

Chapter 4

Human Mast Cell and Basophil/Eosinophil Progenitors

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Abstract

Mast cell, basophil, and eosinophil lineages all derive from CD34⁺ hemopoietic stem cells; however, mast cells are derived from a distinct, nonmyeloid progenitor, while eosinophils and basophils share a common myeloid progenitor. These progenitors likely evolved from an ancestral leukocyte population involved in innate immunity and currently play a central role in the pathology of allergic disease. Advances in isolation and analysis of mast cell and basophil/eosinophil progenitor populations have been critical to understanding lineage commitment, differentiation, function, and transcriptional regulation of these cells and have provided a way of monitoring the effect of novel investigational therapies on these cell populations in samples of blood, bone marrow, and airway secretions.

Key words Mast cell, Basophil, Eosinophil, Progenitor, Tissue culture, Flow cytometry, Cord blood, Bone marrow, Blood, Sputum

1 Introduction

While nonneoplastic human mast cells and basophils perform many similar functions and share a high-affinity IgE receptor, these cells do not share a common progenitor, as opposed to myeloid basophil and eosinophil leukocytes, which do [1]. All three lineages derive from a CD34⁺ hemopoietic stem cell, in response to various differentiation stimuli. CD34 can be found on some differentiated mast cells and eosinophils, as a contributor to cell migration in tissue inflammatory responses [2, 3]. Mast cells are derived from a distinct, nonmyeloid progenitor [4], which likely evolved, along with basophils and eosinophils, from an ancestral leukocyte population involved in innate immunity [5–7]. Major recent advances in mapping human basophil/eosinophil and mast cell growth and differentiation pathways have come from rodent models [8–11], which have underscored key roles of epithelial factors such as TSLP, IL-33, IL-25, and toll-like receptors (TLRs) [12, 13].

The mast cell differentiation-specific stem cell factor (SCF) and its receptor, *c-kit*, and mutations leading to changes in their

expression have guided much recent research into the specific roles of mast cells in a variety of biological processes, including allergic inflammation, angiogenesis and tumor growth, tissue remodeling, graft tolerance, and some autoimmune diseases [14–21]. Advances in isolation and analysis of mast cell and basophil/eosinophil progenitor populations [22–25] have been critical to understanding lineage commitment, differentiation, function, and transcriptional regulation of these cells.

1.1 Mast Cell and Basophil/ Eosinophil Progenitors

Human mast cell differentiation proceeds from an immature CD34⁺, CD38⁺, CD13⁺, c-kit⁺, FcεRI[−], FcγRII/III⁺ cell [4, 26, 27] which can give rise to both mucosal and serosal mast cell phenotypes (Fig. 1). Phenotypic alterations of mast cell populations depend on the tissue milieu and derivation [5, 28]; indeed, cord blood CD34⁺ c-kit⁺ progenitors respond differently to differentiative stimuli than mature FcεRI⁺, c-kit⁺ cells [4, 29], indicating that progenitor and phenotypic maturation are both tissue dependent [30]. Mast cell as well as basophil/eosinophil progenitors can be identified in blood, bone marrow, and various other tissues [31–35].

The recent identification of a novel antigenic marker of mast cells, basophils, and their progenitors [36] has again raised the hypothesis that these cell types may share some lineage characteristics; nonetheless, there is much more solid evidence that human basophils and eosinophils share a common lineage.

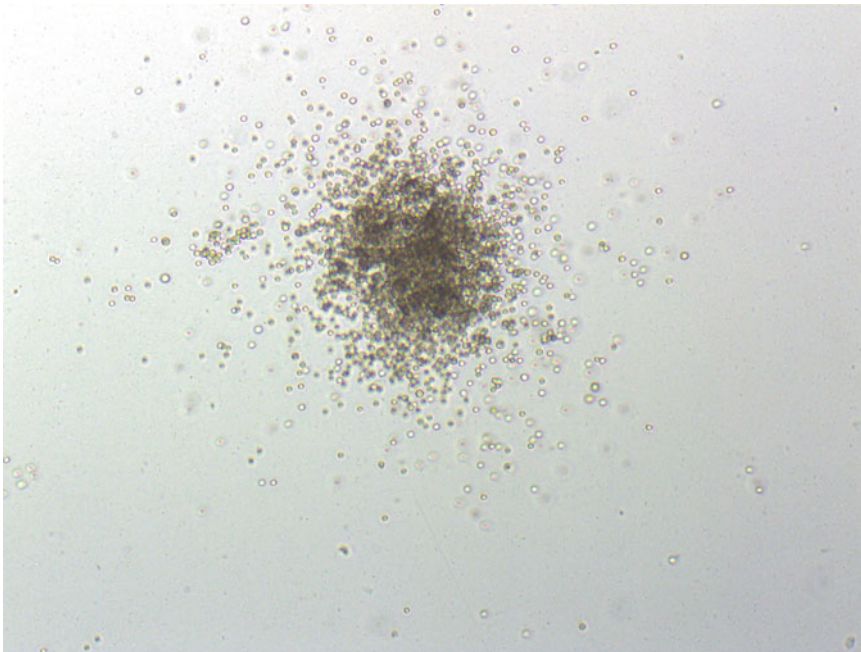


Fig. 1 Eosinophil/basophil colony-forming unit, ×400 magnification, appearing as tightly packed granulated cells

New studies have shown the role of gut microbiota in regulating tissue basophilopoiesis and Th2-dependent IgE responses [37]. Normal and atopic human blood, marrow, and tissues can be used to identify pure or mixed basophil/eosinophil colonies in semi-solid cultures [1, 31, 38] (termed “CFU-baso/eo”) [1, 39].

1.2 Basophil/ Eosinophil Differentiation- Inducing Cytokines

While IL-3 alone is probably involved in human basophil growth and differentiation [40], granulocyte-macrophage colony-stimulating factor (GM-CSF) [1, 31, 38, 41] and IL-5 [39, 42] are directly involved in basophil/eosinophil differentiation. Studies on basophil crisis in chronic myeloid leukemia and the suppressive role of retinoic acid on basophil/eosinophil differentiation support this notion and have further elaborated on these common myeloid pathways [43]. Factors that modulate basophil/eosinophil or mast cell differentiation are listed in Table 1.

Allergic disease is manifested through elevated levels of activated mast cells, eosinophils, and basophils in the affected tissue [44]. In subjects with allergic asthma following allergen challenge, we have observed increased levels of basophils and eosinophils [45] and their progenitors in blood and bone marrow [46–48], and also in the airways [46–49], where these cells may differentiate in situ under the influence of local growth factors. More traditional therapies for treatment of allergic asthma, such as glucocorticoids, have been shown to reduce the level of basophil and eosinophil progenitor cells in bone marrow and blood [46], likely through inhibition of growth factors including IL-3, IL-5, and GM-CSF [50]. Investigational therapies are now emerging which target mast cells, basophils, and eosinophils through pathways critical for cell differentiation. Administration of antibodies to IL-5 has been

Table 1
Cytokines and other factors involved in basophil and mast cell growth and differentiation

Cytokine	Effect
GM-CSF	Basophil/eosinophil growth and differentiation; may downregulate human mast cell differentiation
IL-3	Human basophil growth and differentiation; basophil activation/survival; promotes in vivo basophilia (in primates)
IL-5	Basophil and eosinophil growth and differentiation, activation, and survival
SCF	Primary human mast cell growth and differentiation factor
NGF	A cofactor in human mast cell and basophil/eosinophil differentiation in vitro
RA	RA receptor mutation permits basophil/eosinophil differentiation
Stat5	A regulator in vivo of mast cell differentiation

GM-CSF granulocyte-macrophage colony-stimulating factor, *IL-3* interleukin 3, *IL-5* interleukin 5, *SCF* stem cell factor, *NGF* nerve growth factor, *RA* retinoic acid, *Stat5* signal transducer and activator of transcription 5

shown to reduce the number of eosinophils in circulation [51], presumably by preventing the differentiation of progenitor cells into mature cells, while inhaled antisense therapy targeting the IL-3/5/GM-CSF receptor common beta chain effectively reduced the level of eosinophil progenitors in the airways [52]. Measures of progenitor cells in blood, bone marrow, and tissue are valuable outcome markers of drug efficacy and can be measured using tissue culture or through measurements of cell surface markers by flow cytometry.

2 Materials

2.1 Methylcellulose Colony Assay

For peripheral blood (PB), cord blood (CB), and bone marrow (BM):

1. 6 % dextran (w/v) in PBS (phosphate-buffered saline).
2. McCoy's 5A media.
3. McCoy's 3⁺ media: McCoy's 5A, 10 % fetal bovine serum (FBS), 1 % penicillin-streptomycin, 50 µM 2-mercaptoethanol.
4. Iscove's 2⁺ media: Iscove's medium (IMDM), 1 % penicillin-streptomycin, 50 µM 2-mercaptoethanol.
5. Fetal bovine serum (FBS) (sterile and heat inactivated) (*see Note 1*).
6. Lymphoprep (Accurate Chemical & Scientific).
7. Methylcellulose media (2.1 %) or MethoCult™ (Stem Cell Technologies).
8. Cytokines: hIL-3 (100 ng/mL), hIL-5 (100 ng/mL), and hGM-CSF (1,000 ng/mL) all reconstituted or diluted with PBS + 1 % BSA (bovine serum albumin).
9. Sterile distilled water.
10. Trypan blue (0.4 %).

2.2 Sputum Processing

1. Dulbecco's Phosphate-Buffered Saline (D-PBS).
2. 0.1 % dithiothreitol (DTT) prepared in D-PBS.
3. Trypan blue (0.4 %).
4. Nylon mesh with 48 µm pore size.
5. Funnel.
6. Bench rocker.

2.3 Flow Cytometry for Surface Staining IL-5R α , IL-3R α , and GM-CSFR α in Cord Blood, Peripheral Blood and Bone Marrow

Store the following solutions and reagents at 4 °C:

1. FACS buffer: D-PBS containing 0.1 % sodium azide and 0.5 % bovine serum albumin (BSA).
2. 1 % paraformaldehyde (PFA) in D-PBS or Cytofix™ (BD Biosciences).
3. PerCP-mouse antihuman CD34.

4. FITC-mouse antihuman CD45.
5. PE-mouse antihuman IL-3R α /IL-5R α /GM-CSFR α .
6. Isotype control mouse IgG₁.
7. Fc blocking buffer: FACS buffer with 5 % mouse serum and 5 % human serum.

**2.4 Flow Cytometry
for Surface Staining
of IL-5R α
on Progenitor Cells
in Sputum**

Store the following solutions and reagents at 4 °C:

1. 1 % paraformaldehyde (PFA) in D-PBS (or use Cytofix™).
2. FACS buffer: D-PBS containing 0.1 % sodium azide and 0.5 % BSA.
3. Fc blocking buffer (FACS buffer with 5 % mouse serum, 5 % human serum).
4. PE-mouse antihuman IL-5R α .
5. APC-mouse antihuman CD34.
6. FITC-mouse antihuman CD45.
7. PE-mouse IgG₁ (isotype control).
8. 0.1 % sodium azide in D-PBS.

3 Methods

**3.1 Methylcellulose
Colony Assay
(See Note 2)**

Cord blood (see Note 3), peripheral blood and bone marrow (see Note 4):

1. Dilute sample with McCoy's 5A media (*see Note 5*).
2. Layer up to 25 mL of diluted sample on top of 15 mL of Lymphoprep in a 50 mL conical tube.
3. Centrifuge at 800 $\times g$ at room temperature (RT) for 20 min without brake (*see Note 6*).
4. Using a sterile transfer pipette, remove the mononuclear cell layer at the white interface and transfer to a new 50 mL tube.
5. Top up to 50 mL with McCoy's 5A and mix well.
6. Perform viability count by diluting 1:1 with trypan blue.
7. Wash the cells by centrifuging at 500 $\times g$ at RT for 10 min.
8. Decant supernatant and resuspend the cell pellet in McCoy's 3+ at a concentration of $<1 \times 10^6$ cells/mL.
9. Transfer to a 150 cm² culture flask, and incubate with flask on its side, cap loosened for 2 h at 37 °C in 5 % CO₂ (*see Note 7*).
10. Transfer the nonadherent mononuclear cells from the flask to 50 mL tube(s), and centrifuge at 500 $\times g$ at RT for 10 min.
11. Resuspend the cell pellet in 1–3 mL of Iscove's 2+.

12. Check cell viability by trypan blue exclusion (*see Note 8*).
13. Adjust viable CB and BM cells to a concentration of 1.25×10^6 /mL and PB to a concentration of 2.5×10^6 /mL in Iscove's 2+.
14. Set up the following conditions to make a total volume of 3 mL per tube:
 - Tube 1 (negative control): 1,200 μ L methylcellulose, 600 μ L FBS, 600 μ L Iscove's 2+, 600 μ L cells
 - Tube 2 (IL-3): 1,200 μ L methylcellulose, 600 μ L FBS, 570 μ L Iscove's 2+, 600 μ L cells, 30 μ L IL-3
 - Tube 3 (IL-5): 1,200 μ L methylcellulose, 600 μ L FBS, 570 μ L Iscove's 2+, 600 μ L cells, 30 μ L IL-5
 - Tube 4 (GM-CSF): 1,200 μ L methylcellulose, 600 μ L FBS, 570 μ L Iscove's 2+, 600 μ L cells, 30 μ L GM-CSF
15. Prepare one 10 cm Petri plate (Falcon #3003), containing three 35 mm Petri dishes (Falcon #3001).
16. Using a 3 mL syringe and a 16-gauge needle for each sample, mix gently by drawing methylcellulose/cell mixture up and down. Allow bubbles to settle.
17. Draw up 2 mL of the mixture in the syringe and deliver 1 mL to each of 2–35 mm dishes. Tilt gently to spread evenly over dish bottom. Place the 2 dishes (covered) in the 10 cm dish. Add an open 35 mm dish filled with sterile water.
18. Replace the lid on the 10 cm plate and incubate cell cultures for 14 days (CB and PB) or 10 days (BM) at 37 °C in a 5 % CO₂ incubator.
19. On the final day, assess colony morphology and numbers (1 colony = minimum 40 cells; Eo/B are granulated and clump together (Fig. 1), GM are un-granulated and smaller than Eo/B).

3.2 Sputum Processing

1. Select portions of sputum free from saliva (*see Note 9*).
2. Add a volume of 0.1 % DTT equal to 4 times the weight of the sputum (*see Note 10*).
3. Vortex 15 s, then mix for 15 min at RT on a bench rocker.
4. Add a volume of D-PBS equal to 4 times the weight of the cell plug and mix for 5 additional min on a bench rocker.
5. Filter the solution through 48 μ m nylon mesh.
6. Centrifuge the sample at $500 \times g$ at RT for 10 min and remove the supernatant.
7. Resuspend the cell pellet in the volume of D-PBS to achieve the required cell concentration.

**3.3 Flow Cytometry
for Surface Staining
of IL-5R α , IL-3R α ,
and GM-CSFR α in CB,
PB, and BM**

1. Resuspend 0.5×10^6 cells in 2.5 mL FACS buffer.
2. Wash by centrifugation at $500 \times g$ at 4 °C for 5 min.
3. Remove supernatant and resuspend cell pellet in Fc blocking buffer and vortex for a few seconds.
4. Incubate samples at 4 °C in the dark for 15 min.
5. Add antibodies to each tube and vortex for a few seconds:
 - Tube 1 (compensation): 100 μ l Fc blocking buffer
 - Tube 2 (CD45): 80 μ l Fc blocking buffer, 20 μ l FITC-CD45
 - Tube 3 (CD45): 80 μ l Fc blocking buffer, 20 μ l PE-CD45
 - Tube 4: (CD34): 80 μ l Fc blocking buffer, 20 μ l PerCP-CD34
 - Tube 5: (CD45/CD34/IgG₁): 40 μ l Fc blocking buffer, 20 μ l FITC-CD45, 20 μ l PERCP-CD34, 20 μ l PE IgG₁
 - Tube 6: (CD45/CD34/GM-CSFR α): 40 μ l Fc blocking buffer, 20 μ l FITC-CD45, 20 μ l PERCP-CD34, 20 μ l PE GM-CSFR α
 - Tube 7: (CD45/CD34/IL-3R α): 40 μ l Fc blocking buffer, 20 μ l FITC-CD45, 20 μ l PERCP-CD34, 20 μ l PE-IL-3R α
 - Tube 8: (CD45/CD34/IL-5R α): 40 μ l Fc blocking buffer, 20 μ l FITC-CD45, 20 μ l PerCP-CD34, 20 μ l PE-IL-5R α
6. Incubate at 4 °C in the dark for 30 min.
7. Wash with 2.5 mL FACS buffer. Vortex sample quickly.
8. Centrifuge at $500 \times g$ at 4 °C for 5 min.
9. Discard supernatant and add 350–500 μ l 1 % PFA (or Cytofix™) to all tubes.
10. Cap tubes and wrap in aluminum foil. Store in fridge until sample can be acquired on flow cytometry (*see Note 11*).

**3.4 Flow Cytometry
for Surface Staining
of IL-5R α
on Progenitor Cells
in Sputum**

1. Distribute at least 0.5×10^6 cells per FACS tube over 3 tubes.
2. Add 2 mL of 0.1 % sodium azide in D-PBS to each FACS tube and centrifuge at $500 \times g$ at 4 °C for 10 min.
3. Pour off the supernatant and blot on tissue. Vortex.
4. Add Fc blocking buffer, incubate 10 min, then add antibodies as follows:
 - Tube 1 (D-PBS): 50 μ l Fc blocking buffer
 - Tube 2 (APC-CD34): 45 μ l Fc blocking buffer, 5 μ l APC-CD34
 - Tube 3 (PE-IL-5R): 45 μ l Fc blocking buffer, 5 μ l PE-IL-5R
 - Tube 4 (FITC-CD45): 45 μ l Fc blocking buffer, 5 μ l FITC-CD45
 - Tube 5 (CD34/CD45/PE-IgG₁): 35 μ l Fc blocking buffer, 5 μ l APC-CD34, 5 μ l FITC-CD45, 5 μ l PE-IgG₁

- Tube 6 (CD34/CD45/PE-IL-5R): 35 μ l Fc blocking buffer, 5 μ l APC-CD34, 5 μ l FITC-CD45, 5 μ l PE-IL-5R
5. Vortex. Incubate in the dark, on ice for 30 min.
 6. Wash with 0.1 % sodium azide in D-PBS (2 mL) and centrifuge at $500\times g$ at 4 °C for 10 min.
 7. Pour off supernatant and blot on tissue. Vortex.
 8. Resuspend the cells in 350–500 μ l of 1 % PFA (or Cytofix™).
 9. Cap, vortex, wrap in aluminum foil and store in refrigerator.
 10. Samples are ready for analysis by flow cytometry.

4 Notes

1. FBS is heated to 56 °C for 1 h to inactivate complement.
2. The work is carried out in a class II laminar flow hood to ensure sterility of the sample. All reagents/media (except Lymphoprep which is used at RT) are pre-warmed to 37 °C in water bath before use. Collect samples into sodium heparin (1,000 U/mL) anticoagulant.
3. *Cord blood only*: Add 1 volume cord blood to 5 volumes of 6 % dextran, mix well and incubate in a 37 °C water bath for 20–30 min to separate red blood cells (RBCs) from the sample by sedimentation. Using a transfer pipette, transfer the top (clear) layer to a new 50 mL tube before proceeding.
4. Before diluting BM, gently pass up and down through a 16–18-gauge needle to break up any spicules or clumps.
5. PB and CB are diluted 1:1. BM is diluted 1:5.
6. For density gradients it is necessary to use a swinging bucket rotor rather than a fixed angle rotor. The brake is turned off to allow buckets to come to a more gradual stop, minimizing disruption to the cell layers.
7. Monocytes will adhere to the plastic flask. Cells remaining in suspension are nonadherent mononuclear cells.
8. Using a small aliquot of cells, prepare a 1:1 dilution of cell suspension with 0.4 % trypan blue. Load into a hemocytometer and read after 5 min. Cells that take up the blue stain are considered to be nonviable.
9. The weight of the saliva-free sputum that is selected for processing should be 70–200 mg.
10. For example, sputum weighing 100 mg would require 400 μ l of 0.1 % DTT solution.
11. For best results, sample should be analyzed within a few days.

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