

Chapter 15

Antiinflammatory and Immunosuppressive Functions of Mast Cells

Janet Kalesnikoff and Stephen J. Galli

Abstract

Through the release of biologically active products, mast cells function as important effector and immunoregulatory cells in diverse immunological reactions and other biological responses; for example, mast cells promote inflammation and other tissue changes in immunoglobulin E (IgE)-associated allergic disorders, as well as in certain innate and adaptive immune responses that are thought to be independent of IgE. Despite the mast cell's well-deserved reputation as a promoter of inflammation, others and we have used bone marrow-derived cultured mast cell (BMCMC) engrafted mast cell-deficient *c-kit*-mutant mice (so-called "mast cell knock-in" mice) to show that mast cells can also have important antiinflammatory and immunosuppressive functions in vivo. An early study showed that mast cells can contribute to susceptibility to ultraviolet B (UVB)-induced immunosuppression in one model of contact hypersensitivity (CHS), through effects mediated at least in part by histamine. Subsequently, it was reported that mast cells can mediate negative immunomodulatory effects following *Anopheles* mosquito bites, and in peripheral tolerance to skin allografts; however, the mechanism(s) by which mast cells mediate immunosuppressive functions in these two studies remains to be elucidated. Finally, we showed that mast cells and mast cell-derived IL-10 can limit the magnitude of and promote the resolution of certain CHS responses, and suppress the inflammation and skin injury associated with innate cutaneous responses to chronic low-dose UVB irradiation. This chapter outlines the generation of BMCMCs, a powerful model system commonly used to: (1) identify potential mast cell mediators in vitro; (2) study the mechanisms of mast cell activation and mediator release in response to specific stimuli in vitro; and (3) engraft mast cell-deficient mice to study the effector and immunoregulatory roles of mast cells or specific mast cell mediators in diverse immunological responses in vivo.

Key words: BMMCs, BMCMCs, Mast cell-deficient mice, Engraftment, IL-10

1. Introduction

Mast cells are derived from hematopoietic stem cells, but they do not ordinarily circulate in the mature form; instead, mast cells acquire their mature phenotype locally, following the migration

of their precursors to the vascularized tissues or serosal cavities in which the mast cells will ultimately reside (1–3). Mast cells participate in a wide variety of physiological and pathological processes as a result of their activation by any of an array of receptors. For example, they function as key effector cells in immunoglobulin E (IgE)-associated immune responses, including allergic disorders and certain protective immune responses to parasites. However, mast cells can be activated to perform important effector and immunoregulatory functions by many mechanisms that are independent of IgE (e.g., mast cells can be activated directly by pathogens, microbial products, endogenous and exogenous peptides, cytokines, and other inflammatory mediators) (1, 4).

Activated mast cells can release a diverse array of potent biologically active products (1, 3, 4). Many of these products are considered to be proinflammatory, in that they can elicit, individually or in concert, various features of inflammation (e.g., vasodilation, plasma extravasation, and the recruitment and activation of granulocytes, T cells, B cells, dendritic cells, and monocytes) and promote tissue remodeling. However, upon appropriate activation, mast cells can release products that are known to have several antiinflammatory or immunosuppressive properties, such as IL-10 and TGF- β . Thus, mast cells have the potential to exert positive or negative immunomodulatory functions on immune cells (i.e., influence the development, recruitment, survival, phenotype, or function of immune cells) and thereby enhance or suppress the initiation, magnitude, and/or duration of certain immune responses (1, 2).

Although mice that specifically lack only mast cells have not been reported, *c-kit*-mutant mice that are profoundly deficient in mast cells are often used to assess the contributions of mast cells or specific mast cell products to diverse biological responses in vivo. The mast cell deficiency of these mice can be selectively “repaired” through the adoptive transfer of genetically compatible, in vitro-derived mast cells [e.g., bone marrow-derived cultured mast cells (BMCMCs) from congenic wildtype mice or various transgenic or mutant mice, BMCMCs transduced with short-hairpin RNA to reduce expression of proteins of interest, embryonic stem cell-derived cultured mast cells (ESCMCs), fetal liver-derived mast cells (FLMCs), etc.]. Because *c-kit* mutations affect lineages other than mast cells (see Note 1), it is essential to perform experiments on these so-called “mast cell knock-in mice” to assess the extent to which differences in the biological responses of *c-kit*-mutant mice compared with wildtype mice are due to the lack of mast cells in the *c-kit*-mutant mice (1, 2, 4). Recently, transgenic mice have been generated that express Cre recombinase under the control of the promoter of the gene encoding mouse mast cell

protease 5 (Mcp5) (5) or the gene encoding baboon α -chymase (6). Although the phenotypic features of such mice (as expressed in mast cells and possibly other cell types) remain to be fully characterized, mice with confirmed mast cell-specific Cre expression and mice with inducible mast cell-specific Cre expression may well become powerful genetic models for investigating the contributions of mast cells or mast cell-specific products to health and disease.

Mast cell knock-in mice have been used to identify antiinflammatory and immunosuppressive roles of mast cells during specific immune responses in vivo. An early study showed that mast cells, through effects mediated at least in part by histamine, contribute to the ability of ultraviolet B (UVB) irradiation of the skin to induce systemic immunosuppression of contact hypersensitivity (CHS) responses to the hapten TNCB (2,4,6-trinitrochlorobenzene) (7). Mast cells have also been shown to mediate immunosuppressive functions following *Anopheles* mosquito bites (8), and in peripheral tolerance to skin allografts (which requires the participation of CD4⁺CD25⁺FoxP3⁺ T_{Reg} cells) (9). The mechanism(s) by which mast cells mediate immunosuppressive functions in these two studies remains to be elucidated; however, IL-10 was implicated as a mechanism of immunosuppression in each of these studies, acting either in the draining lymph nodes (8) or at the sites of skin allografts (9). In another study, mast cells and mast cell-derived IL-10 were shown to limit the magnitude of and promote the resolution of CHS responses induced in response to the hapten DNFB (2,4-dinitro-1-fluorobenzene) or urushiol (the hapten-containing sap of poison ivy or poison oak), and to suppress the inflammation and tissue pathology associated with innate cutaneous responses to chronic low-dose UVB irradiation (10). Defining the mechanisms by which mast cells or specific mast cell mediators (such as IL-10 or other mediators) can suppress, rather than enhance, features of specific innate or acquired immune responses and identifying the signals that can elicit such distinct mast cell functions are important goals for future research.

Human mast cell populations and mouse BMCMCs, as well as cultured mast cells derived from other hematopoietic tissues (such as fetal liver) or from embryonic stem cells, are powerful tools to investigate the mechanisms by which mast cells might influence various immunological or other biological responses in vitro. BMCMCs, which can easily be expanded to produce large numbers of mast cells in vitro, are the cell type most commonly used for in vitro studies of mast cell activation events and engraftment into c-*kit*-mutant mast cell-deficient mice. This chapter will describe in detail the generation and characterization of BMCMCs and current protocols for the adoptive transfer of BMCMCs into mast cell-deficient mice.

2. Materials

2.1. Generation of BMCMCs from Mouse Bone Marrow

2.1.1. Bone Marrow Extraction

1. Flushing medium: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% antibiotic-antimycotic solution, and 50 μ M β -mercaptoethanol. Filter sterilized (0.2 μ m bottle top filter).
2. Sterile scissors and forceps.
3. Phosphate buffered saline (PBS).
4. Six-well culture plate and 50 ml Falcon tubes.
5. 5 ml Syringe; 25 gauge needle.

2.1.2. Cell Culture

1. Culture medium: DMEM (see Note 2), supplemented with 10% FBS, 20% Wehi-3 conditioned medium (as a source of IL-3; see Note 3), 2 mM L-glutamine, 1% antibiotic-antimycotic solution, and 50 μ M β -mercaptoethanol. Filter sterilized (0.2 μ m filter).

2.2. Assessing Maturity of BMCMCs

2.2.1. Flow Cytometry

1. Flow cytometry buffer: PBS supplemented with 2% FBS (see Note 4).
2. 5 ml Polystyrene round bottom tubes (compatible with flow cytometer).
3. Purified anti-mouse CD16/32, clone 93 (eBioscience, San Diego, CA; see Note 5).
4. PE conjugated anti-mouse Fc ϵ RI alpha, clone MAR-1 (eBioscience; see Note 6).
5. FITC conjugated anti-mouse CD117 (c-Kit) (2B8) (eBioscience; see Note 6).
6. Propidium iodide; prepare a 1 mg/ml stock.

2.2.2. May-Grünwald/Giemsa Staining

1. Microscope slides, precleaned.
2. PBS.
3. PAP pen.
4. Accustain® May-Grünwald stain (MG1L; Sigma, St. Louis, MO).
5. Accustain® Giemsa stain, Modified (GS1L; Sigma).
6. Mounting medium: CytoSeal 60, DPX, or Permount.

2.3. Adoptive Transfer of BMCMCs into Mast Cell-Deficient Mice

2.3.1. Engraftment of Mast Cell-Deficient Mice

1. DMEM or PBS.
2. 1 ml Syringe; 27 or 30 gauge needle.

2.3.2. Staining and Quantification of Mast Cells

1. 10% Buffered formalin (fixative): 100 ml 40% formalin w/v, 900 ml deionized water, 4 g sodium phosphate monobasic monohydrate ($\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$), 6.5 g sodium phosphate dibasic anhydrous (Na_2HPO_4). Store at room temperature or 4°C .
2. Carnoy's solution (fixative): 3:2:1 v/v/v of ethanol, chloroform, and acetic acid. Prepare fresh.
3. Microscope slides, precleaned.
4. Toluidine blue, 1% aqueous.
5. Alcian blue/Safranin O (Csaba) stain: 900 mg Alcian blue 8GX, 45 mg Safranin O, 1.2 g ferric ammonium sulfate, 250 ml acetate buffer (pH 1.42). Acetate buffer: mix 50 ml solution A (13.6 g sodium acetate in 100 ml deionized water) with 62 ml solution B (8.5 ml 12 N HCl plus 91.5 ml deionized water); make up to 100 ml with deionized water and adjust pH to 1.42. Store Csaba stain at room temperature.
6. Ethanol (70, 95, and 100%).
7. HCl (12 and 1 N).
8. Histo-Clear (National Diagnostics, Atlanta, GA).
9. Mounting medium: CytoSeal 60, DPX, or Permount.

3. Methods

BMCMCs can easily be generated and expanded *in vitro* by culturing mouse bone marrow cells in the presence of Interleukin (IL)-3 for a period of 4–6 weeks. Mature mast cells express Fc ϵ RI (the high-affinity IgE receptor) and Kit (the receptor for stem cell factor, an important regulator of mast cell development, survival, proliferation, and activation) on their surface; these markers can be used to assess the maturity and purity of BMCMC cultures by flow cytometry. Another characteristic feature of mature mast cells is large cytoplasmic granules, which store preformed bioactive mediators of inflammation (such as histamine); BMCMCs can be monitored for metachromatic granule formation by May-Grünwald/Giemsa staining (mast cell granules stain purple). BMCMCs are often used to study the signaling pathways that are activated downstream of specific receptors (by western blot analysis or phosphoflow cytometry), as well as stimulus-induced mast cell functional activation events, including degranulation, and cytokine, chemokine, or eicosanoid lipid mediator release (by various assays including degranulation assays and ELISAs). Although BMCMCs are notoriously difficult to transfect, viruses (e.g., lentiviruses) can readily be used to overexpress or knock-down specific proteins of interest in BMCMCs and the

resulting cells can be expanded and used for both in vitro and in vivo studies (1, 3).

While in vitro studies of mouse BMCMCs can be very useful for investigating mechanisms by which mast cells might exert antiinflammatory or immunosuppressive functions in various immune responses, *c-kit*-mutant mast cell-deficient mice are often used to assess the in vivo relevance and biological importance of such in vitro observations. The two most commonly used models for such studies are WBB6F₁-*Kit*^{W/W^v} or C57BL/6-*Kit*^{W^{sh}/W^{sh}} mice (1, 2). C57BL/6-*Kit*^{W^{sh}/W^{sh}} mice are gaining in popularity for such studies because they have fewer or less-severe phenotypic abnormalities than are observed in WBB6F₁-*Kit*^{W/W^v} mice. For example, C57BL/6-*Kit*^{W^{sh}/W^{sh}} mice are neither anemic nor sterile and therefore can easily be crossed with mice carrying other defined genetic abnormalities of interest (11, 12). However, it is important to consider the effects of other *c-kit*-related phenotypic abnormalities in these mice when interpreting experimental results (see Note 1). To assess the extent to which differences in the expression of biological responses observed in mast cell-deficient and wildtype mice reflect the absence of mast cells or the reduction or absence of specific products expressed by mast cells, BMCMCs can be administered by intravenous (i.v.), intraperitoneal (i.p.), or intradermal (i.d.) injection to generate “mast cell knock-in mice” (1, 4, 11–16). When designing and interpreting experiments, it is important to recognize that the numbers, anatomical distribution, and phenotypic characteristics of adoptively transferred mast cells depend on the injection route or site and the interval of time after injection of the mast cell population into the recipient *c-kit*-mutant mice. Following adoptive transfer of BMCMCs, tissues can be tested for selective repair of mast cell deficiency by identifying mast cells by the staining of their cytoplasmic granules with Toluidine blue (cytoplasmic granules appear purple, defined as “metachromasia”) or Alcian blue/Safranin O (Csaba) stain; the granules of all mature mast cells stain blue with Alcian blue, but some mast cells have granules that stain red with safranin, perhaps reflecting their content of heparin. One also can measure the amounts of mast cell-associated mediators or transcripts in such tissues (17).

3.1. Generation of BMCMCs from Mouse Bone Marrow

3.1.1. Bone Marrow Extraction

1. Pour sterile PBS into six-well culture plate (one well per mouse) and place on ice.
2. Sacrifice donor animal(s) (see Note 7) and remove skin from legs using sterile forceps and scissors (spray animal(s) and/or rinse instruments with 70% ethanol as needed).
3. Remove femur and tibia; scrape away all tissue from bones using sterile scissors (or scalpel blades), discard fibula.
4. Place bones in PBS on ice until all bones are collected. Carry out all remaining steps in tissue culture hood.

5. Using sterile instruments, pick up a bone with forceps and cut off epiphyses (bone ends) with scissors to expose medullary cavity.
6. Using a 5 ml syringe with a 25-gauge needle, flush the marrow out of each bone with flushing medium into a labeled 50 ml collection tube. Pool the femoral and tibial bone marrow from each mouse into one collection tube. Fill tube with flushing medium to wash bone marrow cells.
7. Centrifuge marrow ($500\times g$, 5 min, 20°C).
8. Remove supernatant and resuspend pellet in 10 ml of culture medium per mouse. Place cells in tissue culture flask of appropriate size (e.g., T25 for one mouse, T75 for two mice, etc.).

3.1.2. Cell Culture

1. One to two days after plating cells, transfer medium and suspension cells (leaving debris and adherent cells to stick to bottom of flask) to a new flask and add fresh culture medium (10 ml/mouse).
2. In subsequent weeks, feed cells every 3–4 days (add ~10 ml of culture medium per mouse). Maintain cell density between 2.5×10^5 and 1×10^6 cells/ml. Transfer suspension cells to a new flask once a week until you no longer see adherent cells in the culture flask.
3. Optional (to increase yield of BMCMCs): After 3–4 weeks of culture, centrifuge one-third to half of the culture volume, remove medium, and replace with equal volume of culture medium. Return cells in new medium to original flask, and then dilute cells as necessary.
4. Production of BMCMC populations of suitable phenotype ($\text{Fc}\epsilon\text{RI}^+\text{Kit}^+$ cells with prominent cytoplasmic granules) and numbers (we usually generate $2.5\text{--}10\times 10^7$ BMCMCs per mouse input) takes 4–6 weeks. Test maturity of cells (see [Subheading 3.2](#)) before use in in vitro assays or engraftment into mast cell-deficient mice.

3.2. Assessing Maturity of BMCMCs

3.2.1. Flow Cytometry

1. Need 5×10^4 to 5×10^5 cells/condition. Wash cells with PBS + 2% FBS (see Note 4) in 5 ml polystyrene round bottom tube. Centrifuge at $500\times g$, 5 min, 4°C . Aspirate.
2. Dilute anti-mouse CD16/32 (clone 93; see Note 5) monoclonal antibodies 1/200 in PBS + 2% FBS. Add 10 μl to each pellet (to block nonspecific binding of antibodies). Incubate 5 min on ice.

Protect samples from direct light for all remaining steps (use lid on ice bucket).
3. Prepare staining solution: dilute PE conjugated anti-mouse $\text{Fc}\epsilon\text{RI}$ alpha (clone MAR-1) 1/200 and FITC-conjugated anti-mouse CD117 (c-Kit) (2B8) 1/200 in PBS + 2% FBS

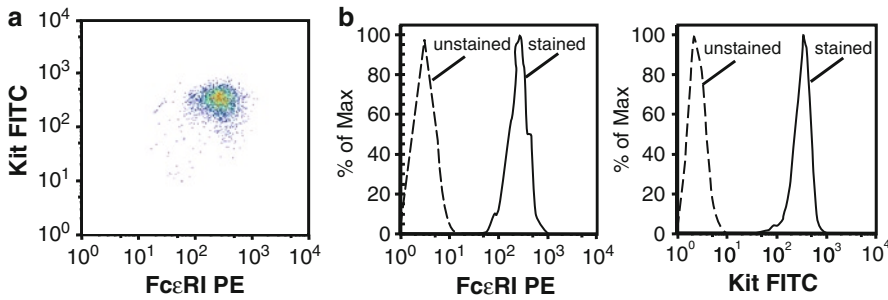


Fig. 1. Analysis of FcεRI and Kit surface expression on BMCMCs by flow cytometry. (a) BMCMCs stained with PE conjugated anti-mouse FcεRI and FITC conjugated anti-mouse Kit. (b) Histogram of FcεRI (*left panel*) or Kit (*right panel*) expression in unstained vs. stained (as in a) BMCMCs.

(in the same tube; see Note 6). Add 20 µl of staining solution to desired tubes from step 2; for each BMCMC population, it is best to have one tube of cells that receives staining solution (“stained”) and one tube of cells that does not receive staining solution (“unstained”). Incubate 15–30 min on ice.

4. Wash cells with 1–3 ml PBS + 2% FBS. Centrifuge at 500 × *g*, 5 min, 4°C. Aspirate.
5. Dilute propidium iodide (PI) stock 1/1,000 in PBS + 2% FBS (final concentration 1 µg/ml). Resuspend cell pellets in 200–400 µl of diluted PI (to exclude dead cells).
6. Analyze cells on flow cytometer.
7. Analyze results using data analysis software (we use FlowJo software; Tree Star, Ashland, OR). An example of the results produced by flow cytometry is shown in Fig. 1.

3.2.2. May-Grünwald/ Giemsa Stain

1. To prepare a cytocentrifuge preparation of BMCMCs (1 × 10⁵ BMCMCs/slide), wash cells once with PBS (centrifuge at 500 × *g*, 5 min, 20°C, then aspirate) and resuspend cells at 1 × 10⁶ cells/ml in PBS. These instructions assume the use of a Shandon Cytospin Cytocentrifuge: label slide and place in slide holder; place slide filter on top of the slide; place sample chamber on top of filter; close slide holder clip; load 100 µl of BMCMC suspension into sample chamber. Spin at 500 rpm, 5 min. Remove slide and air dry (10 min to overnight).
2. Draw a wax circle around cells using a PAP pen.
3. Add May-Grünwald Stain to cover cells. Incubate 5 min.
4. Wash slides in PBS (2–5 min).
5. Dilute Geimsa 1/20 with deionized water. Add to cells and incubate 20 min.
6. Wash slides in tap water (1 min), then air dry slides.
7. Coverslip cells using mounting medium.

8. Examine cells using light microscope. An example of BMCMCs after May-Grünwald/Giemsa staining is shown in Fig. 2.

3.3. Adoptive Transfer of BMCMCs into Mast Cell-Deficient Mice

3.3.1. Engraftment of Mast Cell-Deficient Mice

- 1. Culture a sufficient number of donor BMCMCs for the mice you wish to engraft (see Note 8). The number of BMCMCs that should be used for adoptive transfer into mast cell-deficient mice depends on the route of transfer (Table 1); the following numbers are guidelines, but can be refined, as indicated based on the purpose of the study, for each type of model under investigation.
- 2. Count cells and resuspend in appropriate volume cold DMEM or PBS. Keep cells on ice until ready to inject.

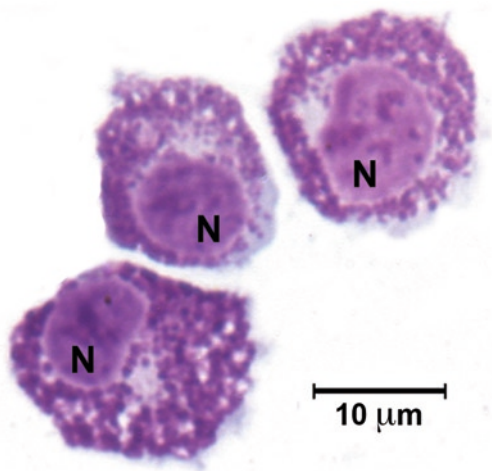


Fig. 2. C57BL/6J BMCMCs stained with May-Grünwald/Giemsa. Note the large number of cytoplasmic granules throughout the cytoplasm. *N* nucleus.

Table 1
Numbers of BMCMCs recommended for adoptive transfer into mast cell-deficient mice

Transfer route	Number of BMCMCs	Injection volume	Needle gauge
Intradermal (i.d.)			
Ear	1–2 × 10 ⁶ cells	50 μl (2 × 25 μl injections; see Note 9)	30 gauge
Back skin	4 × 10 ⁶ cells	400 μl (8 × 50 μl injections in two rows down the length of shaved back)	27 gauge
Intraperitoneal (i.p.)	2–5 × 10 ⁶ cells	200 μl	27 gauge
Intravenous (i.v.)	5–10 × 10 ⁶ cells	100 μl (retroorbital) or 200 μl (tail vein)	30 gauge

See Note 10 regarding the interval after BMCMC transfer to wait before starting experiments to investigate mast cell function

3. Inject BMCMCs [use 27- or 30-gauge needle (see Table 1) and 1 ml syringe] into anesthetized *c-kit*-mutant mice; recipient mice should be 4–6 weeks of age.
4. Time needed for engraftment depends on the route of injection and should be assessed for each experiment (see Note 10). General guidelines are as follows: i.d., 6–8 weeks; i.p., 4–8 weeks; i.v., 8–18 weeks. Wait appropriate amount of time and perform desired in vivo experiment. Part of the analysis should include assessment of the numbers of mast cells that have been engrafted into the anatomical sites of interest (see Subheading 3.3.2 and Note 11).

3.3.2. Staining and Quantification of Mast Cells

1. Euthanize mice and remove tissue(s) of interest (ear pinna, back skin, lung, spleen, lymph nodes, etc.). Place each specimen in labeled histology cassette.
2. Fix specimens in 10% buffered formalin (overnight at 4°C) (see Note 12).
3. Process samples for paraffin embedding (we use an automated processing schedule on the Shandon Hypercentre XP enclosed tissue processor).
4. Embed tissues in paraffin (we use the Shandon Histocentre 2), ensuring a cross-sectional orientation of all tissues, and section tissues at 4 µm.
5. Dewax/rehydrate paraffin sections: 2× Histo-Clear (5 min each), 2× 100% ethanol (2 min each), 1× 95% ethanol (2 min), 1× 70% ethanol (2 min), 3× deionized water (2 min each).
6. Stain tissues with 0.1% toluidine blue or Alcian blue/Safranin O (Csaba) stain (see Note 12).

Toluidine blue. Dilute Toluidine blue 1% aqueous stock 1/10 in 1 N HCl. Add 0.1% Toluidine blue to cover tissue sections. Incubate 1 min. Rinse immediately with tap water. Air dry, then cover slip using DPX or other suitable mounting medium.

3.3.3. Alcian blue/Safranin O (Csaba) stain

Add Alcian blue/Safranin O (Csaba) stain to cover sections. Incubate 20–30 min. Dehydrate/mount in 70% ethanol (30 s), 95% ethanol (1 min), 2× 100% ethanol (2 min each), 2× Histo-Clear (2 min each). Mount using Permunt or other suitable mounting medium.

7. Count mast cells using light microscope. For ear pinna and back skin, average the number of mast cells counted in ten consecutive fields of 1 mm length; express as number of mast cells per horizontal field length (mm). For all other tissues, quantify mast cells according to area (mm²). Report the average number of mast cells present in a 10–25 field count and express as number of mast cells per mm².

3.3.3.1. Mesenteric Windows

1. Carefully arrange mesenteric windows onto slides. Air dry (~4 h).
2. Fix specimen in Carnoy's solution (1 h). Air dry (overnight). After fixation, can store mesenteric windows at 4°C until ready to stain.
3. Carefully remove bowel from mesenteric window. Add Alcian blue/Safranin O (Csaba) stain to cover mesentery. Incubate 20–30 min. Wash slide in tap water. Air dry. Dip in Histo-Clear (optional). Mount using Cytoseal 60 or suitable mounting medium.
4. Count cells as indicated in step 7 above (report mast cells numbers per mm² of mesentery).

4. Notes

1. In addition to lacking mast cells, *c-kit*-mutant mice have other phenotypic abnormalities that may reflect effects of the *c-kit* mutation on other cell lineages. For example, WBB6F₁-*Kit*^{W/W^v} mice, but not C57BL/6-*Kit*^{W-sh/W-sh} mice, are anemic and sterile. Moreover, C57BL/6-*Kit*^{W-sh/W-sh} mice exhibit increased numbers of neutrophils in the spleen, bone marrow, and blood, whereas *Kit*^{W/W^v} mice are neutropenic (16, 18, 19). The *c-kit*-dependent but mast cell-independent phenotypic abnormalities in these mice should be taken into account when using this approach to assess the role of mast cells in various disease models.
2. Can also culture BMCMCs in Iscove's Modified Dulbecco's Medium (IMDM).
3. Can culture BMCMCs in 10 ng/ml interleukin (IL)-3 (instead of 20% WEHI-3 conditioned medium). To generate WEHI-3 conditioned medium: Culture WEHI-3 cells (ATCC: TIB 68) at 1×10⁵ cells/ml in IMDM containing 10% FBS for 3–4 days until the cell concentration reaches 6×10⁵–1×10⁶ cells/ml. Centrifuge culture (500×g, 10 min), then centrifuge supernatant again (1,500×g, 15 min). Filter supernatant through a 0.45 μm filter (Nalgene) and store 100% WEHI-3 conditioned medium at –20°C.
4. Can use PBS, PBS + 0.5% FBS, or PBS + 2% FBS as buffer for flow cytometry staining.
5. Anti-mouse CD16/32 clone 93 or clone 2.4G2 antibodies can be used to block Fc binding.
6. Anti-mouse FcεRI alpha or CD117 (c-Kit; 2B8) antibodies can be coupled to other convenient fluorophores (APC, etc.).
7. We typically generate BMCMCs from mice that are 4–12 weeks of age.

8. Male-derived donor BMCMCs are not suitable for engraftment of female mice. Female-derived donor BMCMCs will successfully engraft into both male and female recipients. Ideally, donor BMCMCs should be generated from mice of the same genetic background as the recipient *c-kit*-mutant mice.
9. Ear (i.d.) engraftment: first injection in the middle of the ear; second injection towards tip of the ear (the fluid remaining from the first injection makes the second injection much easier).
10. After the adoptive transfer of BMCMCs into *c-kit*-mutant mice, the numbers, anatomical distribution, and features of maturation of the adoptively transferred mast cells vary according to the route of injection, the anatomic site, and the interval after transfer (1, 11–13, 15, 20, 21). For example, 4 weeks after the i.p. transfer of BMCMCs into *c-kit*-mutant mice, mast cells numbers are comparable to those observed in wildtype mice (as assessed by the evaluation of peritoneal lavage fluids) and mast cells can be found in the mesentery (14, 15, 20, 22, 23). During the weeks after adoptive transfer, the transferred mast cells acquire phenotypic characteristics that, compared to those of the in vitro-derived mast cells assessed prior to in vivo injection, more closely resemble those of native peritoneal mast cells in the corresponding anatomical sites in wildtype mice (1, 13, 20). However, it is impossible to prove that such adoptively transferred mast cells are literally identical to the native mast cell populations at the corresponding anatomical sites in wildtype mice. Moreover, based on studies of adoptively transferred mast cells in the peritoneal cavity, it appears that the longer the adoptively transferred mast cells remain in a particular anatomical site, the more closely their phenotype resembles that of the corresponding native population of mast cells in wildtype mice of the same age (13). Thus, depending on which aspects of mast cell function are being assessed in each experimental model under investigation, it may be necessary to wait for longer intervals after the transfer of mast cells to initiate the experiment (as the adoptively transferred mast cells may require higher levels of maturity to perform some functions than is required for other functions).
11. For i.p. transfer, we quantify the number of mast cells present in cells washed from the peritoneal cavity and also evaluate the presence and distribution of mast cells in the mesentery of the recipient mice (11, 22, 23). For i.d. transfer, we quantify the number of mast cells per mm² of dermis (11). For i.v. transfer to engraft mast cells in the lungs, we quantify the number of mast cells per mm² of lung parenchyma (11, 24–27). We find that the distribution of mast cells in the peritoneal cavity (after i.p. transfer of BMCMCs) or dermis (after i.d. transfer of BMCMCs) is very similar to that of the native

populations of mast cells in the same anatomical sites in the corresponding wildtype mice. However, after i.v. transfer of BMCMCs, the distribution of mast cells in the respiratory tract of the recipient *c-kit*-mutant mice differs from that in the corresponding wildtype mice in that there are fewer mast cells in the trachea and more in the periphery of the lung in the mast cell knock-in mice than in the corresponding wildtype mice (24). Remarkably, for many features of the “asthma models” analyzed in such mast cell knock-in mice, the results obtained in the mast cell knock-in mice were very similar to (and in many cases statistically indistinguishable from) those in the wildtype mice (24–27). This may reflect the fact that many mast cell functions are mediated by the release of soluble mediators, which can have their effects at some distance from the mast cells that secreted them.

12. Toluidine blue or (Alcian blue/Safranin O (Csaba) stain can be used to quantify mast cells containing cytoplasmic granules in both paraffin and frozen sections. However, if mast cells have recently undergone extensive degranulation, identifying them using stains which bind to the cytoplasmic granules (or by using immunohistochemistry to detect constituents stored in the cytoplasmic granules) may underestimate the numbers of mast cells actually present in the tissues; this is also true when Giemsa staining is used to quantify mast cells in Epon-embedded 1 μ m sections [e.g., in the skin after treatment with phorbol 12-myristate 13-acetate (PMA)] (28).

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