeal and anaphylactic degranulation.

2. Materials and methods

2.1. Patients and control individuals

Birch pollen allergic patients ($n=19$; 7 male; median 41 years (11–61)) were selected based upon history confirmed by skin tests and quantification of sIgE (Aerts et al., 2008). Five healthy control individuals (3 male; median 48 years (16–52)) were also studied. Experiments were performed outside the pollen season with patients free of treatment (including allergen-specific immunotherapy). Approval for this study was obtained from the local ethical committee. Participants were asked to approve an informed consent.

2.2. Basophil activation

Two-hundred μL endotoxin-free heparinized whole-blood were challenged at 37 °C with 200 μL anti-IgE (10 $\mu\text{g}/\text{mL}$; clone G7-18 BD Bioscience, Erembodegem, Belgium), 200 μL activation buffer (Hank's balanced salt solution (Invitrogen, Paisley, UK) with 20 μM HEPES and 7.5% NaHCO_3 , pH 7.4), N-formyl-met-leu-phe (fMLP: 0.5 $\mu\text{g}/\text{mL}$) (Sigma-Aldrich, St-Louis, MO, USA), phorbol 12-myristate 13-acetate (PMA: 10, 100 and 500 ng/mL) without or with ionomycin (IO: 0.25 and 2 $\mu\text{g}/\text{mL}$) (Sigma) and 1 and 10 ng/mL IL-3 (Pepro-Tech, London, England) or recombinant Bet v 1 (rBet v 1: 0.01 $\mu\text{g}/\text{mL}$, kindly provided by Stallergènes, Antony Cedex, France) at different time points (20 s up to 60 min). Reactions were stopped by chilling on ice, adding 1 mL ice-cooled PBS-EDTA 10 mmol/L EDTA and spinning for 5 min (4 °C, 200 g).

2.3. Immunophenotyping

Basophils were stained with 20 μL of monoclonal anti-human IgE (clone GE-1, Sigma Aldrich GmbH, Steinheim, Germany) labeled with Alexa Fluor 405 (Molecular Probes, Invitrogen, Paisley, UK) and 10 μL of monoclonal anti-human CD63-FITC (clone H5C6, BD Biosciences), 10 μL CD203c-APC (clone NP4D6 Biolegend, San Diego, CA, USA) for 20 min on ice. Basophils are identified as anti-IgE and CD203c positive cells.

2.4. Cell fixation and permeabilization for intracellular staining

Different regimens based on paraformaldehyde, methanol, saponin and Triton-X-100 were tested to stain intracellular histamine. Control settings with and without permeabilization and addition of unlabeled DAO to measure specific staining were used. The Phosflow Lyse/Fix Buffer (BD Biosciences) provided the best results, this protocol was adopted for further experiments in which cells were lysed/fixed with 2 mL Phosflow Lyse/Fix Buffer for 20 min (37 °C). Cells were washed with and re-suspended in PBS with 0.1% Triton-X-100 (PBS-TX, pH=7.4). To stain intracellular histamine 10 μL labeled DAO (Sigma-Aldrich) was added and incubated at 37 °C (45 min). Cells were washed and re-suspended in PBS with 0.1% sodium azide.

2.5. Staining of intracellular histamine with labeled diamine oxidase (DAO)

Different fluorochromes were applied to conjugate DAO to study intracellular histamine. Fluorescent nanoparticles (Invitrogen), biotin and streptavidin labeled with PEcy7 (BD), fluorescein isothiocyanate (FITC), Horizon-V500, Phycoerythrin (PE). Except for the nanoparticles, labeling of DAO was performed by BD Biosciences. Finally, the PE-based technique was adopted (*vide infra*).

2.6. Flow cytometric analysis

Flow cytometric analysis was performed on a FACSCanto II flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA). Correct compensation setting for the fluorochromes

used was performed using BD CompBeads (BD Biosciences). As shown in Fig. 1, flow cytometric characterization relied upon a combination of 2 density plots.

Fluorescence minus one (FMO) and DAO staining without permeabilization was used to set a marker between DAO positive and negative cells.

2.7. Standardization

Standardization of intracellular histamine content was performed using standardized fluorospheres (SPHERO Ultra Rainbow Calibration particles, Spherotech, Lake Forest, IL, USA) as described by the manufacturer. Results were expressed as median fluorescence intensity (MFI) and percentage positive/negative cells.

3. Results

3.1. Diamine oxidase specific staining

DAO analysis was performed as described in Fig. 1.

Simultaneous adding of fluorochrome-conjugated and unlabeled DAO in different concentrations results in a dose-dependent inhibition of up to more than 80% of PE-conjugated DAO (Fig. 2, $n=2$) and endorses the specificity of the

histaminase-affinity technique as described by Dvorak et al. (1993, 1997).

3.2. Kinetics of histamine release after IgE/FcεRI cross-linking and stimulation with fMLP, PMA and PMA + ionomycin

Samples are analyzed at different time points to study kinetics of release (Fig. 3). Challenging the cells with anti-IgE, rBet v 1, fMLP and PMA + ionomycin elicits a quick histamine release that starts within minutes, peaks at 5–20 min and remains upon a plateau for at least 1 h. In contrast, stimulation of cells with PMA resulted in a slower histamine release that becomes mostly apparent after 20 min. Stimulation of the cells with ionomycin does not induce histamine release in the experimental conditions we have employed. As this study focuses on IgE/FcεRI-mediated histamine release in comparison with CD63 and CD203c a stimulation time of 20 min was adopted in further experiments.

3.3. Phenotypic patterns

It is confirmed that none of the activation experiments discloses appearance of CD63 in the absence of an up-regulated CD203c expression (Buhning et al., 2004; Hennessy et al., 2005; Chirumbolo et al., 2008). Consequently, activated basophils are further defined as the cells that up-regulate

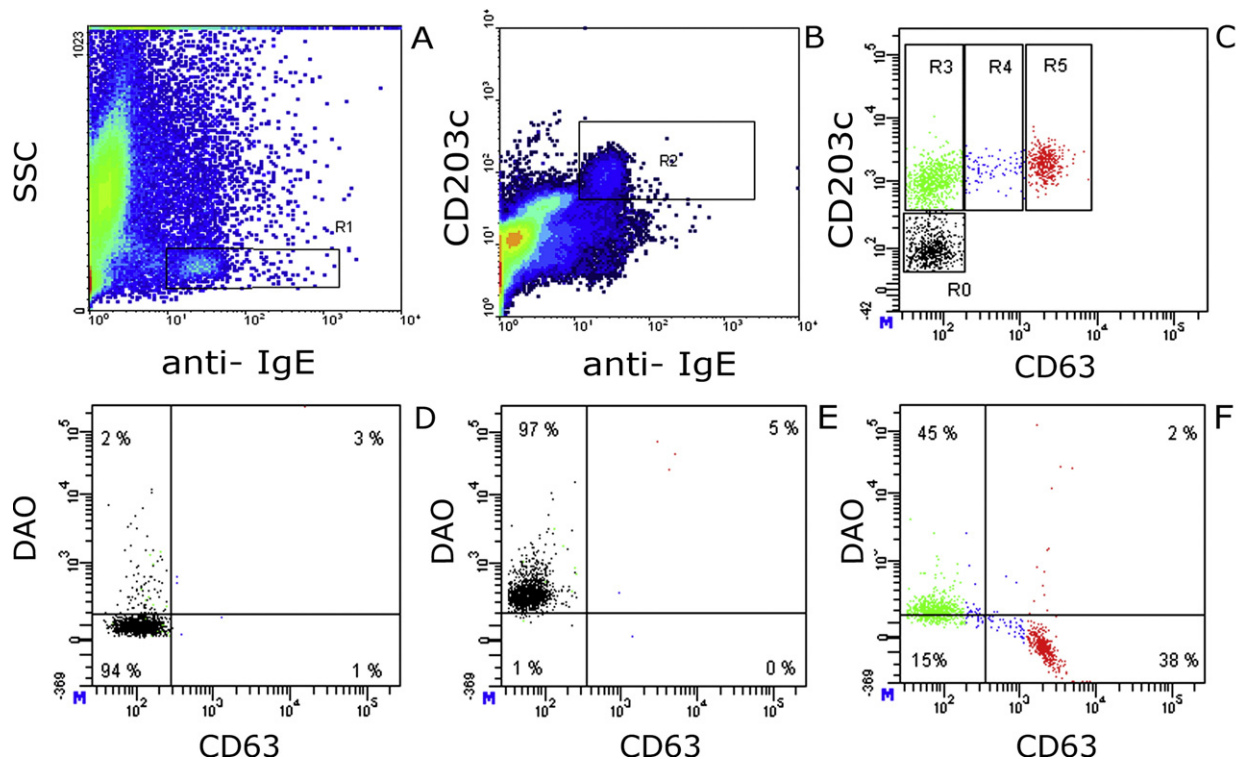


Fig. 1. Histamine release after 20 min by Bet v 1-activated basophils from a birch allergic patient. Basophils are characterized using side scatter (SSC)/anti-IgE (R1) and anti-IgE/anti-CD203c (R2) (A–B). Basophils are defined as anti-IgE and CD203c positive cells, co-expression of CD203c and CD63 is analyzed and reveals 3 subpopulations of distinct CD63 expression; i.e. CD203c^{bright}+CD63[−] (R3, green), CD203c^{bright}+CD63^{dim}+ (R4, blue) and CD203c^{bright}+CD63^{bright}+ (R5, red). Gate R0 denotes non-activated basophils (C). Histamine-containing basophils are defined as DAO⁺ cells (D–F). Histamine staining without permeabilization of the cells (D). Histamine staining with permeabilization and buffer (E) and allergen stimulation (F). In this sample, 38% of the basophils demonstrate complete release of their histamine content (DAO[−]) and are situated in the lower-right quadrant of panel F. Note the “Logicle” display method of panels C–F (Parks et al., 2006) and that panel F shows only CD203c^{bright}+ cells (R3–5).

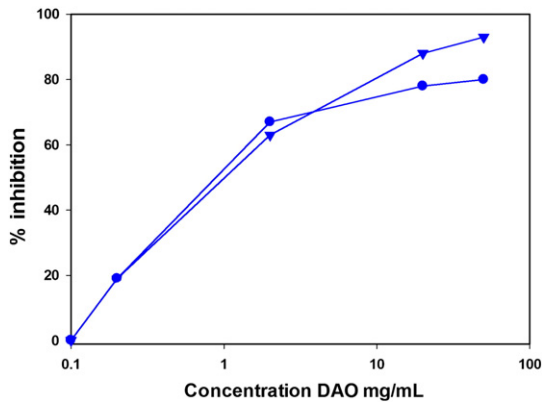


Fig. 2. Diamine oxidase (DAO) inhibition. Adding unlabeled DAO resulted in a dose-dependent inhibition of up to more than 80% of PE-conjugated DAO ($n=2$).

CD203c (CD203^{bright+}) (Fig. 1C). Depending on the stimulus and stimulation conditions, 3 subtypes of CD63 expression are delineable: i) a CD203c^{bright+}CD63^{dim+} subpopulation without CD63 expression, ii) a CD203c^{bright+}CD63^{dim+} population with moderate appearance of CD63 and, iii) a CD203c^{bright+}CD63^{bright+} subpopulation with distinct CD63 expression (Fig. 1C, respectively R3, R4 and R5). Histamine-containing basophils are defined as DAO⁺ cells (Fig. 1D–F). Histamine staining without permeabilization of the cells (D). When challenged with buffer no histamine release is demonstrable (E). When challenged with allergen pronounced histamine release in basophils is generally restricted to the CD203c^{bright+}CD63^{bright+} cells (F).

3.4. Activation patterns after IgE/FcεRI cross-linking and stimulation by fMLP

Table 1 displays the median (range) percentages of histamine releasing cells (CD203^{bright+}CD63^{bright+}DAO⁺ subpopulation) upon stimulation with buffer, anti-IgE, fMLP and rBet v 1. Cross-linking of IgE/FcεRI by anti-IgE or challenging with

fMLP in controls and patients elicits similar percentages of histamine releasing cells. Only patients demonstrate histamine releasing cells upon stimulation with allergen.

Fig. 4 shows that cross-linking of IgE/FcεRI by anti-IgE and rBet v 1 in patients and challenging cells with fMLP almost invariably results in a significant up-regulation of CD203c expression (Fig. 4, R3 + 4 + 5). In these CD203^{bright+} cells, the distribution of CD63 expression is bimodal. However, in most cases the CD63⁺ cells comprise a CD63^{dim+} and CD63^{bright+} subpopulation (respectively gated in R4 and R5). CD203c^{bright+}CD63^{bright+} cells demonstrate a clear histamine release, whereas CD203c^{bright+}CD63^{dim+} cells show less histamine release. Note the higher DAO labeling of rBet v 1 stimulated cells that do not degranulate as compared to buffer stimulation (cells gated out in R3 and displayed in the upper left quadrant of the DAO plots).

3.5. PMA, ionomycin and PMA + ionomycin-induced activation patterns

Fig. 5 displays a representative sample of cells stimulated with PMA with or without ionomycin for 20 min. PMA elicits a different pattern as compared with IgE/FcεRI cross-linking or fMLP, with a dose-dependent up-regulation of CD203c and release of histamine (3-fold lower content) but without CD63 expression. In contrast, PMA + ionomycin induces a significant expression of CD203c and dose-dependent CD63 with histamine release, indicating a shift to anaphylactic degranulation as a result of Ca⁺⁺ influx. Incubation with ionomycin alone during 20 min has no effect.

3.6. Effect of IL-3 incubation

Fig. 6 summarizes the potential effects of pre-incubation experiments with IL-3 with and without rBet v 1. IL-3 up-regulates CD203c without expression of CD63 or apparent release of histamine. Actually, in some of the patients up-regulation of CD203c by IL-3 is associated with a doubling of the DAO fluorescence intensity as compared to resting basophils (upper left quadrants of DAO plots in Fig. 6B). Furthermore Fig. 6 shows that the potential of short pre-activation with IL-3 to facilitate basophil responsiveness is not absolute and demonstrable in all patients. In the patient displayed in Fig. 6A, demonstrating 81% of basophil activation (CD63⁺ cells) for rBet v 1 stimulation, no priming effect of 15 minute IL-3 pre-incubation is demonstrable. In contrast, Fig. 6B, showing a patient with only 16% of basophil activation for rBet v 1, discloses that IL-3 can promote basophil responsiveness to allergen, with a shift from isolated CD203c up-regulation to an appearance of CD63 and release of histamine.

As already disclosed in Fig. 4, Fig. 6B shows that in some patients allergen alone can augment DAO labeling of activated but non-degranulating basophils up to a 5-fold (cells gated out in R3 and displayed in the upper left quadrant of the DAO plots). Finally, the panels displaying pre-incubation with IL-3 and subsequent stimulation with rBet v 1 reveal that histamine release can dissociate from CD63 up-regulation, as histamine content in the CD203c^{bright+}CD63^{dim+} cells (R3) returns to basal conditions.

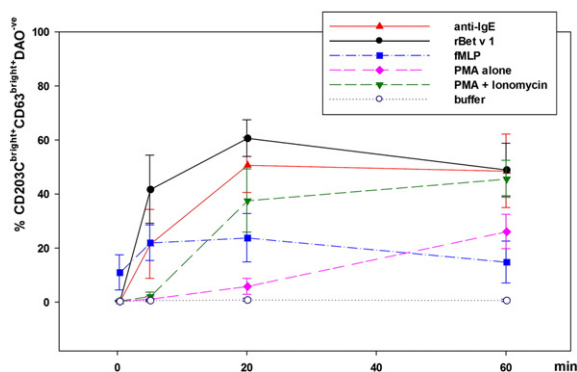


Fig. 3. Characteristics of histamine release. Kinetics of histamine release (% DAO⁺ cells) by CD203^{bright+}CD63^{bright+} cells after stimulation with buffer, anti-IgE, rBet v 1, fMLP, PMA 100 ng/mL and PMA 100 ng/mL + ionomycin 2 μg/mL (bottom) ($n=3$). Samples are analyzed at 4 different time points (20 s, 5–20–60 min). Results are expressed as mean ± SEM.

Table 1
Median (range) percentages of histamine releasing cells (i.e. CD203^{bright}+CD63^{bright}+DAO[−] subpopulation). Stimulation with buffer, anti-IgE, fMLP and major birch pollen allergen rBet v 1.

	Buffer	Anti-IgE	fMLP	Bet v 1 (1 µg/mL)	Bet v 1 (0.01 µg/mL)	Bet v 1 (0.001 µg/mL)
Controls (n = 5)	<1 (all<1)	32 (2–51)	12 (1–51)	<1 (all<1)	<1 (all<1)	<1 (all<1)
Patients (n = 19)	<1 (<1–2)	27 (4–64)	26 (6–60)	17 (4–54)	54 (2–86)	39 (<1–76)

4. Discussion

This study shows that intracellular histamine and its release by in vitro activated basophils can successfully be analyzed by flow cytometry applying an enzyme-affinity-fluorochrome method, based on the well-established affinity of the histaminase DAO for its substrate histamine (Dvorak et al., 1993). Traditionally, histamine release is quantified using techniques that measure its concentration in the supernatant of activated cells. Together with morphometric studies and flow-assisted analysis of surface activation markers, these assays have disclosed fundamental insights into distinct basophil degranulation patterns such as piecemeal degranulation and anaphylactic degranulation. We anticipate that flow-assisted dual

analysis of activation markers and histamine release at a single cell level might capture data that are inaccessible for traditional techniques that require homogeneous cell populations and of which results represent an average of isolated cells analyzed. This study reveals that flow cytometry provides qualitative data concerning dose-responses and kinetics of histamine release consistent with other analyses (Tanizaki et al., 1984; Rotz et al., 1991; MacGlashan, 1995; Mochizuki et al., 2003; Gibbs et al., 2006, 2007). Challenging the cells with anti-IgE, allergen, fMLP and PMA + ionomycin elicits a rapid histamine release that peaks within 5–20 min and lasts for at least 1 h. Histamine release by the phorbol ester PMA is slower, starting around 20 min and rising subsequently for minimally 1 h (Dvorak et al., 1992; Lie et al., 1998).

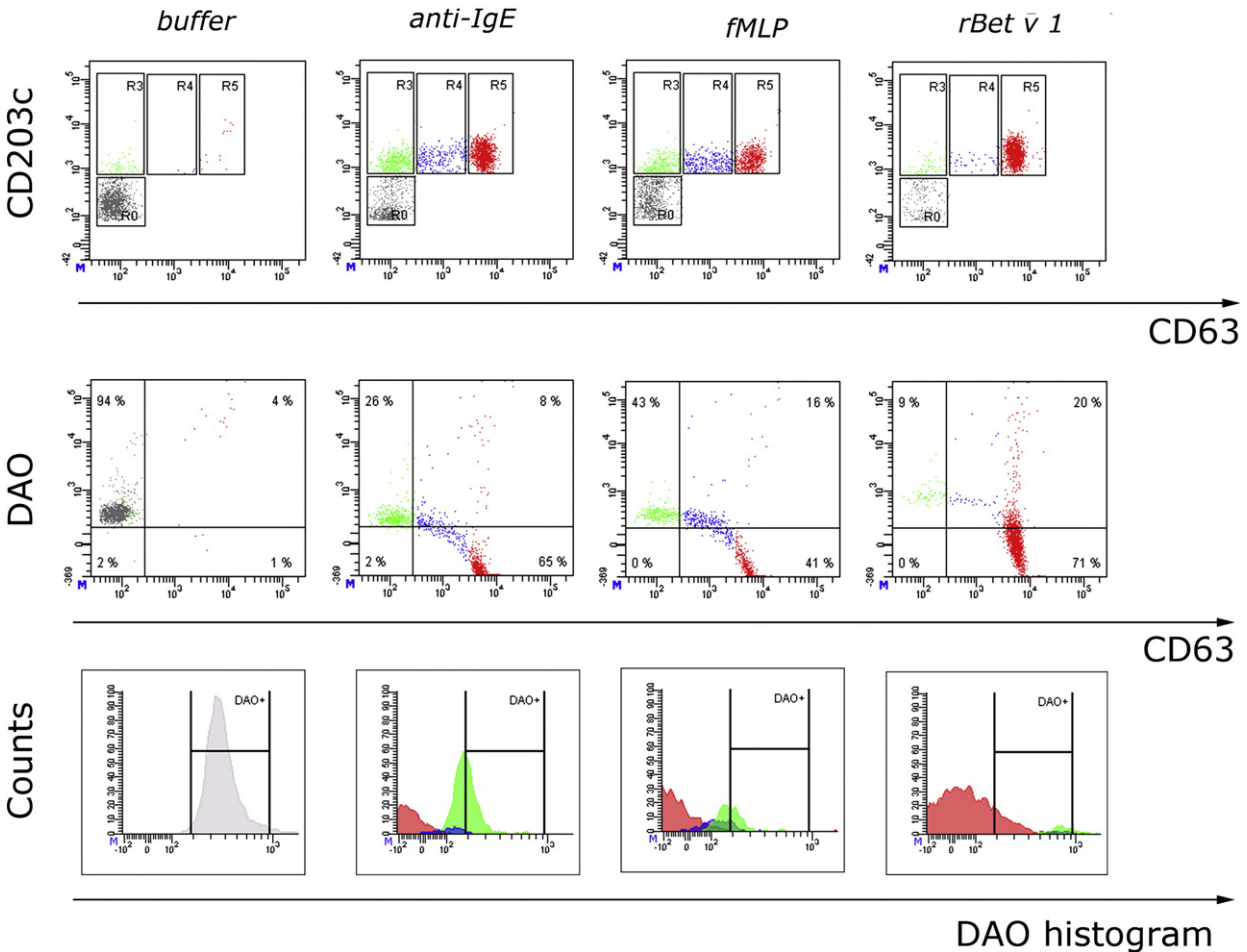


Fig. 4. Representative sample (n = 19) of histamine release after IgE/FcεRI cross-linking and activation by fMLP. CD203c expression upon stimulation with buffer, anti-IgE, fMLP and recombinant birch pollen allergen component rBet v 1. Upon stimulation with rBet v 1, 71% of the basophils completely released their histamine content (DAO[−] cells) and are situated in the lower-right quadrant of the bottom panel. The third panel shows the DAO histogram (y-axis in second panel). Note the “Logicle” display method of panels.

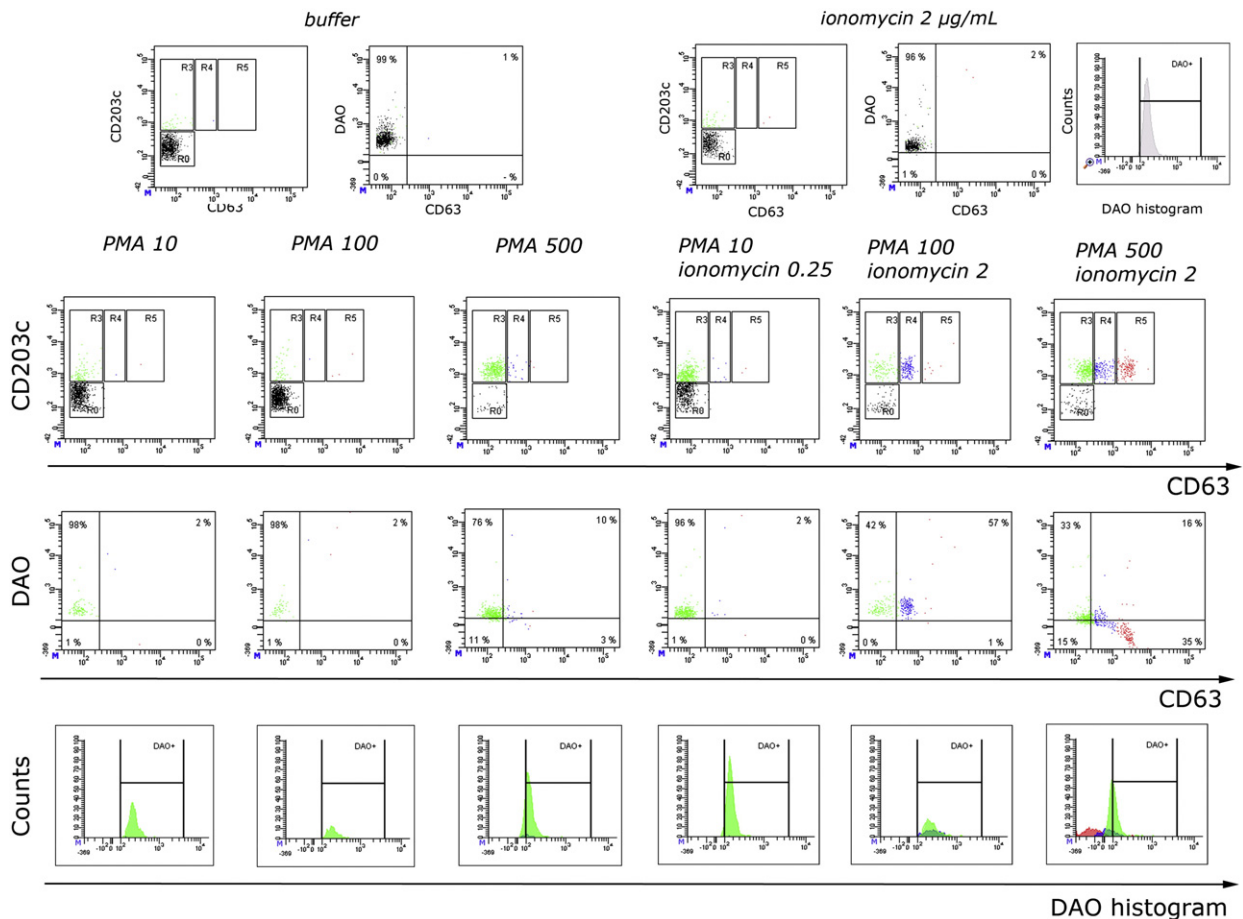


Fig. 5. Representative case ($n = 3$) of histamine release by PMA + ionomycin. CD203c expression upon stimulation with ionomycin (IO) 2 $\mu\text{g/mL}$, PMA 10, 100 and 500 ng/mL and PMA + ionomycin 10 ng/mL–0.25 $\mu\text{g/mL}$, 100 ng/mL–2 $\mu\text{g/mL}$ and 500 ng/mL–2 $\mu\text{g/mL}$. Histamine-containing basophils are defined as DAO⁺ cells. The third panel shows the DAO histogram (y-axis in second panel). Note the “Logicle” display method of panels.

CD63 and CD203c have become well-accepted surface markers of basophil activation. However, the relationship between the expression of these markers and mediator release remains elusive. Comparative analyses between flow-assisted phenotyping and quantification of extracellular histamine have revealed that appearance of these markers can be dissociated from mediator release (MacGlashan, 2010, 1995). It has been proposed that the total amount of histamine released is the sum of piecemeal and anaphylactic degranulation, but that the appearance of CD63 is restricted to anaphylactic degranulation (MacGlashan, 2010). The prevailing hypothesis is that histamine release constitutes a continuum that starts with piecemeal degranulation and subsequently (rapidly) converts to anaphylactic degranulation (Dvorak et al., 1991). In this model the switch to anaphylactic degranulation temporarily coincides with the peak influx of calcium, whereas the strength of the cytosolic calcium response governs the relative amount of anaphylactic vs. piecemeal degranulation (MacGlashan, 2010).

Our experiments confirm that basophils that up-regulate CD203c upon stimulation with anti-IgE, allergen or fMLP, generally exhibit a bi-modal distribution of CD63 expression with a CD63[−] and CD63⁺ subpopulation (Buhning et al., 2004; Hennessdorf et al., 2005; Chirumbolo et al., 2008). However, as shown in Figs. 1, 4–6, the individual cell responses of CD63

expression are heterogeneous, varying from absent (CD63[−]) over weak (CD63^{dim+}) to strong (CD63^{bright+}). This heterogeneity aligns with observations by others, demonstrating low and high CD63 expressing basophilic subpopulations upon IgE-mediated stimulation of the cells (Buhning et al., 2004; Hennessdorf et al., 2005; Chirumbolo et al., 2008). In contrast, PMA-induced CD63 expression is confirmed to be poor (MacGlashan, 2010), stressing phorbol esters not to induce significant anaphylactic degranulation (Dvorak et al., 1992). However, adding ionomycin to PMA shifted the response from piecemeal degranulation to anaphylactic degranulation.

The most relevant observations relate to the simultaneous analyses of activation markers and histamine release at the individual cell level that explicitly show the DAO labeling to be bi-modal, like appearance of CD63. Actually, synthesis of the available data reveals prominent release of histamine with eventually complete emptying of basophils to be restricted to anaphylactic degranulation of cells up-regulating both CD203c and CD63, and is observed for IgE/Fc ϵ R1 cross-linking and stimulation with fMLP or higher doses of PMA + ionomycin. In contrast, CD203c^{bright+}CD63[−] and CD203c^{bright+}CD63^{dim+} cells generally exhibit no manifest release of histamine in our settings. Except, for stimulation with PMA and the lowest dose of PMA + ionomycin in which release of histamine can be

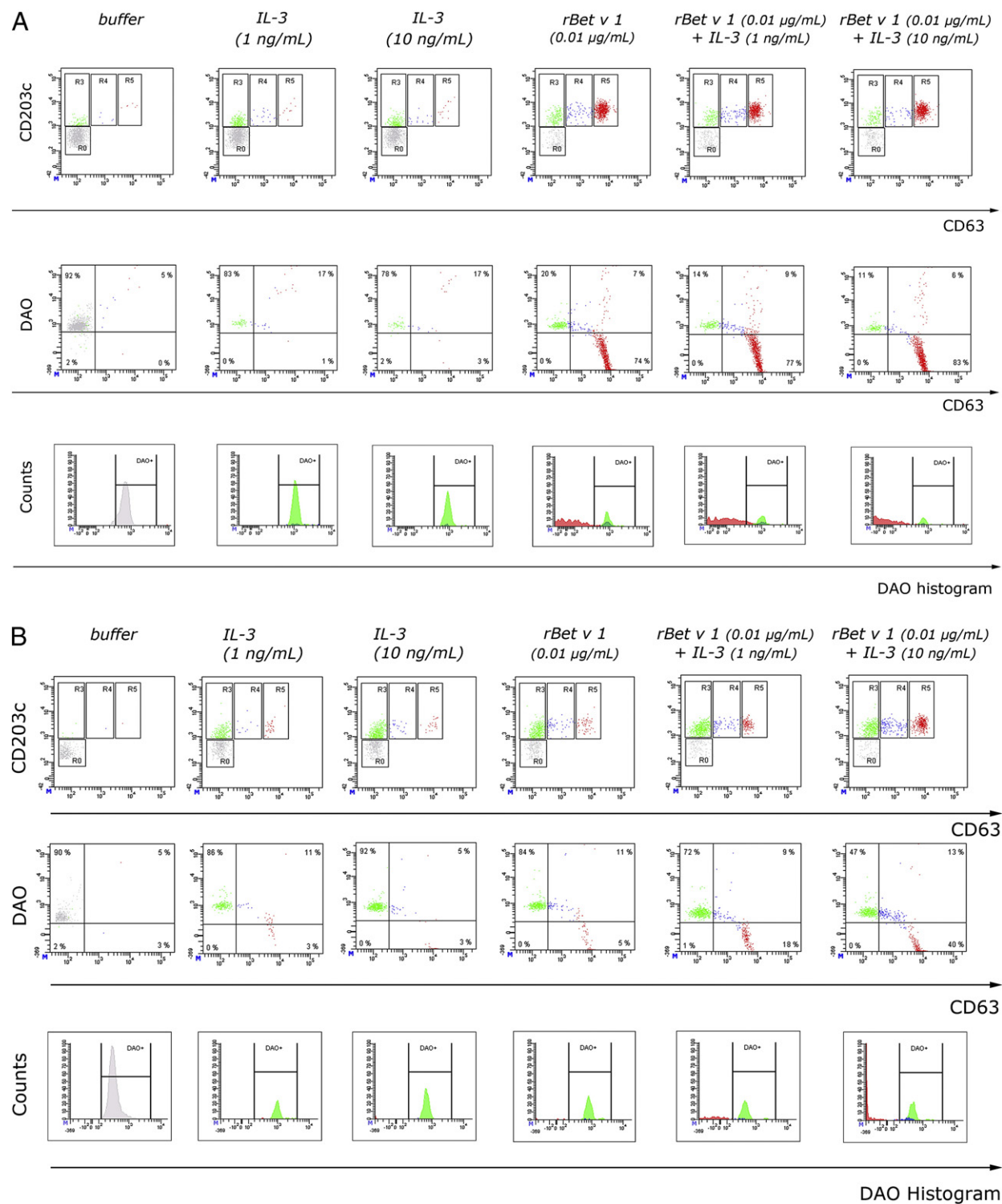


Fig. 6. Two representative cases of histamine release by IL-3 ± rBet v 1 (n = 19). CD203c expression, CD63 appearance and histamine release upon stimulation with buffer, IL-3 (1 ng/mL and 10 ng/mL), rBet v 1 0.01 µg/mL, and pre-incubation with IL-3 (1 ng/mL and 10 ng/mL) with subsequent activation with rBet v 1 0.01 µg/mL for 20 min in a patient demonstrating 81% of basophil activation (CD63⁺ cells) for rBet v 1 stimulation (A) and a patient with only 16% of basophil activation for Bet v 1 (B), respectively. Histamine-containing basophils were defined as DAO⁺ cells. The third panel shows the DAO histogram (y-axis in second panel). Note the "Logic" display method.

observed in the CD203c^{bright}+CD63[−] cells, and for the 2 highest doses of PMA + ionomycin that elicit leaking of histamine from CD203c^{bright}+CD63[−] and CD203c^{bright}+CD63^{dim}+ cells. These findings parallel with the observations that histamine leaking by phorbol esters can result from alternative mechanisms such as piecemeal degranulation and a fruste form of anaphylactic degranulation (Dvorak et al., 1992) and emphasize that histamine release can dissociate from appearance of CD63 (MacGlashan, 2010, 1995).

IL-3 has commonly been recognized to enhance the basophil response to both IgE/FcεRI-dependent and IgE/FcεRI-independent stimuli and has also been shown to trigger piecemeal degranulation in cultured human umbilical-cord blood-cell-derived basophils (Dvorak et al., 1989). Our data confirm that pre-incubating of basophils with IL-3 can prime basophils of some patients (Merget et al., 1990; Miadonna et al., 1997; Redrup et al., 1998; Yoshimura et al., 2002; Ferrer et al., 2003; Chen et al., 2003; Hauswirth et al., 2007; Lourenco et al., 2008; Schroeder et al., 2009) with a distinct up-regulation of CD203c by cells (Buhning et al., 2004; Hauswirth et al., 2007) but without expression of CD63 (Buhning et al., 2004; Yoshimura et al., 2002; Ebo et al., 2007b) nor significant histamine release (Lie et al., 1998; Merget et al., 1990; Redrup et al., 1998; Yoshimura et al., 2002; Tedeschi et al., 1999). Actually, incubation with IL-3 resulted in heterogeneous responses with a rise of DAO labeling by primed basophils (CD203^{bright}+CD63[−] cells) in some patients. The exact reason for this observation remains elusive and speculative. It could perhaps relate to a change(s) in accessibility of DAO to the histamine stores, de novo synthesis by an enhanced L-histidine decarboxylase activity. However, to the best of our knowledge, IL-3-induced expression of L-histidine decarboxylase has currently only been reported in immature progenitor cells and to require longer incubations (Schneider et al., 1987; Minkowski et al., 1990; Lebel et al., 1990; Dy et al., 1993; Schneider et al., 1999). Fig. 6B also reveals that cells that do not up-regulate CD63 upon challenging with allergen in the absence of exogenous IL-3, can demonstrate a rise of intracellular DAO labeling. The explanation of this phenomenon remains to be disentangled but it could relate to autocrine and/or paracrine priming by IL-3, as this cytokine is rapidly synthesized after IgE/FcεRI cross-linking (Schroeder et al., 2009). An alternative explanation would be that the analyses at 20 min are too early and that not all granules are fused to the plasma membrane and that the contents of an un-extruded granule could be more accessible to DAO because of the ongoing process. Finally, the pre-incubation experiments displayed in Fig. 6B disclose that the rise of intracellular histamine in CD203c^{bright}+ cells can return to baseline values without appearance of CD63. This dissociation, joined to significant release of histamine by CD63^{bright}+ basophils in the same sample, validate the proposal that histamine release constitutes a continuum that starts with piecemeal degranulation and converts to anaphylactic degranulation.

In conclusion, this study demonstrates that intracellular histamine and its release can be analyzed by flow cytometry. Moreover, the technique enables integration in a multi-parameter analysis with simultaneous quantification of different surface activation markers. Syntheses of our data suggest an association between an isolated up-regulation of CD203c and small vesicle fusion with piecemeal degranulation and slow leaking of

histamine from the activated cells, whereas appearance of CD63 reflects anaphylactic degranulation with significant release. However, it is also demonstrated that expression of both markers can dissociate from histamine release. Up-regulation of CD203c does not per se mirror histamine release, whereas histamine release can occur without appearance of CD63. The priming effect of IL-3 on basophils is not restricted to facilitating basophil responsiveness to subsequent challenging but might also involve the uploading of the cells with histamine, but this hypothesis remains to be confirmed in further studies. Finally, we anticipate that coupling this technique to staining of intracellular signaling molecules and/or inhibitory receptors might bring new insights to the understanding of the mechanisms that govern the different degranulation profiles.

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