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## ***Ex vivo and in vitro Primary Mast Cells***

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## Summary

Mast cells are cells of the innate immunity whose biological responses are markedly modulated by effector molecules of adaptive immunity *i.e.* antibodies. They thus contribute to anti-infectious defense, but also to antibody-dependent inflammatory responses. They are especially well known as inducers of the allergic reaction. They are widely distributed in most tissues, but in low numbers. They are not readily purified, and with a poor yield. For these reasons, means to generate large numbers of homogenous non-transformed mast cells have been developed. We describe here 1) fractionation methods suitable for purifying mouse or rat peritoneal mast cells and for purifying human mast cells of various origins, and 2) conditions for generating pure cultured mast cell populations from mouse, rat and human tissues.

## Key-words

Mouse, rat, human, mast cells, purification, culture

## 1. INTRODUCTION

Like many other cells of the myeloid lineage, mast cells stand at the interface between innate and adaptive immunity. They contribute to anti-infectious defense when pathogens interact directly with specific receptors [1] or indirectly through the binding of bacterial products to Toll-like receptors (TLRs) [2, 3]. They initiate the allergic reaction when their high-affinity IgE receptors (FcεRI) [4], which are constitutively occupied by IgE antibodies, are aggregated by a multivalent allergen [5]. They also initiate IgG-dependent inflammatory responses when immune complexes aggregate their low-affinity IgG receptors (FcγRIIIA in mice) in murine models of autoimmune diseases [6]. Mast cell biology has therefore been actively investigated, in mice, rats and humans.

Mast cells are widely distributed among tissues, in virtually all organs [7]. In all cases, however, mast cells represent a minor population. Sources of homogenous mast cells have therefore been searched for and used for experimental studies. Methods based on differential sedimentation in high-density medium have proved useful to obtain reasonably pure *ex vivo* mature tissue mast cells [8-10]. One such method is described here. The major limitation of these techniques is their poor yield, making biochemical analyses difficult. For this reason, transformed mast cell lines of various origins have been extensively used. The most popular has been the Rat Basophilic Leukemia (RBL) cell line, which was understood to be a mucosal-type immature rat mast cell line [11, 12]. RBL cells have been invaluable for the biochemical characterization and the cloning of FcεRI subunits [13], as well as the dissection of proximal signaling following FcεRI aggregation [14]. RBL has also been widely used for transfection of cDNAs and for the mutagenesis analysis of the functional properties of a variety of membrane receptors. A few mouse and human tumor mast cell lines have been described, often lacking FcεRI [15-20]. In any case, tumor mast cells are not suitable for addressing issues such as differentiation and proliferation. Cultured mast cells, derived from various sources of precursor cells permitted such studies. Hematopoietic stem cell-derived Mast Cells from mice (mBMHC) [21], rats (rBMHC) [22] or from humans (HMC) [23] have been instrumental for

analyzing growth factor requirements and intracellular signaling leading to mast cell proliferation and activation. Considerable numbers of pure primary mouse mast cells can indeed be generated and kept in culture for several months, enabling *in vitro* biochemical analyses and *in vivo* studies, especially by reconstituting mast cell-deficient mice with cultured mast cells derived from genetically modified mice [24].

## **2. MATERIALS**

### **2.1. Mouse mast cells**

#### ***2.1.1. Ex vivo mouse mast cells***

1. 6-9 week-old mice
2. Metrizamide
3. Tris-A-EDTA buffer containing 0.025 M Tris-HCl, 0.12 M NaCl, 0.005 M KCl, 0.01 M EDTA and 0.5 mg/mL human serum albumin
4. siliconized 10 mL glass tubes
5. Toluidine blue solution containing 0.5% toluidine blue, 30% ethanol pH 1

#### ***2.1.2. Cultured mouse mast cells***

##### ***2.1.2.1. Mouse Bone Marrow-derived Mast Cells (mBMMC)***

1. 6-9 week-old mice
2. RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 IU/mL) and streptomycin (100 µg/mL) (complete RPMI)
3. Recombinant mouse IL-3<sup>1</sup>
4. Wright-Giemsa stain

5. Mota's fixative prepared as follows: add 4 g of lead acetate (basic) to 50 mL of distilled deionized water; stir at slow speed and add 2-4 mL of glacial acetic acid to dissolve the lead acetate and make the solution clear; add 50 mL of absolute ethanol; keep tightly closed and store at room temperature; prepare fresh every 1-2 month.

## **2.2. Rat mast cells**

### ***2.2.1. Ex vivo rat mast cells***

1. 10-15 week-old rats
2. Metrizamide
3. Tris-A-EDTA buffer containing 0.025 M Tris-HCl, 0.12 M NaCl, 0.005 M KCl, 0.01 M EDTA and 0.5 mg/mL human serum albumin
4. siliconized 10 mL glass tubes
5. Toluidine blue solution containing 0.5% toluidine blue, 30% ethanol pH 1

### ***2.2.2. Cultured rat mast cells***

1. 10-15 week-old rats
2. Iscove's modified dulbecco's medium supplemented with  $5 \times 10^{-5}$ M 2-Mercaptoethanol, penicillin (100 IU/mL), streptomycin (100  $\mu$ g/mL), 20% heat-inactivated fetal calf serum serum and 2-10 ng/mL of recombinant rat IL-3 (rRIL-3)<sup>2</sup> (complete IMDM)
3. Hayem's solution

## **2.3. Human mast cells**

### ***2.3.1. Ex vivo human mast cells***

1. Tyrode buffer containing 137 mM NaCl, 2.7 mM KCl, 0.36 mM Na<sub>2</sub>HPO<sub>4</sub> and 5.55 mM glucose
2. TE buffer : Tyrode buffer containing 2 mM EDTA

3. Hepes buffer contains 20 mM Hepes, 125 mM NaCl, 5 mM KCl and 0.5 mM glucose
4. HA buffer : Hepes Buffer plus 0.25 mg/mL BSA
5. Phosphate Buffered Saline (PBS) (without MgCl<sub>2</sub>/CaCl<sub>2</sub>)
6. Turk's staining solution contains 0.01 % crystal violet in 3 % acetic acid
7. Percoll gradients
8. Macs C1 column

### **2.3.2. Cultured human mast cells derived from hematopoietic progenitors**

1. Serum-free mast cell culture medium (SFCM): Iscove modified dulbecco's medium supplemented with L-glutamine ( $2 \times 10^{-3}$  mol/L), penicillin (100 IU/L), streptomycin (100 µg/L),  $7.5 \times 10^{-5}$  mol β-mercaptoethanol, bovine serum albumin ( $2 \times 10^{-4}$  mol/L), iron-saturated human transferrin ( $5 \times 10^{-7}$  mol/L), insulin ( $1.7 \times 10^{-6}$  mol/L), 80 ng/mL recombinant human stem cell factor (rhSCF)<sup>3</sup>, 50 ng/mL recombinant human Interleukin-6 (rhIL-6) and 2 ng/mL recombinant human Interleukin-3 (rhIL-3; first week only).
2. Magnetic cell separator MiniMACS, MidiMACS, VarioMACS or SuperMACS.
3. MACS column(s) type MS+/RS+, LS+/VS+ or XS+ (plus RS+, VS+ or XS+ column adapter).
4. Pre-Separation filters.
5. Ficoll-Paque® (d:1.077 g/mL) and phosphate buffered saline (PBS) supplemented with 2 mM EDTA or 0.6 % anticoagulants citrate dextrose-formula A (6 % ACD-A: 22.3 g/L glucose, 22 g/L sodium citrate and 8 g/L citric acid in H<sub>2</sub>O).
6. Buffer: PBS pH 7.2, supplemented with 0.5 % bovine serum albumin and 2 mM EDTA or 0.6 % ACD-A. Degas buffer by applying vacuum.
7. FcR blocking reagent
8. CD34 microbeads
9. Fluorochrome-conjugated CD34 antibody (e.g. CD34-PE) and fluorochrome-conjugated CD45 antibody (e.g. CD45-FITC) for control of CD34 progenitor cell isolation.

10. PBS containing 0,1% human AB serum

11. PBS containing 0.1% NaN<sub>3</sub> and 2% paraformaldehyde

### **3. METHODS**

#### **3.1. Mouse mast cells**

##### ***3.1.1. Ex vivo mouse mast cells***

Mouse mast cells can be purified from cells recovered by peritoneal washing, among which they represent 3-4% of total cells.

1. Lethally anesthetized 6-9 week-old mice are injected intraperitoneally with 2 mL Tris-A-EDTA buffer. Following careful massage of the abdomen, the abdominal cavity is opened and the fluid is recovered with a Pasteur pipette.
2. Cells are washed once and resuspended at  $1 \times 10^7$ /mL in the same buffer at room temperature.
3. Aliquots of 2 mL peritoneal cell suspension are gently layered over 1.5 mL of 22.5% metrizamide dissolved in Tris-A-EDTA buffer, in siliconized tubes, and centrifuged at 270 g for 10 min at room temperature. Most cells sediment at the interface above metrizamide, while mast cells sediment at the bottom of the tube.
4. The upper layer, the interface (containing cells) and most of the metrizamide solution are VERY carefully discarded, leaving 50-100  $\mu$ L above the small mast cell pellet<sup>4</sup>. Pelleted cells are gently resuspended and transferred into new tubes.
5. Under these conditions,  $1 \times 10^5$  mast cells (>95% pure) can be recovered per mouse.

##### ***3.1.2. Cultured mouse mast cells***

### *3.1.2.1. Mouse Bone Marrow-derived Mast Cells (mBMMC)*

Bone marrow (BM) cells are harvested from the femurs of 6-9 week-old mice.

1. After lethal anesthesia of the animals, femurs are carefully dissected to eliminate any muscle fragment.
2. One extremity is cut with sterile scissors, and the needle of a 10-mL syringe, previously filled with sterile RPMI is inserted in the medullar canal. Bone marrow cells are flushed out and collected in a sterile 15-mL tube.
3. After sedimentation of aggregates, cells in suspension are collected in another 15-mL tube, centrifuged, and the equivalent of cells from two femurs are resuspended in 60 mL complete OptiMEM supplemented with 1ng/ml recombinant murine IL-3.
4. The cell suspension is transferred into culture flasks and placed at 37°C in a humidified CO<sub>2</sub> incubator.
5. Every 3-4 days (i.e. twice a week), non-adherent cells are centrifuged, resuspended at 3x10<sup>5</sup> viable nucleated cells/mL in fresh IL-3-containing culture medium, and placed in new culture flasks.
6. After 3 weeks, cultures consist of virtually pure (>90%) FcεRI<sup>+</sup>, Kit<sup>+</sup> mast cells as judged by staining and immunofluorescence.
7. mBMMC thus obtained keep growing in IL-3 containing medium for at least 2-3 months<sup>5,6</sup>.

### *3.1.3. Identifying and phenotyping mouse mast cells*

#### *3.1.3.1. Wright-Giemsa staining*

1. Cells are resuspended at 2x10<sup>5</sup> cells/mL in RPMI.
2. One hundred μL suspension are placed in cytopsin chambers, mounted onto clean slides. Slides are spun at 14 g for 5 min.

3. They are air-dried and stained with Wright-Giemsa before they are mounted in 1-2 drops Permout under a cover slip.

### *3.1.3.2. Toluidine Blue staining*

1. Cytospin slides prepared as above are fixed by adding several drops of Mota's fixative to cover the cells for 10 min. NB: Mota's evaporates quickly, so additional drops must be added once or twice to prevent crystal formation.
2. Fixative is removed by slowly running water down, not directly on cells, and slides are dried.
3. Cells are stained for 20 min with 2-3 drops of acidic toluidine blue.
4. Stain is removed by running water down the slide.
5. Slides are air dried and mounted in 1-2 drops Permout under a cover slip<sup>6</sup>.

### *3.1.3.3. Analysis of the Expression of FcεRI and Kit by Immunofluorescence*

1. Aliquots of  $5 \times 10^5$  cells are incubated in 50μL complete RPMI containing 10μg/mL mouse IgE or rat anti-mouse Kit mAb (ACK2) for 1 hr at 0°C in Eppendorf tubes.
2. They are washed twice in PBS and incubated for 30 min at 0°C in 50μL complete RPMI containing an appropriate dilution of F(ab')<sub>2</sub> fragments of FITC-labeled rat anti-mouse immunoglobulin (for mouse IgE) or FITC-labeled mouse anti-rat immunoglobulin (for ACK2). Negative controls are incubated with FITC-labeled secondary F(ab')<sub>2</sub> only.
3. Cells are washed once and resuspended in 500 μL PBS.
4. Fluorescence is analysed by flow cytometry.

## **3.2. Rat mast cells**

### **3.2.1. *Ex vivo rat mast cells***

The same procedure as that described for mouse mast cells can be applied to rat peritoneal cells. Due to the body weight of rats, mast cells can be obtained in relatively high numbers<sup>4</sup>.

### **3.2.2. *Cultured rat mast cells***

1. Bone marrow cells are obtained from outbred male Wistar rats, 10-15 week-old (weight 225-250 g) with the same method as for mouse mast bone marrow cells except for the following points.
2. Complete IMDM is used as culture medium;
3. Mononuclear cell counts are performed by dilution of the cell suspension (1:10) in Hayem's solution and numeration in a hemocytometer; cell suspension is therefore adjusted to  $2.5 \times 10^5$  viable nucleated BM cells/mL in complete IMDM.
4. A final concentration of  $2.5 \times 10^5$  viable nucleated BM cells/mL is suspended in complete Iscove medium in 175 cm<sup>2</sup> flasks that are kept at 37°C in a humidified incubator with 5% CO<sub>2</sub> in air.
5. These cultures are refed and restimulated with fresh complete medium containing recombinant rat IL-3 every 4-6 days.
6. Percentage of mast cells is determined by differential counts (400 cells) on Wright-Giemsa and toluidine blue stained cytocentrifuge preparations.
7. Rat BMCM proliferate rapidly initially, representing 85% of cells after approximately 15 days. After this, the percentage of BMCM increases slowly to almost 100% before cell viability declines around the fifth week<sup>7,8</sup>.

## **3.3. Human mast cells**

### **3.3.1. *Ex vivo human mast cells***

Mast cells with different phenotypes can be purified from various human tissues, including lung, intestine, uterus, skin or foreskin. The general protocol comprises a first step of enzymatic treatment of the desired tissue, in order to obtain a monocellular suspension. This enzymatic treatment is followed by a first step of purification by centrifugation over Percoll gradient and mast cells are further enriched by separation from contaminating cells using anti c-kit immunomagnetic beads <sup>9</sup>.

### 3.3.1.1. Tissue processing

Human mast cells are isolated under sterile conditions by a four-step enzymatic tissue dispersion method.

1. Macroscopically normal human tissue is obtained from surgical specimens.
2. The tissue is placed in TE buffer at 4°C immediately after resection until dissection is started.
3. If present (human intestine) the mucosa is separated mechanically from the submucosa/muscular layers. Always for intestine specimen, mucus is removed by incubation with acetylcysteine at 1 mg/mL, and epithelial cells are detached with 5 mM EDTA.
4. Whatever the nature of the specimen used, the tissue is enzymatically digested by a four-step incubation (each for 30 min) with four enzymes (3 mg/mL pronase corresponding to 21 U/mL, 0.75 mg/mL chymopapain corresponding to 0.39 U/mL, 1.5 mg/mL collagenaseD corresponding to 0.405 U/mL, and 0.15 mg/mL elastase corresponding to 15.75 U/mL). During the first digestion step, the tissue (mucosa, in case of intestinal specimen) is chopped finely with scissors. For the third and fourth digestion steps, the incubation buffer is supplemented with DNase at 15 µg/mL corresponding to 15 U/mL.
5. The cells freed after the last two digestion steps are separated from tissue fragments by filtration through a polyamide Nybolt filter (pore size, 300 µm), washed, pooled, and counted after staining with Turk's solution.
6. The viability of cells is measured by dye exclusion using trypan blue staining

### 3.3.1.2. *Enrichment of mast cells*

Cells are fractionated on discontinuous Percoll gradients and further enriched in mast cells by separation from contaminating cells using immunomagnetic beads.

1. Five mL of cell suspension resuspended in HA buffer are layered carefully over 20 mL of Percoll solution (density = 1.037 g/mL) in a 50 mL polypropylene tube.
2. After centrifugation (500 X g, 20°C, 15 min), the cell sediment is harvested, washed twice with HA buffer and counted.
3. For immunomagnetic separation, cells are resuspended in 1 mL of HA buffer containing 1 mg/mL albumin and 5  $\mu$ g/mL mAbYB5.B8 (directed against human c-kit) and incubated for 30 min at 4°C with gentle rolling.
4. The cells are then washed in HA buffer and resuspended in 500  $\mu$ L of HA buffer containing 1 mg/mL albumin.
5. Finally, the cell suspension is incubated with a goat-anti mouse IgG Ab coupled to paramagnetic beads for 30 min at 4°C. During incubation the tubes are gently rolled.
6. After washing in HA buffer, mast cells are enriched by magnetic separation of the cells using a MACS C1 column placed in magnetic field.
7. After separation, cells are counted, and washed in culture medium without antibiotics.

### 3.3.2. *Cultured human mast cells derived from hematopoietic progenitors*

Pure populations of human mast cells (HMC) are obtained by long-term culture of human normal hematopoietic progenitors (CD34+ cells) in the continuous presence of Stem Cell Factor (SCF). Sources of these CD34+ cells are mainly bone marrow, cord blood or peripheral blood. Usually, mononuclear cells from bone marrow and cord blood contain 0.5-1% of CD34+ cells, whereas peripheral blood contains less than 0.1% of CD34+ cells. Hematopoietic progenitor cells can be rapidly and efficiently enriched to a purity of about 85-98 % using positive selection of

magnetically labeled CD34+ cells. Mononuclear cells from peripheral blood (PBMC), cord blood, or bone marrow are obtained by density gradient centrifugation over Ficoll Paque®. For immunomagnetic separation, CD34+ hematopoietic progenitor cells are magnetically labeled using MACS CD34 MicroBeads. The magnetically labeled cells are enriched on positive selection columns in the magnetic field <sup>10</sup>.

### *3.2.3.1. Purification of CD34 positive progenitors from human cord blood, bone marrow or peripheral blood cells*

#### 3.2.3.1.1. Sampling of Cord Blood Cells

1. Dilute anticoagulated (preservative-free heparin sodium, 1.0 mL for 50 mL of sample) cord blood 1:4 with PBS.

#### 3.2.3.1.2. Sampling of Bone Marrow Cells

1. Collect bone marrow in 50 mL tubes containing 5 mL PBS containing 2 mM EDTA or 0.6 % ACD-A or 200 U/mL heparin and store at 4°C if the cells cannot be processed immediately.
2. For release of the cells from aggregates, dilute in 10x excess of RPMI 1640 medium containing 0.02 % collagenase B and 100 U/mL DNase and shake gently at room temperature for 45 min.
3. Pass cells through 30 µm nylon mesh or filter. Wet filter with buffer before use.

#### 3.2.3.1.3. Sampling of Peripheral Blood Cells

1. Start with fresh human blood treated with an anticoagulant, e.g. heparin, citrate, ACD-A or citrate phosphate dextrose (CPD) or leukocyte-rich buffy coat not older than 8 hrs. Dilute cells with 2-4 volumes of PBS containing 2 mM EDTA or 0.6 % ACD-A.
2. Obtention of a mononuclear cell suspension: Carefully layer 35 mL of diluted cell suspension over 15 mL of Ficoll-Paque®. Centrifuge for 35 min at 400xg at 20°C in a

swinging-bucket rotor (without brake). Aspirate the upper layer leaving the mononuclear cell layer undisturbed at the interphase. Carefully collect interphase cells and wash twice in PBS containing 2 mM EDTA or 0.6 % ACD-A. Centrifuge for 10 min at 300xg at 20°C. Resuspend cell pellet in a minimal volume of buffer. Mononuclear cell counts are performed by dilution of the cell suspension (1:100) in Hayem's solution and numeration in a hemocytometer. Adjust the cell suspension at 300  $\mu$ l of buffer per  $10^8$  total cells. For less than  $10^8$  total cells, use 300  $\mu$ l. Proceed to magnetic labeling.

3. Magnetic Labeling of CD34+ Progenitor Cells: Add 100 $\mu$ l FcR Blocking Reagent per  $10^8$  total cells to the cell suspension to inhibit unspecific or Fc-receptor mediated binding of CD34 MicroBeads to non-target cells. Label cells by adding 100  $\mu$ l CD34 MicroBeads per  $10^8$  **total** cells, mix well and incubate for 30 min at 6°-12°C. Wash cells carefully and resuspend in appropriate amount of buffer (MS+/RS+ column: 500-1000  $\mu$ l; LS+/VS+ column: 1–10 mL, max.  $2 \times 10^8$  cells per mL). Proceed to magnetic separation.
4. Magnetic Separation of  $<2 \times 10^9$  Mononuclear Cells: Choose a column type (MS+/RS+ or LS+/VS+) according to the number of total unseparated cells and place it (with column adapter) in the magnetic field of the MACS separator. Fill and rinse with buffer (MS+/RS+: 500  $\mu$ l; LS+/VS+: 3 mL). Pass mononuclear cells through 30  $\mu$ m nylon mesh or Pre-Separation Filter to remove clumps. Wet filter with buffer before use. Apply cells to the column, allow cells to pass through the column and wash with buffer (MS+/RS+:  $3 \times 500$   $\mu$ l; LS+/VS+:  $3 \times 3$  mL). Remove column from separator, place column on a suitable tube and pipette buffer on top of column (MS+/RS+: 1 mL; LS+/VS+: 5 mL). Firmly flush out retained cells with pressure using the plunger supplied with the column. Repeat magnetic separation step: apply the eluted cells to a new prefilled positive selection column (for  $<10^7$  CD34+ cells: MS+/RS+; for  $<10^8$  CD34+ cells: LS+/VS+), wash, and elute retained cells in buffer (MS+/RS+: 500  $\mu$ l; LS+/VS+: 2.5 mL).
5. Magnetic Separation of  $2 \times 10^9$ - $2 \times 10^{10}$  Mononuclear Cells: Assemble XS+ column and place

it in the column holder of the SuperMACS using XS+ column adapter. Turn 3-way-stopcock to position “fill”. Fill the column from the bottom with buffer from the syringe until the buffer reaches the syringe cylinder. Turn the 3-way-stopcock to position “run” and rinse column by filling from the top with buffer. Allow buffer to run into the column. Then, add more buffer. Rinse with 50 mL of buffer. Close 3-way-stopcock; leave the syringe attached during separation, except when refilling with buffer. Move column in the magnetic field of the SuperMACS by turning the handle. Pass cells through 30  $\mu$ m nylon mesh or filter to remove cell clumps. Apply cells into the syringe cylinder that is set up on the XS+ column and turn 3-way-stopcock to position “run”. Allow the cells to pass through the column. Remove flow resistor and wash with 4x30 mL buffer. Close 3-way-stopcock and remove column out of the magnetic field of the SuperMACS by turning the XS+ adapter handle backward. Detach syringe from the 3-way-stopcock, fill with buffer and attach to port A of the XS+ column. Elute retained cells with 20 mL buffer using the syringe. Repeat magnetic separation step: apply the eluted cells to a new prefilled XS+ column or VS+ column, wash, and elute retained cells in buffer.

6. Evaluation of Hematopoietic Progenitor Cell Purity: The purity of the isolated hematopoietic progenitor cells can be evaluated by flow cytometry or fluorescence microscopy. Fluorescent staining of CD34+ cells can be accomplished by direct immunofluorescent staining using an antibody recognizing an epitope different from that recognized by the CD34 monoclonal antibody QBEND/10 (e.g. CD34-PE). For optimal discrimination of CD34+ cells from other leukocytes, counterstain cells with an antibody against CD45 (e.g. CD45-FITC). CD34+ cells express CD45 at a lower level as compared to lymphocytes. Use the antibodies in appropriate concentrations recommended by the manufacturers. Typically, staining for 5 min at 6°-12°C should be sufficient. After fluorescence staining, cells should be washed and resuspended in buffer.

### 3.2.3.2. Culture conditions

1. CD34 positive cells are cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> at a starting density of 1 x 10<sup>5</sup> cells/mL in SFCEM.
2. CD34<sup>+</sup> cells may initially proliferate 100 times or more the starting number of cells if rhSCF is combined with rhIL-3 and rhIL-6. Luxurious growth is observed during the first 2-3 wk, although debris will begin to accumulate because of non-mast cell lineage apoptosis and necrosis.
3. Adherent macrophages also may start increasing in number by 2 wk. Check cultures weekly and separate nonadherent mast cell-committed progenitors from adherent cells and debris. Gently pipette and remove nonadherent cells to a new flask or centrifuge nonadherent cells and supernatant at 150g for 5 min, resuspend cells in complete media with growth factors every 5 to 7 days.
4. The number and percentage of mast cells (MC) in the cultures are assessed weekly using Wright-Giemsa and acidic toluidine blue (see mouse mast cell culture section) staining of cytocentrifuged samples. Usually, 10<sup>6</sup> CD34<sup>+</sup> cells at 8-10 wk may give rise to 5-15 x10<sup>7</sup> HHPMC with less than 5% contamination with other cell types.
5. Immunostaining for tryptase can be also used to further identify mast cells in the cultures. Briefly, cytocentrifuged preparations are fixed in cold acetone for 30 min, air-dried, then stored at -20°C. Fixed samples are then treated with mouse IgG1 anti-tryptase monoclonal antibodies, and finally submitted to a Fast-Red immunostaining.
6. After >10 weeks in culture, more than 95 % of the cells are identified as mast cells by their morphological features, the presence of metachromatic granules and their positivity for tryptase. These cells can then used for subsequent experiments as pure normal HMC <sup>11 12</sup>.
7. Since CD34 positive-derived mast cells usually do not express spontaneously a significant amount of FcεRI at their membrane, they can be incubated for 5 additional days with 2-10 μg/mL of human myeloma IgE and 20 ng/mL of recombinant human IL-4 (rhIL-4) in order to enhance the expression of FcεRI on their membrane.

### 3.2.3.3. Phenotyping human mast cells for Kit and FcεRI expression

1. For flow cytometric analysis,  $5 \times 10^5$  cells are incubated for 60 min at 4°C with the monoclonal receptor antibodies (mouse IgG against the  $\alpha$ -chain of the high-affinity IgE receptor (FcεRI) or against Kit (YB5.B8)) in 50  $\mu$ l phosphate-buffered saline (PBS), containing 0.1% human AB serum.
2. After two washes with PBS, cells are incubated for 45 min at 4°C with a 1:20 dilution of anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC).
3. Following two washes, cells are suspended in 400  $\mu$ l of PBS containing 0.1% NaN<sub>3</sub>, fixed with 2% paraformaldehyde solution, and analyzed on an EPICS Profile flow cytometer (Coulter, Krefeld, Germany).
4. Negative controls are done in the absence of antibody, with IgG and isotype-matched desmin antibody D33 (DAKO).
5. For flow cytometric analysis, Elite Workstation software (Coulter) is used.

## 4. NOTES

1. Recombinant IL-3 can be replaced by 4-20% conditioned medium made of culture supernatant of cells stably transfected with cDNA encoding IL-3.
2. Recombinant rat IL-3 (rRIL-3) can be replaced by 5-20% conditioned medium made of culture supernatant of rat mononuclear splenocytes ( $10^6$  cells/mL) stimulated with 1mg/L Concanavaline A for 5-7 days.
3. Recombinant human SCF can be replaced by 5-20% conditioned medium made of culture supernatant of cells stably transfected with cDNA encoding rhSCF.
4. Because mast cells are a minor population amongst peritoneal cells, even a minimal contamination will drastically alter mast cell purity. For this reason, all cells at the interface

MUST be discarded. This is why siliconized tubes are used. Carefully wiping tube walls before collecting mast cell pellets may also improve purity.

5. Bone marrow cells can be kept frozen in liquid nitrogen before they are cultured. When frozen, mBMMC quickly die upon thawing.
6. Mast cell numbers are calculated by determining the percentage of acidic toluidine blue positive cells out of total Wright-Giemsa-positive cells. Toluidine blue should stain mast cells red-purple (metachromatic staining) and the background blue (orthochromatic staining). Metachromasia, tissue elements staining a different color from the dye solution, is due to the pH, dye concentration and temperature of the basic dye. Blue or violet dyes will show a red color shift, and red dyes will show a yellow color shift with metachromatic tissue elements.
7. Rat bone marrow cells can be kept frozen in liquid nitrogen before they are cultured, but it is impossible to keep rBMMC frozen.
8. Mast cell numbers are calculated exactly as described above for mBMMC cultures.
9. Isolated human mast cells cannot be frozen using classical techniques
10. Once purified, CD34+ cells can be kept frozen in liquid nitrogen before they are cultured, whereas it is impossible to keep mature HMC frozen.
11. Isolated human MC can be maintained in culture for up to 3 months in the continuous presence of recombinant human SCF. MC purity increases during culture and reached nearly 100%. During the first week of culture, MC numbers decrease, but after that time they start to proliferate. Cultured MC do not change their histamine content, phenotype or morphology. They are even more responsive towards IgE-dependent stimulation, which causes the release of high amounts of histamine, leukotrienes and cytokines such as TNF-alpha and IL-5.

12. Once pure, HMC cultures stop growing at 8-10 weeks. They nevertheless keep alive without any significant changes in their phenotype for another 2-5 months provided that they are maintained in SCF-containing medium.

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