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## Research paper

## Seven week culture of functional human mast cells from buffy coat preparations

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## ABSTRACT

Functional, mature human mast cells have been generated by *in vitro* differentiation of CD133<sup>+</sup>/CD34<sup>+</sup> progenitor cells isolated from e.g. cord blood, peripheral blood, bone marrow or fetal liver. However, the protocols published so far require long term cultivation, i.e. up to 15 weeks for mast cell differentiation, which makes such approaches not only laborious but also costly. Here, we have developed a protocol for generating functional human mast cells from peripheral blood already within 7 weeks. Human CD133<sup>+</sup> progenitors were isolated from buffy coat preparations of peripheral blood and cultured in the presence of stem cell factor (SCF) and IL-6 for 7 weeks. IL-3 was added to the culture medium during the first 3 weeks, and fetal calf serum (FCS) added during the last week. *In vitro* differentiated CD133<sup>+</sup> cells exhibited multiple characteristics of mature mast cells. Thus, cells contained tryptase and expressed functional levels of FcεRI. Anti-IgE stimulation induced significant release of histamine and PGD<sub>2</sub> and also of chemokines including MCP-1, IL-8, MIP-1α, and MIP-1β. The fact that our *in vitro* differentiated mast cells are derived from a generally available source of progenitor cells makes this novel protocol widely applicable to any patient group, irrespective of age. Moreover, this progenitor source is more readily available than e.g. bone marrow or cord blood-derived progenitors. Consequently, our protocol has great potential in studies on mast cell biology and mast cell pathology, and e.g. on evaluation of drug effects.

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## 1. Introduction

Mast cells play an important role as the primary effector cells in immediate-type hypersensitivity reactions. Central to their

role in allergy is their expression of the high affinity Fc receptor for IgE (FcεRI) (Kinet, 1999). This receptor becomes occupied by allergen-specific IgE produced by B cells. Aggregation of IgE-occupied FcεRI by antigen coordinates activation of intracellular signalling pathways and results in degranulation of preformed mediators and synthesis and secretion of *de novo* synthesized lipid mediators, cytokines, and chemokines (Galli et al., 2005). Mediators derived from mast cells such as histamine, leukotrienes, and prostaglandins are responsible for the IgE-dependent allergic reactions clinically recognized as anaphylactic reactions, acute asthma, and allergic rhinitis (Ishizaka et al., 1983).

Mast cells are derived from CD34<sup>+</sup> pluripotent progenitor cells that are defined as c-kit (CD117)<sup>+</sup>, CD133<sup>+</sup>, and FcεRI<sup>+</sup>, and they lack T-cell (CD2), B-cell (CD19, CD20), macrophage (CD14), and eosinophil lineage surface markers (Kirshenbaum et al., 1991; Rodewald et al., 1996; Chen et al., 2005). In contrast to other

**Abbreviations:** PBS; phosphate buffered saline; BSA; bovine serum albumine; SCF; stem cell factor; FCS; fetal calf serum; APAAP; alkaline phosphatase anti-alkaline phosphatase; FACS; fluorescence-activated cell sorter; MCP-1, monocyte chemotactic protein 1; MIP-1α; macrophage inflammatory protein-1 alpha; MIP-1β; macrophage inflammatory protein-1 beta; GM-CSF; granulocyte macrophage colony stimulating factor.

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granulocytes that differentiate and mature within the bone marrow, mast cells leave the bone marrow as progenitors and undergo their terminal differentiation in the tissue. Therefore, mature mast cells do not circulate in the peripheral blood. The phenotype of mast cells varies in different anatomical compartments. These phenotypically distinct populations of human mast cells are referred to as mast cell heterogeneity (Galli et al., 2005).

Primary human mast cells are difficult to isolate because only limited amounts of cells can be retrieved from human tissues. Moreover, current methods are quite laborious (Schulman et al., 1982; Sellge and Bischoff, 2006). Instead, alternative means of obtaining human mast cells by *in vitro* differentiation have been successful using various sources of progenitor cells and variable culture conditions (Saito et al., 1996; Valent et al., 1992; Irani et al., 1992).

However, comparative studies of long term cultured mast cells (Iida et al., 2001) reveal significant quantitative as well as qualitative differences between mast cells depending on their source. Thus, yields between cord blood progenitor-derived and adult progenitor-derived human mast cells differ greatly. Neonatal hematopoietic progenitor cells generate more descendants than their adult counterparts. Functionally, the adult-type progenitor-derived mast cells are reportedly superior evaluated by their ability to release histamine upon activation (Inomata et al., 2005). It appears, therefore, that peripheral blood is a preferable source for many studies.

Previously published protocols are based on long term cultures (Dahl et al., 2002; Wang et al., 2006b; Yamaguchi et al., 2007), up to 15 weeks, which makes *in vitro* differentiation of mast cells extremely laborious, costly but also vulnerable to contaminations. Thus, the potential advantages of reduced culture time are evident. The aim of this study was to reduce culture time for *in vitro* differentiation of human mast cells and thereby introduce a novel protocol that is more generally applicable to progenitor cell donors of different ages and with different diseases with aberrant mast cell function.

Here, we report a seven week suspension culture method for generating human mast cells from *in vitro* differentiated progenitors isolated from peripheral blood. In the current study we used CD133<sup>+</sup> progenitor cells (Dahl et al., 2002), because CD133 defines a biologically distinct subset of haematopoietic stem cells that includes not only CD34<sup>+</sup> (bright) cells, but also a subset of CD34<sup>+</sup> (dim) cells. Such CD133<sup>+</sup> cells possess long term repopulating potential and could be critical in human mast cell development (Miraglia et al., 1997; Yin et al., 1997; Bauer et al., 2007). The *in vitro* differentiated cells were morphologically and functionally comparable to previously described cultured human mast cells as they stained negative for CD14 and CD23, but positive for CD117, CD203c and tryptase, and at variable levels for FcεRI. Moreover, the cells responded to FcεRI activation by releasing histamine and PGD<sub>2</sub>. Finally, we demonstrate that IgE cross-linking also induces significant production of chemokines such as MCP-1, IL-8, MIP-1α, and MIP-1β.

## 2. Materials and methods

### 2.1. Cytokines and reagents

Ficoll–Paque cell separation solution was purchased from GE Healthcare (Uppsala, Sweden). AC133 cell isolation kit and

LS+ cell separation columns were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). StemSpan culture medium was from Stem Cell Technologies (Vancouver, Canada). Fetal calf serum (FCS) and penicillin/streptomycin were purchased from GIBCO BRL (Grand Island, NY, USA). Human recombinant stem cell factor (rhSCF), human rhIL-6, and human rhIL-3 were purchased from R&D Systems (Abingdon, UK). Human rIL-4 was purchased from PeproTech (London, England). IgG1 antibodies against chymase and tryptase were purchased from Chemicon (Temecula, CA, USA). IgG1 RPE conjugated monoclonal antibody (mAb) against CD117 (clone 104D2), and its isotype control were from Serotec (Kidlington, UK). CD23/FITC (clone MHM6) and CD14/RPE (clone R0864) and their isotypes were purchased from Dako Cytomation (Glostrup, Denmark). CD203c/PE (clone 97A6) and its isotype were from Immunotech (Marseille, France). Myeloma IgE (supernatant from the B-cell line U266) was a kind gift from Lars K. Poulsen, National University Hospital, Allergy Unit. Anti-FcεRIα mAb, CRA-1 was purchased from Kyokuto Pharmaceuticals (Tokyo, Japan). The alkaline phosphatase anti-alkaline phosphatase (APAAP) kit used was from Dako Cytomation (Glostrup, Denmark). DAPI, 4',6-diamidino-2-phenylindole was purchased from Sigma-Aldrich.

### 2.2. Cell preparation

Human mast cells were generated from CD133<sup>+</sup> cells from buffy coat preparations. With the acceptance from the local ethical committee, fresh buffy coats were obtained from the blood bank of Aarhus University Hospital, Denmark. From a total volume of 470 ml full blood the concentrated leukocyte suspension (buffy coat) was collected via Optipress II (Baxter Healthcare, Deerfield, IL, USA). The 50 ml buffy coat was diluted with triple volume PBS and gently layered on a Ficoll–Paque gradient before centrifugation at room temperature for 30 min, 450 ×g. The interface layer of mononuclear cells was harvested and washed in PBS at 4 °C for 5 min, 450 ×g.

### 2.3. Purification of CD133<sup>+</sup> cells

CD133<sup>+</sup> cells were separated using AC133 cell isolation kit and a magnetic LS+ separation column according to the manufacturer's instructions. Briefly, the mononuclear cells were resuspended in 300 μl MACS buffer (PBS containing BSA, EDTA, pH 7.2) and pre-treated with FcR blocking reagent at 100 μl/10<sup>8</sup> cells. Cells reacted with AC133 MicroBeads and incubated for 30 min at 4 °C. After incubation, the cells were washed in MACS buffer. The pellet was resuspended in 500 μl MACS buffer and passed through the separation column. The column was washed four times with 3 ml MACS buffer in the magnetic field, and CD133<sup>+</sup> cells were retained in the column as the lineage negative cells were eluted. The column was removed from the magnet and the CD133<sup>+</sup> cells were collected and counted in Trypan blue.

### 2.4. Cell cultures

Purified CD133<sup>+</sup> cells were suspended at 5 × 10<sup>5</sup> cells/ml in StemSpan medium supplemented with 100 ng/ml rhSCF, 50 ng/ml rhIL-6, 1 ng/ml rhIL-3, 100 μg penicillin/

streptomycin per ml, and grown for 3 weeks after which IL-3 was omitted from the culture media. From week 6, 10% FCS was added, and mature PBMC were analysed in week 7. In order to evaluate whether culture time had implications for the phenotype and function of the cells,  $n=6$  batches of cells were cultured for 11 weeks and analysed in weeks 7, 9 and 11.

Cells were cultured in suspension for the entire period. Medium was totally renewed weekly. Cells were spun at 4 °C for 5 min, 450  $\times g$ , the culture medium gently aspirated and replaced by fresh medium. Weekly, cell cultures were stained with Trypan blue (0.5% in saline, Pharmacy, University Hospital of Aarhus, Denmark) to assess cell viability and with Alcian blue to assess the purity and numbers of mast cells by metachromasi (Gilbert and Ornstein, 1975).

Studies on the influence of cell density on mast cell phenotype and survival were carried out (data not shown). Four batches of CD133<sup>+</sup> cells were split and cultured at three different cell densities:  $5 \times 10^5$  cells/ml,  $7.5 \times 10^5$  cells/ml, and  $1 \times 10^6$  cells/ml. With increasing cell densities, cell phenotype was not affected with respect to surface receptor expression, protease content, total histamine content or histamine release, but the yield of mature cells decreased. Thus, in the present study progenitor cells were suspended in culture

medium at  $5 \times 10^5$  cells/ml and this cell density was maintained during the entire culture period.

## 2.5. Immunocytochemical staining

Immunostaining for intracellular stores of tryptase was performed using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method described by Craig et al. (1986). Cytospins were prepared by cytocentrifugation (250  $\times g$ ) using a Shandon Cytospin 3. The cytopins were air-dried, fixed in methanol/acetone solution, and were incubated overnight in a humidified chamber at 4 °C with mAb against tryptase. After washing with Tris buffered saline (TBS), the cytopins were incubated with rabbit anti-mouse IgG for 30 min at room temperature. After washing, the cytopins were incubated with the alkaline phosphatase mouse anti-alkaline phosphatase for 30 min. Negative controls were performed using isotype-matched mouse IgG1 instead of the primary antibody and by the T-cell line Jurkat.

Cell surface expression of FcεRI  $\alpha$ -chain was assessed by both flow cytometry and immunostaining as this receptor is essential for the use of cell cultures in allergen-mediated

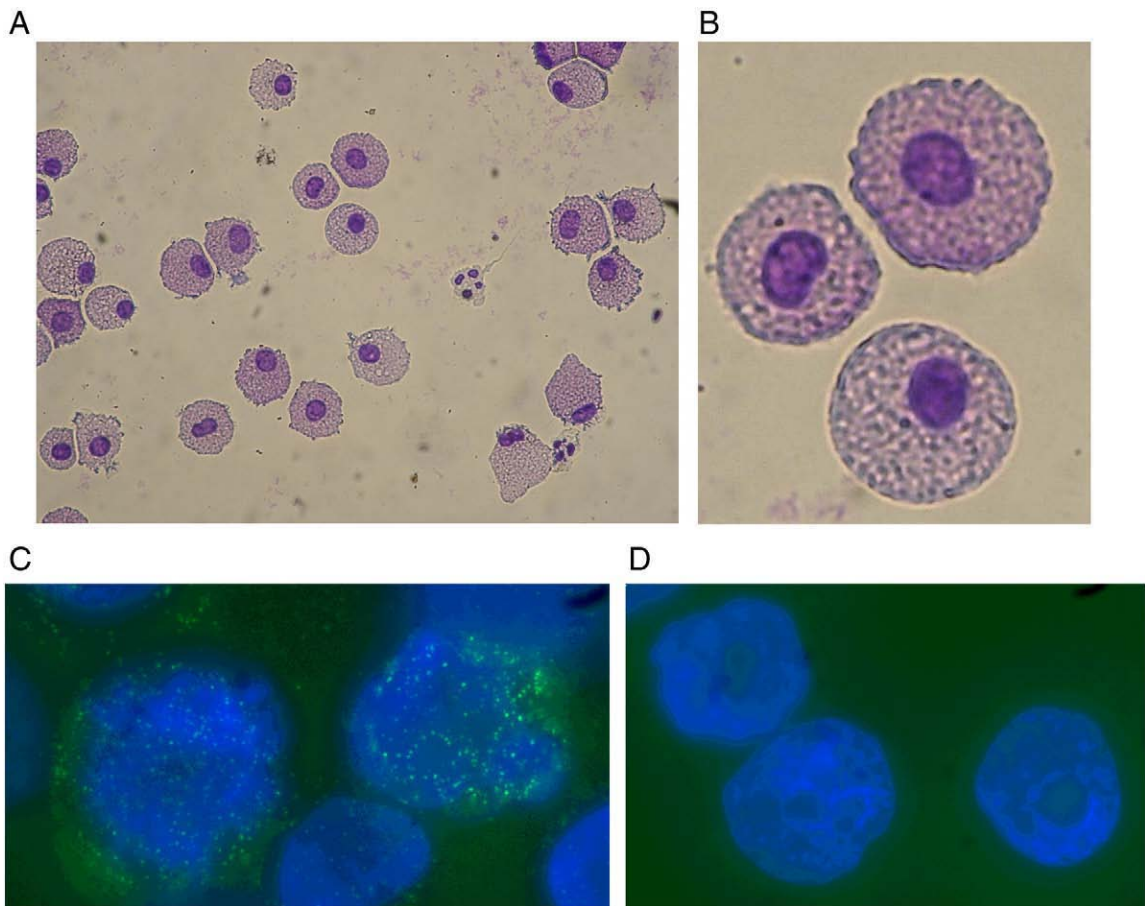


**Fig. 1.** CD133<sup>+</sup> peripheral blood-derived mast cell, 7 weeks. A and B. Human peripheral blood-derived mast cells (PBMC) cultured in serum-free StemSpan medium for 6 weeks and with 10% FCS for 1 week. Stem cell factor, IL-6, and IL-3 was added to the media but IL-3 was omitted after the first week of culture. Cells stained with May–Grünwald–Giemsa show contents of cytoplasmic granules. C. Cell surface expression of FcεRI  $\alpha$ -chain by immunostaining with CRA-1 (green) or D. An isotype control. Cells were counterstained with DAPI, 4',6-diamidino-2-phenylindole, which is a fluorescent stain that binds strongly to nuclear DNA (blue).

studies. Unsensitized cells were incubated with CRA-1 (0.1 µg) primary monoclonal antibody against the high affinity IgE receptor for 30 min at 4 °C and washed with PBS containing 0.1% HSA followed by incubation for 30 min at 4 °C with secondary FITC labelled antibody. Isotype control was performed using an isotype-matched IgG2b instead of CRA-1. Cells were counterstained with DAPI, 4',6-diamidino-2-phenylindole (Fig. 1C and D).

## 2.6. May–Grünwald–Giemsa staining

Cytospins were prepared by cytocentrifugation (250 ×g) using a Shandon Cytospin 3 and air-dried. They were incubated in May–Grünwald solution (Merck 1424, Darmstadt, Germany) for 4 min and washed in PBS (pH 6.4) for 4 min. After rinsing in water cytospins were stained in Giemsa (Merck 9204) diluted 1/10 in PBS, pH 6.4, for 8 min and rinsed in water. Cytospins air-dried and were seen under a light-microscope (Olympus, BX40 (Fig. 1A and B).

## 2.7. Flowcytometry

Surface expression of CD117, FcεRI, CD14, CD23, and CD203c was assessed by flow cytometry using a FACS Calibur (BD, San José, Ca, USA). Human cultured mast cells were sensitized with human myeloma IgE (2 µg/ml) for 24 h prior to the analysis. Cells were washed twice in PBS containing 0.1% HSA before labelling with antibody in dilution 1/10. Cells were incubated for 30 min at 4 °C with conjugated monoclonal receptor antibodies. The CRA-1 labelled cells, after the primary antibody incubation, were again incubated at 4 °C for 30 min with secondary FITC-conjugated anti-mouse IgG2b antibody after two washes in PBS with 0.1% HSA. Cell viability was assessed by exclusion of propidium iodide. Data were analysed with FCS-express. Overton Subtraction was used to calculate the net percentage of positive cells (Overton, 1988).

## 2.8. Mediator release from in vitro differentiated human mast cells

The presence of mast cells was assessed by assays for release of substantial amounts of histamine and PGD<sub>2</sub> in response to cross-linking of the FcεRI. PGD<sub>2</sub> is considered a characteristic marker for mast cells, whereas histamine is also released by basophils in less quantity.

Mature mast cells were incubated with human myeloma IgE (2 µg/ml) for 24 h at 37 °C in StemSpan medium supplied with 100 ng/ml rhSCF and 50 ng/ml rhIL-6. The sensitized mast cells were washed and 10<sup>4</sup> cells were resuspended (10<sup>5</sup> cells/ml) in 100 µl pipes buffer (10 mM Pipes, 150 mM Na-acetate, 5 mM K-acetate, 0.6 mM CaCl<sub>2</sub>, 1.1 mM MgCl<sub>2</sub>, glucose 1 mg/ml, human serum albumine 0.3 mg/ml, and heparin 15 IE/ml, pH 7.4) with rhSCF and rhIL-6. Cells were activated by incubation with 100 µl anti-IgE (Dako, Denmark, 5 µg/ml) for 30 min at 37 °C. Histamine release was stopped by the addition of 100 µl ice-cold buffer pipes buffer (10 mM Pipes, 150 mM Na-acetate, 5 mM K-acetate, 0.6 mM CaCl<sub>2</sub>, 1.1 mM MgCl<sub>2</sub>, pH 7.4) followed immediately by centrifugation (450 ×g) for 5 min at 4 °C. The cell pellets and supernatants (150 µl) were collected separately for the histamine assay. For

determination of histamine release, a microfiber-based method was used (Skov et al., 1985). The method is based on chemical extraction of histamine to ground glass micro-fibers and measures histamine by fluorescence of a histamine-*o*-phthaldehyde complex (Skov et al., 1985).

For the quantitation of total histamine 10<sup>5</sup> unstimulated mast cells were washed in PBS at 500 ×g for 5 min at 4 °C and resuspended in 100 µl PBS. To stabilize histamine perchloric acid was added (40 µl, 7%), and incubated at room temperature for 5 min before the solution was neutralized by the addition of 400 µl pipes buffer. The intracellular histamine content is determined by the above described method (Skov et al., 1985). The sensitivity of the assay is 0.63 ng histamine, 2 HCl.

Measurement of prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) from anti-IgE stimulated mast cells was measured using a PGD<sub>2</sub>-MOX-EIA kit (Cayman Chemicals, Ann Arbor, MI) according to manufacturer's instructions. The sensitivity of the assay was 6 pg/ml. The intra-assay coefficient of variation was less than 10%.

## 2.9. Analysis of chemokine secretion from in vitro differentiated human mast cells

Cells were sensitized for 3–5 days with rhIL-4, 10 ng/ml, prior to analysis of chemokine secretion. Media were renewed before stimulation in order to quantify constitutive as well as activation dependent chemokine secretion from human mast cells. Cells were incubated 24 h with human myeloma IgE (2 µg/ml) at 37 °C before the FcεRI were cross linked for a period of 2 h, 4 h, 8 h, or 24 h. The spent media were collected by centrifugation and accumulated chemokines were quantified using the Luminex® technology (Langouche et al., 2005) and a Bio-Plex® human cytokine assay (Bio-Rad, CA, USA) for simultaneous quantitation. A Bio-Rad cytokine assay (Breuel and De Ponti, 2006) was used for determination of human chemokines in media collected from stimulated mast cells. Beads were read on the Bio-Plex® suspension array system. Samples were read in duplicate. Sensitivity was 2 pg/ml and the intra-assay coefficient of variation varied from 4–13%.

## 2.10. Statistics

All results are expressed as mean±standard error of the mean values for *n* independent batches of mast cell cultures.

**Table 1**

Surface receptor expression of the high affinity IgE receptor (FcεRI), CD117, and CD203c on PBMC

| Receptor expression | Positive difference (%) | netMFI     |
|---------------------|-------------------------|------------|
| FcεRI               | 31.4±3.3                | 77.4±7.8   |
| CD117 (c-kit)       | 88.3±2.2                | 649.2±73.6 |
| CD203c              | 72.6±3.2                | 215.6±19.0 |

CD133<sup>+</sup> PBMC were cultured for 7 weeks with SCF, IL-6 and initially with IL-3. Cells preincubated for 24 h with myeloma IgE (2 µg/ml) before incubation with anti-FcεRIα (CRA-1) or an isotype control for 30 min, washing, and labelling with FITC-conjugated anti-IgG2b antibody. Expression of CD117/RPE, and CD203c/PE was assessed in unstimulated cells by primary conjugated antibodies compared to an isotype control. Flow cytometry was performed using a FACS Calibur. Data are presented as mean±SEM of MFI (mean fluorescence intensity) and percentage of positive cells calculated by Overton Subtraction.

Basic phenotypic and functional analyses such as immunostaining, surface receptor expression, and histamine release were performed on  $n=57$  batches of cell cultures. These data confirmed a high degree of reproducibility of the independent batches. Throughout this manuscript  $n=57$  unless otherwise indicated. In addition, we present data from  $n=9$  batches that have been extensively analysed for multiple phenotypic markers as well as for kinetics of chemokine gene activation. These batches are representative for the cells generated by our novel protocol.

### 3. Results

#### 3.1. Phenotypic analysis of *in vitro* differentiated human mast cells

Following Ficoll–Paque sedimentation and subsequent positive selection with antibodies specific for the AC133 antigen we harvested  $2.5 \pm 0.2 \times 10^6$  CD133<sup>+</sup> progenitors from a buffy coat preparation. Preliminary results showed an optimal

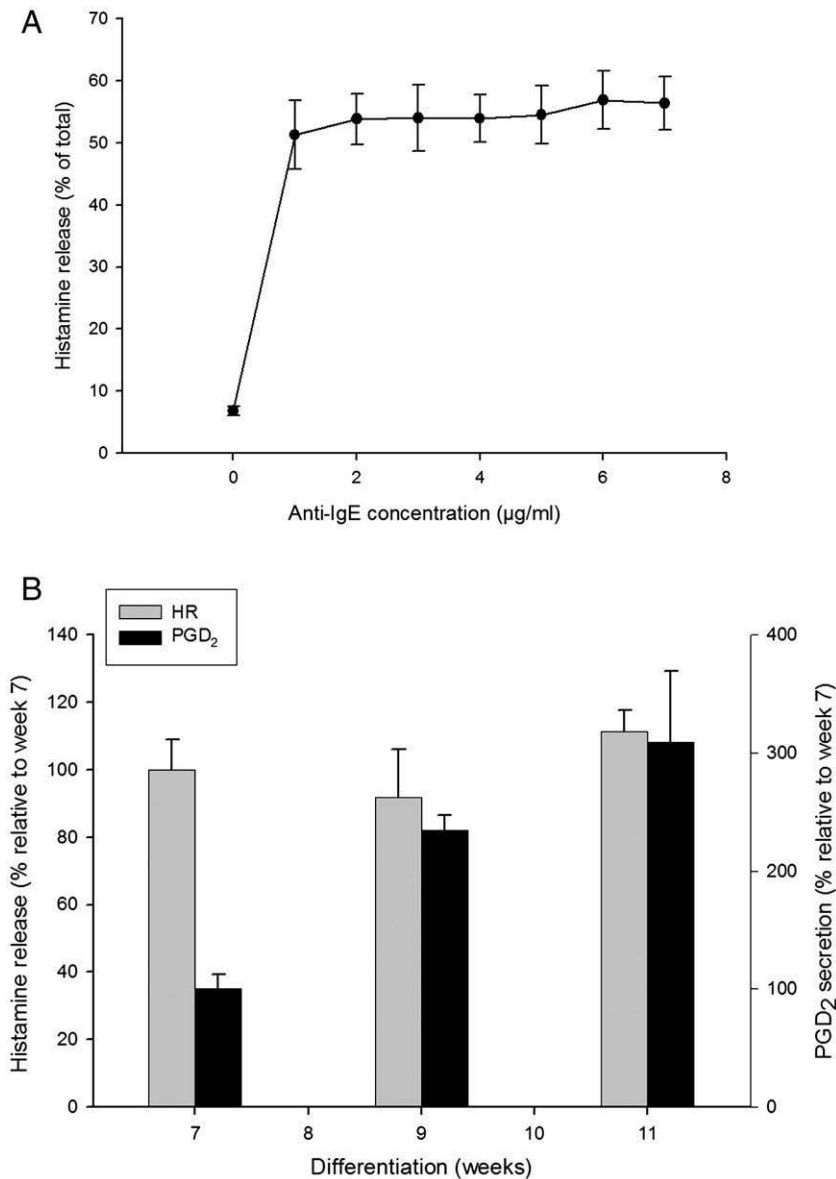


**Fig. 2.** IgE-dependent histamine release and secretion of PGD<sub>2</sub> from PBMC. A. CD133<sup>+</sup> PBMC cultured for 7 weeks were preincubated with myeloma IgE (2 µg/ml) before IgE receptor cross-linking for 30 min. with anti-IgE in increasing concentrations ((µg/ml, DAKO). IgE stimulated cells served as controls ( $n=6$ ). Histamine release was determined by a microfiber-based method and illustrated as % histamine released of total histamine per cell. B. Functional analyses of CD133<sup>+</sup> cells *in vitro* differentiated for 7, 9 or 11 weeks. Anti-IgE induced histamine release in % of total histamine in the cells, and histamine release from 9 to 11 weeks cells is illustrated relatively to histamine release from cells cultured for 7 weeks (left y-axis). Spontaneous histamine release induced by IgE alone was  $7.7 \pm 2.2\%$  in week 7;  $6.7 \pm 0.8\%$  in week 9;  $9.7 \pm 2\%$  in week 11. IgE-dependent secretion of PGD<sub>2</sub> was measured by PGD<sub>2</sub>-MOX-EIA kit (Cayman Chemicals). PGD<sub>2</sub> secretion from  $10^4$  cells is calculated in ng/ml, and expressed relatively to PGD<sub>2</sub> secretion from cells cultured for 7 weeks (right y-axis),  $n=6$ . Spontaneous secretion of PGD<sub>2</sub> induced by IgE alone was  $5.5 \pm 2\%$  in week 7;  $2.1 \pm 0.6\%$  in week 9;  $4.5 \pm 1.5\%$  in week 11.

culture period of 7 weeks and these cells were therefore extensively analysed for various surface markers and functionality. However, to elucidate mast cell phenotype after prolonged culture period cells were also analysed in weeks 9 and 11 for expression of FcεRI and for receptor-mediated release of histamine and PGD<sub>2</sub> ( $n=6$ ). The cells were *in vitro* differentiated with 100 ng/ml SCF and 50 ng/ml IL-6 as described in the Materials and methods section. During the final week, i.e. week 7, 9 or 11, FCS (10% v/v) was added to the culture media. After 7 weeks were generated  $8.2 \pm 1.2 \times 10^6$  cells (Fig. 1). Following metachromatic staining with Alcian blue 99.4±0.3% of the cells were positive in this simple and indicative test for a mast cell phenotypic marker.

The protease tryptase is a major mast cell characteristic. By immunostaining, 80.0±5.4% of the cells were tryptase positive after 7 weeks of culture and no significant changes were observed in weeks 9 and 11. High intracellular stores of histamine is another mast cell characteristics. The mean total histamine content of the peripheral blood-derived mast cells was  $15.5 \pm 5.3$  pg/cell.

The sensitized cells were also analysed by FACS for expression of a broad range of surface markers attributed to different lineages in the hematopoietic system. At 7 weeks of culture 88.3±2.2% of the cells were positive for CD117, 31.4±3.3% of the cells were FcεRIα positive (Table 1) and the activation marker CD203c was expressed on 72.6±3.2% of the cells. The density on the cells of the same receptors (mean fluorescent intensity or MFI) was 649.2±73.6 for CD117, 77.4±7.8 for FcεRIα, and 215.6±19.0 for CD203c, respectively. The cells were CD14 and CD23 negative, demonstrating that they are not monocytic. Collectively, these analyses indicate that the cultures are of mast cell lineage.

Six of the 57 cell batches were propagated for a total of 11 weeks, and the FcεRI expression was assessed on a single-cell

basis using immunofluorescence microscopy (Fig. 1C and D). Using this approach,  $50.8 \pm 9.2\%$  of the cells were FcεRI positive in week 7, declining to  $36.7 \pm 10.6\%$  in week 11. Staining was neither visible on mast cells stained with an isotype (Fig. 1C) nor on human Jurkat T cells stained with the FcεRI antibody. The apparent discrepancy between the estimated numbers of FcεRI positive cells in the FACS and the microscopical analyses in week 7 may be explained by the rather modest separation of the histograms generated from the isotype and FcεRI specific antibodies in the FACS analysis, which results in an underestimation of the size of the positive cell population. In fact, the proportion of FcεRI positive cells scored in microscopical analyses has consistently been larger than that obtained in the FACS analyses.

### 3.2. Mediator release from activated human mast cells

Mast cell functionality was assessed by quantification of histamine released from cell secretory granules into the medium, and concomitantly by generation of the significant mast cell arachidonic acid product, prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) after 7, 9, or 11 weeks of differentiation. Cells cultured for 7 weeks were incubated with 2 µg/ml human myeloma IgE for 24 h followed by anti-IgE stimulation for 30 min. Cells sensitized with IgE released  $1.3 \pm 0.1\%$  of the total histamine content whereas subsequent anti-IgE stimulation increased histamine release to  $53.5 \pm 3.0\%$  corresponding to the release of  $8.9 \pm 1.4$  pg histamine/cell. Thus, the cells release substantial amounts of their total histamine content within the range of that previously published for mast cells (Kinoshita et al., 1999; Kikuchi et al., 2002; Lappalainen et al., 2007). Mast cell functionality assessed by anti-IgE mediated histamine release was not altered by prolonged culture time (Fig. 2B, left y-axis). The IgE induced leak of PGD<sub>2</sub> was low ( $0.3 \pm 0.1$  ng PGD<sub>2</sub>/ml

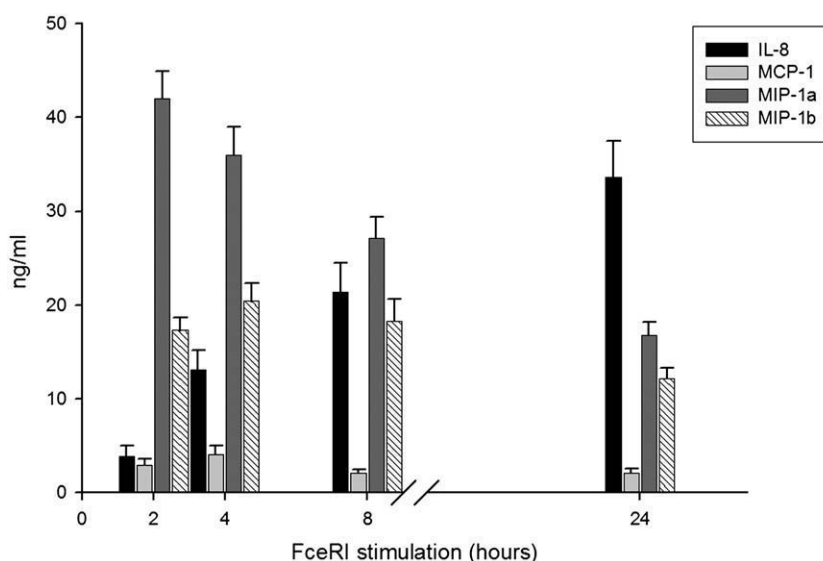


**Fig. 3.** PBMC release chemokines upon anti-IgE stimulation. CD133<sup>+</sup> PBMC cultured for 7 weeks were preincubated for 3–5 days with rhIL-4 (10 ng/ml) and for 24 h with myeloma IgE (2 µg/ml) before addition of anti-FcεRIα (CRA-1) for indicated times of 0, 2 h, 4 h, 8 h, or 24 h ( $10^6$  cells/ml). Culture media were collected after centrifugation of the cells and accumulated chemokines quantified using the Luminex® technology and a Bio-Plex® human cytokine assay for simultaneous quantitation. Beads were read on the Bio-Plex® suspension array system. Samples were read in duplicate. Spontaneous release of chemokines at 0 h was:  $0.5 \pm 0.3$  ng IL-8/ml,  $0.7 \pm 0.2$  ng MCP-1/ml,  $0.03 \pm 0.01$  ng MIP-1α/ml, and  $0.2 \pm 0.05$  ng MIP-1β/ml.

from  $10^4$  cells), and the spontaneous release was even below this level (data not shown). However, anti-IgE stimulation induced secretion of  $8.1 \pm 0.7$  ng PGD<sub>2</sub>/ml from  $10^4$  cells, and the PGD<sub>2</sub> secretion increased significantly from cells *in vitro* differentiated for 9 and 11 weeks (Fig. 2B right y-axis,  $p < 0.05$ ). This suggests an optimal phenotype of the mast cells in weeks 7 to 9.

### 3.3. Effect of mast cell activation on chemokine release

Mast cells are effector cells contributing to the inflammatory reaction through directly released mediators and widespread communication with other inflammatory cells. The experiments on chemokine secretion from mast cells are focused on 7 week cultures of cells.

We have previously shown that *in vitro* differentiated cord blood-derived mast cells produce chemokines in response to FcεRI activation (Holm et al., 2006). Here we assessed chemokine production in untreated cultures or following FcεRI activation for up to 24 h, the kinetics of which is displayed in Fig. 3. Whereas constitutive chemokine production was very low, significant quantities accumulated during activation. Thus, e.g. at 8 h after FcεRI cross-linking *in vitro* differentiated peripheral blood-derived mast cells ( $10^6$ /ml) produced:  $2.1 \pm 0.4$  ng MCP-1/ $10^6$  cells,  $21.4 \pm 3.1$  ng IL-8/ $10^6$  cells,  $27.1 \pm 2.3$  ng MIP-1α/ $10^6$  cells, and  $18.3 \pm 2.3$  ng MIP-1β/ $10^6$  cells ( $n = 7$ ).

Collectively, these data show that the *in vitro* differentiated cells derived from buffy coat preparations are functionally intact mast cells in a 7 to 9 week window but already from week 7 the cells are highly responding to activation via the high affinity receptor for IgE by releasing mediators involved in chemotaxis and activation of inflammatory cells in an inflammatory reaction.

## 4. Discussion

We have efficiently generated functional human mast cells within 7 weeks of *in vitro* differentiation of CD133<sup>+</sup> precursors isolated from buffy coat preparations. Expression of mast cell specific receptors, secretion of histamine and PGD<sub>2</sub> in response to FcεRI cross-linkage, and expression of tryptase all confirmed that these cells are mast cells. Previously published *in vitro* differentiation protocols (Wang et al., 2006b; Valent et al., 1992; Dahl et al., 2002) require long term cultures, up to 15 weeks, which makes cultivation of mast cells not only very laborious but also expensive and extremely vulnerable to infections. Advantages of our novel method are a) greatly reduced culture periods and b) rather easy accessibility to donors. Here, we also simplified existent protocols (Wang et al., 2006b; Iida et al., 2001; Saito et al., 2006) by maintaining the cells in suspension during the entire culture period. However, the most remarkable advantage of the generated mast cells is their high level of immunological functionality compared to the cells generated by recently published protocols (Lappalainen et al., 2007; Yamaguchi et al., 2007). Lappalainen et al. (2007) report an anti-IgE mediated histamine release of about 35% ( $n = 6$ ) of total histamine compared to 53% ( $n = 57$ ) in this study. This illustrates the impact of culture conditions on mast cell phenotype and the various factors influencing the outcome of

cells (i.e. culture time, serum-supplemented cultures) are commented on in this discussion.

Mature mast cells are generated in only 7 weeks by this protocol. However, to address whether mast cell phenotype or functionality alters with increasing culture time, we further analysed the cells after 9 and 11 weeks of differentiation ( $n = 6$ ). We found an optimal mast cell phenotype after 7 to 9 weeks of *in vitro* differentiation as FcεRI expression decreased with prolonged culture period. However, the altered FcεRI expression had no impact on mast cell functionality assessed by histamine release suggesting a low receptor threshold for induction of an anti-IgE mediated response in mast cells. This is further illustrated by the rapid dose response in anti-IgE mediated histamine release shown in Fig. 2A.

Previously, we (Dahl et al., 2002) and others (Kinoshita et al., 1999; Ahn et al., 2000) have shown that human mast cells proliferate *in vitro* for a longer period of time in serum-free conditions than in fetal calf serum-supplemented conditions, but for maximal differentiation of *in vitro* generated mast cells FCS is required. Therefore, the addition of FCS and the selection of an optimal time point for the addition are crucial for reducing culture time but yet maintaining an adequate yield of highly functional mature mast cells. Lappalainen et al. (2007) present a protocol using serum-free culture conditions.

In the present study a final cell number of  $8.2 \pm 1.2 \times 10^6$  mature mast cells were generated from one buffy coat preparation of 50 ml without cytokine priming of the donor. This is comparable with the yield of cells reported by Iida et al. (2001) from 12–14 weeks cultures of peripheral blood progenitor cells, but inferior to the yield reported in more recent protocols (Wang et al., 2006b; Lappalainen et al., 2007) where  $2.0 \pm 1.0 \times 10^7$  mast cells were cultured in 9 to 12 weeks. In our study prolonged culture time had no significant effect on the yield. Thus, the yield of mature mast cells is dependent on differences in culture conditions other than culture time. Characteristically, mast cells contain histamine which is released by degranulation. The mean intracellular histamine level in the cultured cells was  $15.5 \pm 5.3$  pg/cell. Comparable levels of total histamine ( $15.5 \pm 3$  pg/cell) were reported in mast cells also cultured with SCF 100 ng/ml and IL-6 50 ng/ml (Kinoshita et al., 1999).

Sensitized, resting cells released low levels of the arachidonic acid product PGD<sub>2</sub> with significant induction following cross-linkage of FcεRI (5 μg/ml,  $8.1 \pm 0.7$  ng PGD<sub>2</sub>/ml/ $10^4$  cells). Compared hereto long term cultured peripheral blood-derived mast cells are reported to secrete PGD<sub>2</sub> in a nine-fold reduced level upon activation (Wang et al., 2006a). This reduced level of PGD<sub>2</sub> secretion is within the range of that previously found in cord blood-derived mast cells ( $2.4 \pm 0.3$  ng PGD<sub>2</sub>/ $10^4$  cells,  $n = 14$ , unpublished), (Obata et al., 1996). Thus, short term culture generates functionally intact human mast cells with a remarkable capability of PGD<sub>2</sub> secretion. This functional difference may be related to decreased cell surface expression of FcεRI on long term differentiated mast cells but no data on receptor expression was reported.

Tryptase is the most specific mast cell marker (Sommerhoff, 2001). Whereas some mast cells express only tryptase (MC<sub>T</sub>), others express both chymase and tryptase (MC<sub>TC</sub>). Immunocytochemical staining studies showed that the peripheral blood-derived mast cells phenotypically

resemble the subtype of mast cells referred to as MC<sub>T</sub> that predominate in the human alveolar wall, the epithelium of the lung, and small intestinal mucosa (Forsythe and Ennis, 2000).

Following mast cell activation via the FcεRI, the transcriptional levels of several CC chemokines are markedly increased (Nakajima et al., 2002; Gonzalez-Espinosa et al., 2003). In accordance with these studies we previously demonstrated a significant augmentation in the accumulation of the chemokines MCP-1, MIP-1β, and IL-8 from cord blood-derived mast cells (Holm et al., 2006). Using our novel protocol we now demonstrate that mast cells generated from buffy coat progenitors also secrete IL-8, MIP-1α, and MIP-1β but in quantities far exceeding those from CBMC (Holm et al., 2006).

The amplitudes of FcεRI-mediated IL-8 and MIP-1α production from mast cells sensitized with IgE and IL-4 differ from that reported on 12 week cultures of PBMC activated under similar conditions (Wakahara et al., 2001). Thus, culture conditions appear to influence the level of chemokine production from PBMC but, importantly, the kinetics of FcεRI-mediated chemokine production is comparable in the studies (Wakahara et al., 2001). Kinetics of MCP-1 and MIP-1β are parallel as maximal accumulation of these chemokines occurs at 4 h of activation after which the accumulation reaches a steady-state level. To our knowledge, this is the first report on FcεRI-mediated kinetics of MCP-1 and MIP-1β in peripheral blood-derived mast cells.

In conclusion, we have developed a method to *in vitro* differentiate human mast cells derived from CD133<sup>+</sup> progenitors in buffy coat preparations, in a manner that considerably reduce time and consequently reduce labour time, and therefore also the risk of culture infections. This short term culture protocol will be useful for the evaluation of mast cell biology and effect of drugs on these cells. Moreover, as the method only requires an easily accessible blood sample it provides the possibility to study mast cells from individual mast cell populations and mast cell pathology in different groups of patients.

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