

Chapter 4

Generation of a Human Allergic Mast Cell Phenotype from CD133⁺ Stem Cells

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Abstract

Cultured human mast cells are a useful tool for research into innate immune responses as well as allergic mechanisms. Mast cells cultured from peripheral blood can provide information on immune mechanisms of known, selected individuals. With the method presented here eight million mast cells can be cultured from ca. one million stem cells purified from one unit (450 mL) of human peripheral blood. Culture with IgE and IL4 optimizes an allergic phenotype of the mast cells.

Key words Mast cell, Human stem cells, Buffy coat, Cultured mast cells, SCF, CD63, FcεRI

1 Introduction

Mast cells are tissue-resident cells first described by Ehrlich in 1878 that derive from bone marrow-derived hematopoietic stem cells. These CD34⁺ C117⁺ CD133⁺ cells released into circulation can be purified and cultured to resemble tissue mast cells. Mature mast cells contain large amounts of preformed heparin, histamine, and a number of cytokines, and continue to express the stem cell factor receptor CD117.

Mast cells are present in most tissues, characteristically proximal to blood vessels, nerves, and epithelia. Early work [1] identified two subtypes of mast cells based on the expression of chymase. Recent work has described distinguishable phenotypes of mast cells associated with anatomical compartments of the human lung [2] that were identified by protein markers (FcεRI, IL9-R, HDAC, and others). Mast cells can be purified from tissue or cultured from purified stem cells [3–6]. In both instances, they are dependent on SCF [7].

2 Materials

2.1 Isolation and Culture of CD133⁺ Human Stem Cells

1. Human peripheral blood (100–450 mL), or fresh buffy coat, can be citrated or in EDTA (*see Note 1*).
2. Ficoll Paque (GE Health Care).
3. Sterile 50 mL tubes.
4. Sterile PBS.
5. Low-speed centrifuge.
6. AC133⁺ cell isolation kit (Miltenyi).
7. MACS buffer (Miltenyi).
8. LS Separation columns (Miltenyi).
9. StemSpan culture medium (Stem Cell Technologies, Vancouver, Canada).
10. Penicillin/streptomycin.
11. Foetal bovine serum (FBS).
12. Human recombinant stem cell factor (rhSCF) (R&D Systems, Abingdon, UK).
13. Human rhIL-3 (R&D Systems, Abingdon, UK).
14. Human rhIL-6 (R&D Systems, Abingdon, UK).
15. Recombinant IL-4 (Peprotech EC Ltd., London, UK).
16. Human monoclonal IgE, produced by various companies.
17. CM: Stem Span culture medium with IL-6 (50 ng/mL), SCF (100 ng/mL), and penicillin/streptomycin (100 µg/mL).

2.2 Reagents for Determining Mast Cell Purity and Reactivity

1. May–Grünwald solution (Merck, Darmstadt, Germany).
2. Giemsa (Merck) diluted 1/10 in PBS, pH 6.4.
3. Microslides and cover slips.

2.2.1 May–Grünwald– Giemsa Staining

2.2.2 Detection of Density of FcεRI, Mast Cell Reactivity, and Kit Surface Receptor Expression by Flow Cytometry

1. Polystyrene round-bottom tubes (5 mL).
2. PBS + 0.1 % BSA (4 °C).
3. Anti-CD63 FITC (Biolegend, San Diego, CA, clone H5C6).
4. CD203c PE (Biolegend, San Diego, CA, clone NP4D6).
5. Qifikit (Dako, Glostrup, Denmark).
6. Anti-human IgE (BD Pharmingen, San Jose, CA, clone G7-18).

3 Methods

All work is to be done in a sterile lab bench, except mast cell activation tests and staining (Subheadings 3.3 and 3.4).

3.1 Purification of CD133⁺ Stem Cells from Peripheral Blood

1. Bring Ficoll and PBS to room temperature (22 °C), and aliquot 13 mL Ficoll into a sterile 50 mL tube for each 24 mL blood to be processed (*see Note 2*).
2. Dilute blood with 0.25 volumes of PBS in a sterile flask, and carefully layer blood on the Ficoll layer (*see Note 3*). Centrifuge for 30 min at room temperature and 450×*g* without acceleration or brake to preserve the integrity of the gradient interface.
3. Using a sterile pipette or pastette, remove the plasma layer above the interface. Collect PBMC at the interface and wash with 20 mL cold PBS for each gradient tube. Centrifuge for 5 min at 4 °C and 450×*g*. Pool all cells into one 10 mL tube, wash them again in 10 mL MACS buffer, and count the number of leukocytes in the sample.
4. To count the cells, dilute 20 µL cells with 80 µL trypan blue, and count in a Bürker-Türk or Neubauer modified chamber. While counting, centrifuge as before (5 min, 4 °C, 450×*g*).
5. Resuspend all cells in 300 µL MACS buffer (maximally 10⁸ cells per 300 µL) in a 10 mL tube and chill on ice.
6. Add 100 µL Fc blocking reagent, and 100 µL CD133 microbeads per 300 µL cell suspension.
7. Incubate on ice for 2 min, and at 4 °C for 28 min. Dilute cells with 10 mL MACS buffer, and centrifuge for 5 min at 4 °C and 450×*g*. Resuspend in 500 µL MACS buffer per 10⁸ cells.
8. Wipe the MidiMACS with 70 % ethanol and place it in the sterile bench.
9. Fill each vial with 300 µL cells up to 10 mL with MACS buffer. Centrifuge for 5 min at 450×*g* and 4 °C. Pour off the supernatant and then resuspend the pellet in 5 mL MACS buffer.
10. Apply 3 mL MACS solution to an LS column in a MACS magnet. When the MACS solution has reached the top of the column, add the cell suspension. Collect the eluate in a clean tube. Wash the column with 3×5 mL MACS buffer, collecting the eluate each time. Remove the column from the magnet and pass 5 mL MACS buffer through to elute retained cells from the column. Count the number of cells in the eluate in a Bürker-Türk chamber or other hemocytometers. Centrifuge all tubes for 5 min at 450×*g* and 4 °C. There should be no visible pellet in the second last tube, and all CD133⁺ cells in the last tube.

3.2 Expansion of Stem Cells and Maturation of Mast Cells

1. Resuspend CD133⁺ stem cells in CM with IL-3 at $5 \cdot 10^5$ cells/mL and culture at 37 °C in a humidified CO₂ incubator.
2. Inspect the cultures twice a week for growth and sterility (*see Note 4*). If the medium appears spent, as indicated by an orange or even yellow color, add some more CM to continue supporting growth until the next medium change.
3. Change medium on a weekly basis. Transfer all cells and medium to a sterile centrifuge tube, and centrifuge for 5 min at $450 \times g$ and 4 °C. Remove the supernatant. Resuspend up the cell pellet in 1 mL CM and count in a Bürker-Türk chamber. Resuspend the cells at $5 \cdot 10^5$ cells/mL in the appropriate medium and transfer to a fresh well or flask of appropriate size.
4. IL-3 should be added for the first 3 weeks to expand cell lines. In weeks 4–6, the cells are cultured in CM only. In weeks 7–8, FBS (10 %), IL-4 (10 ng/mL), and IgE (80 kU/L) are added to mature mast cells (*see Note 5*).
5. At the end of the culture period, evaluate mast cell quality, purity, and reactivity as described in the next section.

3.3 Mast Cell Staining with May–Grünwald–Giemsa by Microscopy

1. Prepare a cytopsin with 10^3 – 10^4 mast cells. Centrifuge at 1,000 rpm for 5 min. Air-dry until cells appear dry.
2. Stain with May–Grünwald solution for 4 min, and wash in PBS for 4 min.
3. Rinse in water, stain with Giemsa solution for 8 min, and rinse again in water.
4. Air-dry cytopsin and examine mast cells in a light microscope to confirm the appearance of granules in the cytoplasm (Fig. 1).

3.4 Mast Cell Reactivity and Phenotyping by Flow Cytometry

1. Prepare five log dilutions of anti-IgE or (APC-labelled) anti-FcεRI antibody in PBS from 1 ng/10 μL as well as a blank tube (*see Note 6*).
2. Add 90 μL mast cell preparation containing at least 5,000 cells.
3. Incubate at 37 °C for 30 min.
4. Add antibodies to CD63 (FITC) and CD203c (PE) and incubate at 4 °C for 20 min (*see Note 4*).
5. Wash with 2 mL PBS, and centrifuge at $450 \times g$ and 4 °C.
6. Acquire data on a flow cytometer; plot a histogram of CD63 for all cells for all tubes, and set a threshold at 2 % of the blank. Plot the fraction of positive cells against anti-IgE or anti-FcεRI concentration (*see Note 7*).
7. Add titrated amounts of antibody to FcεRI, and CD117 to aliquots of mast cell culture. Use QiFikit according to the manufacturer's instructions to determine the density of receptors on cells.

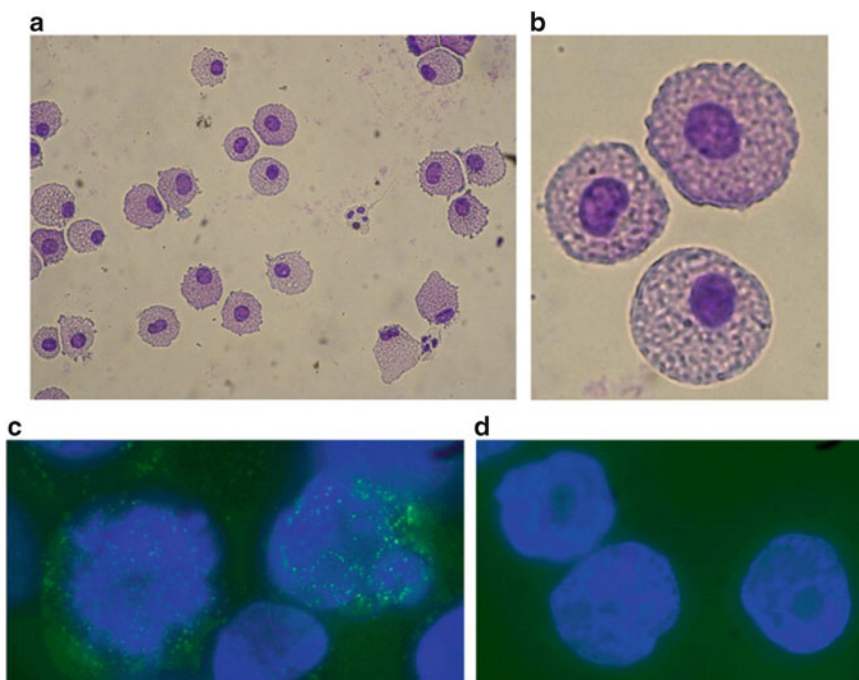


Fig. 1 CD133⁺ peripheral blood-derived mast cell, 7 weeks. **(a)** and **(b)** Human peripheral blood-derived mast cells (PBMC) cultured in serum-free StemSpan medium for 6 weeks and with 10 % FCS for 1 week. Stem cell factor, IL-6, and IL-3 were added to the media but IL-3 was omitted after the first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

4 Notes

1. We have not purified CD133⁺ cells for mast cell culture from heparinized blood.
2. Typical yields of stem cells per mL blood are 3,800 for fresh blood, 2,500 for buffy coats, and blood 24 h old (unpublished observations).
3. This can take some practice. It is important to avoid disturbing the surface of the Ficoll layer. Find a comfortable and relaxed position, e.g., resting the elbow on the laminar flow bench edge. Use a pipetting aid which allows you to set the speed of dispensing to low, and use a constant flow rate, holding the tube at an angle of approx. 45–60°.
4. If there was a sign of contamination, the flask was removed or the well was emptied and cleansed with 70 % ethanol in water.
5. IgE can be obtained from myeloma, or as recombinant products. We have used recombinant human IgE [8].

6. Optimization of antibody concentrations should be performed by titration prior to the start of the experiment.
7. We use log SS (230V) and FS (40V) on a BD instrument to focus mast cells in the scatter plot. If APC-labelled anti-FcεRI is used to stimulate cells, the fraction of cells expressing FcεRI as well as the relationship between FcεRI expression and activation can be examined.

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