

Chapter 11

Isolation and Characterization of Human Intestinal Mast Cells

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Abstract

Mast cells are granulated immune cells typically located at barrier sites of the body, such as the skin and the mucosa of the respiratory, urogenital, and gastrointestinal tract. They are well known for their capacity to participate in the orchestration of inflammatory and immune responses by releasing a broad array of mediators as a consequence of IgE-dependent and IgE-independent activation. Mast cells derive from myeloid progenitors, but in contrast to other myeloid cells, they leave the bone marrow in an immature state; therefore, mast cells are not visible in the blood under normal conditions. For full maturation, the tissue environment is necessary. Thus, mature mast cells can be only isolated from tissue such as skin or mucosal sites, which makes mast cell isolation complicated. This chapter describes methods to isolate, purify, and culture mast cells from the human intestinal mucosa. Human mucosal mast cells can be used to characterize their mediators and to study the mechanisms of human mast cell activation, signal transduction, and exocytosis in response to specific stimuli.

Key words Mast cells, Human, Intestinal, Gut, Bowel, Cell isolation, Cell culture, Cell activation, Mediator release assay

1 Introduction

Mast cells are heavily granulated immune cells that are dispersed throughout the body. They originate from immature, bone marrow-derived CD34⁺ hematopoietic stem cells circulating in the peripheral blood as committed progenitors before homing [1–3]. Unlike other immune cells, mature mast cells are only found in tissues, not in the blood. They are located at sites of the host–environment interface, such as the skin and the mucosa of the respiratory, urogenital, and gastrointestinal tract [1–4]. The normal human gastrointestinal tract contains numerous mast cells. The largest number is found in the *lamina propria*, where 2–3 % of the cells are mast cells [4]. According to their protease content, human mast cells have been divided into two subtypes, namely, cells containing both tryptase and chymase (MC_{TC}, similar to the so-called

connective tissue-type mast cells in rodents) and mast cells containing only tryptase (MC_T, similar to the so-called mucosal-type mast cells in rodents). Most of mast cells found in the *lamina propria* belong to the tryptase-positive, chymase-negative subtype (MC_T).

Mature mast cells are involved in physiological processes such as host defense against bacteria and tissue remodeling [1–5]. Apart from these functions, mast cells are known to be of particular importance in the pathophysiology of immediate-type allergic reactions and of other chronic inflammatory diseases. They exert their biological functions by releasing a broad array of mediators as a consequence of IgE-dependent and IgE-independent activation. These mediators are either preformed such as histamine, chymase, and tryptase and released from cytoplasmic granules or de novo synthesized such as various lipid compounds (leukotrienes and prostaglandins) with pleiotropic inflammatory and chemotactic functions as well as a number of cytokines, chemokines, and growth factors [1–3]. Cross-linking of the high-affinity IgE receptor (FcεRI) by receptor-bound IgE and antigen is the major trigger for mast cell activation [6]. In addition, particular cytokines such as the mast cell growth factor/stem cell factor (SCF) and IL-4 have been identified as important regulators of human mast cell function [7–13]. In the gut, mast cells interact with nerves and epithelial cells to regulate various physiological processes such as intestinal motility, intestinal permeability, or ion and fluid secretion. Furthermore, intestinal mast cells are involved in intestinal inflammation, e.g., in the course of allergic enteritis or IBD [4, 14, 15].

Most studies aimed to analyze mechanisms regulating mast cell-driven diseases or the physiological importance of mast cells have been performed in rodent mast cells. However, to understand how these responses pertain to human physiology or disease, human mast cell models are of particular interest. Because of the restricted FcεRI expression and the very low levels of proteases, human mast cell lines such as HMC-1 and LAD2 can serve as surrogates of tissue mast cells to a very limited degree only [16]. Thus, a number of systems have been developed to obtain sufficient quantities of primary human mast cells. The newly developed method for obtaining mast cells from human embryonic stem cells is not allowed in all countries [17]. Mast cells generated from immature precursors derived from peripheral blood or cord blood release the appropriate suite of inflammatory mediators in response to mast cell activators including antigen and are frequently used [18]. However, mast cells undergo their final phase of differentiation in the tissues and display functional diversity depending on the tissue in which they differentiate. We have established methods to isolate and purify human intestinal mast cells, providing a unique source of tissue-derived mature human mast cells. Similar methods have been used for the isolation of human mast cells from other organs such as the lung or the skin [19–21].

The following protocols describe the isolation of cells from the human intestinal mucosa by a combination of mechanical fragmentation and enzymatic digestion, the purification of mast cells by magnetic cell separation, and the culture of mast cells. Furthermore, we describe methods for the characterization of effector functions of human intestinal mast cells with the focus on mediator release, cytokine production, and activation of signaling molecules.

2 Materials

2.1 Cell Isolation

1. Shaking water bath (37 °C).
2. Tyrode's buffer: 137 mM NaCl, 2.7 mM KCl, 0.36 mM Na₂HPO₄, 5.55 mM glucose; adjust to pH 7.4 and store at 4 °C.
3. Tissue storage buffer: Tyrode's buffer, ampicillin 0.5 mg/mL, gentamicin 0.2 mg/mL, and metronidazole 0.2 mg/mL. Store at 4 °C.
4. TE buffer: Tyrode's buffer containing 2 mM ethylenediamine-tetraacetic acid (EDTA); store at 4 °C.
5. TGMD buffer: Tyrode's buffer supplemented with gelatin (1 mg/mL) and 1.23 mM MgCl₂. Make fresh as required.
6. Enzyme solution PCh: 25 mL of TE buffer, 75 mg of pronase (*Roche Diagnostics*), and 13 mg of chymopapain (*Sigma-Aldrich*); make fresh as required.
7. Enzyme solution Co: 50 mL of TGMD buffer, 75 mg of collagenase NB 4G (*Serva*), 15 µg/mL DNase (*Roche Diagnostics*); make fresh as required.
8. GIBCO® Media RPMI 1640 with phenol red, GlutaMAX, and 25 mM HEPES (*Life Technologies*).
9. Culture medium: GIBCO® Media RPMI 1640 supplemented with 10 % (v/v) heat inactivated fetal calf serum (FCS), 100 µg/mL streptomycin, 100 µg/mL gentamicin, 100 U/mL penicillin, and 2.5 µg/mL amphotericin B.
10. Trypan blue.
11. May-Grünwald/Giemsa stain.
12. Giemsa (Azure Eosin Methylene Blue).
13. Tweezers and scissors, kept in sterile beaker with 70 % ethanol.
14. Bottle-top filter.
15. Nylon mesh, pore size 250 and 100 µm.
16. 50 mL conical tubes.
17. *N*-Acetyl-L-cysteine.

2.2 Purification of Intestinal Mast Cells

1. 80 cm² or 175 cm² cell culture flask.
2. Auto MACS Rinsing Solution (*Miltenyi Biotec*).
3. Bovine serum albumin (BSA).
4. Dead Cell Removal Kit (*Miltenyi Biotec*).
5. CD117 human MicroBead Kit directed against human *c-Kit* (*Miltenyi Biotec*).
6. MACS LS Columns (*Miltenyi Biotec*).
7. MidiMACS Separation Unit (*Miltenyi Biotec*).
8. MACS Multi Stand (*Miltenyi Biotec*).
9. Nylon mesh, pore size 30 μ m.
10. Trypan blue, May–Grünwald/Giemsa stain, bottle-top filter, 50 mL conical tubes, culture medium (*see* Subheading 2.1).

2.3 Culture of Intestinal Mast Cells

1. CO₂ incubator.
2. Human recombinant SCF (rhSCF).
3. Human recombinant IL-4 (rhIL-4).
4. 6-, 12-, 24-, 48-, or 96-well flat-bottom plates.
5. Trypan blue, May–Grünwald/Giemsa stain, culture medium (*see* Subheading 2.1).

2.4 Stimulation of Intestinal Mast Cells and Measurement of Mediators and Signaling Molecules

1. Human myeloma IgE, antihuman IgE antibodies (provided by Dr. U. Blank, INSERM Unite 699, Paris, France) or antihuman Fc ϵ RI alpha antibodies or Ionomycin and PMA.
2. Stimulation buffer with 135 mM NaCl, 5 mM KCl, 5.6 mM glucose, 10 mM HEPES (pH 7.3), 1.8 mM CaCl₂, 1 mM MgCl₂, and 0.5 mg/mL BSA.
3. Citrate buffer: 0.05 M trisodium citrate-dihydrate dissolved in 50 mL bi-distilled water; adjust to pH 4.5.
4. 4-Nitrophenyl-N-acetyl- β -D-glucosaminide (pNAG) solution: 1.3 mg/mL pNAG in 0.05 M citrate buffer. Store at –20 °C.
5. Glycine buffer: 0.2 M glycine solution; adjust to pH 10.7 and store at 4 °C.
6. Extraction buffer: 25 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.05 % Triton X-100, 10 mM β -mercaptoethanol, supplemented with the protease inhibitor cocktail Complete™ Mini (*Roche Diagnostics*); make fresh as required.
7. RNeasy Mini Kit (*Qiagen*).
8. Histamine enzyme-linked immunosorbent assay (ELISA).
9. Cysteinyl leukotriene ELISA.
10. Cytokine ELISA.

11. Cytokine assays.
12. Proteome Profiler Array (*R&D Systems*).

2.5 Inhibition of Intracellular Proteins

1. Water bath.
2. Crushed ice.
3. Calcium-free Hanks' balanced salt solution (HBSS) with 30 mM HEPES; adjust to pH 7.0.
4. Streptolysin O.
5. Neutralizing antibodies.
6. RPMI containing 2 mM CaCl₂ and 10 % FCS.

3 Methods

3.1 Isolation of Cells from the Intestinal Mucosa

For the isolation of cells from the human intestinal mucosa, we use the modified four-step enzymatic tissue dispersion method originally described by Schulman et al. [19]. Other investigators have succeeded in isolating intestinal cells using simpler methods [22, 23]. In our hands, the method described here is superior in terms of cell yield, mast cell percentage, and cell integrity.

1. Obtain intestinal tissue from surgical specimens of patients who underwent bowel resection (*see Note 1*).
2. Wash specimen with water and remove adherent mesentery and fat from tissue using tweezers and scissors. Place specimen immediately in tissue storage buffer (*see Note 2*).
3. Separate mucosa from the submucosa/muscular layer using tweezers and scissors. Discard submucosa/muscularis.
4. Transfer mucosa to a 50 mL conical tube containing 30–40 mL of TE buffer + 1 mg/mL *N*-acetyl-L-cysteine. Incubate for 10 min in a shaking water bath at 37 °C. After incubation, agitate tube gently to remove mucus. Repeat **step 4** if mucus is not adequately removed.
5. Transfer mucosa in a 50 mL conical tube containing 30–40 mL of Tyrode's buffer + 5 mM EDTA. Incubate for 20 min in a shaking water bath at 37 °C. After incubation, agitate tube gently to remove epithelial cells and residual mucus.
6. Place tissue in a plastic Petri dish containing 15 mL TE buffer and cut with scissors into 1 mm³ pieces.
7. Place tissue suspension on a bottle-top filter containing nylon mesh with a pore size of 250 µm. Wash with 50 mL of TE buffer (*see Note 3*). Discard filtrate. Close the bottle-top filter with the stopper.
8. Suspend tissue in 25 mL of enzyme solution PCh and transfer suspension in a 50 mL conical tube. Incubate 30 min in a shaking

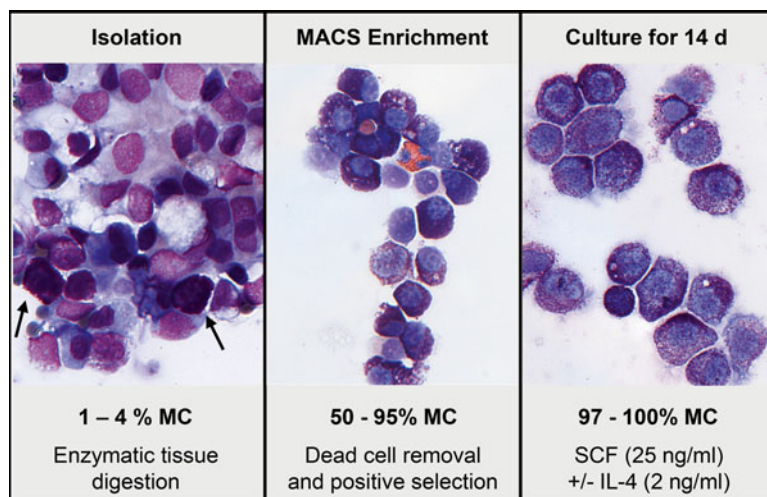


Fig. 1 May–Grünwald/Giemsa stain of cell fractions obtained after cell isolation (arrows, mast cells), MACS enrichment, and culture for 14 days (d) in the presence of SCF (25 ng/mL)

water bath at 37 °C. Filter as in **step 7** and wash with 50 mL of TGMD buffer. Discard filtrate.

9. Suspend tissue in 25 mL of enzyme solution Co and transfer suspension in a 50 mL conical tube. Incubate 30 min in a shaking water bath at 37 °C. Filter as in **step 7** and wash with 25 mL of TGMD buffer. Centrifuge filtrate ($300\times g$, 10 min), remove supernatant, resuspend cells in 1–2 mL of cell culture medium, and place at 4 °C.
10. Repeat **step 9**.
11. Pool cells collected in **steps 9** and **10**, resuspend in 20 mL of RPMI, and filtrate through a bottle-top filter containing nylon mesh with a pore size of 100 μ m. Wash with 30 mL of RPMI. Centrifuge ($300\times g$, 10 min) and resuspend in 10 mL of culture medium.
12. Count the cells after staining with trypan blue. Prepare cyto-centrifuge smears and stain with May–Grünwald/Giemsa to perform a differential count (*see Note 4*, Fig. 1).

3.2 Enrichment of Intestinal Mast Cells by Dead Cell Removal and Positive Selection Using Magnetic Cell Separation

Enrichment of human intestinal mast cells can be performed by using magnetic cell sorting (MACS). In general, mast cells are 50–75 % pure after positive sorting by immunomagnetic labeling of c-Kit. In some cell preparations, the purity can be greater than 90 %. Further purification, as much as 100 %, is possible by culture of the cells (*see Subheading 3.3*). Purification of mast cells can be achieved also by long-term culture of non-enriched cell fractions [24]. This approach has the advantage of higher cell numbers and a better culture sufficiency, but the purity is often poor and the

required culture period quite long. Thus, MACS is the method of choice for the purification of mast cells.

1. Culture freshly isolated intestinal cells at 4×10^6 /mL in culture medium for 1–2 h or overnight in 75 cm² (up to 1.2×10^8 cells) or 150 cm² (up to 2.5×10^8 cells) tissue culture flasks (*see Note 5*).
2. Harvest cells by gently shaking (*see Note 6*). Count after staining with trypan blue (*see Note 7*).
3. To remove clumps, pass cells through a 30 μ m filter. Centrifuge cells (300 $\times g$, 10 min, 4 °C) and remove supernatant.
4. Resuspend cells in *Dead Cell Removal MicroBeads* solution (100 μ L per 1×10^7 cells) and incubate for 15 min at room temperature (RT).
5. Place a positive selection column-type LS in the magnetic field of an appropriate MACS separator. Wash the column with 5 mL of $1 \times$ *Binding Buffer* according to the manufacturer's instructions. Discard eluate.
6. Give 1–10 mL of $1 \times$ *Binding Buffer* onto the cell suspension, mix gently, and apply the mixture onto the column.
7. Let the negative cells pass through and wash the column four times with 500 μ L $1 \times$ *Binding Buffer*. Collect effluent as live cell fraction.
8. Count the cells after staining with trypan blue. Prepare cyto-centrifuge smears and stain with May–Grünwald/Giemsa to perform a differential count.
9. Centrifuge cells, remove supernatant, and resuspend cells in *Auto MACS Rinsing Solution* (300 μ L/ 10^8 cells) and add 100 μ L *FcR Blocking Reagent* (human IgG) and 100 μ L of CD117 MicroBeads. Mix well, and incubate for 15 min in a refrigerator at 4 °C.
10. Wash cells with 10–20 mL of *Auto MACS Rinsing Solution* containing 5 mg/mL BSA, centrifuge, remove supernatant, and resuspend in *Auto MACS Rinsing Solution* containing 5 mg/mL BSA (1–2 mL) again.
11. Place a positive selection column-type LS in the magnetic field of an appropriate MACS separator. Wash with 5 mL *Auto MACS Rinsing Solution* containing 5 mg/mL BSA according to the manufacturer's instructions. Discard eluate.
12. Transfer cell suspension as many as 10^8 cells to the top of the MACS column (*see Note 8*). Once the cell suspension has completely entered, start washing the column with at least 10 mL *Auto MACS Rinsing Solution* containing 5 mg/mL BSA. Collect the effluent in a 50 mL conical tube (mast cell-depleted fraction).

13. Remove MACS column from the separator. Fill column with *Auto MACS Rinsing Solution* containing 5 mg/mL BSA (as much as 7 mL), firmly flush out the positive fraction using the supplied plunger, and collect cells in an appropriate tube.
14. Centrifuge cells and resuspend in culture medium. Count the cells after staining with trypan blue. Prepare cytocentrifuge smears and stain with May–Grünwald/Giemsa to perform a differential count (*see* **Note 9**, Fig. 1).

3.3 Culture of Intestinal Mast Cells

In the absence of growth factors, MACS-enriched mast cells die completely within 3–7 days. Long-term culture of human intestinal mast cells can be achieved in the presence of recombinant SCF preventing mast cell apoptosis and inducing proliferation [24, 25]. Additional factors such as IL-3 and IL-4 enhance mast cell growth in the presence of SCF by decreasing apoptosis (IL-3) or increasing proliferation (IL-4). In the absence of SCF, IL-4 has no effect and IL-3 has only a minimal effect on mast cell survival. The addition of IL-3, IL-4, or IL-3 + IL-4 to the culture medium in combination with SCF can enhance mast cell numbers after 2–3 weeks of culture approximately two-, three-, or fourfold, respectively, in comparison with mast cells cultured with SCF alone [8, 26].

The purity of mast cells largely increases during culture. If the mast cells are enriched by MACS before culture, purity is generally 85–95 % after 1 week and 95–100 % after 2 weeks of culture (Fig. 1).

Cultured mast cells release much higher amounts of mediators in response to Fcε receptor I (FcεRI) cross-linking than freshly isolated mast cells, which is most likely related to the fact that the isolation and purification procedure causes a reversible damage of the cells that reduces their functional capacities. During culture, mast cells regain the full capacity to respond to FcεRI cross-linking. This suggests that cultured mast cells reflect more accurately the phenotype of mast cells *in vivo* than freshly isolated mast cells [8, 24, 25].

1. Culture mast cells in culture medium. Adjust the cell concentration to $1\text{--}2 \times 10^5$ mast cells/mL for MACS-enriched cells or to 2×10^6 of total cells/mL for unpurified cells. Culture of intestinal mast cells can be performed in 96-, 48-, 24-, 12-, or 6-well flat-bottomed plates in 0.2, 0.5, 1, 2, or 5 mL of cell culture medium, respectively (*see* **Note 10**).
2. Add SCF at a concentration of 25 ng/mL. If required, add other cytokines (*see* **Note 11**).
3. Maintain cells in a humidified atmosphere containing 5 % CO₂ at 37 °C.
4. Change 50 % of the culture medium twice during the first week and then once a week thereafter. Add new growth factors each time.
5. Subculture cells if required (*see* **Note 12**).

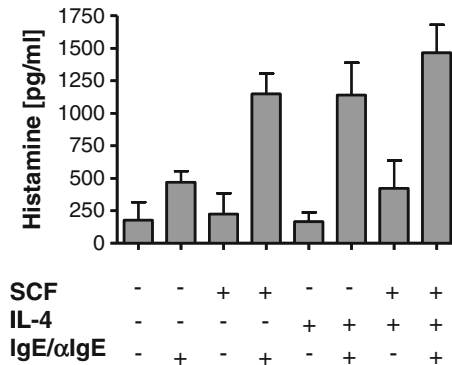


Fig. 2 Combined activation by FcεRI cross-linking, SCF stimulation, or IL-4 priming induces potentiated release of histamine in human intestinal mast cells. Mast cells were cultured with SCF alone or with IL-4 (+) in addition to SCF for 10 days (d). SCF was withdrawn overnight and cells were treated with myeloma IgE for 90 min and stimulated with antihuman IgE (IgE/αIgE) (+), SCF (+) or a combination of both

6. After an appropriate culture period (*see* text above), harvest mast cells by gently mixing with a pipette. Count the cells after staining with trypan blue. Prepare cytocentrifuge smears and stain with May–Grünwald/Giemsa to perform a differential count.

3.4 Mediator Release and Cytokine Production in Human Intestinal Mast Cells

The procedure of isolation, purification, and culture of mast are powerful methods to obtain pure tissue-derived mature human mast cells. These cells can be used to identify mast cell mediators and to study both the mechanisms of mast cell activation and signal transduction as well as the molecular process of exocytosis during mediator release in response to specific stimuli. For the study of mast cell mediator release and cytokine production in response to cell activation, we have been using protocols optimized to use as small cell numbers as possible. Figure 2 shows that combined activation by FcεRI cross-linking, SCF stimulation, or IL-4 priming induce potentiated release of inflammatory mediators such as histamine in human intestinal mast cells [13].

Stimulation Assay for the Study of Expression and Release of Mediators and Activation of Signaling Molecules

1. Wash mast cells twice in PBS (*see* Note 13).
2. Resuspend mast cells in stimulation buffer (50–1,000 μL per condition as required), and transfer them into appropriate tubes (*see* Note 14).
3. Prepare cell lysates for the detection of total β-hexosaminidase or histamine (*see* Subheading “Preparation of Mast Cell Lysates”).
4. Incubate tubes at 37 °C for 10 min without agonists.
5. Add trigger/agonist of interest (e.g., for FcεRI cross-linking add IgE (1 μg/mL)) and incubate at 37 °C for 90 min. Leave control untreated or add appropriate isotype control.

6. Wash cell suspension two times with PBS (discard supernatant) and resuspend cells in stimulation buffer.
7. Add anti-IgE and incubate at 37 °C for 10 min to determine phosphorylation status of signaling molecules, for 90 min to analyze the mRNA expression of cytokines/chemokines, or for 6 h to examine the release of cytokines/chemokines (*see Note 15*).
8. Harvest cells after time of interest and transfer them to an RNase free 1.5 mL microfuge tube. Centrifuge cells (300 ×g, 10 min, 4 °C) and collect supernatants. Store aliquots at -80 °C. Resuspend pellet immediately in RLT buffer provided by the RNeasy Mini Kit to isolate total RNA and to determine mRNA expression by real-time reverse transcription polymerase chain reaction. Resuspend pellet immediately in extraction buffer to analyze protein expression or activation by SDS-PAGE and Western blot (*see Note 16*).
9. Measure mediators of interest in the supernatants by β -hexosaminidase release assay (*see Subheading “ β -Hexosaminidase Release Assay”*), appropriate ELISA, or multiplex bead immunoassay according to the manufacturer’s instructions and using an appropriate *cytokine assay*. Dilution of samples might be required (in particular for histamine). Calculate degranulation as: (β -hexosaminidase activity in the supernatant fraction/total β -hexosaminidase activity in the cellular and supernatant fraction) × 100. Assay mRNA levels by real-time reverse transcription polymerase chain reaction. Analyze cell protein extracts by SDS-PAGE and Western blot or *Proteome Profiler Arrays*.

Preparation of Mast Cell Lysates

1. Place 100 μ L of the cell suspension in an appropriate tube and add 100 μ L of water.
2. Freeze cell suspension at -80 °C.
3. Before determination of mediators, thaw suspension and sonicate for 3–5 min.
4. Centrifuge cell debris down and use supernatant for analysis.

β -Hexosaminidase Release Assay

1. Place 50 μ L of pNAG solution in a well of a 96-well plate.
2. Add 20 μ L of supernatant or cell lysates of 1×10^6 stimulated cells/mL to the well and incubate for 90 min at 37 °C.
3. After incubation stop reaction by adding 150 μ L of glycine buffer.
4. Determine optical density with a photometer at $\lambda = 405$ nm.

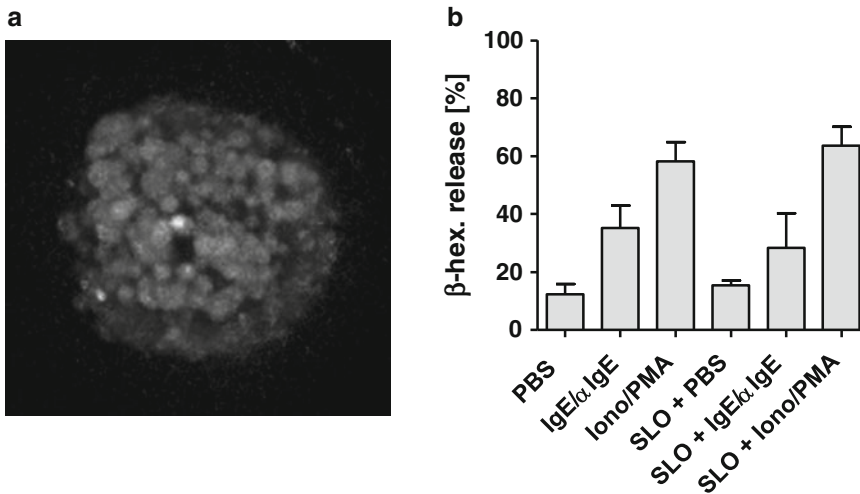


Fig. 3 Staining of intracellular vesicle-associated membrane protein (VAMP)-3 after SLO treatment (a) and β -hexosaminidase (β -hex) release in response to Fc ϵ RI cross-linking (IgE/ α IgE) or to Ionomycin (Iono)/PMA (each 1 μ M) following SLO treatment (b)

*Inhibition of Intracellular
Proteins Prior to Cell
Activation*

Inhibition of proteins is required to show their functional impact. We used an alternative approach to gene silencing (*see* **Note 17**) by administration of specific neutralizing antibodies to analyze the role of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) in mast cell mediator release [27, 28]. Antibodies directed against different SNARE isoforms recognize cytoplasmic NH₂-terminal region required for SNARE binding. The bacterial-derived toxin streptolysin O (SLO) allows a temporary permeabilization of vital cells in a dose-dependent manner to deliver antibodies [29]. Figure 3 shows that SLO treatment allows intracellular vesicle staining (a) but does not affect responsiveness of the cells after activation by Fc ϵ RI cross-linking or administration of Ionomycin and PMA (b).

1. Wash cells with calcium-free HBSS and transfer into appropriate tubes.
2. Add SLO at concentrations yielding approximately 60–80 % trypan blue staining of the cells (20–40 μ g/mL) and the neutralizing antibodies at 20 μ g/mL.
3. Incubate at 37 °C for 15 min in a water bath.
4. Stop the reaction and seal the pores by addition of at least threefold ice cold RPMI medium containing 2 mM CaCl₂ and 10 % FBS (3:1) and incubate on ice for 60 min.
5. Centrifuge the cell suspension, discard the supernatant, and resuspend the cells in appropriate buffer.
6. Go on performing the stimulation assay.

4 Notes

1. Tissue can be obtained from all parts of the bowel. Most specimens we have obtained have come from patients who underwent resection because of large bowel cancer.
2. Tissue can be stored at 4 °C overnight, although immediate processing is preferential. In our hands, numbers and viability of isolated mast cell are only slightly impaired after overnight storage of the tissue specimen.
3. Tissue suspension needs to be stirred using tweezers or with other appropriate instruments to avoid obstruction of the filter. If residual mucus completely clogs the mesh, transfer tissue suspension to a new bottle-top filter.
4. Approximately $1\text{--}2.5 \times 10^7$ cells can be obtained from 1 g of mucosal tissue. As much as 10 g can be used in one preparation. If more tissue is available, perform two (or more) preparations in parallel. Normally isolated cells contain 1–4 % mast cells, but the percentage can be higher in selected patients. Cell preparations contain 10–40 % erythrocytes, which has to be taken in account by calculating mast cell numbers.
5. Do not add SCF to the culture medium because it downregulates *c-Kit* in mast cells and, therefore, diminishes the enrichment efficiency and recovery.
6. Mast cells are semi-adherent. Cells can be harvested by resuspending the cells with the pipet only.
7. Normally, 20–50 % of the cells are trypan positive after overnight culture (mast cell survival is generally higher). If more than 60 % of the cells are trypan positive, we do not recommend performing a positive selection by MACS because the enrichment efficiency and mast cell recovery will be very low.
8. Although it is passed through a 30 μm filter, the cell suspension might obstruct the MACS column, which is related to the high amount of cell clumps and residual mucus. We recommend applying cells in small fractions of 1–2 mL. In the case of obstruction, remove the rest of the cell suspension on the top of the column and replace it with 2 mL of MACS buffer. The obstruction can be overcome by pulling up and down the buffer with a pipet. Continue with this column as recommended in the protocol. For the remaining cells, use a new column.
9. Metachromatic staining of mast cells after MACS is sometimes poor, and exact quantification is difficult. Generally, between 0.5 and 3×10^6 mast cells can be obtained from 5 to 10 g of mucosa.
10. More than the half of the MACS-enriched cell preparation dies completely within the first days of culture. This may be related to the damage of the cells acquired during the cell separation.

11. To obtain high amounts of pure mast cells, we recommend adding IL-4 at a concentration of 2 ng/mL (in combination with SCF) to the culture. Keep in mind that IL-4 changes the mast cell phenotype [8, 9, 13] (Fig. 2). IL-4 can be added at the beginning of the culture or later. The *IL-4 phenotype* will be obtained after 1–2 weeks.
12. In some cultures mast cells proliferate strongly in particular if IL-4 is added to the culture medium. Proliferation will stop if mast cell numbers exceed $0.5\text{--}1 \times 10^6/\text{mL}$. Then, split cells in a new culture plate.
13. Washing of cultured mast cells is very important because culture supernatants contain high amounts of histamine.
14. For the measurement of β -hexosaminidase, histamine, or LTC₄ after Fc ϵ RI cross-linking, cell concentrations $0.5\text{--}1 \times 10^6$ cells/mL are used. We perform experiments in 50–100 μL in tubes. For the measurement of cytokines after Fc ϵ RI cross-linking, cell concentrations 1×10^6 cells/mL are used. We perform experiments in 100–300 μL in tubes or in a 96 or 48 or 24-well plate. For mRNA studies, we use at least 8×10^4 mast cells/condition (preferable is 1×10^5).
15. For the analysis of mRNA induction for most cytokines upon Fc ϵ RI cross-linking, the optimal time point is 90 min. Upregulation of mRNA is still detectable after 6 h. To measure cytokines in the supernatants, we recommend incubation periods of 6 h or longer. If you wish to analyze only degranulation and eicosanoid production, stimulation for 30–60 min is enough. We usually stop the reaction after 30 min in a standard assay for the investigation of degranulation and eicosanoid production in response to Fc ϵ RI cross-linking. For histamine, approximately 3–5 min and for leukotrienes approximately 10–15 min are required for maximal release [7]. Degranulation and eicosanoid release can also be studied after longer stimulation time such as 6 h. Because of longer incubation time, spontaneous histamine release is higher and eicosanoids might be partly degraded.
16. For the analysis of protein expression or activation, at least 2×10^5 cells/condition are lysed in 50 μL extraction buffer. Cell debris should be pelleted ($14,000 \times g$ for 5 min at 4 °C) and supernatants stored at -80 °C until used for analysis.
17. We undertook extensive efforts to transfect primary human mast cells with siRNA. In contrast to SLO permeabilization, the cells must be viable for at least 48–72 h if not longer after transfection to block the transcription of the corresponding proteins. We tested a number of different transfection systems either without seeing efficient transfection or the transfection was accompanied by a loss of responsiveness to cell activation.

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