

Ex Vivo and In Vitro Primary Mast Cells

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Summary

Mast cells are cells of the innate immune system 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 allergic reactions. 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 immunoglobulin (Ig)E 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

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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 permit such studies. Mouse (21) or rat (22) bone marrow-derived mast cells (BMMCs) and human mast cells (HMCs) derived from hematopoietic stem cells (23) have been instrumental in 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: 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: 0.5% toluidine blue and 30% ethanol, pH 1.

2.1.2. Cultured Mouse Mast Cells

2.1.2.1. MOUSE BMMCs

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 interleukin (IL)-3 (*see Note 1*).
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: 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 and 30% ethanol, pH 1.

2.2.2. Cultured Rat Mast Cells

1. 10–15 week-old rats.
2. Iscove's modified Dulbecco's medium (IMDM) supplemented with 5×10^{-5} M 2-mercaptoethanol, penicillin (100 IU/ml), streptomycin (100 µg/ml), 20% heat-inactivated fetal calf serum, and 2–10 ng/ml of recombinant rat IL-3 (rRIL-3) (*see Note 2*) (complete IMDM).
3. Hayem's solution.

2.3. HMCs

2.3.1. *Ex Vivo* HMCs

1. Tyrode buffer: 137 mM NaCl, 2.7 mM KCl, 0.36 mM Na₂HPO₄, and 5.55 mM glucose.
2. TE buffer: Tyrode buffer containing 2 mM EDTA.
3. HEPES buffer: 20 mM HEPES, 125 mM NaCl, 5 mM KCl, and 0.5 mM glucose.
4. HA buffer: HEPES buffer plus 0.25 mg/ml bovine serum albumin (BSA).
5. Phosphate-buffered saline (PBS) (without MgCl₂/CaCl₂).
6. Turk's staining solution containing 0.01% crystal violet in 3% acetic acid.
7. Percoll gradients.
8. MACS C1 column.

2.3.2. Cultured HMCs Derived from Hematopoietic Progenitors

1. Serum-free mast cell culture medium (SFCM): IMDM supplemented with L-glutamine (2×10^{-3} mol/l), penicillin (100 IU/l), streptomycin (100 µg/l), 7.5×10^{-5} M β -mercaptoethanol, BSA (2×10^{-4} mol/l), iron-saturated human transferrin (5×10^{-7} mol/l), insulin (1.7×10^{-6} mol/l), 80 ng/ml recombinant human stem cell factor (rhSCF) (see **Note 3**), 50 ng/ml recombinant human IL-6 (rhIL-6), and 2 ng/ml 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 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 H₂O).
6. Buffer: PBS pH 7.2, supplemented with 0.5% BSA 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-phycoerythrin (CD34-PE)] and fluorochrome-conjugated CD45 antibody [e.g., CD45-fluorescein isothiocyanate (CD45-FITC)] for control of CD34 progenitor cell isolation.
10. PBS containing 0.1% human AB serum.
11. PBS containing 0.1% NaN₃ 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 1×10^7 cells/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 $270 \times g$ for 10 min at room temperature. Most cells sediment at the interface above the metrizamide, whereas 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 µl above the small mast cell

pellet (see **Note 4**). Pelleted cells are gently resuspended and transferred into new tubes.

5. Under these conditions, 1×10^5 mast cells (>95% pure) can be recovered per mouse.

3.1.2. Cultured Mouse Mast Cells

3.1.2.1. MOUSE BMMCs

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. BM 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 and centrifuged, and the equivalent of cells from two femurs are resuspended in 60 ml complete OptiMEM supplemented with 1 ng/ml recombinant murine IL-3.
4. The cell suspension is transferred into culture flasks and placed at 37°C in a humidified CO₂ incubator.
5. Every 3–4 days (i.e., twice a week), non-adherent cells are centrifuged, resuspended at 3×10^5 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⁺, Kit⁺ mast cells as judged by staining and immunofluorescence.
7. Mouse BMMCs (mBMMCs) thus obtained keep growing in IL-3-containing medium for at least 2–3 months (see **Notes 5** and **6**).

3.1.3. Identifying and Phenotyping Mouse Mast Cells

3.1.3.1. WRIGHT-GIEMSA STAINING

1. Cells are resuspended at 2×10^5 cells/ml in RPMI.
2. 100 µl of the suspension, mounted onto clean slides, is placed in cytopsin chambers. Slides are spun at $14 \times g$ for 5 min.
3. They are air-dried and stained with Wright–Giemsa before they are mounted in one to two drops Permount under a cover slip.

3.1.3.2. TOLUIDINE BLUE STAINING

1. Cytopsin 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 two to three drops of acidic toluidine blue.
4. Stain is removed by running water down the slide.
5. Slides are air-dried and mounted in one to two drops Permount under a cover slip.

3.1.3.3. ANALYSIS OF THE EXPRESSION OF FcεRI AND KIT BY IMMUNOFLUORESCENCE

1. Aliquots of 5×10^5 cells are incubated in 50 μl complete RPMI containing 10 μg/ml mouse IgE or rat anti-mouse Kit mAb (ACK2) for 1 h at 0°C in Eppendorf tubes.
2. They are washed twice in PBS and incubated for 30 min at 0°C in 50 μl complete RPMI containing an appropriate dilution of F(ab')₂ 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')₂ only.
3. Cells are washed once and resuspended in 500 μl PBS.
4. Fluorescence is analyzed 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. Because of the body weight of rats, mast cells can be obtained in relatively high numbers.

3.2.2. Cultured Rat Mast Cells

BM cells are obtained from outbred male Wistar rats, 10–15 week-old (weight 225–250 g), with the same method as for mouse mast BM cells except for the following points.

1. Complete IMDM is used as culture medium.
2. 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 2.5×10^5 viable nucleated BM cells/ml in complete IMDM.
3. A final concentration of 2.5×10^5 viable nucleated BM cells/ml is suspended in complete IMDM in 175-cm² flasks that are kept at 37°C in a humidified incubator with 5% CO₂ in air.
4. These cultures are refed and restimulated with fresh complete medium containing rIL-3 every 4–6 days.
5. Percentage of mast cells is determined by differential counts (400 cells) on Wright-Giemsa and toluidine blue-stained cytocentrifuge preparations.

6. Rat BMMCs (rBMMCs) proliferate rapidly initially, representing 85% of cells after approximately 15 days. After this, the percentage of BMMCs increases slowly to almost 100% before cell viability declines around the fifth week (*see Notes 7 and 8*).

3.3. HMCs

3.3.1. Ex Vivo HMCs

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 (*see Note 9*).

3.3.1.1. TISSUE PROCESSING

HMCs 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 in human intestine, the mucosa is separated mechanically from the submucosa/muscular layers. Always for intestinal specimens, 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 the case of an 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. 5 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 ($500 \times g$, 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 µg/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 µl of HA buffer containing 1 mg/ml albumin.
5. Finally, the cell suspension is incubated with a goat anti-mouse IgG antibody 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 HMCs Derived from Hematopoietic Progenitors

Pure populations of HMCs 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 BM, cord blood, or peripheral blood. Usually, mononuclear cells from BM and cord blood contain 0.5–1% of CD34⁺ cells, whereas peripheral blood contains <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 (PBMcs), cord blood, or BM 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 (see **Note 10**).

3.3.2.1. PURIFICATION OF CD34⁺ PROGENITORS FROM HUMAN CORD BLOOD, BM, OR PERIPHERAL BLOOD CELLS

3.3.2.1.1. 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.

3.3.2.1.2. Sampling of BM Cells.

1. Collect BM 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 10× 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.

3.3.2.1.3. 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 h. Dilute cells with two to four 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 $400 \times g$ 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 $300 \times g$ 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 to 300 µl of buffer per 10^8 total cells. For $<10^8$ total cells, use 300 µl. Proceed to magnetic labeling.
3. Magnetic labeling of CD34⁺ progenitor cells: Add 100 µl 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 µl 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 µl; LS+/VS+ column: 1–10 ml, maximum 2×10^8 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 µl; LS+/VS+: 3 ml). Pass mononuclear cells through 30-µm 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+: 3×500 µl; LS+/VS+: 3×3 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 µl; LS+/VS+: 2.5 ml).
5. Magnetic separation of 2×10^9 to 2×10^{10} mononuclear cells: Assemble XS+ column and place it in the column holder of the SuperMACS using XS+ column

adapter. Turn three-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 three-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 three-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- μ 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 three-way stopcock to position "run." Allow the cells to pass through the column. Remove flow resistor and wash with 4×30 ml buffer. Close three-way stopcock and remove column out of the magnetic field on the SuperMACS by turning the XS+ adapter handle backward. Detach syringe from the three-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: Evaluate the purity of the isolated hematopoietic progenitor cells 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 with lymphocytes. Use the antibodies at appropriate concentrations recommended by the manufacturers. Typically, staining for 5 min at 6–12°C should be sufficient. After fluorescence staining, wash and resuspend the cells in buffer.

3.3.2.2. CULTURE CONDITIONS

1. CD34⁺ cells are cultured at 37°C in a humidified atmosphere containing 5% CO₂ at a starting density of 1×10^5 cells/ml in SFCM.
2. CD34⁺ cells may initially proliferate to generate 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 weeks, 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 weeks. Cultures are checked weekly, and non-adherent mast cell-committed progenitors are separated from adherent cells and debris. Non-adherent cells are gently pipetted and removed to a new flask, or non-adherent cells and supernatant are centrifuged at $150 \times g$ for 5 min, and cells are resuspended in complete media with growth factors every 5–7 days.

4. The number and percentage of mast cells in the cultures are assessed weekly using Wright–Giemsa and acidic toluidine blue (see **Subheading 3.1.3.**) staining of cytocentrifuged samples. Usually, 10^6 CD34⁺ cells at 8–10 weeks may give rise to 5×10^7 to 15×10^7 HHPMC with <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, and 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, >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 HMCs (see **Notes 11** and **12**).
7. Because CD34⁺-derived mast cells usually do not express spontaneously a significant amount of Fc ϵ RI at their membrane, they can be incubated for 5 additional days with 2–10 μ g/ml human myeloma IgE and 20 ng/ml rhIL-4 to enhance the expression of Fc ϵ RI on their membrane.

3.3.2.3. PHENOTYPING HMCs FOR KIT AND Fc ϵ RI EXPRESSION

1. For flow cytometric analysis, 5×10^5 cells are incubated for 60 min at 4°C with the monoclonal receptor antibodies [mouse IgG against the α -chain of the high-affinity IgE receptor (Fc ϵ RI) or against Kit (YB5.B8)] in 50 μ l 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 FITC.
3. Following two washes, cells are suspended in 400 μ l PBS containing 0.1% NaN₃, 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, Trappes, France).
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. rIL-3 can be replaced by 5–20% conditioned medium made of culture supernatant of rat mononuclear splenocytes (10^6 cells/ml) stimulated with 1 mg/l Concanavalin A for 5–7 days.
3. rhSCF 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. Although primary BM cells can be kept frozen in liquid nitrogen before they are cultured, if frozen, mBMMCs 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 color different 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 BM cells can be kept frozen in liquid nitrogen before they are cultured, but it is impossible to keep rBMMCs frozen.
8. Mast cell numbers are calculated exactly as described above for mBMMC cultures.
9. Isolated HMCs 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 mast cells can be maintained in culture for up to 3 months in the continuous presence of rhSCF. Mast cell purity increases during culture and reaches nearly 100%. During the first week of culture, mast cell numbers decrease, but after that time they start to proliferate. Cultured mast cells do not change their histamine content, phenotype, or morphology. They are even more responsive toward IgE-dependent stimulation, which causes the release of high amounts of histamine, leukotrienes, and cytokines such as tumor necrosis factor (TNF)- α and IL-5.
12. Once pure, HMC cultures stop growing at 8–10 weeks. They nevertheless stay 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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