

Molecular and stimulus-response profiles illustrate heterogeneity between peripheral and cord blood-derived human mast cells

Bettina M. Jensen,^{*,1} Pernille M. Frandsen,[†] Ellen M. Raaby,[†] Peter Oluf Schiøtz,[†]
Per S. Skov,^{*} and Lars K. Poulsen^{*}

^{*}Allergy Clinic, Copenhagen University Hospital–Gentofte Hospital, Hellerup, Denmark; and [†]Department of Paediatrics, Aarhus University Hospital, Aarhus, Denmark

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ABSTRACT

Different protocols exist for in vitro development of HuMCs from hematopoietic stem cells, which results in distinct mast cells regarding molecular markers and activation patterns. Here, we introduce a SR profile using immunological, neurogenic, and pharmacological stimuli to characterize cellular functionality. Mast cells were obtained from three culture protocols using two types of PBdMCs (CD34⁺ PBdMC or CD133⁺ PBdMC) and one type of CBdMC (CD133⁺ CBdMC). We analyzed resting cells for specific mast cell markers at protein and mRNA levels, thereby creating a molecular profile. To characterize the SR profile, we stimulated cells with anti-IgE, C3a, C5a, Substance P, or Compound 48/80 and measured the release of histamine and cytokines (IL-10, IL-13, GM-CSF, TNF- α). Molecular profiling revealed that CD133⁺ CBdMC expressed less chymase, Fc ϵ RI α , and CD203c but more CD117 compared with CD34⁺ and CD133⁺ PBdMC. The SR profile for histamine release illustrated a functional heterogeneity between PBdMC and CBdMC. PBdMC released >10% histamine upon stimulation with anti-IgE, C3a, Substance P, and Compound 48/80, whereas CBdMC only reacted to C3a. Cytokine secretion was only detected after anti-IgE stimulation. Here, the SR profile identified the CD133⁺ PBdMC as the most active cells regarding secretion of IL-10, IL-13, GM-CSF, and TNF- α . Cells from all three culture protocols, however, produced IL-10 spontaneously at comparable levels. We recommend validating mast cell cultures by means of molecular and SR profiles to characterize the mast cells and enhance consensus among studies. *J. Leukoc. Biol.* 95: 000–000; 2014.

Introduction

Mast cells are granulocytes with a heterogeneous composition of intracellular enzymes, which has led to a crude division of mast cells into MC_T and MC_{TC} [1]. In the murine system, these phenotypes have been linked to mucosal or connective tissue mast cells, respectively. In the human system, MC_{TC} have been associated with skin and MC_T with lung mast cells [1, 2]. However, the discovery that mast cells with similar protease profiles can react differently has increased the complexity. Examples of this functional heterogeneity are found in human skin and heart mast cells. Both cell types are considered to be MC_{TC}, and both release histamine when stimulated with Compound 48/80. However, only the skin mast cells respond to Substance P [3]. Therefore, HuMC display a tissue-specific heterogeneity, resulting in a large variation between different mast cell populations in terms of activation patterns, which cannot always be explained by MC_T versus MC_{TC} phenotypes.

Mast cell research improved greatly when in vitro stem cell-derived mast cells were introduced [4–6]. However, this also added another dimension of complexity as the different culture protocols available gave rise to distinct mast cells, which may have led to conflicting results (e.g., differences in response to TLR ligands [7–9]). Very few reports have compared these different culture systems regarding functionality, and the resemblance of in vitro-differentiated mast cells with specific tissue types is still an unsolved question [10–12].

Experimental conditions, such as progenitor source (e.g., peripheral blood, cord blood, bone marrow, fetal liver), progenitor type (CD34⁺, CD133⁺), and culturing protocol (medium with or without serum, liquid or methylcellulose medium, cytokine combinations, and duration of culture), may influence the type of mast cells produced and thereby, their response to a certain stimuli. Until now, in vitro-cultured mast cells have mainly been characterized by cell morphology, histamine, and protease content and receptor expression, but this

Abbreviations: C3a/5a=complement 3a/5a; CBdMC=cord blood-derived mast cells; C_T=cycle threshold; HClO₄=perchloric acid; HDC=histidine decarboxylase; HuMC=human mast cells; MC_T=tryptase-positive mast cells; MC_{TC}=tryptase- and chymase-positive mast cells; PBdMC=peripheral blood-derived mast cells; SCF=stem cell factor; SR=stimulus response

1. Correspondence: Allergy Clinic, Dept. 22, First floor, Gentofte Hospital, Niels Andersens vej 65, DK-2900, Hellerup, Denmark. E-mail: bettina.margrethe.jensen@regionh.dk

cannot necessarily be transformed into mast cell functionality. Therefore, we suggest that a functional validation of the in vitro-derived mast cells should be applied to each culture protocol to enable comparison of results between the different mast cells.

In the present study, we analyzed HuMC generated from three culture protocols using different combinations of culture systems and progenitors, resulting in two types of PBdMC (CD34⁺ PBdMC or CD133⁺ PBdMC) and one type of CBdMC (CD133⁺ CBdMC). We characterized the expression of typical molecular mast cell markers (proteases and receptors) on protein and mRNA level to obtain a molecular profile. The functional validation was done by generating a SR profile for each mast cell culture protocol. We applied commonly used mast cell stimuli and created a SR profile for histamine release and cytokine secretion (IL-10, IL-13, GM-CSF, TNF- α). The stimuli used were divided into: (1) immunological IgE-dependent, using anti-IgE—mediating activation through the high-affinity IgE receptor Fc ϵ RI, which activates most mast cells to release mediators, such as histamine and cytokines [13]; (2) immunological non-IgE-dependent, using C3a and C5a anaphylatoxins generated via complement activation that interacts with GPCRs and activate mast cells in the skin, heart, and lung (subtypes of mast cells) [3, 14–18]; (3) neurogenic, using Substance P, a peptide probably working via a GPCR and a PKC-dependent mechanism that seems to activate skin and purified lung mast cells but not sinus mucosa, heart, tonsil, or colon mast cells [3, 13, 16, 19–23]; and (4) pharmacological, using Compound 48/80, a synthetic polyamine that activates mast cells via G proteins and the PLC pathway and for which different results are found in the literature regarding lung mast cells, but skin and heart mast cells seem to be activated by this substance [3, 16, 19, 24].

The characterization of the molecular profile and SR profiles revealed that progenitor source and to a smaller extent, culturing protocol are important for the type of mast cells generated.

MATERIALS AND METHODS

Cultures of HuMC

HuMC were developed from CD34⁺ peripheral blood-derived mononuclear cells, as described by Kirshenbaum et al. [6], or from CD133⁺ mononuclear cells from peripheral blood or cord blood, as described by Holm et al. [25] and Dahl et al. [26]. In brief, CD34⁺ cells purified from buffy coat blood (60 ml) using CD34⁺ microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were cultured in StemPro-34 culture media (Invitrogen, Carlsbad, CA, USA) containing 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μ g/ml streptomycin (Invitrogen), 100 ng/ml IL-6 (PeproTech, Paris, France), and 100 ng/ml SCF (PeproTech). IL-3 (30 ng/ml; R&D Systems, Minneapolis, MN, USA) was included for the 1st week only. At Week 7 of culture, the HuMC were used for experiments.

CD133⁺ cells were purified from fresh heparinized cord blood (100 ml) or buffy coat blood (50 ml) using CD133⁺ microbeads (Miltenyi Biotec GmbH) and cultured in StemSpan (Stem Cell Technologies, Vancouver, Canada) medium, containing 100 U/ml penicillin (Invitrogen), 100 μ g/ml streptomycin (Invitrogen), 50 ng/ml IL-6, and 100 ng/ml SCF (both from R&D Systems). In addition, the cultures were given 1 ng/ml IL-3 (R&D Systems) for the first 3 weeks and 10% FCS (Invitrogen) from Week 6, and

the HuMC were used for experiments from Week 7. Maturity and purity (metachromatic stain and surface marker expression of CD117 and Fc ϵ RI validated by flow cytometry) were in accordance with previous reports [6, 10, 26].

Immunocytochemical staining of tryptase and chymase

HuMC were analyzed for tryptase and chymase on cytospin preparations containing 50,000 cells (centrifugation procedure 300 g, 5 min, room temperature). Air-dried slides were fixed in Carnoy's buffer (Hospital Pharmacy, Copenhagen University Hospital, Denmark) for 10 min, washed in PBS (Invitrogen), and incubated overnight at 4°C in a humid chamber with mouse anti-HuMC tryptase (Chemicon, Millipore, Billerica, MA, USA), mouse IgG1 isotype control (BD PharMingen, BD Biosciences, Franklin Lakes, NJ, USA), mouse anti-HuMC chymase-biotin (Chemicon, Millipore), or mouse IgG1-biotin isotype control (BD PharMingen, BD Biosciences). Slides were washed in PBS, incubated for 1 h in the dark at 4°C with FITC-conjugated goat anti-mouse IgG antibody (tryptase and isotype control; Dako, Glostrup, DK) or streptavidin-PE conjugate (chymase and isotype control; BD PharMingen, BD Biosciences), again washed in PBS, and then coated with Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined using a BX60 fluorescence microscope (Olympus, Ballerup, Denmark). Images were recorded with an F-View II camera using the Image Analysis software system (version 3.2; Olympus).

Flow cytometry analysis of surface molecules

The HuMC were analyzed for surface marker expression using the following antibodies: Fc ϵ RI α (Cosmo Bio, Tokyo, Japan) and FITC-conjugated goat anti-mouse IgG antibody (Dako); CD88-FITC (AbD Serotec, Oxford, UK); CD117-PE, CD123-FITC, CD124-PE (all from BD PharMingen, BD Biosciences); and CD203c-PE (Beckman Coulter, Marseille, FR). Isotype controls used were: mouse IgG2b isotype (Dako); and mouse IgG2a-FITC and IgG1-PE (BD PharMingen, BD Biosciences). HuMC were incubated with antibodies for 30 min in the dark at 4°C and washed in PBS + 0.5% HSA (CSL Behring GmbH, Marburg, Germany) + 2 mM EDTA (Applied Biosystems, Foster City, CA, USA). For secondary antibodies, another 30 min was added, and the HuMC were then analyzed on a Cytomics FC 500 MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA). MXP Acquisition and Analysis software version 2.2 was used to acquire data, and data analysis was performed using FlowJo 8 for Macintosh (Tree Star, Ashland, OR, USA). Results are given as geometric mean, adjusted for isotype control.

Analysis of mRNA expression

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified on a Qubit fluorometer (Invitrogen). RNA (0.017 μ g or 0.1 μ g) was then submitted to first-strand cDNA synthesis using SuperScript II RT (Invitrogen), as described earlier [27], followed by real-time PCR using primers (Applied Biosystems) for tryptase (Hs02576518_gH), chymase (Hs00156558_m1), HDC (Hs00157914_m1), C5aR (Hs00704891_s1), and Fc ϵ RI α (Hs00758600_m1), all with a 6-carboxyfluorescein (FAMTM) probe and β -actin (#4310881E, VIC/TAMRA probe). Each gene analysis was run in triplicates (45 cycles; 50°C for 2 min $\rightarrow$ 95°C for 10 min $\rightarrow$ 45 $\times$ 95°C for 15 s $\rightarrow$ 60°C for 60 s) on a Taqman ABI Prism Sequence 7700 with Sequence Detector v1.7a software or on a StepOnePlus Real-Time PCR with StepOne v2.1 software (Applied Biosystems). C_T values were correlated to β -actin [C_T (corr) = C_T (gene of interest) – C_T (β -actin)], and the expression was calculated as: $2^{-C_T \text{ (corr)}} \times C_T$ values for β -actin; 24.36 ± 2.84 for CD34⁺ PBdMC; 27.15 ± 1.87 for CD133⁺ PBdMC; and 29.76 ± 2.90 for CD133⁺ CBdMC.

Histamine release assay

HuMC (300,000 cells/ml) were sensitized overnight using 1 μ g/ml IgE (Calbiochem, Merck Chemicals, Beeston, Nottingham, UK), washed in

PIPES buffer (Hospital Pharmacy) + 0.5% HSA, and resuspended to a cell concentration of 10^5 cells/ml. Reaction was made with 50 μ l cell suspension and 50 μ l stimulant (anti-IgE; KPL, Gaithersburg, MD, USA), C3a or C5a (both R&D Systems), Substance P (Sigma-Aldrich, St. Louis, MO, USA), or Compound 48/80 (Sigma-Aldrich; for concentrations, see figures or text). After 1 h, 25 μ l supernatant was transferred to glass fiber-coated microtiter plates, and the histamine released was detected using a fluorescence method (RefLab, Copenhagen, Denmark).

The total cellular histamine content was measured by lysing 50 μ l cell suspension with 7% HClO_4 for 5 min at room temperature, followed by neutralization with 400 μ l PIPES buffer. The results are given as percent histamine released.

Cytokine production and measurement

HuMC (500,000 cells/ml) were sensitized overnight using 1 μ g/ml IgE, washed in culture medium, and resuspended to a cell concentration of 10^6 cells/ml. Cells were stimulated with 5 μ g/ml anti-IgE (KPL), 1 μ g/ml C3a or 1 μ g/ml C5a (both R&D Systems), 10 μ M Substance P (Sigma-Aldrich), or 50 μ g/ml Compound 48/80 (Sigma-Aldrich) for 18 h. Supernatant was then harvested and analyzed for the following cytokines, with minimum detection levels indicated as (pg/ml): IL-5 (3), IL-10 (2), IL-13 (3), GM-CSF (2), and TNF- α (7) content, using Bio-Plex Pro cytokine assay (Bio-Rad Laboratories, Berkeley, CA, USA) and Bio-Plex 200 system with Bio-Plex manager 5.0 (Bio-Rad Laboratories). The assays were run according to the manufacturer's description except that only one-quarter of the bead and antibody concentration was used.

Statistical analysis

Data from the three groups were analyzed using InStat (GraphPad Software, La Jolla, CA, USA) and Mann-Whitney test with two-tailed P value. The error bars in figures indicate sd values. Statistical significance was graded as not significant, $P > 0.05$; $*0.05 \geq P > 0.01$; $**0.01 \geq P > 0.001$; and $***P \geq 0.001$.

RESULTS

Heterogeneity in molecular surface marker expression among in vitro-derived mast cells

We initiated mast cell validation by a molecular profiling characterizing surface receptor expression to better understand a possible difference in activation. As seen in **Fig. 1A**, all three mast cell cultures expressed Fc ϵ RI and CD117 but not CD123, confirming that the cells are mast cells. The expression of Fc ϵ RI and CD117 was, however, different among the three cultures. CD133 $^+$ CBdMC expressed significantly less Fc ϵ RI compared with the two types of PBdMC but significantly more CD117 (Fig. 1B), which has been described earlier by Andersen et al. [10]. No significant difference was found in the expression of Fc ϵ RI and CD117 between the two types of PBdMC (Fig. 1B).

C5aR (CD88), known to be expressed on skin mast cells and lung mast cells, positive to both tryptase and chymase, was found near the detection limit on mast cells from all three culture protocols. The receptor for IL-4 (CD124), which has been shown to be highly expressed on lung mast cells (MC $_T$) but shows only low expression on skin mast cells (MC $_{TC}$), was found to be expressed more on both types of CD133 $^+$ -derived mast cells with a significant difference between CD133 $^+$ PBdMC and CD34 $^+$ PBdMC.

Mast cells from all three culture protocols expressed CD203c, but the two types of PBdMC expressed a higher level with CD34 $^+$ PBdMC, showing significantly more compared with CD133 $^+$ CBdMC. We also immunostained the cells for tryptase and chymase and found that all three protocols gener-

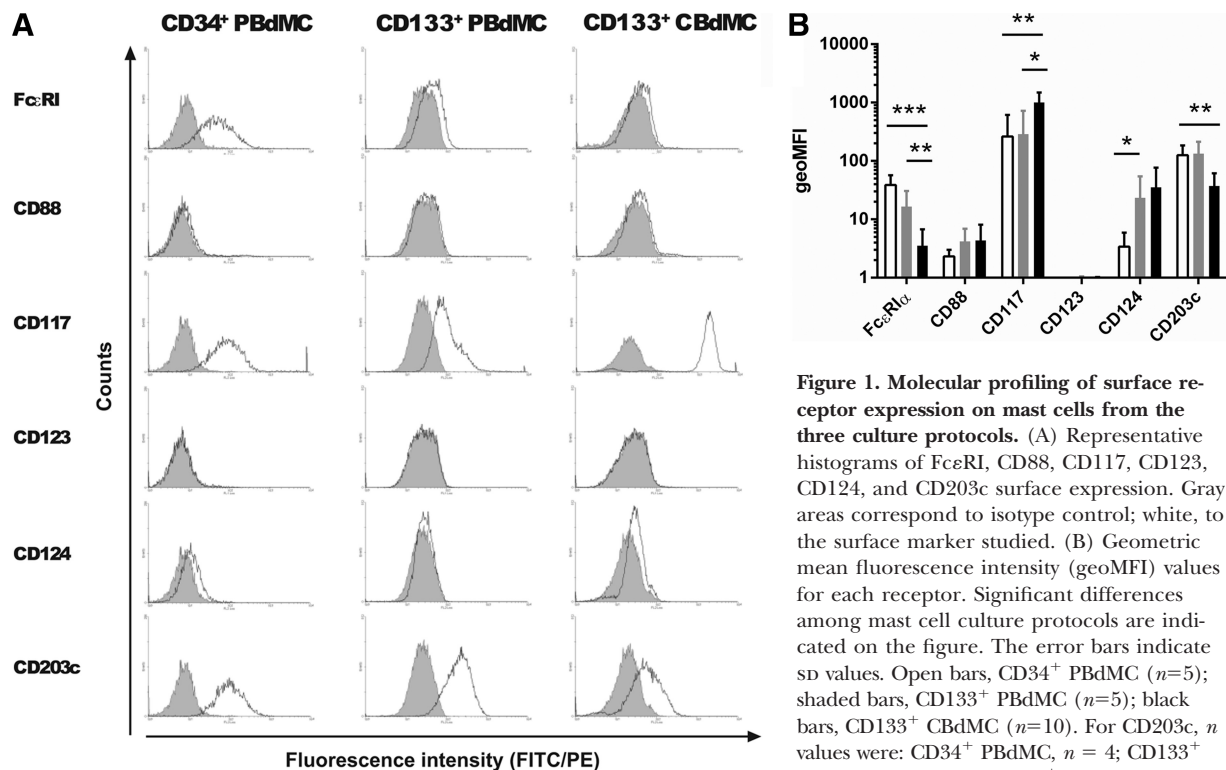


Figure 1. Molecular profiling of surface receptor expression on mast cells from the three culture protocols. (A) Representative histograms of Fc ϵ RI, CD88, CD117, CD123, CD124, and CD203c surface expression. Gray areas correspond to isotype control; white, to the surface marker studied. (B) Geometric mean fluorescence intensity (geoMFI) values for each receptor. Significant differences among mast cell culture protocols are indicated on the figure. The error bars indicate sd values. Open bars, CD34 $^+$ PBdMC ($n=5$); shaded bars, CD133 $^+$ PBdMC ($n=5$); black bars, CD133 $^+$ CBdMC ($n=10$). For CD203c, n values were: CD34 $^+$ PBdMC, $n=4$; CD133 $^+$ PBdMC, $n=4$; and CD133 $^+$ CBdMC, $n=6$.

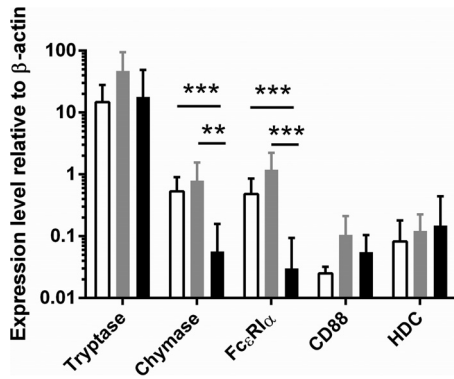


Figure 2. Molecular profiling of mRNA expression of tryptase, chymase, FcεRIα, CD88, and HDC within the three mast cell culture protocols. Results are correlated to β-actin expression. Significant differences among mast cell culture protocols are indicated on the figure. The error bars indicate SD values. Open bars, CD34⁺ PBdMC ($n=8$); shaded bars, CD133⁺ PBdMC ($n=8$); black bars, CD133⁺ CBdMC ($n=14$).

ated cells expressing both proteases (the expression of chymase varied within the cultures) but with a trend of CD133⁺ CBdMC less bright in the fluorescence intensity for the chymase stain (data not shown). The molecular profiling of surface receptors illustrated that the PBdMC expressed the same markers at a similar magnitude except for CD124, which seemed down-regulated on the CD34⁺ PBdMC.

PBdMC have a common molecular profile regarding immune receptor mRNA and mediator content

To characterize further differences between culture protocols, we extended the molecular profiling to include an analysis of mRNA expression of tryptase, chymase, FcεRIα, CD88, and HDC in resting cells. All three types of cultured mast cells had a strong expression of tryptase, with CD133⁺ PBdMC reaching the highest level (Fig. 2). Chymase was also found in all mast cell cultures but at a significantly lower level in CD133⁺ CBdMC, which supports the trend seen with the immunostaining. In correlation to the flow cytometry analysis of surface receptors, CD133⁺ CBdMC had a significantly reduced expression of FcεRIα mRNA. Expression of CD88 mRNA was found to be low in all of the mast cells, which again, supports the low surface receptor expression.

As a result of an earlier report of CD133⁺ CBdMC containing less histamine [10], we also measured HDC, but found no differences among the three mast cell cultures. Nevertheless, when measuring the total cellular histamine content, the CD133⁺ CBdMC had ~50% less than the two types of PBdMC: CD34⁺ PBdMC ($n=8$), 11.1 ± 8 pg/cell; CD133⁺ PBdMC ($n=8$), 11.8 ± 7 pg/cell; CD133⁺ CBdMC ($n=14$), 5.2 ± 3 pg/cell. Significant differences were found between CD133⁺ PBdMC and CD133⁺ CBdMC ($P=0.005$) and CD34⁺ PBdMC and CD133⁺ CBdMC ($P=0.021$).

Molecular profiling using mRNA expression supported the observation that PBdMC are more alike with regard to the expression of typical mast cell markers when compared with CBdMC.

CBdMC are inferior with regard to histamine release

To generate a SR profile of the in vitro-derived mast cells, we used a panel of potential mast cell activators and started out by looking at the SR profile for histamine release. With the activation of the mast cells via their FcεRI, using anti-IgE, we found that IgE-sensitized CD133⁺ CBdMC were very poor releasers of histamine (maximum 10% histamine release; Fig. 3), whereas both types of PBdMC showed a stronger and highly significant ($0.01 \geq P > 0.001$) histamine release, with a maximum ~30%. These results support the FACS and mRNA data, where CD133⁺ CBdMC showed the lowest expression of FcεRIα, making them less likely to respond to FcεRI activation.

We also stimulated cells with immunological non-IgE stimuli using the anaphylatoxins C3a or C5a. The strongest histamine release was found when using C3a, and here, CD133⁺ PBdMC were significantly different from the CD133⁺ CBdMC but not from the CD34⁺ PBdMC (Fig. 4). C5a induced low and comparable histamine release for all three types of mast cells (10% or less). Neurogenic stimulation using Substance P activated the CD133⁺ CBdMC to a histamine release below 10%, whereas the PBdMC showed a histamine release of 20–30%.

The pharmacological stimulus, Compound 48/80, which has been used as a general mast cell activator, was found to activate both types of PBdMC to release significantly more histamine than CD133⁺ CBdMC, which showed a mean release below 10%. Generating a SR profile, by looking at histamine release, revealed that CBdMC are poor releasers, only activated by C3a. In contrast, the PBdMC reacted to all compounds tested, except for C5a, again illustrating a similar profile.

Cytokine release from CBdMC appeared limited to spontaneous IL-10 production

We next investigated the SR profile for cytokine secretion from the three mast cell cultures and found that IgE-sensitized

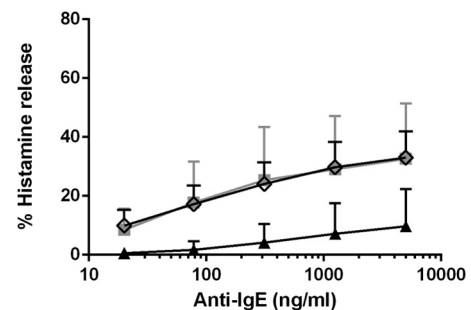


Figure 3. SR profiling using anti-IgE-mediated histamine release. IgE-sensitized mast cells were stimulated for 1 h with anti-IgE (5000, 1250, 312, 78, or 20 ng/ml), and histamine was determined as percent histamine released using HClO₄-lysed cells as a total histamine reading. Significant differences were found between CD34⁺ PBdMC and CD133⁺ CBdMC ($0.01 \geq P \geq 0.001$) and CD133⁺ PBdMC and CD133⁺ CBdMC ($P \leq 0.001$) for all anti-IgE concentrations. The error bars indicate SD values. Open diamonds, CD34⁺ PBdMC ($n=8$); shaded squares, CD133⁺ PBdMC ($n=8$); black triangles, CD133⁺ CBdMC ($n=14$).

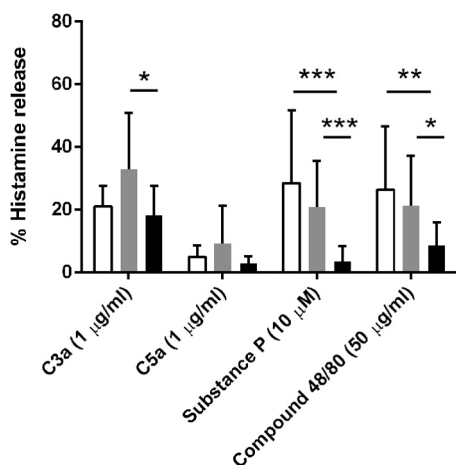


Figure 4. SR profiling using non-IgE-mediated histamine release.

HuMC (IgE-sensitized) were stimulated with 1 µg/ml C3a, 1 µg/ml C5a, 10 µM Substance P, or 50 µg/ml Compound 48/80. Histamine was determined as percent histamine released using HClO₄-lysed cells as a total histamine reading. Significant differences among mast cell culture protocols are indicated on the figure. The error bars indicate sd values. Open bars, CD34⁺ PBdMC (*n*=8); shaded bars, CD133⁺ PBdMC (*n*=8); black bars, CD133⁺ CBdMC (*n*=14).

mast cells from both types of the PBdMC released IL-10, IL-13, GM-CSF, and TNF-α after anti-IgE stimulation but not with any of the other stimuli (Fig. 5). We saw some indication of IL-5 release from CD34⁺ and CD133⁺ PBdMC, but the results were not reproducible with different Bio-Plex kits and are therefore not included. The CBdMC did not, in general, respond to any stimuli, but a spontaneous IL-10 secretion could be detected, which was comparable with the release from the two PBdMC (16±10 pg/10⁶ cells from CD34⁺ PBdMC, 9±6 pg/10⁶ cells from CD133⁺ PBdMC, 21±16 pg/10⁶ cells from CD133⁺ CBdMC; *n*=4 for all three mast cell cultures). Interestingly, mRNA analysis of resting and anti-IgE-stimulated cells did not differ (results not shown), indicating a constitutive nature of the spontaneous IL-10 production/secretion.

As illustrated in Fig. 6, we found a large heterogeneity in the amount of cytokine released after anti-IgE stimulation among different donors (each point in the figure corresponds to one donor) within the same mast cell culture protocol. Some were even found to have a response below the cytokine detection level, as seen with IL-13 and TNF-α secretion from the CD34⁺ PBdMC, where secretion from three out of four donors was below the detection level. For the CD133⁺ CBdMC, one donor was found to secrete a small amount of IL-10 and IL-13; the rest was below the detection limit.

The use of cytokine release to generate a SR profile illustrated the same pattern as found with histamine release—that CBdMC appeared unresponsive except for the spontaneous IL-10 release. The PBdMC released cytokines but only in response to anti-IgE stimulation. In contrast to the histamine SR profile and the molecular profile, the SR profile for cytokine secretion illustrated a difference between the PBdMC, with CD133⁺ PBdMC producing significantly more GM-CSF com-

pared with CD34⁺ PBdMC, but also, the IL-13 and TNF-α secretion was more prevalent among the CD133⁺ PBdMC.

DISCUSSION

Many different protocols for the development of HuMC exist, which might result in distinct mast cell subtypes. An overview of the difference among 12 published mast cell culturing protocols, regarding cell yield, histamine and protease content, and receptor expression (CD117 and FcεRI), was published by Andersen et al. [10] and illustrates the great heterogeneity among in vitro-derived mast cells. The present study takes the characterization of in vitro-derived HuMC one step further, by generating a molecular profile and a functional validation by creating SR profiles.

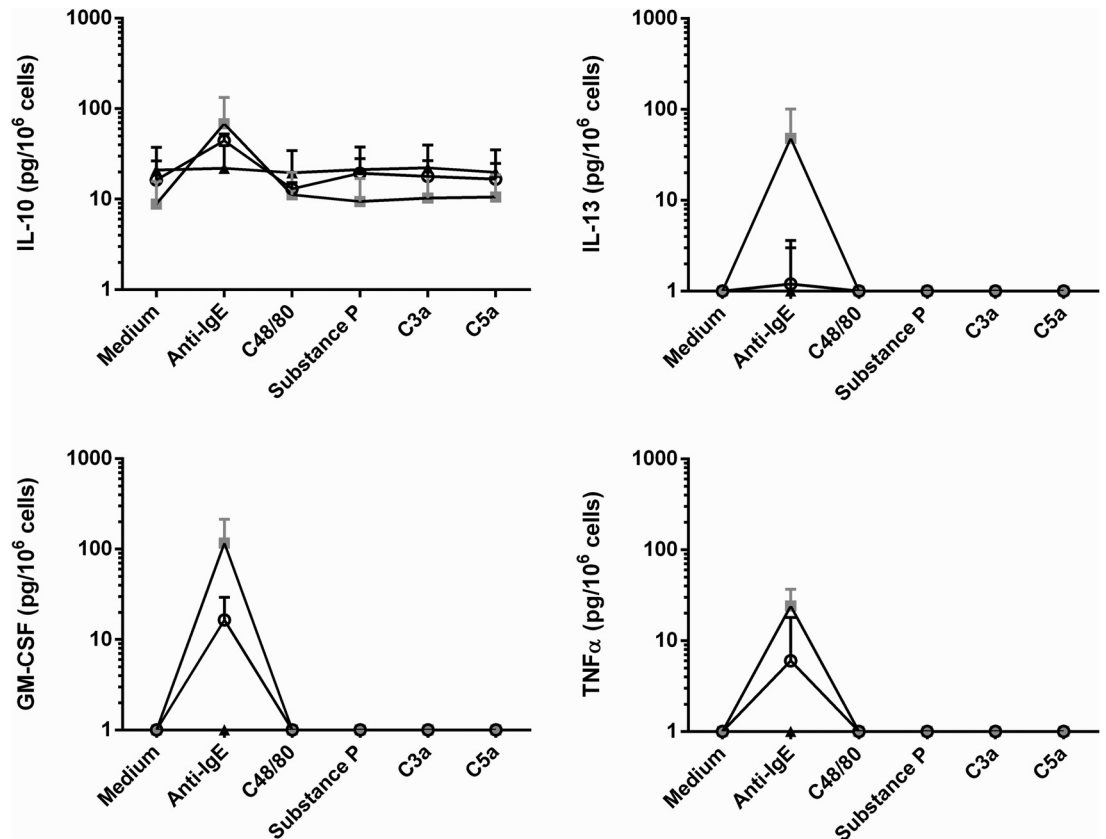
Molecular profiling was done by characterizing the expression of typical mast cell markers at protein and mRNA levels. We found that the PBdMC were quite similar in both aspects, except for a difference in CD124 surface expression. It has been reported that lung mast cells and CBdMC express a high level of CD124, whereas skin mast cells have a low expression [28, 29]. As the CD34⁺ PBdMC were found to have a low expression of CD124, they might resemble skin mast cells, whereas the CD133⁺ CBdMC and PBdMC could be more similar to lung mast cells based on the molecular profile.

To generate a SR profile, we used five different mast cell activators that have been widely described in the literature and represent different activation routes, e.g., immunological, neurogenic, and pharmacological. We did not include calcium ionophore (A23187 raises cytosolic-free calcium ion concentration, which activates mast cells to mediator release), even though it is used on a regular basis in mast cell research. All mast cells (in vivo- and in vitro-derived) react to calcium ionophore, and it is therefore not relevant to use when examining differences in activation [13, 30].

The first part of creating a SR profile was done by investigating the histamine release. Anti-IgE stimulation has been considered to be an activator of all mast cells, but as shown here, CD133⁺ CBdMC were very poor in histamine release, in contrast to both types of PBdMC and could be classified as nonreleasers as a result of a release below 10% [31]. This lack of histamine-releasing capability is likely correlated to the low expression of FcεRI on these cells. However, mast cells with a nonreleaser profile and a normal FcεRI/IgE level have been found in lung tissue, indicating that releasability is not just correlated to FcεRI expression level [31, 32]. In addition, these nonreleasing mast cells still react to A23187, again illustrating that this compound is not optimal when generating a SR profile [31].

The CD133⁺ CBdMC also had a nonreleasing profile when using non-IgE-mediated stimuli, which the two types of PBdMC were also found to have in relation to C5a-mediated histamine release. The CD34⁺ and CD133⁺ PBdMC were not significantly different using any of the tested stimuli, thus giving them the same SR profile for histamine release with IgE- and non-IgE-mediated stimuli. The CD133⁺ CBdMC only had a histamine release above 10% using C3a stimulation and were therefore quite different in the histamine-release SR profile.

Figure 5. SR profiling of cytokine release with IgE-dependent and non-IgE stimuli. IgE-sensitized HuMC were stimulated for 18 h with 5 $\mu\text{g/ml}$ anti-IgE, 50 $\mu\text{g/ml}$ Compound 48/80, 10 μM Substance P, 1 $\mu\text{g/ml}$ C3a, or 1 $\mu\text{g/ml}$ C5a. Culture supernatant was then analyzed for IL-10, IL-13, GM-CSF, and TNF- α content. The cytokine level is given as $\text{pg}/10^6$ cells. The error bars indicate SD values. Open circles, CD34 $^{+}$ PBdMC ($n=4$); shaded squares, CD133 $^{+}$ PBdMC ($n=4$); black triangles, CD133 $^{+}$ CBdMC ($n=4$).



Characterization of the cytokine SR profile for the three types of mast cells illustrated a limited cytokine release. Only anti-IgE-mediated stimulation resulted in release of cytokines from the two types of PBdMC. This is in contrast to a report

from Kulka et al. [33], where CD34 $^{+}$ PBdMC are shown to release GM-CSF and TNF- α after Substance P and Compound 48/80 stimulation. These investigators used 10–50 times less stimulant and a different cytokine detection assay, which could affect the results. Nevertheless, this difference in cytokine release, despite the use of the same type of mast cell culture protocol, illustrates the importance of using a SR profile to obtain consensus between laboratories.

Even though CD133 $^{+}$ CBdMC did not, in general, release cytokines, they spontaneously released IL-10 comparable with the two types of PBdMC.

Human lung mast cells and CBdMC have been reported to spontaneously release IL-10, which seems to be potentiated in the presence of SCF [34–36]. As all of our mast cell stimulations were done in the presence of SCF, this was in favor of IL-10 secretion. However, the amount of spontaneously secreted IL-10 that we found is lower compared with other reports (lung mast cells and CBdMC secrete in the range of 200 $\text{pg}/10^6$ cells [34, 35], whereas we detected 9–21 $\text{pg}/10^6$ cells).

Interestingly, anti-IgE-mediated stimulation, which resulted in IL-10 release from the PBdMC, did not change the IL-10 mRNA level (data not shown), even though it has been reported for lung mast cells and CBdMC [34, 35]. A constitutive mRNA level could indicate a continuous accumulation of IL-10 into a preformed pool of cytokine, as has been found in CBdMC [35] and which may be released upon stimulation, as seen with the two types of PBdMC.

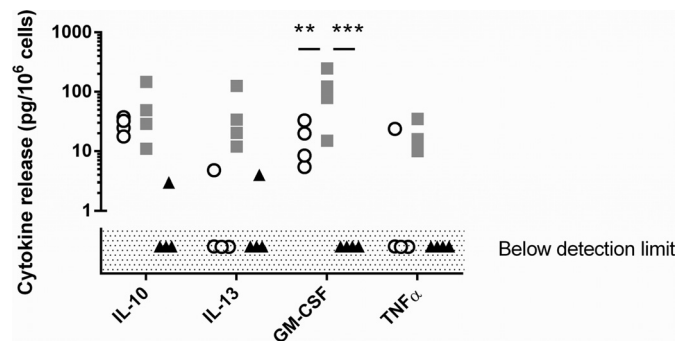


Figure 6. Heterogeneity in cytokine release after anti-IgE stimulation. IgE-sensitized HuMCs were stimulated for 18 h with 5 $\mu\text{g/ml}$ anti-IgE. Culture supernatants were then analyzed for IL-10, IL-13, GM-CSF, and TNF- α content. The cytokine level is given as $\text{pg}/10^6$ cells corrected for spontaneous release. Significant differences among mast cell culture protocols are indicated on the figure. Each point corresponds to a mast cell culture from one donor; four donors are used within each culture protocol. Donors with cytokine secretion below the assay detection level are located in the gray zone. Detection level for each cytokine is given in Materials and Methods. Open circles, CD34 $^{+}$ PBdMC; shaded squares, CD133 $^{+}$ PBdMC; black triangles, CD133 $^{+}$ CBdMC.

The concentration of the cytokines detected in this study was lower compared with other reports, as discussed above. Recently, several publications have suggested that large variances can be found among, but also within, quantitative cytokine assays [37–39]. Unfortunately, when comparing different multiplex assays, it seems that the Bio-Rad Bio-Plex assay is less efficient when it comes to reproducibility (batch-to-batch variation) and correlation to results obtained with other multiplex assays [37–39]. In particular, IL-10, IL-13, TNF- α , and GM-CSF detection involves large variation and low cytokine concentrations [38]. As these are the cytokines we are measuring, we may have detected too low a concentration, even though all samples used for Figs. 5 and 6 were analyzed on the same day with the same assay batch, which would exclude day and batch-to-batch variation. However, the low quantity of cytokine does not change the fact that there is a difference in cytokine secretion among the three mast cell cultures after anti-IgE stimulation.

Speculations can be made regarding an autocrine regulation by IL-10, as IL-10 secreted from CBdMC was found to down-regulate cytokine release (e.g., TNF- α), which furthermore, was reversed by anti-IL-10 antibody treatment [35, 36]. As the three mast cell cultures had the same level of spontaneously secreted IL-10, it is probably not IL-10 that mediates the difference found in anti-IgE-mediated cytokine secretion among the cultures. Nevertheless, IL-10 might affect the cytokine quantity released by the PBdMC and thereby, also play a part in the reason for the low levels of cytokines detected. Furthermore, an autocrine regulation by IL-10 could also explain why our CD34⁺ PBdMC did not respond to Substance P and Compound 48/80.

Cord blood progenitors give the highest mast cell yield, but these mast cells seem to be less responsive. This unresponsiveness might be a result of a more immature profile, as has been suggested by others [10, 40]. An immature status could also be indicated by the morphology of the cells, which seems to differ between PBdMC (CD34⁺ and CD133⁺) and CD133⁺ CBdMC, with the CBdMC having bilobed or multilobed nuclei (unpublished observations). This phenomenon has also been described in

relation to systemic mastocytosis, where cells classified as promastocytes (atypical mast cell type II) are found with bilobed or multilobed nuclei [41–43]. A study by Sperr et al. [43], furthermore, describes the morphology of CBdMC (derived from CD34⁺ stem cells) during their development and illustrates that after 42 days, bilobed or multilobed cells are found, classified as premature cells. After continued culturing until Day 80, the cells now appear more mature with a round nucleus (mononuclear) [43]. This indicates that CBdMC develop more slowly than PBdMC, which might be a consequence of the progenitor cells used; i.e., progenitor cells from peripheral blood are more mature than cells from cord blood. An extension in culture time might mature the CBdMC and thereby improve their responsiveness.

In contrast to the progenitor source, the progenitor type seems to have less impact on the mast cells, which is illustrated by the molecular profile and the SR profile. The profiles of CD34⁺ and CD133⁺ PBdMC are much more similar compared with CD133⁺ CBdMC and PBdMC.

According to the SR profile presented here for the CD133⁺ CBdMC, these mast cells are not optimal for activation studies using immunological (IgE-dependent or independent), neurogenic, and pharmacological pathways when looking at histamine and to some degree, cytokine release. However, other stimulatory pathways (e.g., through chemokine, cytokine, or TLRs) may have a stronger impact on the CD133⁺ CBdMC activation. Moreover, alternative readouts, such as the release of chemokines, leukotrienes, prostaglandins, or the induction of adhesion and migration, may reveal other functions of these cells. Nevertheless, to generate an example of a simple but relevant SR profile of in vitro-derived HuMC, we have limited our study to histamine release and cytokine secretion. Histamine release, in particular, has been used when studying in vivo-derived mast cells, and much information is available for tissue-specific mast cells. We have summed up results from the literature regarding histamine release and the compounds used in this study and compared the results with our findings to reveal a possible similarity between our in vitro-derived mast cells and a specific tissue type (Table 1). For this comparison,

TABLE 1. An Overview of HuMCs from Different Tissues and Their Histamine Release SR Profile When Stimulated with Anti-IgE, C3a, C5a, Substance P, or Compound 48/80

Tissue	Anti-IgE	C3a	C5a	Substance P	Compound 48/80
Skin [13, 14, 19, 44, 45]	+	+	+	+	+
Lung [3, 13, 15, 18, 24, 46, 47]	+	–	–	–	–
Lung MC _{TC} [16]			+	+	+
Lung MC _T [16]			–	+	–
Heart [3, 15, 17]	+		+/-	–	(+) ^a
Nasal mucosa [20, 48]				+	–
Sinus mucosa [22, 49]	+			–	–
Colon/intestine [13]	+			–	–
Tonsils [13, 15]	+			–	–
Adenoids [13]	+			–	–
CD34 ⁺ PBdMC	+	+	–	+	+
CD133 ⁺ PBdMC	+	+	–	+	+
CD133 ⁺ CBdMC	–	+	–	–	–

+, Histamine release when stimulated; –, histamine release absent or below 10%. ^aOnly histamine release with concentrations >100 μ g/ml.

cells releasing <10% histamine are considered as nonreleasers. As illustrated in the table, both types of PBdMC might have the highest resemblance to skin mast cells, despite being unresponsive to C5a. In contrast, the CD133⁺ CBdMC do not resemble any tissue-type mast cell, as they only react to C3a. So, although we detect similarities, our findings illustrate that it is not possible to generate tissue-specific HuMCs in vitro using these three protocols.

In conclusion, we have characterized three well-established human mast cell culture protocols with regard to their molecular profile and SR profile for histamine release and cytokine secretion. We found that CD34⁺ and CD133⁺ PBdMC were more alike, despite a difference in CD124 expression and levels of secreted GM-CSF, IL-13, and TNF- α . Both types of PBdMC appear highly competent for mast cell research, even though they do not share an identical SR profile with specific tissue-type mast cells. In contrast, the CD133⁺ CBdMC had a low expression of several of the mast cell markers and appeared almost insensitive toward all of the stimuli tested, revealing the limited use for these cells in mast cell research.

We suggest that in vitro-generated mast cells are validated not only by a molecular profile but also with a SR profile to reach consensus among results obtained with different mast cell cultures.

AUTHORSHIP

B.M.J., P.S.S., L.K.P. conceived of and designed the experiments. B.M.J., P.M.F., E.M.R. performed the experiments. All authors were involved in the interpretation of the data and outline of the manuscript. B.M.J. wrote the first version of the manuscript.

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