

Chapter 19

Assay of Mast Cell Mediators

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Abstract

Mediator release from activated mast cells is a major initiator of the symptomology associated with allergic disorders such as anaphylaxis and asthma. Thus, methods to monitor the generation and release of such mediators have widespread applicability in studies designed to understand the processes regulating mast cell activation and for the identification of therapeutic approaches to block mast cell-driven disease. In this chapter, we discuss approaches used for the determination of mast cell degranulation, lipid-derived inflammatory mediator production, and cytokine/chemokine gene expression as well as cytokine release.

Key words Mast cell, IgE sensitization, Degranulation, PGD₂, LTC₄, Cytokines, Cytometric bead array, RT-PCR, Chemokines, ELISA

Abbreviations

BMMC	Bone marrow-derived mast cells
FcεRI	High-affinity IgE receptor
HuMC	Human mast cell
IL	Interleukin
LT	Leukotriene
PG	Prostaglandin
TNF-α	Tumor necrosis factor-alpha

1 Introduction

The inappropriate or exaggerated release of proinflammatory mediators from activated mast cells represents a central event in the initiation and perpetuation of allergic reactions associated with asthma, allergic rhinitis, anaphylaxis, and certain allergic dermatological and ocular disorders [1, 2]. Such events are generally a consequence of binding of antigen (Ag)-specific IgE molecules to high-affinity receptors for IgE (FcεRI) expressed on the mast cell

surface, and subsequent aggregation of these receptors as a result of Ag-mediated cross-linking of the FcεRI-bound IgE [3]. However, under specific circumstances, other stimuli can also contribute to mast cell activation [4]. The ability to measure the release of mast cell mediators is thus fundamental to the understanding of how mast cells can be activated in health and disease and for the identification of potential therapeutic approaches for the treatment of allergic disorders.

Although mast cells release a wide array of mediators in this manner, these mediators can generally be considered to belong to three major categories based on their mode of production, storage, and/or release: (1) pre-synthesized mediators which are stored in cytoplasmic granules, the contents of which are released by the process of exocytosis; (2) lipid-derived mediators such as the eicosanoids prostaglandin (PG)D₂ and leukotriene (LT)C₄, which are synthesized *de novo* then immediately released; and (3) cytokines, chemokines, and growth factors which are generated following induction of gene expression then subsequently released [2–4]. Histamine is certainly the most important pre-synthesized mast cell granule component in terms of disease, and extremely sensitive competition-based assays are available which can effectively measure histamine release from very few cells. Nevertheless, the relative cost of these assays precludes their use for wide-scale studies and for high-throughput screening aimed at identifying inhibitors of mast cell degranulation. For this reason, most laboratories monitor degranulation through determination of the release of the granule component β-hexosaminidase which can be readily assayed by a simple colorimetric determination of its enzymatic activity. Although not as sensitive as the currently available assays for histamine, it is sufficiently sensitive to allow determination of degranulation in relatively small numbers of mast cells and is amenable to a 96-well format. Due to the differences in granularity, however, this is highly dependent on the source of the mast cells; human mast cells (HuMCs), including the LAD2 human mast cell line, requiring approximately 1×10^4 cells per well; mouse bone marrow-derived mast cells (BMMCs), 3×10^4 to 5×10^4 cells per well; and the rat RBL 2H3 mast cell line, approximately 4×10^4 cells per well.

The traditional means of assay of eicosanoid generation and release through preloading of cells with the radiolabeled precursor, arachidonic acid, has now largely been circumvented by the widespread use of sensitive ELISA-based assays for the quantitation of released LTC₄ and PGD₂. These assays have a high degree of sensitivity, requiring only 1×10^4 cells per well for both assays. Besides the increased sensitivity, a distinct advantage of this assay format is that it avoids the use of radioactivity. Nevertheless, the ELISA kits are fairly expensive so studies must be conducted on a more selective basis than for degranulation.

There is much more flexibility in the approaches that can be used to monitor cytokine/chemokine production and hence release than those utilized for measuring either degranulation or eicosanoid generation. Both mRNA and protein determination have been utilized to assess cytokine generation based on the assumed correlation between mRNA levels and the amount of protein generated and released from the mast cells. It must be remembered, however, that mRNA levels do not always predict protein production. Several platforms are available that can simultaneously quantitate mRNA or protein levels of multiple cytokines/chemokines. The production or release of individual cytokines can subsequently be respectively assessed by quantitative real-time PCR and ELISA. These assays however require more cells (1×10^6 and 0.5×10^6 , respectively) than for the determination of degranulation and eicosanoid production.

In this chapter, we describe protocols that will allow researchers to readily monitor mast cell degranulation, eicosanoid release, and cytokine generation and release. These methods are highly reproducible and can be adapted to monitoring mediator release in mast cells from multiple sources. We, however, will primarily focus our discussions on mediator release from mouse bone marrow-derived mast cells (BMMCs) and from human mast cells (HuMCs) derived from CD34⁺ peripheral blood progenitors.

2 Materials

2.1 *IgE Sensitization and Stimulation*

1. Mouse anti-DNP IgE, clone SPE-7 (Sigma-Aldrich, St Louis, MO, USA).
2. Human myeloma IgE (Calbiochem, Millipore, Billerica, MA, USA) biotinylated as described [5] or by using an appropriate commercially available biotinylation kit.
3. Streptavidin (Sigma-Aldrich).
4. Goat anti-human IgE (KPL, Gaithersburg, MD, USA).
5. DNP (dinitrophenol)-HSA (Sigma-Aldrich).
6. Anti-Fc ϵ RI α antibody (eBioscience, San Diego, CA, USA).

2.2 *β -Hexosaminidase Release Assay (Degranulation)*

1. HEPES degranulation buffer (pH 7.4): 10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM sodium phosphate (dibasic), 5.6 mM glucose, 1.8 mM calcium chloride, 1.3 mM magnesium sulfate. To make 1 L, add 2.38 g HEPES, 8.00 g NaCl, 0.2 g KCl, 0.103 g Na₂HPO₄·7H₂O, 1.008 g glucose to 800 mL distilled H₂O (dH₂O). Adjust pH to 7.4 with NaOH (5 M) and add 0.265 g CaCl₂·2H₂O (1.8 mM), 0.32 g MgSO₄·7H₂O (1.3 mM). Adjust volume to 1 L with dH₂O, sterilize by filtration, and store at 4 °C.

2. Citrate buffer (pH 4.5): 40 mM citric acid, 20 mM sodium phosphate dibasic. To make 200 mL, add 1.681 g citric acid and 1.072 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (20 mM) to 180 mL dH_2O . Adjust pH to 4.5 with NaOH (5 M), add dH_2O to 200 mL, sterilize by filtration, and store at 4 °C.
3. Glycine (400 mM, pH 10.7): Add 15.1 g glycine to 480 mL dH_2O , and adjust pH to 10.7 with NaOH (5 M). Adjust volume to 500 mL and sterilize by filtration and store at RT.
4. HEPES buffer (pH 7.4) with 0.04 % BSA: Add 0.04 g BSA (bovine serum albumin) to 100 mL HEPES buffer degranulation buffer (pH 7.4).
5. *p*-Nitro-*N*-acetyl- β -D-glucosaminide (pNAG) solution: 3.5 mg/mL pNAG (Sigma-Aldrich) solution is made up in citrate buffer and needs to be sonicated to dissolve.
6. 0.1 % (v/v) Triton X-100: Add 100 μL of Triton X-100 to 100 mL dH_2O . Cut the end of the pipette tip prior to pipetting Triton X-100.
7. 96-well cell culture plate (Nunc, Rochester, NY, USA).

2.3 Cytokine/ Chemokine Expression by qPCR

1. RNeasy Mini Kit (Qiagen).
2. RNase-free water (UltraPure grade).
3. QIAshredders (Qiagen).
4. QuantiTect reverse transcription kit containing genomic DNA elimination reagent (Qiagen).
5. SYBR green primer-probe sets including TNF- α , IL-8, etc. (Qiagen).
6. QuantiTect SYBR green PCR mastermix (containing HotStarTaq DNA polymerase) (Qiagen).

2.4 Multiplex Cytokine Bead Array (Flow Cytometry)

1. BD Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA) or appropriate array kit for human samples.
2. 24- or 48-well cell culture plate (Costar).
3. BMMC medium without IL-3 [6].
4. Human mast cell culture medium [7].
5. A flow cytometer capable of detecting and distinguishing fluorescence emission at 576 and 670 nm and BD CellQuest™ or BD CellQuest Pro software.
6. 15 mL conical polypropylene tubes.
7. BD Falcon™ 12×75 mm sample acquisition tubes or equivalent.
8. BD Calibrite™ 3 beads.
9. BD Calibrite APC beads.
10. FCAP Array software.

**2.5 Cytokine
Secretion Assay
by ELISA**

1. Cytokine assay kit (e.g., R&D Systems).
2. 24- or 48-well cell culture plate (Costar).
3. BMMC medium without IL-3 [6].
4. Human mast cell culture medium [7].

**2.6 Eicosanoid
Generation Assay**

1. HEPES buffer+0.04 % BSA (same as for β -hexosaminidase assay).
2. LTC₄ enzyme immunoassay (Cayman Chemicals).
3. PGD₂ MOX enzyme immunoassay (Cayman Chemicals).

3 Methods**3.1 IgE Sensitization**

Mast cell stimulation can be Fc ϵ RI dependent and Fc ϵ RI independent. Fc ϵ RI-dependent stimulation might require IgE sensitization prior to stimulation as follows:

1. Prepare mature human or rodent mast cells (*see Note 1*). Propagate a sufficient number of mast cells (HuMCs or mouse BMDCs) needed for the specific analysis (*see Note 2*) and resuspend in cell culture medium at a concentration between 2×10^5 and 1×10^6 cells/mL (*see Note 3*).
2. Add human or mouse IgE (*see Note 4*) to a final concentration of 100 ng/mL.
3. Sensitize cells with IgE for a minimum of 1 h up to 18 h as desired.
4. Remove the cell culture medium by centrifugation ($300 \times g$, 10 min, RT).
5. Wash cells to remove free IgE by resuspending in culture medium or appropriate buffer, and centrifuge $300 \times g$ for 10 min and repeat once more.
6. The cells are now ready for Fc ϵ RI-mediated stimulation—resuspend at the desired concentration in the appropriate buffer or medium (*see following sections*) where different approaches can be used: anti-IgE antibodies; IgE-specific Ag; or in the case of biotinylated IgE, streptavidin). Mast cells can also be stimulated directly through Fc ϵ RI using an anti-Fc ϵ RI α antibody. *See Note 5* for examples of non-Fc ϵ RI-mediated stimulation.

**3.2 Monitoring
Degranulation by
Measuring
 β -Hexosaminidase
Release (See Note 6)**

All procedures are conducted at room temperature (RT) unless otherwise specified.

1. Use human mast cells at a concentration of 0.5 to 1×10^4 cells per well in a 96-well reaction plate (U-bottom). BMDCs are used at a concentration of 3 to 5×10^4 cells per well. For IgE sensitization and cytokine starvation, *see* Subheading 3.1 and Notes 2 and 3.

2. Pre-warm freshly prepared HEPES degranulation buffer with 0.04 % BSA to 37 °C.
3. To remove excess IgE, wash sensitized cells three times by adding HEPES degranulation buffer with 0.04 % BSA (up to 10×10^6 cells in 10 mL buffer), then centrifuge at $300 \times g$ for 10 min, and repeat twice, as described in Subheading 3.1.
4. Resuspend cells so that 90 μ L contains the final cell number needed for each well (total final volume is 100 μ L). Aliquot cells into a U-bottomed 96-well plate (reaction plate). Place the plate at 37 °C (non-gassed hot air oven or equivalent) for 10 min.
5. Prepare stimulants in HEPES + 0.04 % BSA: Make dilutions at $10 \times$ final concentration. Add 10 μ L buffer (vehicle control) or stimulant and incubate for 30 min at 37 °C.
6. Meanwhile, prepare 3.5 mg pNAG/mL citrate buffer solution. Remember to make sufficient pNAG solution for both supernatants and cell lysates. Aliquot 100 μ L per well in a flat bottom 96-well plate. Make one plate for supernatant and one for lysate.
7. After incubation, spin plate for 5 min, $300 \times g$ at 4 °C, to terminate the reaction and sediment the cells.
8. Remove 50 μ L supernatant and add to supernatant-pNAG plate (“supernatant”) and incubate for 90 min at 37 °C.
9. Lyse the remaining cells in the reaction plate by adding 150 μ L 0.1 % Triton X-100—use a multichannel pipette and pipette up and down 5 times, and change tips for each row. Leave plate at 37 °C for 10 min and transfer 50 μ L of lysis solution to lysate-pNAG plate (“lysate”) and incubate for 90 min at 37 °C.
10. Stop reaction in both the supernatant and lysate plates by adding 100 μ L of a 400 mM glycine solution (a yellow color indicates presence of β -hexosaminidase) and read absorbance using a plate reader at $\lambda = 405$ nm with reference filter $\lambda = 620$ nm.
11. To calculate β -hexosaminidase release (degranulation), determine the relative amount of β -hexosaminidase in the supernatant as a percentage of the total β -hexosaminidase in the cells prior to stimulation as follows:

$$\beta\text{-Hexosaminidase release (\%)} = \frac{[\beta\text{-hexosaminidase released}]}{[\text{total } \beta\text{-hexosaminidase present in supernatant and cell contents}]} = \frac{2 \times (\Delta \text{supernatant}_{(A405 \text{ nm})})}{(\Delta \text{supernatant}_{(A405 \text{ nm})}) + (4 \times \Delta \text{cell lysate}_{(A405 \text{ nm})})} \times 100 \%$$

See **Note 7** for equation explanation.

Typical examples of degranulation values and kinetics of release are shown in Fig. 1.

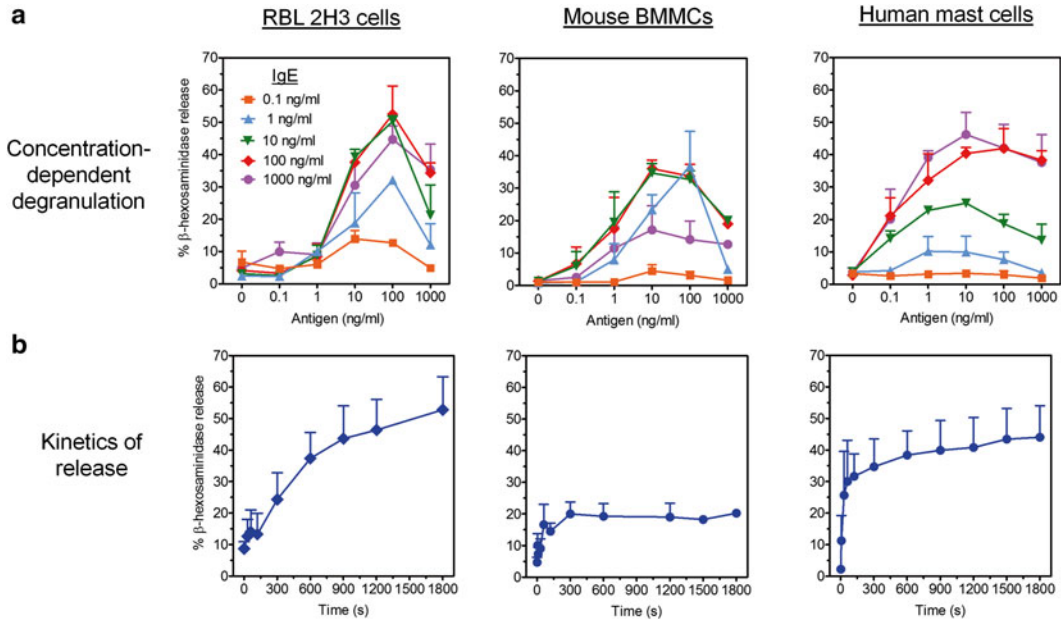


Fig. 1 Typical titration curves and degranulation kinetic of mast cells stimulated with IgE plus antigen. **(a)** Titration curves obtained for IgE and antigen in RBL 2H3 cells, mouse BMMCs, and HuMCs. Cells were sensitized overnight in the indicated concentrations of IgE and then challenged for 30 min with the indicated concentrations of antigen. **(b)** Kinetics of degranulation of IgE (100 ng/mL)-sensitized RBL 2H3 cells, mouse BMMCs, and HuMCs in response to an optimal concentration of antigen (100 ng/mL). Results are means $\pm$ SE of 2–4 experiments

3.3 Measuring Cytokine and Chemokine Gene Expression by Quantitative PCR

Isolation of Total RNA from Mast Cells Using RNeasy Mini Kit

Typically, mast cells are stimulated for 2–24 h prior to processing of samples for gene expression analysis.

The use of the commercially available RNeasy Mini Kit (Qiagen) for isolating RNA from mast cells typically yields 8–16 μ g per 10^6 cells for HuMCs and 8–14 μ g per 10^6 cells for BMMCs and produces RNA of excellent quality for quantitative PCR (qPCR) and SuperArrays (*see Note 8*). A brief description of the protocol is described below (refer to manufacturer's instructions for more detail).

1. After the desired stimulation, transfer mast cells to conical tubes (15 mL) and centrifuge at $300 \times g$ for 10 min. Remove supernatant, resuspend cell pellet by flicking the tube, and then lyse cells by adding RLT lysis buffer (350 μ L).
2. Further lyse cells by pipetting up and down 5 times prior to transferring directly onto a QIAshredder spin column in a 2 mL collection tube. Centrifuge at $8,000 \times g$ for 2 min.

3. Add 70 % ethanol (350 μ L) to the cell homogenate. Mix well by pipetting prior to transferring to an RNeasy spin column placed in a 2 mL collection tube. Centrifuge at $8,000\times g$ for 30 s. Ethanol is added to the cell homogenate to allow optimum binding of RNA to the silica membrane within the spin column.
4. Remove the supernatant and wash the silica membrane by adding RW1 buffer (700 μ L) to the spin column and centrifuge at $8,000\times g$ for 30 s.
5. Remove the supernatant and add RPE buffer (500 μ L) to the spin column and centrifuge at $8,000\times g$ for 2 min.
6. Transfer the spin column to a 1.5 mL microtube and add 40 μ L of RNase- and DNase-free water and centrifuge at $8,000\times g$ for 2 min.
7. Remove the spin column and retain the supernatant containing the eluted RNA in water. This can be stored at -80°C until required or used immediately for cDNA preparation.
8. Assess RNA yield ($[\text{RNA}] = \text{absorbance at } \lambda = 260 \text{ nm} (\text{OD}_{260\text{nm}}) \times 40 \mu\text{g/mL}$ (1 $\text{OD}_{260\text{nm}}$ unit)) and purity ($\text{OD}_{260\text{nm}}/\text{OD}_{280\text{nm}}$ ratio) using a nanodrop spectrophotometer. A typical yield of RNA from 1 million BMMC or HuMC is 300 $\mu\text{g/mL}$ with an $\text{OD}_{260\text{nm}}/\text{OD}_{280\text{nm}}$ ratio of 1.8–2.0.

*Preparation of cDNA for
Quantitative PCR*

1. Transfer 1 μg of total cellular RNA from mast cells to a thin-walled microtube (0.2 mL) containing 2 μL of genomic wipe-out buffer (7 $\times$) and make up to a volume of 14 μL with RNase-free water.
2. Vortex the samples to ensure proper mixing of the reagents, centrifuge briefly to collect the liquid at the bottom of the microtube, and then heat samples at 42°C for 2 min using a thermocycler machine.
3. While samples are heating, prepare a stock of reverse transcription mastermix (1 $\times$) in a thin-walled microtube (200 μL) by aliquoting reverse transcriptase (RT) enzyme (1 $\mu\text{L}/\text{sample}$, contains Omniscript and Sensiscript RT with RNase inhibitor), RT buffer (4 $\mu\text{L}/\text{sample}$, containing dNTPs), and RT primer mix (1 $\mu\text{L}/\text{sample}$, containing oligo-dTs). Make enough RT mastermix stock to obtain 6 μL per genomic DNA (gDNA)-free RNA sample.
4. Take the gDNA-free RNA sample (14 μL), add 6 μL of reverse transcription mastermix, vortex, and then centrifuge briefly to collect the liquid at the bottom of the microtube. Total volume of reaction is 20 μL .
5. Transfer to a thermocycler and incubate at 42°C for 15 min and 95°C for 3 min, followed by 4°C for 10 min. The cDNA is now ready to be used in qPCR or can be stored at -20°C until required.

*Measuring
Gene Expression
by Quantitative PCR*

Gene expression by mast cells can be quantified using reagents from various companies. We routinely use Qiagen SYBR green real-time PCR reagents for individual primer/probe sets and SuperArray for determining numerous cytokine genes expressed at once.

1. Prepare individual PCR reaction mix stock solutions for each primer/probe set of interest by adding QuantiTect SYBR green PCR mastermix (12.5 μ L, containing the HotStarTaq DNA polymerase enzyme, SYBR green buffer, and SYBR I and ROX reference dyes) with the appropriate QuantiTect primer/probe sets of genes of interest (2.5 μ L) or housekeeping gene (12.5 μ L), and aliquot 15 μ L per well in triplicate to thin-walled PCR microtubes (200 μ L).
2. Dilute mast cell cDNA (1 μ g in 20 μ L) 1 in 10 with RNase-free water (180 μ L) to give a total volume of 200 μ L and final concentration of 50 ng/10 μ L.
3. Take 10 μ L aliquots (100 ng per reaction) of cDNA and add to PCR microtubes containing the PCR reaction mix to give a total volume of 25 μ L. Unused cDNA can be stored at -20°C .
4. As a control, each RNA sample, which had not been reverse transcribed to cDNA, is used in a PCR reaction to determine whether nonspecific amplification of contaminating DNA in RNA samples is present.
5. Seal the microtubes and transfer to a real-time PCR cycler machine (ABI PRISM 7700, Applied Biosystems) and perform the following reaction:
95 $^{\circ}\text{C}$ for 15 min (PCR activation step), followed by 40 cycles of:
 - (a) 95 $^{\circ}\text{C}$ for 15 s (denaturation step).
 - (b) 5 $^{\circ}\text{C}$ for 30 s (annealing step).
 - (c) 72 $^{\circ}\text{C}$ for 34 s (extension, data collection period).
6. After each PCR reaction, perform a dissociation curve on each sample to determine that each PCR product has a single peak relating to the SYBR green fluorescence. Products that have a double peak should be discounted.

Gene expression is analyzed using the RT-PCR cycler ABI PRISM 7700. The relative fold expression of genes of interest is calculated as follows: for each sample, the threshold cycle (C_t) is determined and normalized to the housekeeping gene (β -actin or GAPDH) (ΔC_t). For differences in gene expression between samples, the ΔC_t of treated cells is subtracted from untreated control cells ($\Delta\Delta C_t$), and the relative fold expression is calculated using the formula $2^{\Delta\Delta C_t}$. Complementary DNA stocks are taken from three different batches of BMMC or HuMC to determine relative quantities across multiple runs.

3.4 Measuring Cytokine Generation by Multiplex

One advantage of using multiplex cytometric bead arrays to measure cytokine release is the use of fewer samples as compared to the conventional ELISA method. In addition, multiple cytokine proteins in one research sample can be detected simultaneously. From **step 6**, this protocol is according to the manufacturer's instructions for measuring mouse cytokines (BD Cytometric Bead Array Cytokine Kit) with some minor adjustments. All procedures are conducted at room temperature, and the incubations are performed in a humidified 37 °C, 5 % CO₂ incubator.

1. Sensitize BMMCs in cytokine-free medium with mouse anti-DNP IgE as described in Subheading 3.1. If HuMCs are used, sensitize overnight with 100 ng/mL human biotinylated IgE in complete HuMC medium (*see Note 9*).
2. The next day, wash the cells three times with 5–10 mL pre-warmed (37 °C) BMMC or HuMC medium, respectively, to remove excess of IgE as described in Subheading 3.1.
3. Resuspend BMMCs in cytokine-free medium at a concentration of 0.5 to 1×10^6 cells/mL. Place the plate at 37 °C for 10 min. HuMCs are resuspended in complete medium, and aliquot cells into 24- or 48-well plates (culture plate) at a concentration of 1×10^6 cells/mL (in a volume of 450 µL for 48-well plates and 900 µL for 24-well plates).
4. Prepare stimulant in cytokine-free BMMC medium or HuMC medium: Make stock solutions of stimulants at 10× final concentration. Add 50 µL (for 48-well plate) or 100 µL (for 24-well plate) buffer or stimulant and incubate for 4–8 h at 37 °C (*see Note 10*).
5. After incubation, transfer the cells into 1.5 mL microcentrifuge tubes and centrifuge for 5 min at $1,000 \times g$, 4 °C. Collect the cell-free supernatants. Samples can either be used directly or stored at –80 °C until assayed.
6. BD Cytometric Bead Array is used to measure multiple cytokines. Prepare cytokine standards (20–5,000 pg/mL) according to the manufacturer's instructions.
7. Mix the cytokine-capture beads according to the manufacturer's instructions, and add 10 µL of each bead suspension, for each tube to be analyzed, into a 15 mL Falcon tube marked "mixed capture beads." Vortex the mixture beads carefully.
8. Thaw and dilute your samples if necessary by using the appropriate volume of assay diluent and mix carefully.
9. Add 50 µL mixed capture beads to all assay tubes.
10. Add 50 µL of the cytokine standard dilutions to the tubes marked "standard" according to the manufacturer's instructions.
11. Add 50 µL of your unknown sample to the labeled "sample" tubes.

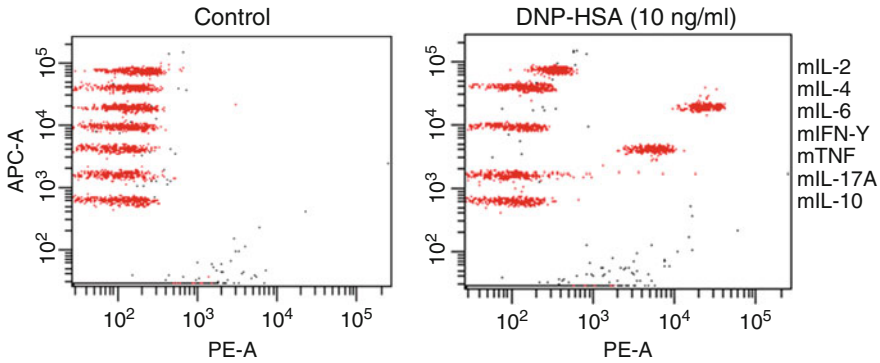


Fig. 2 Typical sample data from BMMCs obtained by BD Cytometric Bead Array Cytokine Kit for mouse Th1/Th2 cytokine profile. Cells were sensitized overnight with IgE-DNP (100 ng/mL) and then stimulated for DNP-HSA (10 ng/mL) for 6 h (control is unstimulated BMMCs). Samples were acquired using BD CellQuest software

12. Add 50 μ L of the detection reagent to all assay tubes, and incubate for 2 h at room temperature, in the dark. In the meantime, perform cytometer setup as described in the manufacturer's instructions for the cytometric bead array.
13. After 2 h incubation, add 1 mL of wash buffer to each assay tube and centrifuge at $200\times g$ for 5 min.
14. Discard the supernatant from each assay tube and add 300 μ L wash buffer.
15. Acquire your samples on the flow cytometer according to manufacturer's instruction.

Data are acquired using BD CellQuest software on a BD FACSaria™ II cytometer. FCAP Array software is used to analyze the cytokine data. Typical sample data from BMMCs obtained by BD Cytometric Bead Array Cytokine Kit for mouse Th1/Th2 cytokine profile are seen in Fig. 2.

3.5 Measuring Cytokine Release by ELISA

All procedures are performed at room temperature and the incubations are performed in a humidified 37 °C, 5 % CO₂ incubator.

1. Prepare sufficient HuMCs or BMMCs to perform cytokine measurements at least in duplicate. HuMCs or BMMCs are sensitized overnight in appropriate medium as described in Subheading 3.1.
2. The next day, wash the cells three times with 5–10 mL pre-warmed (37 °C) HuMC medium or BMMC medium, respectively, to remove excess of IgE as described in the sensitization protocol (Subheading 3.1).
3. Resuspend HuMCs at the desired concentration for the appropriate assay in complete medium, and aliquot the cells into 24- or 48-well plates (culture plate) at a concentration of

1×10^6 cells/mL (in a volume of 450 μ L for 48-well plates and 900 μ L for 24-well plates). Resuspend BMMCs in cytokine-free medium at a concentration of 0.5 to 1×10^6 cells/mL. Place the plate at 37 °C for 10 min.

4. Prepare stimulant in HuMC medium or in cytokine-free BMMC medium. Make dilutions 10 $\times$ final concentration. Add 50 μ L (for 48-well plates) or 100 μ L (for 24-well plates) buffer or stimulant, and incubate for 4–8 h at 37 °C (*see* **Note 10**).
5. After incubation, pipette the supernatant(s) into 1.5 mL microcentrifuge tubes, and centrifuge for 5 min at 1,000 $\times g$, 4 °C; then collect the cell-free supernatants. Store the supernatants at –80 °C until assayed.
6. Measure the cytokine or chemokine content using an appropriate kit according to the manufacturer's instructions (*see* **Note 11**).

3.6 Measuring Eicosanoid Generation

The protocol below describes the measurement of the release of prostaglandin D₂ (PGD₂) and leukotriene C₄ (LTC₄) from Ag-stimulated mast cells of human and mouse origin using commercially available kits (Cayman Chemicals). Generally, PGD₂ release is lower than LTC₄ release following Ag stimulation, and BMMCs release greater amounts of LTC₄ than do HuMCs. Therefore, cell numbers have to be adjusted accordingly. Since the assay is based on a competitive enzyme immunoassay, it is critical to determine the optimum cell number and activation period. Since there are further dilutions of supernatants after stimulation, it is feasible to use the same cell samples for analysis of both eicosanoids.

Preparing Sample Supernatants for Eicosanoid Measurement

Human or mouse mast cells are sensitized overnight in appropriate medium as described in Subheading 3.1.

1. Warm freshly prepared HEPES + 0.04 % BSA (assay buffer) to 37 °C.
2. Wash cells in pre-warmed HEPES + 0.04 % BSA (10 mL) by centrifugation at 300 $\times g$ for 10 min. Remove supernatant carefully, resuspend cells in HEPES + 0.04 % BSA, and centrifuge again.
3. Repeat wash one more time (**step 2**).
4. Resuspend cells for PGD₂ and LTC₄ assays as follows. Prepare HuMCs or the LAD2 human mast cell line to a density of 2×10^3 cells per 80 μ L or BMMCs to a density of 1×10^5 cells per 80 μ L.
5. Aliquot cells (80 μ L per well) into a U-bottomed 96-well plate (reaction plate) and place at 37 °C for 10 min.

6. While cells are equilibrating to 37 °C, prepare stimulants in HEPES + 0.04 % BSA: Make working concentrations of stimulants at 5× final concentrations. Add 20 µL assay buffer or stimulant and incubate for 20 min at 37 °C.
7. After 20 min incubation, spin plate at 4 °C for 5 min, 300 × *g*.
8. For PGD₂ assay, take supernatants (25 µL) and transfer to thin-walled tubes (200 µL) containing freshly prepared methoximating (MOX) reagent (25 µL), and derivatize samples at 60 °C for 30 min using a thermocycler machine (as per manufacturer's instructions). Also add freshly prepared MOX reagent to an aliquot of PGD₂ stock (25 µL, 40 ng/mL) to produce a PGD₂-MOX stock of 20 ng/mL. These MOX samples can either be used directly in the EIA or stored for up to 6 weeks at 4 °C.
9. For LTC₄ assay, take supernatants (30 µL) and place into 200 µL microcentrifuge tubes. These samples can either be used directly in the EIA or stored at -80 °C.

Measuring PGD₂ in Cell-Free Supernatants

This protocol is a brief summary of the manufacturer's instructions (Cayman Chemicals) with some minor adjustments for cell density relevant to mast cell activation. The reader is advised to refer to the manufacturer's instructions for a complete and detailed protocol.

1. For HuMC or LAD2 samples, add 10 µL of MOX-cell-free supernatants to EIA plate (pre-coated with mouse anti-rabbit IgG) containing 40 µL HEPES + 0.04 % BSA buffer. For BMMC samples, dilute MOX-cell-free supernatants 1 in 10 prior to adding 10 µL of cell-free supernatants to EIA plate containing 40 µL HEPES + 0.04 % BSA buffer.
2. Prepare PGD₂ standards (7.8–1,000 pg/mL) from the PGD₂-MOX stock of 20 ng/mL and add 50 µL per well in duplicate to EIA plate.
3. Add 50 µL per well of PGD₂-MOX AChE tracer.
4. Add 50 µL per well of PGD₂-MOX antiserum, seal plate with an adhesive plastic film, and incubate for 18 h at 4 °C.
5. The following day, wash the microplate 5× with EIA wash buffer (200 µL/well).
6. Add 200 µL of freshly prepared Ellman's reagent to each well, seal plate with adhesive plastic film, and incubate on orbital shaker in the dark at room temp for 60 min.
7. Read plate at a wavelength of 405–420 nm when absorbance of B₀ well is in the range 0.3–0.8 AU (after blank subtracted).

The amount of PGD₂ in each sample is then calculated from the standard curve.

Typical examples of PGD₂ release are shown in Fig. 3.

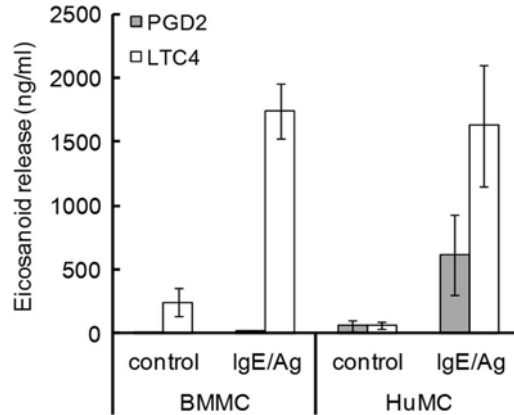


Fig. 3 Eicosanoid release by mouse and human mast cells following $Fc\epsilon RI$ -dependent activation. Mouse BMMCs or HuMCs were sensitized overnight with IgE-DNP (100 ng/mL) or biotinylated IgE (100 ng/mL), respectively, and then challenged for 20 min respectively with DNP-HSA (30 ng/mL) or streptavidin (100 ng/mL). Results are means $\pm$ SE of 4 independent experiments performed in duplicate

Measuring LTC₄ in Cell-Free Supernatants

This protocol is a brief summary of the manufacturer's instructions (Cayman Chemicals) with some minor adjustments for cell density relevant to mast cell activation. The reader is advised to refer to the manufacturer's instructions for a complete and detailed protocol.

1. For HuMC or LAD2 samples, add 25 μ L of cell-free supernatants to EIA plate (pre-coated with mouse anti-rabbit IgG) containing 25 μ L HEPES + 0.04 % BSA buffer. For BMMC samples, dilute 1 in 100 (two serial dilutions of 1 in 10) prior to adding 50 μ L of cell-free supernatants to EIA plate.
2. Prepare LTC₄ standards (7.8–1,000 pg/mL) from the LTC₄ stock of 20 ng/mL and add 50 μ L per well in duplicate to EIA plate.
3. Add 50 μ L per well of LTC₄-AChE tracer.
4. Add 50 μ L per well of LTC₄ antiserum, seal plate with an adhesive plastic film, and incubate for 18 h at RT.
5. The next day, remove liquid from wells and wash 5 $\times$ with EIA wash buffer (200 μ L/well).
6. Add 200 μ L of freshly prepared Ellman's reagent to each well, seal plate with plastic film, and incubate on orbital shaker in the dark at room temperature for 60 min.
7. Read plate at 405–420 nm when absorbance of B₀ well is in the range 0.3–0.8 AU (blank subtracted).

The amount of LTC₄ in each sample is then calculated from the standard curve. Typical examples of LTC₄ release are shown in Fig. 3.

4 Notes

1. For the examples provided in this chapter, we prepared primary HuMCs from CD34⁺ peripheral blood progenitors [7] isolated from healthy volunteers after informed consent under a protocol (NCT00001756) approved by the NIH Institutional Review Board. BMMCs were prepared from mouse bone marrow [6] under a protocol approved by the NIAID Animal Care and Use Committee.
2. Recommended cell concentrations (Table 1) and stimulation times (Table 2) for different mast cell assays.
3. For some experiments, cytokines normally present in the mast cell maintenance/growth culture medium should be omitted (e.g., SCF/IL-3, if the effect of SCF/IL-3 stimulation is investigated). Cytokine starvation should not exceed 24 h.
4. When thawing an aliquot of IgE, the suspension should be centrifuged at high speed ($>16,000 \times g$) for 60 min in order to

Table 1
Recommended cell concentrations for specific mast cell assays

Cell concentrations necessary for specific assays		
	Human (cell/sample)	Mouse (cell/sample)
Degranulation	5,000–10,000	30,000–50,000
Cytokine release	500,000	500,000
Eicosanoids: LTC ₄	1,000	10,000
PGD ₂	2,000	100,000
mRNA	1,000,000 50–100 ng/reaction	1,000,000 50–100 ng/reaction

Table 2
Stimulation time for specific mast cell assays

Stimulation time for specific assays (<i>see also</i> Note 10)		
	Human	Mouse
Degranulation	30–60 min	30–60 min
Cytokine release	4–24 h	4–24 h
Eicosanoids	20 min	20 min
mRNA	2–24 h	2–24 h

remove aggregated IgE. Aggregated IgE may activate the cell during sensitization.

5. The following are some examples of traditional stimulants and working concentrations used for mast cell stimulation: compound 48/80 (0.05–5 mg/mL), substance P (1–10 μ M), complement 5a (0.1–1 μ g/mL), and LPS (100 ng/mL). Stimulation time depends on which mediators are being examined.
6. Besides β -hexosaminidase release, degranulation can also be monitored by histamine (fluorometric assay from RefLab ApS or EIA from Immunotech) and tryptase release (Phadia).
7. Equation explanation for β -hexosaminidase release: Δ supernatant (OD_{405nm}): absorbance reading of supernatant (OD_{405nm})—absorbance reading of buffer only (i.e., blank (OD_{405nm})). Δ cell lysate (OD_{405nm}): absorbance reading of cell lysate (includes product from $\frac{1}{2}$ of supernatant)—absorbance reading of buffer only (i.e., blank(OD_{405nm})). Value “2” in the nominator: correction factor applied since only $\frac{1}{2}$ of the supernatant volume is sampled (with the other $\frac{1}{2}$ remaining with the cell lysate). Value of “4” in the denominator: correction factor since only $\frac{1}{4}$ of cell lysate is sampled. This cell lysate contains the intracellular contents plus $\frac{1}{2}$ of the supernatant. Note that only $1\times$ (Δ supernatant) appears in the denominator since the “ Δ cell lysate” value includes the equivalent of $1\times$ (Δ supernatant).
8. RNA extraction from mast cells can be performed by the TRIzol® method or use of an appropriate lysis buffer. For real-time PCR, we found the use of the Qiagen RNeasy kits provided the best quality RNA (OD_{260nm}/OD_{280nm} ratios of 1.95–2.07) with Ct values for housekeeping genes lower than with TRIzol extraction.
9. In HuMCs, antigen or SCF alone fails to generate sufficient cytokine release. Thus, in order to generate sufficient cytokine release, it is recommended to stimulate HuMCs with both antigen and SCF.
10. The stimulation time for cytokine secretion (and also mRNA synthesis or stability) is dependent on the specific cytokines investigated.
11. The amount of cytokine or chemokine release can vary depending on cell condition; thus, if the samples absorbance (OD) is above the standard range, the sample needs to be diluted.

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