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Phenotypic and functional characterization of *in vitro* cultured human mast cells

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Abstract

Background

Mast cell progenitor cells, derived from $CD34^+$ hematopoietic stem cells, enter the circulation and subsequently mucosal or connective tissues where they mature to mast cells. Upon activation, mast cells increase the expression of activation markers, e.g. CD63, and release histamine amongst other mediators. Traditionally, release of these mediators is quantified using assays measuring their extracellular concentration in the supernatant of stimulated cells.

Methods

Human mast cells were cultured from peripheral blood, phenotypically characterized, passively sensitized with allogenic IgE antibodies and finally stimulated by anti-IgE that cross-links IgE/FcεRI complexes. Alterations in the number of cells positive for CD63 and release of histamine were quantified simultaneously by flow cytometry.

Results

In culture, two distinct $CD45^+$ cell populations were identified: $CD117^+CD203c^{+hi}$ and $CD117^-CD203c^{+low}$ cells. Both populations showed positivity for FcεRI, tryptase and chymase, and contained histamine. Activation resulted in a significant increase of cells positive for $CD63^+$ up to 21% (range: 11–39) for $CD117^+CD203c^{+hi}$ cells (P=0.005), and 27% (18–55) $CD63^+$ for $CD117^-CD203c^{+low}$ cells (P=0.02). Baseline histamine content was higher for $CD117^+CD203c^{+hi}$ cells than for $CD117^-CD203c^{+low}$ cells, respectively 994 (695–6815) Molecules of Equivalent Specific Fluorochrome V500 per cell (MESF-V500/cell) and 797 (629–4978) MESF-V500/cell (P=0.02). After activation, $CD117^+CD203c^{+hi}$ cells showed significant histamine release of 578 (366–1521) MESF-V500/cell, whilst $CD117^-CD203c^{+low}$ cells resulted in 310 (217–366) MESF-V500/cell histamine release.

Conclusion

This study discloses that culturing HuMC from $CD34^+$ progenitors yields 2 phenotypically distinct cell populations that display a greatly similar response upon cross-linking of IgE/FcεRI complexes.

Introduction

Mast cell progenitor cells (MCPC), derived from $CD34^+$ hematopoietic stem cells, enter the circulation and subsequently mature to mast cells after migration into the mucosal or connective tissues [1-3]. Unlike MCPC, mature tissue-resident mast cells do not express CD34, demonstrate a higher level of CD117 (c-kit, mast/stem cell growth factor receptor), express the high affinity IgE receptor (FcεRI) and the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP-3) CD203c [3-5]. Mature mast cells also contain granules loaded with mediators like histamine, heparin and cytokines and depending on the tissue in which they mature, tryptase (mucosal mast cells) or both tryptase and chymase (connective tissue mast cells) [6-8]. Mast cells can be activated, either IgE-mediated, i.e. by IgE/FcεRI cross-linking, or non-IgE-mediated (e.g. through cytokines and growth factors, anaphylatoxins, immune complexes and physical triggers) [9]. Upon activation, mast cells exhibit an increased membrane expression of activation markers such as CD63, CD203c and CD107, and release various mediators such as histamine, tryptase, prostaglandins, leukotrienes and cytokines [10,11]. Traditionally, release of these mediators is quantified measuring their extracellular concentration in the supernatant of all stimulated cells [10,12].

Unlike peripheral blood basophils, mast cells are tissue-resident cells that are present only in small numbers and are thus poorly accessible for *ex vivo* and *in vitro* analyses. Therefore, studies on human mast cells (HuMC) have mainly focused on mast cell cultures [13-18]. Previously, we provided a proof-of-concept that histamine content and release by individual basophils can be studied using multi-colour flow cytometry in a technique designated as HistaFlow® [19]. Moreover, as the technique allows a simultaneous immunophenotyping of the cells, it was shown that the technique enables to study the functional behaviour of different subpopulations and individual cells [19,20].

Here we sought to investigate whether this technique could also be applicable for functional analysis of *in vitro* cultured HuMC.

Materials and methods

In vitro culture of HuMC

HuMC were cultured according to Schmetzer et al. (2014) and Wang et al. (2006) with modifications [12,21]. Briefly, buffy coat cell concentrates were isolated from 500 mL healthy donor blood (supplied by Red Cross Donor Centre,

Belgium). Next, peripheral blood mononuclear cells were isolated from these buffy coats using Histopaque-1077 (Sigma-Aldrich, St. Louis, Missouri, USA). $CD34^+$ cells were enriched using the EasySep Human CD34 Selection Kit (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's instructions. Isolated $CD34^+$ progenitor cells with a minimal purity of 80% were cultured in a serum-free methylcellulose-based medium (MethoCult SF H4236, Stemcell Technologies) supplemented with penicillin (100 units/mL) (Life Technologies, Waltham, USA), streptomycin (100 µg/mL) (Life Technologies), low-density lipoprotein (LDL, 10 µg/mL) (Stemcell Technologies), 2-mercaptoethanol (55 µmol/L) (Life Technologies), stem cell factor (SCF, 100 ng/mL) (Miltenyi Biotec, Bergisch Gladbach, Germany) and interleukin-3 (IL-3, 100 ng/mL) (PeproTech, Rocky Hill, USA). Cells were plated into a 6-well plate at a density of 1 to 2 x 10^5 cells/mL and kept at 37°C in a humidified CO_2-incubator for 14 days. At day 3, 7 and 10 cells were nourished by adding 300 µL Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies) containing penicillin (100 units/mL), streptomycin (100 µg/mL), 1% Insulin-Transferrin-Selenium (Life Technologies), 0.1% Bovine Serum Albumin (Sigma-Aldrich), SCF (20 ng/mL) and IL-3 (20 ng/mL) as a thin layer covering the MethoCult well. At day 14, cells were retrieved from the MethoCult and maintained in IMDM containing only SCF (IMDM + SCF 10 ng/mL) at a density of 0.5 x 10^6 cells/mL for a 7 days resting phase. At day 21, medium was replaced with fresh IMDM containing SCF. A total of 34 cell cultures were performed for which, depending on the yield of each cell culture, a different numbers of experiments could be accomplished.

Flow cytometric analysis

Immunophenotyping

At day 21 of culture, viability of the cells was assessed using a 7-AAD viability staining solution. Cells were stained for surface makers with monoclonal anti-human CD45-peridinin chlorophyll (CD45-PerCP) (BioLegend, San Diego, USA), CD117-phycoerythrin (CD117-PE) (BD Biosciences) (n = 34), CD203c-allophycocyanin (CD203c-APC) (n = 31) (Pharmingen, BD Biosciences, Erembodegem, Belgium), FcεRI-phycoerythrin (FcεRI-PE) (eBioscience, San Diego, USA) (n = 25) and CD63-fluorescein isothiocyanate (CD63-FITC) (BD Biosciences) (n = 10).

Intracellular markers were determined as the number of cells positive for histamine (n = 21), tryptase (n = 9) and chymase (n = 9). Therefore, mast cells were fixed with 1 mL of 4% paraformaldehyde (PFA) solution for 30 minutes at room temperature, washed with and resuspended in PBS with 0.05% Triton-X-100 (PBS-TX, pH=7.4)

(Sigma-Aldrich). Next, mast cells were stained with diamine oxidase-V500 (DAO-V500) (BD Biosciences) [19], monoclonal anti-human tryptase-Alexa Fluor 700 (anti-tryptase-AF700) (BioLegend) and monoclonal anti-human chymase-Alexa Fluor 700 (anti-chymase-AF700) (BioLegend), respectively, and incubated at 37°C for 30 minutes. Cells were washed and suspended in 300 µL PBS with 0.1% sodium azide and measured.

Flow cytometric analysis was performed on a FACSCanto II flow cytometer (BD Immunocytometry Systems, San Jose, CA) equipped with three lasers (405 nm, 488 nm and 633 nm). Correct compensation settings for antibodies conjugated with fluorochromes were performed using BD CompBeads (BD Biosciences). Since DAO is not an antibody, compensation with labelled DAO was executed manually by aligning the medians of positive as well as negative stained cell populations. Flow cytometric data were analyzed using Kaluza Analysis 1.3 software (Beckman Coulter, USA). A fluorescence minus one (FMO) sample was used to set a marker between positive and negative cells according to the 99th percentile. The percentages refer to the net number of cells positive for each parameter.

Functional analysis

At day 21 until day 35 function of the HuMC was evaluated in 10 experiments by passively sensitizing the cells with allogenic sera containing elevated titres of total IgE (400 - 2000 kU/L) during 30 minutes at 37°C in a humidified CO_2-incubator. Next, cells were centrifuged (200 g, 5 minutes, 20°C) and the cell pellet was resuspended in pre-warmed (37°C) Tyrode's buffer (Sigma-Aldrich) at a density of 0.5 x 10^6 cells/mL. Subsequently, 100 µL passively sensitized HuMC were incubated with 100 µL Tyrode's buffer as a negative control or 100 µL of 2 µg/mL monoclonal anti-IgE (BD Biosciences) as a positive control during 20 minutes at 37°C. Reactions were stopped by chilling on ice and spinning for 5 minutes (4°C, 200 g). The supernatant was removed and cells were stained with monoclonal anti-human CD45-PerCP, CD203c-APC, CD117-PE and CD63-FITC for 20 minutes at 4°C. Finally, cells were washed and resuspended in PBS with 0.1% sodium azide and measured.

Histamine content was determined before and after activation as described above. A total of 7 experiments were conducted in duplicate. DAO staining with and without permeabilisation was used to set a marker between DAO positive and negative cells according to the 99th percentile. Standardization was performed using standardized fluorospheres (SPHERO Ultra Rainbow Calibration particles, Spherotech, Lake Forest, IL, USA) as described by the

manufacturer. Briefly, the measured median fluorescence intensity (MFI) was converted to a calibrated value using linear regression. Results were expressed as the mean of the duplicates in Molecules of Equivalent Specific Fluorochrome V500 (MESF-V500) for histamine content or Molecules of Equivalent Specific Fluorochrome APC (MESF-APC) for CD203c density. Histamine release was calculated by subtracting the intracellular content from activated cells from the content from non-activated cells.

Statistical analysis

Results are expressed as median and range (min-max). IBM SPSS Statistics version 23 software was used for data analysis. Non-parametric statistical analysis was performed where appropriate. A P-value of < 0.05 was considered significant.

Results

Mast cell characterization

Over all, CD45 positive cells showed cell viability of 95% (92 – 99) (figure 1B). As demonstrated in figure 1C, cells were subsequently characterized based upon expression of CD117 and CD203c. Two clear distinct cell populations could be identified: $CD117^{+}CD203c^{+hi}$ cells and $CD117^{-}CD203c^{+low}$ cells representing respectively 50% (21 - 97) and 50% (3 – 79) of the cells in culture. $CD117^{+}CD203c^{+hi}$ cells showed a basal expression of CD203c of 133 (86 – 432) MESF-APC/cell, whilst $CD117^{-}CD203c^{+low}$ cells showed a basal expression of CD203c of 70 (42-117) MESF-APC/cell (Wilcoxon: $P = 0.02$).

$CD117^{+}CD203c^{+hi}$ cells showed a significantly higher number of cells expressing the FcεRI as compared to $CD117^{-}CD203c^{+low}$ cells (figure 1D and 2A) (Wilcoxon: $P < 0.0001$), respectively 96% (87 – 99) and 86% (43 – 98) with a median difference of 11% (1 - 44). Besides, $CD117^{+}CD203c^{+hi}$ cells showed a significantly higher intracellular histamine content than $CD117^{-}CD203c^{+low}$ cells (figure 1E and 2B) (Wilcoxon: $P < 0.0001$), respectively 1388 MESF-V500/cell (187 – 10473) and 1011 MESF-V500/cell (198 – 8016) with a median difference of 414 MESF-V500/cell (11 – 2457). Both subpopulations showed an equal number of cells positive for tryptase (figure 1F and 2D), respectively 96% (66 – 100) and 96% (58 – 99) with a median difference of 2% (0 – 25). Lastly, $CD117^{-}CD203c^{+low}$ cells showed a slightly higher number of cells positive for chymase (Wilcoxon: $P = 0.01$) (figure 1G and 2C), respectively 97% (74 – 100) and 90% (61 – 99) with a median difference of 5% (0 – 25).

Functional analysis

Expression of CD63 and upregulation of CD203c

Activation of passively IgE-sensitized cells with anti-IgE resulted in a significant upregulation in a net number of cells positive for $CD63^+$ of 21% (11 – 39) for $CD117^+CD203c^{+hi}$ cells (Wilcoxon: P = 0.005) and 27% (18 – 55) for $CD117^-CD203c^{+low}$ cells (Wilcoxon: P = 0.02) (n= 10). A discrete higher CD203c density up to 55 MESF-APC/cell (32 – 110) for $CD117^+CD203c^{+hi}$ cells (Wilcoxon: P = 0.005) and 62 MESF-APC/cell (19 – 187) for $CD117^-CD203c^{+low}$ cells (Wilcoxon: P = 0.02) could be observed.

Histamine content

Histamine content and release are illustrated in figure 3. Histamine content of non-stimulated cells was higher for $CD117^+CD203c^{+hi}$ cells as compared to $CD117^-CD203c^{+low}$ cells, respectively 994 (695 – 6815) MESF-V500/cell and 797 (629 – 4978) MESF-V500/cell (Wilcoxon: P = 0.02).

Stimulation of $CD117^+CD203c^{+hi}$ cells with anti-IgE resulted in a histamine release of 578 (366 – 1521) MESF-V500/cell (Wilcoxon: P = 0.04) in 5 cultures (figure 3C-D, left). In the remaining 2 cultures, no release of histamine was demonstrable.

Stimulation of $CD117^-CD203c^{+low}$ cells with anti-IgE resulted in a histamine release of 310 (217 – 366) MESF-V500/cell in 3 experiments (Wilcoxon: P = 0.11) (figure 3C-D, right). However, in the remaining 4 cultures no release of histamine could be observed.

Discussion

Studies on human mast cells have mainly been using cells cultured from different origins [12-14,17,18,21,22], as mast cells are tissue-resident cells, only present in small numbers. Here we confirm that human mast cells can be cultured from peripheral blood $CD34^+$ progenitor cells. However, these cultures yield two phenotypically distinct cell populations. Firstly, mature mast cells that express CD45, CD117 (c-kit), CD203c and FcεRI on their surface and contain histamine, tryptase and chymase [4,23], thereby resembling the connective tissue phenotype of mast cells [8,24]. Secondly, a $CD117^-CD203c^{+low}$ subpopulation displaying characteristics of HuMC with a lower number of cells positive for FcεRI and a lower histamine content, but equal number of cells positive for tryptase and a

slightly higher number of cells positive for mast cell specific chymase. We speculate these $CD117^{-}CD203c^{+low}$ cells to be immature mast cells with an internalized c-kit receptor upon binding of its ligand. This c-kit disappearance already has been described for HuMC as a mechanism by which the availability of the c-kit receptor is controlled [25-28]. Functional analysis reveals that both, the $CD117^{+}CD203c^{+hi}$ and $CD117^{-}CD203c^{+low}$ subpopulation, display a significantly upregulated number of cells positive for CD63 and upregulated expression of CD203c after IgE-mediated cross-linking of their FcεRI, but in the vast majority of $CD117^{-}CD203c^{+low}$ cells this upregulation was not accompanied by a quantifiable histamine release. This dissociation between CD63 upregulation and histamine release already has been described for basophils [29-31], and could relate to stimulation conditions such as supra-optimal stimulation with anti-IgE.

To our knowledge this is the first description of flow cytometric quantification of histamine release by cultured HuMC. Our data reveal the existence of 2 phenotypically distinct cell populations that display a greatly similar response upon traditional activation through cross-linking of IgE. Furthermore, as this flow cytometric technique enables integration in a multi-parameter analysis with simultaneous quantification of various surface activation and degranulation markers, we suggest that it might capture data that cannot be obtained with traditional release tests. Especially on a single cell level or with some subpopulations, expanding experiments with intracellular signaling [32-35] or inhibitory receptors [36-39] might provide further insights into different pathophysiological mechanisms.

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References

1. Dahlin JS, Hallgren J. Mast cell progenitors: Origin, development and migration to tissues. Mol Immunol 2014.
2. Matarraz S, Lopez A, Barrena S, Fernandez C, Jensen E, Flores J, Barcena P, Rasillo A, Sayagues JM, Sanchez ML and others. The immunophenotype of different immature, myeloid and B-cell lineage-committed CD34+ hematopoietic cells allows discrimination between normal/reactive and myelodysplastic syndrome precursors. Leukemia 2008;22:1175-83.

3. Sanchez-Munoz L, Teodosio C, Morgado JM, Escribano L. Immunophenotypic characterization of bone marrow mast cells in mastocytosis and other mast cell disorders. Methods Cell Biol 2011;103:333-59.
4. Valent P, Schernthaner GH, Sperr WR, Fritsch G, Agis H, Willheim M, Buhring HJ, Orfao A, Escribano L. Variable expression of activation-linked surface antigens on human mast cells in health and disease. Immunol Rev 2001;179:74-81.
5. Buhring HJ, Streble A, Valent P. The basophil-specific ectoenzyme E-NPP3 (CD203c) as a marker for cell activation and allergy diagnosis. Int Arch Allergy Immunol 2004;133:317-29.
6. Harvima IT, Levi-Schaffer F, Draber P, Friedman S, Polakovicova I, Gibbs BF, Blank U, Nilsson G, Maurer M. Molecular targets on mast cells and basophils for novel therapies. J Allergy Clin Immunol 2014.
7. Lappalainen J, Lindstedt KA, Kovanen PT. A protocol for generating high numbers of mature and functional human mast cells from peripheral blood. Clin Exp Allergy 2007;37:1404-14.
8. Ahn K, Takai S, Pawankar R, Kuramasu A, Ohtsu H, Kempuraj D, Tomita H, Iida M, Matsumoto K, Akasawa A and others. Regulation of chymase production in human mast cell progenitors. J Allergy Clin Immunol 2000;106:321-8.
9. da Silva EZ, Jamur MC, Oliver C. Mast cell function: a new vision of an old cell. J Histochem Cytochem 2014;62:698-738.
10. MacGlashan DW, Jr. Basophil activation testing. J Allergy Clin Immunol 2013;132:777-87.
11. Siraganian RP. Mast cell signal transduction from the high-affinity IgE receptor. Curr Opin Immunol 2003;15:639-46.
12. Wang XS, Yip KH, Sam SW, Lau HY. Buffy coat preparation is a convenient source of progenitors for culturing mature human mast cells. J Immunol Methods 2006;309:69-74.
13. Krohn IK, Sverrild A, Lund G, Dahl R, Erjefalt JS, Backer V, Hoffmann HJ. Cultured mast cells from patients with asthma and controls respond with similar sensitivity to recombinant Der p2-induced, IgE-mediated activation. Scand J Immunol 2013;78:352-6.
14. Kirshenbaum AS, Kessler SW, Goff JP, Metcalfe DD. Demonstration of the origin of human mast cells from CD34+ bone marrow progenitor cells. J Immunol 1991;146:1410-5.
15. Maaninka K, Lappalainen J, Kovanen PT. Human mast cells arise from a common circulating progenitor. J Allergy Clin Immunol 2013;132:463-9 e3.
16. Dichlberger A, Schlager S, Lappalainen J, Kakela R, Hattula K, Butcher SJ, Schneider WJ, Kovanen PT. Lipid body formation during maturation of human mast cells. J Lipid Res 2011;52:2198-208.
17. Radinger M, Jensen BM, Kuehn HS, Kirshenbaum A, Gilfillan AM. Generation, isolation, and maintenance of human mast cells and mast cell lines derived from peripheral blood or cord blood. Curr Protoc Immunol 2010;Chapter 7:Unit 7 37.
18. Siebenhaar F, Falcone FH, Tiligada E, Hammel I, Maurer M, Sagi-Eisenberg R, Levi-Schaffer F. The search for mast cell and basophil models - are we getting closer to pathophysiological relevance? Allergy 2015;70:1-5.
19. Ebo DG, Bridts CH, Mertens CH, Hagendorens MM, Stevens WJ, De Clerck LS. Analyzing histamine release by flow cytometry (HistaFlow): a novel instrument to study the degranulation patterns of basophils. J Immunol Methods 2012;375:30-8.
20. Cop N, Uyttebroek AP, Sabato V, Bridts CH, De Clerck LS, Ebo DG. Flow cytometric analysis of drug-Induced basophil histamine release. Cytometry B Clin Cytom 2015.
21. Schmetzer O, Valentin P, Smorodchenko A, Domenis R, Gri G, Siebenhaar F, Metz M, Maurer M. A novel method to generate and culture human mast cells: Peripheral CD34+ stem cell-derived mast cells (PSCMCs). J Immunol Methods 2014.
22. Lowman MA, Rees PH, Benyon RC, Church MK. Human mast cell heterogeneity: histamine release from mast cells dispersed from skin, lung, adenoids, tonsils, and colon in response to IgE-dependent and nonimmunologic stimuli. J Allergy Clin Immunol 1988;81:590-7.
23. Walls AF, Amalinei C. Detection of mast cells and basophils by immunohistochemistry. Methods Mol Biol 2014;1192:117-34.
24. Xia HZ, Kepley CL, Sakai K, Chelliah J, Irani AM, Schwartz LB. Quantitation of tryptase, chymase, Fc epsilon RI alpha, and Fc epsilon RI gamma mRNAs in human mast cells and basophils by competitive reverse transcription-polymerase chain reaction. J Immunol 1995;154:5472-80.

25. Babina M, Rex C, Guhl S, Thienemann F, Artuc M, Henz BM, Zuberbier T. Baseline and stimulated turnover of cell surface c-Kit expression in different types of human mast cells. Experimental Dermatology 2006;15:530-537.
26. Shimizu YJ, Ashman LK, Du ZM, Schwartz LB. Internalization of Kit together with stem cell factor on human fetal liver-derived mast cells - New protein and RNA synthesis is required for reappearance of Kit. Journal of Immunology 1996;156:3443-3449.
27. Yee NS, Langen H, Besmer P. Mechanism of Kit-Ligand, Phorbol Ester, and Calcium-Induced down-Regulation of C-Kit Receptors in Mast-Cells. Journal of Biological Chemistry 1993;268:14189-14201.
28. Yee NS, Hsiau CWM, Serve H, Vosseller K, Besmer P. Mechanism of down-Regulation of C-Kit Receptor - Roles of Receptor Tyrosine Kinase, Phosphatidylinositol 3'-Kinase, and Protein-Kinase-C. Journal of Biological Chemistry 1994;269:31991-31998.
29. MacGlashan D, Jr. Expression of CD203c and CD63 in human basophils: relationship to differential regulation of piecemeal and anaphylactic degranulation processes. Clin Exp Allergy 2010;40:1365-77.
30. Hauswirth AW, Natter S, Ghannadan M, Majlesi Y, Schernthaner GH, Sperr WR, Buhring HJ, Valenta R, Valent P. Recombinant allergens promote expression of CD203c on basophils in sensitized individuals. J Allergy Clin Immunol 2002;110:102-9.
31. MacGlashan DW, Jr. Graded changes in the response of individual human basophils to stimulation: distributional behavior of events temporally coincident with degranulation. J Leukoc Biol 1995;58:177-88.
32. Verweij MM, Sabato V, Nullens S, Bridts CH, De Clerck LS, Stevens WJ, Ebo DG. STAT5 in human basophils: IL-3 is required for its FcepsilonRI-mediated phosphorylation. Cytometry B Clin Cytom 2012;82:101-6.
33. Aerts NE, Dombrecht EJ, Bridts CH, Hagendorens MM, de Clerck LS, Stevens WJ, Ebo DG. Simultaneous flow cytometric detection of basophil activation marker CD63 and intracellular phosphorylated p38 mitogen-activated protein kinase in birch pollen allergy. Cytometry B Clin Cytom 2009;76:8-17.
34. Ebo DG, Dombrecht EJ, Bridts CH, Aerts NE, de Clerck LS, Stevens WJ. Combined analysis of intracellular signalling and immunophenotype of human peripheral blood basophils by flow cytometry: a proof of concept. Clin Exp Allergy 2007;37:1668-75.
35. Verweij MM, De Knop KJ, Bridts CH, De Clerck LS, Stevens WJ, Ebo DG. P38 mitogen-activated protein kinase signal transduction in the diagnosis and follow up of immunotherapy of wasp venom allergy. Cytometry B Clin Cytom 2010;78:302-7.
36. Sabato V, Verweij MM, Bridts CH, Levi-Schaffer F, Gibbs BF, De Clerck LS, Schiavino D, Ebo DG. CD300a is expressed on human basophils and seems to inhibit IgE/FcepsilonRI-dependent anaphylactic degranulation. Cytometry B Clin Cytom 2012;82:132-8.
37. Nakahashi-Oda C, Tahara-Hanaoka S, Shoji M, Okoshi Y, Nakano-Yokomizo T, Ohkohchi N, Yasui T, Kikutani H, Honda S, Shibuya K and others. Apoptotic cells suppress mast cell inflammatory responses via the CD300a immunoreceptor. J Exp Med 2012;209:1493-503.
38. Nakahashi-Oda C, Tahara-Hanaoka S, Honda S, Shibuya K, Shibuya A. Identification of phosphatidylserine as a ligand for the CD300a immunoreceptor. Biochem Biophys Res Commun 2012;417:646-50.
39. Bachelet I, Munitz A, Moretta A, Moretta L, Levi-Schaffer F. The inhibitory receptor IRp60 (CD300a) is expressed and functional on human mast cells. J Immunol 2005;175:7989-95.

Figure 1. **Phenotypic characterization of cultured cells.**

Selection of single cells for analysis based on FSC-H (forward scatter-Height) and FSC-A (forward scatter-Area). All CD45 positive cells were evaluated for viability using 7-AAD staining. Viable $CD45^+$ cells were gated for further analysis **(A-B)**. Two distinct $CD45^+$ cell populations were identified: $CD117^+CD203c^{+hi}$ and $CD117^-CD203c^{+low}$ cells **(C)**. Both cell populations showed positivity for FcεRI **(D)**, histamine **(E)**, tryptase **(F)** and chymase **(G)**. The percentages refer to the number of cells positive for each parameter.

Figure 2. **Cell characterization based on expression of CD117 and CD203c.**

$CD117^+CD203c^{+hi}$ cells showed a significantly higher number of cells positive for FcεRI **(A)** on their cell membrane and a significantly higher histamine content **(B)** in comparison with $CD117^-CD203c^{+low}$ cells. $CD117^+CD203c^{+hi}$ cells showed a slightly lower number of cells positive for chymase **(C)** than $CD117^-CD203c^{+low}$ cells. The number of cells positive for tryptase **(D)** was equal in both subpopulations. The lines denote the medians. P values were calculated using Wilcoxon signed rank test. The N in the lower right corner represents the number of experiments.

Figure 3. **Histamine content and release after stimulation with anti-IgE.**

Comparison of two subpopulations: $CD117^+CD203c^{+hi}$ cells **(left)** and $CD117^-CD203c^{+low}$ cells **(right)**. DAO specificity was ensured by staining without **(A)** and with permeabilisation **(B)**. Evaluation of cell function: Non-stimulated cells (negative control) showed neither $CD63^+$ upregulation, nor histamine release **(C)**. $CD117^+CD203c^{+hi}$ cells stimulated with anti-IgE showed a significant upregulation of cells positive for $CD63^+$ of 32%, with a histamine release of 496 MESF-V500/cell **(D, left)**. $CD117^-CD203c^{+low}$ cells stimulated with anti-IgE resulted in a significant upregulation of cells positive for $CD63^+$ of 41%, with histamine release of 365 MESF-V500/cell **(D, right)**. For more information, see results section.