Chapter 8

Isolation and Characterization of Mast Cells in Mouse Models of Allergic Diseases

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Abstract

After their activation, mast cells release a variety of bioactive mediators that contribute to characteristic symptoms of allergic reactions. Ex vivo analysis of mast cells derived from their progenitors or isolated from mice is an indispensable tool for the development of newer and more effective therapies of allergic syndromes. Here, we describe the differentiation and isolation of mouse mast cells from different sources including differentiation from bone marrow, differentiation from fetal liver, and isolation of residential connective tissue-type mast cells from the peritoneum. These techniques are valuable tools for the study of mast cell function and their contribution to allergic reactions.

Key words Mast cells, Allergy, Peritoneum, Bone marrow, Fetal liver

1 Introduction

Mast cells play a central role in allergic reactions. Their response to an allergen underlies the symptoms seen in acute and chronic allergic disease. In allergic disease, mast cells are most frequently activated by an allergen-specific IgE, which is produced by B cells during a Th2 response to allergen exposure. FcεRI on the mast cell surface binds the allergen-specific IgE with high affinity. This high-affinity binding results in a half-life of cell-bound IgE that is on the order of days [1, 2]. Therefore sensitization of mast cells is persistent when serum IgE levels are increased. Cross-linking of the FcεRI-bound IgE with the specific allergen activates mast cells and leads to the release of histamine, serotonin, and a variety of other biologically active mediators that are stored in preformed granules. This process is called degranulation, and elicits the immediate hypersensitivity. Mast cells also produce a variety of cytokines and lipid mediators, such as leukotrienes and prostaglandins that are major contributors to the late or chronic phase of allergic disease [3].

Mast cells have essential roles in mediating allergic diseases and have been studied with a focus on understanding the molecular

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mechanism of their activation. Likewise, mast cells are often sought-after target for the development of treatments and management of allergic diseases.

Ex vivo studies using mast cells mainly depend on a reliable source of large numbers of cells. Mast cells are tissue-resident cells that are present only in small numbers. In the mouse system, protocols were developed to allow differentiation and culture of mast cells from bone marrow mast cell progenitors using mast cell growth factors and cytokines. This approach is especially useful when combined with the availability of mouse lines carrying mutations in virtually all known genes. By culturing bone marrow from these animals, using established protocols described in this chapter, mast cells lacking a specific gene or carrying a specific mutation could be obtained and the impact of the mutation on mast cell function can be easily determined. However, in some cases, the mutation of interest results in an embryonic lethal phenotype or death early after birth, making isolation of bone marrow progenitors from those mice inaccessible. In these cases, two alternatives can be used: (1) derivation of mast cells from embryonic stem cells [4], or (2) to obtain progenitors for mast cell differentiation from fetal or neonatal liver, if available [5, 6].

Although bone marrow-derived mast cells are a useful model for mast cell studies, they are phenotypically different from residential mast cell populations, including differences associated with the level of maturation and the level of mast cell protease expression. For example, bone marrow-derived mast cells are similar to mucosal mast cells in vivo and stain by alcian blue due to the high expression of chondroitin sulfate. However, peritoneal mast cells have a phenotype that is more consistent with connective tissue mast cells. The peritoneal mast cells express high amounts of heparin and stain with safranin, but not alcian blue [7]. In vivo, mast cells derive from a distinct precursor in the bone marrow and mature under local tissue microenvironmental factors [8], which make each population unique and hard to model in vitro. Resident mast cells can be relatively easy to isolate from the peritoneal cavity using a Percoll gradient. Although this isolation technique provides only a small amount of mast cells, the recovered cells are a valuable source of fully differentiated connective tissue mast cells that matured in a specific tissue microenvironment.

The characterization of mast cells is essential for the evaluation of mast cell differentiation or isolation. Expression of FcεRI on the cell surface is not only crucial for mast cell function in allergic reactions, but it can also be used together with c-Kit expression as a landmark for successful differentiation and maturation of bone marrow-derived mast cell cultures. In rodents, expression of the FcεRI receptor is limited to mast cells and basophils. Thus, this receptor can be used as a marker for the quality of mast cells

isolated from the peritoneal cavity. Another important aspect of mast cell culture and isolation is the quality of the granules. This can be assessed either by their specific staining or by functional assays that test the cells' ability to degranulate. Together, this chapter provides protocols allowing for the efficient differentiation, isolation, and characterization of mast cells for studies evaluating the molecular mechanisms of mast cell activation.

2 Materials

2.1 Mast Cell Differentiation from Bone Marrow or Fetal Liver

1. Surgical tools: Two forceps and two scissors.
2. 70 μm cell strainer.
3. Low-linting paper wipers.
4. 15 ml conical tube.
5. 1× PBS.
6. 70% EtOH.
7. 25 G needle.
8. 5 cc syringe.
9. Tissue culture plates (6-well, 3 cm, and 10 cm).
10. Centrifuge.
11. Laminar flow hood.
12. CO_2-humidified incubator.
13. Mast cell medium: 500 ml of Iscove's modified Dulbecco's medium (IMDM), 6 ml of 100 × penicillin–streptomycin–glutamine, 6 ml of 1 M HEPES buffer, 6 ml of 100 × nonessential amino acids, 6 ml of 100 mM sodium pyruvate, 2 μl of 2-mercaptoethanol, 60 ml of heat-inactivated fetal bovine serum, 5 ng/ml of mouse recombinant IL-3, and 10 ng/ml of mouse recombinant SCF (*see* **Note 1**).

2.2 Peritoneal Mast Cell Isolation

1. Surgical tools: Two forceps and two scissors.
2. 1× PBS.
3. 70% EtOH.
4. 25 G needle.
5. 5 cc syringe.
6. Disposable transfer pipettes (5 ml).
7. Centrifuge.
8. 50 ml of Peritoneal mast cell medium: 46.5 ml of Dulbecco's modified Eagle medium (DMEM), 2.5 ml of fetal bovine serum (final concentration of 5% v/v), and 1.0 ml of 1 M HEPES (final concentration of 20 mM) (*see* **Note 1**).

Table 1
Antibodies for staining FcεRI and c-kit on mast cells differentiated

Sample number	Type of staining	Antibody used (clone)
1.	Isotype control for FITC and PE	Mouse IgG1-FITC (P3.6.2.8.1.); rat IgG2b-PE (A95-1)
2.	FITC IgE specific + PE isotype	Anti-mouse FcεRI alpha (MAR-1); rat IgG2b-PE (A95-1)
3.	PE c-Kit specific + FITC isotype	Anti c-Kit-PE (2B8); mouse IgG1-FITC (P3.6.2.8.1)
4.	FITC and PE specific	Anti-mouse FcεRI alpha (MAR-1); anti c-Kit-PE (2B8)

9. 70% Percoll solution: 7 ml of Percoll, 1 ml of 10× PBS, 0.1 ml of heat-inactivated fetal bovine serum, and 1.9 ml of H_2O (*see* **Note 1**).
10. 1 L of 10× Phosphate-buffered saline (PBS): 80.0 g of NaCl, 11.6 g of Na_2HPO_4, 2.0 g of KH_2PO_4, 2.0 g of KCl, bring the volume up to 1 L with H_2O, and pH to 7.0.

2.3 Material for Characterization of Mast Cells

1. Mouse IgE (monoclonal, clone SPE-7).
2. FACS staining buffer (1% BSA in PBS).
3. HBSS buffer with calcium and magnesium.
4. Blocking antibody (anti-mouse FcγIII/II).
5. Specific antibodies to c-Kit and FcεRI, fluorescently labeled (Table 1).
6. Centrifuge.
7. Fluorescence-activated cell sorting instrument.
8. 5-ml polypropylene tubes.
9. Cytospin.
10. Microscopic slides.
11. Xylene.
12. Permount mounting solution.
13. Toluidine working solution: Mix 0.5 ml of toluidine blue stock solution (0.5 g toluidine blue O in 50 ml of 70% EtOH) in 4.5 ml of 1% sodium chloride (0.5 g NaCl in 50 ml ddH_2O).
14. Alcian blue staining solution (0.5% of Alcian blue in 0.3% of acetic acid): Dissolve 50 mg of Alcian blue 8 GX in 10 ml of ddH_2O with 30 μl of glacial acetic acid.

15. Safranin staining solution (0.1% safranin O in 0.1% acetic acid): Dissolve 10 mg of safranin O in 10 ml of ddH_2O with 10 μl of glacial acetic acid.
16. Glycine/carbonate buffer (0.2 M glycine, 0.1 M Na_2CO_3, pH 10.0): 1.06 g of Na_2CO_3, 1.50 g of glycine, and bring the volume up to 100 ml with H_2O.
17. Citrate buffer (0.1 M sodium citrate, 0.1 M citric acid, pH 4.5): Dissolve 2.94 g of sodium citrate in 100 ml H_2O. Dissolve 2.10 g of citric acid in 100 ml of H_2O. Mix 25 ml of 0.1 M citric acid and 20 ml of 0.1 M sodium citrate to get 45 ml of citrate buffer.
18. p-NAG solution (2.5 mM s *p*-nitrophenyl *N*-acetyl-β-d-glucosaminide in citrate buffer): 38.7 mg in 45 ml of citrate buffer.

3 Methods

3.1 Isolation and Differentiation of Bone Marrow-Derived Mast Cells from Mast Cell Progenitors

1. Euthanize mice 6–12 weeks of age by CO_2 inhalation and saturate the mouse with 70% ethanol.
2. Clip the abdominal skin below the sternum, grab on both sides of the incision with forceps, and remove the skin from the lower part of the body including the legs. Dissect the legs away from body with scissors.
3. Remove muscle from the legs with scissors and cut off the fibula. Clean the remaining tissue from a tibia using low-linting paper wipers. It is important to remove all the tissue to prevent contamination of the bone marrow preparation.
4. Separate the tibia from the femoral bone at the knee joint. Ensure that the tibia is intact to prevent contamination of the bone marrow. Saturate the tibia with 70% ethanol and place it on a 3 cm plastic plate with sterile ice-cold PBS. From this point, it is necessary that all procedures are carried out in a laminar flow hood with sterile tools, material, and solutions.
5. Grip the tibia with sterile forceps and cut off each end of bone.
6. Using a 25 G needle and a 5 cc syringe filled with mast cell medium (*see* **Note 2**), expel the bone marrow from both ends of the bone with a jet of medium directed into a 15 ml conical tube.
7. Centrifuge at 300 × *g* for 10 min at 4 °C and resuspend cells in 4 ml of mast cell medium.
8. Culture mast cells in 1 well of a 6-well tissue culture plate. Incubate cells at 37 °C in a humidified incubator under 5% (v/v) CO_2 for 4 weeks (*see* **Note 3**).

9. Change medium every 5–7 days or any time the medium changes color to orange or yellow. Regularly remove adherent cells from the culture by transferring the mast cell culture to a new plate. Remove excess mast cells or split the cell culture into bigger tissue culture plates if necessary (*see* **Note 3**).

3.2 Isolation and Differentiation of Mast Cell Progenitors from Newborn Liver

1. Isolate the liver and keep it on ice in IMDM medium.
2. Immediately after isolation, cut the liver into small pieces and pass them through the 70 μm nylon cell strainer by mincing with the tip of a 10 ml pipette containing mast cell medium.
3. Spin the cell suspension at 300 × *g* for 10 min at room temperature.
4. Wash cells once with 5 ml of mast cell medium.
5. Resuspend cells in mast cell medium and transfer them to a 10 cm tissue culture plate with 20 ml of complete media.
6. Change medium every 5–7 days or any time the medium changes color to orange or yellow for 4 weeks. Regularly remove adherent cells from the culture by transferring the cells in suspension to a new plate.

3.3 Isolation of Peritoneal Mast Cells

1. Prepare a 5 ml syringe fitted with a 25 G short needle and filled with 3 ml of PBS. Leave approximately 1 ml of air in the syringe (*see* **Note 4**).
2. Euthanize mice by CO_2 inhalation.
3. Make an incision into the abdominal skin below the sternum, taking care not to clip the peritoneal wall.
4. Grab the two sides of the cut using forceps and gently pull apart the abdominal skin, exposing the sternum and the pelvis.
5. Hold the peritoneum with forceps and gently fill the abdominal cavity with PBS and the air without disturbing blood vessels. The peritoneum should self-seal after removal of the needle.
6. Shake the mouse body gently about 20 times to increase the yield of cells in the peritoneal fluid.
7. Hold the peritoneal wall with forceps and make a small hole using scissors to insert a transfer plastic pipette into the air pocket in the peritoneal cavity. The previously injected air minimizes the loss of fluid in this step (*see* **Note 5**).
8. Express the air from the pipette in the cavity and aspirate the medium and the peritoneal cells.
9. While still holding the peritoneum, longitudinally open the abdominal wall using scissors. Collect the remaining peritoneal fluid.

10. Collect peritoneal cells from five mice (*see* **Note 6**).
11. Centrifuge the cell suspension for 5 min at 300×*g* at room temperature and remove supernatant.
12. Resuspend the pellet in 8 ml of 70% isotonic Percoll solution and transfer to a 15 ml conical tube (*see* **Note 7**).
13. Gently overlay the 70% Percoll solution with 2 ml of peritoneal mast cell (PMC) medium and centrifuge for 15 min at 700×*g* at room temperature. Mast cells and red blood cells (RBC) will form a pellet, while other cells will form a layer on the Percoll/PMC medium interface.
14. Carefully remove and discard the top layer and Percoll gradient without disturbing the mast cell pellet at the bottom of the tube.
15. Resuspend the mast cell pellet with 0.5 ml of PMC medium and transfer to a clean 15 ml conical tube with 10 ml of PMC medium (*see* **Note 8**).
16. Centrifuge for 5 min at 400×*g* and resuspend the pellet in 1 ml of PMC medium (*see* **Note 9**).

3.4 Characterization of Mast Cells Based on FcεRI and c-Kit Receptor Expression Using Fluorescence-Activated Cell Sorting (See Fig. 1)

1. Harvest 2×10^6 cells from each tested culture of mast cells.
2. Centrifuge cells for 5 min at 300×*g* at room temperature, and resuspend the cell pellet in 4 ml of fluorescence-activated cell sorting (FACS) staining buffer.
3. Separate cells into four 5 ml polypropylene tubes, each of which will contain 1 ml of cell suspension (5×10^5 cells).
4. Centrifuge the cells for 5 min at 300×*g* at room temperature, resuspend the cells in 250 μl of 1:100 diluted blocking antibody

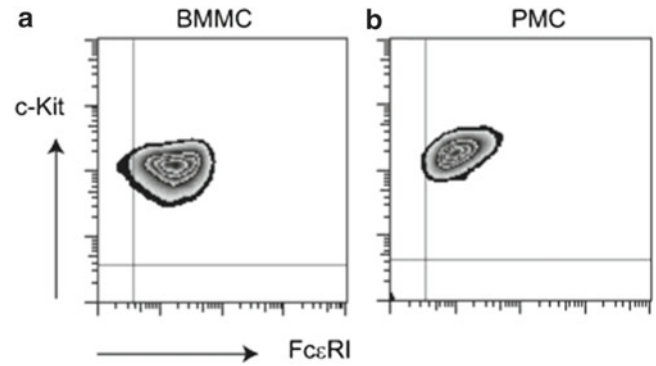

Fig. 1 Analysis of FcεRI and c-Kit expression on the surface of mast cells using fluorescence-activated cell sorting. Mast cells were differentiated from bone marrow (**a**) or isolated from mouse peritoneal cells (**b**). Cells were stained with fluorescently labeled specific antibodies and analyzed on a FACS Cyan (Beckman Coulter). Surface expression of FcεRI and c-Kit is similar in both types of mast cells

(anti-mouse FcγIII/II) in FACS staining buffer, and incubate for at least 5 min at room temperature.

5. Add 250 μl of 1:100 dilution of appropriate specific Ab in FACS staining buffer into each tube, as shown in Table 1 (*see* **Note 10**).
6. Incubate for at least 30 min at room temperature.
7. Wash once with 2 ml of FACS staining buffer.
8. Resuspend the cells in 0.5 ml of FACS staining buffer and measure fluorescence using a FACS instrument. More than 95% of mast cells in culture should be positive for both c-Kit and FcεRI in order to be ready for experiments.

3.5 Characterization of Mast Cells Based on Hexosaminidase Release

1. Incubate 3×10^6 mast cells with 1 μg of IgE overnight.
2. Wash cells two times in HBSS buffer with calcium and magnesium and 0.1% bovine serum albumin (BSA).
3. Resuspend cells in 2.0 ml of HBSS buffer.
4. Dilute antigen (HSA–BSA) by twofold serial dilution in HBSS buffer with calcium and magnesium and 0.1% BSA. The highest concentration recommended is 100 ng/ml. Prepare 0.2% Triton X-100 by mixing 40 μl of Triton 10% solution in 2 ml of PBS.
5. Pre-warm the cell suspension at 37 °C and transfer 100 μl of the cell suspension (150,000 cells) to each well of a 96-well plate.
6. Add 100 μl of diluted antigen, 0.2% Triton X-100 for positive control, or HBSS buffer for negative (basal release) control.
7. Incubate at 37 °C for 30 min.
8. Spin the plate in a centrifuge at $300 \times g$ for 3 min.
9. Transfer 10 μl of supernatant from each sample to a new 96-well plate.
10. Add 50 μl of p-NAG solution and incubate for 90 min at 37 °C.
11. Stop the reaction by adding 200 μl glycine/carbonate buffer.
12. Read the absorbance at 405 nm.
13. Calculation: For net release, subtract the basal release of non-activated cells from all of the results. The release in 0.2% Triton X-100 equals 100% (*see* **Note 11**).

3.6 Staining Mast Cell Granules Using a Cytospin Preparation

1. Wash 10^5 cells from the mast cell cultures or isolations in cold 1% BSA/PBS once and resuspend in 100 μl of cold 1% BSA/PBS.
2. Place marked slides and filters into appropriate slots in the cytospin with filters facing the center of the cytospin.

3. Aliquot 100 μl of each sample into the appropriate wells of the cytospin.
4. Place the cytospin lid over the samples and spin at 300 × *g* for 3 min.
5. Remove the filters and slides.
6. Examine each slide under the microscope to ensure that the cells are reasonably dispersed.
7. Air-dry the slides overnight. Do not fix the cells for toluidine and alcian blue/safranin staining.

3.7 Characterization of Mast Cells Based on Alcian Blue: Safranin O Staining

1. Incubate the air-dried cytospin for 10 min with a solution of 0.5% alcian blue in 0.3% acetic acid (pH 3).
2. Wash slides in PBS two times.
3. Incubate slides for 10 min with a solution of 0.1% safranin O in 0.1% acetic acid (pH 4).
4. Wash with PBS.
5. Air-dry.
6. Mount with mounting medium.

3.8 Characterization of Mast Cells Based on Toluidine Blue Staining (See Fig. 2)

1. Stain air-dry cytospins in toluidine blue working solution for 5 min.
2. Wash in PBS three times.
3. Dehydrate quickly in 100% EtOH.
4. To further clear slides, wash slides in xylene three times.
5. Air-dry in a chemical hood.
6. Mount with mounting medium.

4 Notes

1. Media are prepared under sterile conditions in a laminar flow hood to prevent contamination. After mixing all components, medium must be filtered through a 0.22 μm filter to sterilize and store up to 1 month at 2–8 °C.
2. This protocol is using mast cell medium based on IMDM. Alternatively, RPMI can be used. However, to reach the same efficiency of proliferation and differentiation of mast cell cultures as with IMDM, the concentrations of IL-3 in RPMI medium should be increased to 20 ng/ml.
3. Mast cell progenitors are expanding exponentially during differentiation. The amount of mast cells can be increased by transferring mast cell cultures to bigger tissue culture plates. Volume of culture medium can be increased at any time during

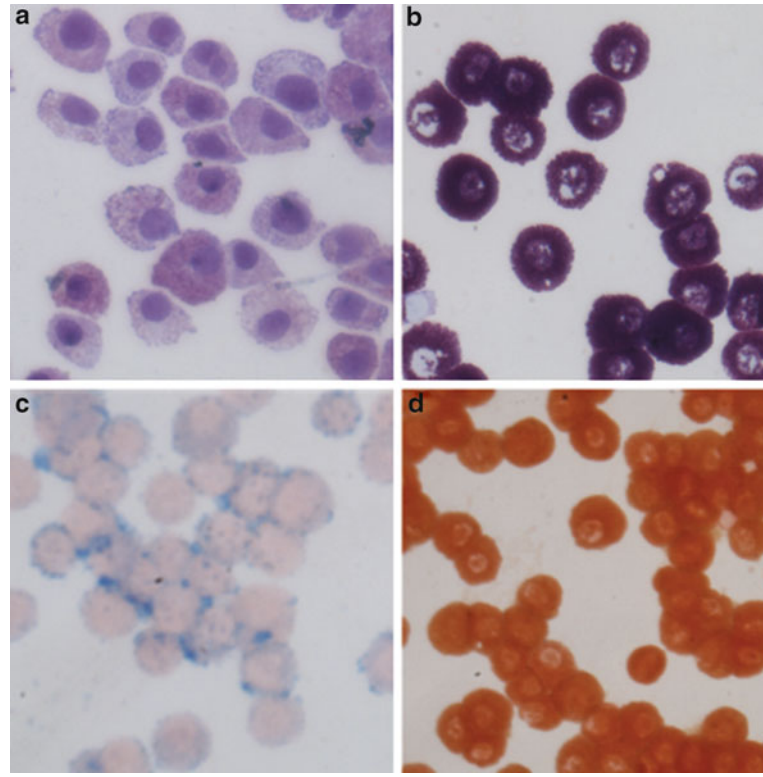

Fig. 2 Mast cell staining and evaluation of mast cell maturity and protease content in mast cell granules. Mast cells were obtained either by differentiation from bone marrow (**a**, **c**) or isolated from mouse peritoneal cells (**b**, **d**). Cells were stained with toluidine blue (**a**, **b**) or with alcian blue/safranin (**c**, **d**). Bone marrow-derived mast cells have only minimal amount of toluidine-stained granules compared to peritoneal mast cells. Bone marrow-derived mast cells stained with alcian blue, but not with safranin. Peritoneal mast cells have high heparin content and their granules can be stained with safranin

the culture process. For this reason, the protocol only uses the tibia for bone marrow isolation and 4 ml of medium for culture. This provides a sufficient amount of mast cells for most applications. For high-volume mast cell cultures, bone marrow from both femurs of the mouse and larger volumes of medium can be used at the beginning of differentiation.

4. Injected air makes collection of the fluid from the peritoneal cavity easier and minimizes fluid loss.
5. In this protocol, RBC are isolated together with mast cells. Thus, it is important to avoid any disturbance of blood vessels on the peritoneal wall during the isolation of peritoneal cells.
6. On average, the mouse peritoneal cavity contains only 10^5 mast cells. For effective isolation, five mice have to be pooled on one Percoll gradient.

7. The Percoll solution must be of room temperature during the cell separation for effective isolation of mast cells from the other peritoneal cells.
8. Mast cells are isolated together with RBC. RBC-lysis buffer can remove RBC. However, this treatment interferes with some applications (i.e., assessments of mast cell apoptosis). Small numbers of RBC do not affect most mast cell studies. Thus, for most applications RBC lysis is not necessary.
9. Isolated peritoneal mast cells can be immediately used for experiments; alternatively, PMC can be cultured in mast cell medium for several weeks. Their expansion, however, is minimal compared to mast cells derived from mast cell progenitors.
10. Samples 2 and 3 in Table 1 are not necessary for each culture tested. Those samples are needed only for compensation during the setup of the FACS instrument.
11. Mast cell degranulation typically ranges from 15 to 80% depending on many factors including the type of stimulation, mouse strain [9], and amount of cytokines in the culture medium [10].

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