Video Article

Isolation of Peritoneum-derived Mast Cells and Their Functional Characterization with Ca^{2+}-imaging and Degranulation Assays

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URL: https://www.jove.com/video/57222
DOI: doi:10.3791/57222

Keywords: Immunology and Infection, Issue 137, Peritoneal mast cells, murine mast cells, intracellular free calcium concentration, fluorescence imaging, degranulation, mediator release, beta-hexosaminidase

Date Published: 7/4/2018

Citation: Tsvilovskyy, V., Solis-Lopez, A., Öhlenschläger, K., Freichel, M. Isolation of Peritoneum-derived Mast Cells and Their Functional Characterization with Ca^{2+}-imaging and Degranulation Assays. *J. Vis. Exp.* (137), e57222, doi:10.3791/57222 (2018).

Abstract

Mast cells (MCs), as a part of the immune system, play a key role in defending the host against several pathogens and in the initiation of the allergic immune response. The activation of MCs via the cross-linking of surface IgE bound to high affinity IgE receptor (FcεRI), as well as through the stimulation of several other receptors, initiates the rise of the free intracellular Ca^{2+} level ($[Ca^{2+}]_i$) that promotes the release of inflammatory and allergic mediators. The identification of molecular constituents involved in these signaling pathways is crucial for understanding the regulation of MC function. In this article, we describe a protocol for the isolation of murine connective tissue type MCs by peritoneal lavage and cultivation of peritoneal MCs (PMCs). Cultures of MCs from various knockout mouse models by this methodology represent a useful approach to the identification of proteins involved in MC signaling pathways. In addition, we also describe a protocol for single cell Fura-2 imaging as an important technique for the quantification of Ca^{2+} signaling in MCs. Fluorescence-based monitoring of $[Ca^{2+}]_i$ is a reliable and commonly used approach to study Ca^{2+} signaling events, including store-operated calcium entry, which is of utmost importance for MC activation. For the analysis of MC degranulation, we describe a β-hexosaminidase release assay. The amount of β-hexosaminidase released into the culture medium is considered as a degranulation marker for all three different secretory subsets described in MCs. β-hexosaminidase can easily be quantified by its reaction with a colorigenic substrate in a microtiter plate colorimetric assay. This highly reproducible technique is cost-effective and requires no specialized equipment. Overall, the provided protocol demonstrates a high yield of MCs expressing typical MC surface markers, displaying typical morphological and phenotypic features of MCs, and demonstrating highly reproducible responses to secretagogues in Ca^{2+}-imaging and degranulation assays.

Video Link

The video component of this article can be found at https://www.jove.com/video/57222/

Introduction

MCs play a prominent role during innate and acquired immune responses. Specifically, MCs participate in the killing of pathogens, such as bacteria and parasites, and also degrade potentially toxic endogenous peptides or components of venoms (for review see Galli *et al.* 2008[1]). The physiological role of MCs in innate and adaptive immunity is the subject of heated debate. Therefore, the numerous data discrepancies in the studies performed with different MC-deficient mouse models require a systematic re-evaluation of immunological functions of MCs beyond allergy[2]. Mature MCs are mostly localized in tissues and organs such as the skin, lung, and gut, and are usually found only in small numbers in the blood. MCs derive from hematopoietic precursors, such as MC progenitors, and complete their differentiation and maturation in the microenvironments of almost all vascularized tissues[1]. T-cell-derived factor interleukin (IL)-3 selectively promotes the viability, proliferation, and differentiation of a pluripotent population of mouse MCs from their hematopoietic progenitors[3]. Stem cell factor (SCF) is produced by structural cells in the tissues and plays a crucial role in the MC development, survival, migration, and function[4]. The properties of individual MCs may differ depending on their ability to synthesize and store various proteases or proteoglycans. In mice, the so-called connective tissue-type MCs are distinguished from mucosal MCs according to their anatomic localization, morphology, and content of heparin and proteases[5].

In MCs, an increase of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) is indispensable for the degranulation and production of eicosanoids, as well as for the synthesis of cytokines and activation of transcription factors in response to antigen and various secretagogues[6]. A major downstream target of these stimuli is phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate. DAG activates protein kinase C and IP3 releases ions of Ca^{2+} from the endoplasmic reticulum. Depletion of these stores activates Ca^{2+} influx through plasma membrane Ca^{2+} channels, leading to store operated calcium entry (SOCE). This process is evoked through the interaction of the Ca^{2+}-sensor, stromal interaction molecule-1 (Stim1), in the endoplasmic reticulum with Orai1[7] as well as through the activation of transient receptor potential canonical (TRPC) channel proteins (for review see Freichel *et al.* 2014[8]) in the plasma membrane.

To study the physiological role of these channels, several pharmacological (application of channel blockers) or genetic approaches are typically used. In the latter case, suppression of protein expression is achieved by targeting mRNA (knockdown) or genomic DNA editing with global or tissue-specific[9] deletion of an exon coding a pore forming subunit of a channel (knockout). The availability of a blocker with sufficient specificity

for these channels is limited. In addition, the knockdown approach requires careful control of its effectivity using, *e.g.*, Western blot analysis, and is hampered by the unavailability of specific antibodies for the targeted channel protein in many cases. Thus, the usage of knockout mouse models is still considered as the gold standard for such analysis. A preferred *in vitro* model for the investigation of MC functions is cultivation of PMCs that can be isolated *ex vivo* as a fully mature population (as opposed to differentiating MCs *in vitro* from, *e.g.*, bone marrow cells)[10]. As compared to bone marrow derived MCs (BMMCs), which are also commonly used to study MC function *in vitro*, the stimulation of FcεRI and beta-hexosaminidase release is increased 8-fold and 100-fold in PMCs, respectively. In this article, we describe a method of isolation and cultivation of murine PMCs, which allows to obtain after 2 weeks of culturing a high number of pure connective tissue type MCs, sufficient for further analysis.

Despite recent progress in the development and application of genetically encoded calcium indicators, their usage is still limited by difficulties in delivery of genes to target cells, specifically, in highly-specialized cells such as primary cultured MCs. In addition, this group of fluorescent indicators for $[Ca^{2+}]_i$ measurements still lacks ratiometric dyes with a high dynamic range. For these reasons, the preferred method for ratiometric $[Ca^{2+}]_i$ measurement is still the use of the fluorescent dye "Fura-2"[11].

Currently, the most commonly used approach for the evaluation of MC activation and degranulation is the measurement of β-hexosaminidase activity. β-hexosaminidase, an allergic mediator, is one of the MC granule components that is co-released with histamine in constant proportion from MCs[12]. β-hexosaminidase can be readily and accurately measured through its reaction with a specific substrate, which produces a measurable quantity of a colorigenic product that is easily detectable in a microplate colorimetric assay. In this article, an application of this technique for the analysis of PMCs degranulation in response to a variety of stimuli is reported.

Aggregation of the high-affinity plasmalemmal IgE receptor (FcεRI) activates in MCs a versatile intracellular signaling pathway, leading to a release of secretory granule content in the surrounding extracellular space. In addition to the specific antigen, many other stimuli can activate MCs to release a diverse array of immunomodulatory mediators, such as complement anaphylatoxins (*e.g.*, C3a and C5a)[13], the vasoconstrictor peptide endothelin 1 (ET1)[14], as well as numerous cationic substances and drugs provoking pseudoallergic reactions (*e.g.*, icatibant)[15] by binding to MRGPRX2[16]. Intracellular signaling pathways involved in MRGPR-induced MCs degranulation are poorly characterized as compared to FcεRI-mediated intracellular signaling; these pathways only started to be intensively studied during the last few years[17] after the receptor identification[16]. Largely, the plasma membrane ion channels involved in calcium entry followed by MRGPRX2 stimulation remain to be understood. Therefore, the present article also focuses on intracellular calcium signaling and degranulation of MCs stimulated with MRGPR agonists.

Protocol

All animal procedures were performed according to the German legislation guidelines for care and use of laboratory animals (officially approved by the Karlsruhe regional council).

1. PMC Isolation and Cultivation by Intraperitoneal Lavage

1	Ice
2	10 mL syringes
3	27 G needles
4	20 G needles
5	Styrofoam block and pins
6	Collection tubes (50 mL Plastic Centrifuge Tubes)
7	70% ethanol
8	RPMI Medium (Pre chilled and kept on ice)
9	PMC Medium: RPMI Medium + 20% FCS (Fetal Calf Serum) + 1% Penicillin Streptomycin solution (Pen-Strep)
10	Dulbecco's phosphate buffered saline - DPBS (without Ca^{2+} and Mg^{2+})
11	Culture Flasks (25 cm)
12	Growth factors stock solutions (IL-3: 1 µg/µL; SCF: 2.5 µg/mL)
13	Serological pipettes (10 mL)
14	Pipettes tips sterile (20–200 µL)
15	Hemocytometer
16	Bench Centrifuge
17	Scissors and forceps
18	CO_2 chamber for mice
19	Open sterile hood
20	Closed sterile hood
21	Cell incubator (37 °C and 5% CO_2)
22	Transfer pipettes (20–200 µL)

Table 1: Materials for Step 1.

1. **PMC isolation by intraperitoneal lavage**
    1. Prior to the cell isolation, prepare the materials listed in **Table 1**.
    2. Use 8 to 14 week-old male mice for PMC isolation. Euthanize a mouse by CO_2 inhalation, and confirm the death by loss of reflexes. Spray the mouse with 70% ethanol and fix it on a foam block using pins (dorsal side down). Remove the ventral skin of the mouse using blunt edge scissors. Avoid damaging the peritoneal cavity.
    NOTE: We use typically this protocol for mice with a C57BL/6N strain genetic background.
    3. Inject 7 mL of the ice-cold RPMI medium and 5 ml of air in the peritoneal cavity using a 10 mL syringe equipped with a 27 G needle. Push the needle carefully in the peritoneum and do not perforate any organs. Use a spot in the region of the epididymal fat to reduce the risk of an organ perforation.
    4. After injection, shake the mouse for 1 min to detach peritoneal cells into the RPMI medium. Do not shake the mouse too strongly, to avoid damaging the internal organs and contaminating the peritoneal cavity with the blood.
    5. Reuse the 10-mL syringe by equipping it with a new 20 G cannula. Shift the inner organs to one side by tilting the foam block and gently tapping it on the bench to make medium aspiration easier from the other side. Insert a 20 G needle, bevel up, and aspirate the fluid from the abdomen gently and slowly (~0.5 mL/s) to avoid clogging by the inner organs. Collect as much fluid as possible (typically 5–6 mL).
    6. Remove the needle from the syringe and transfer the collected cell suspension in a collection tube on ice. Discard a sample tube if there is visible blood contamination.
    7. Centrifuge the tubes with the cell suspension at ~300 x *g* for 5 min. Under a sterile hood aspirate the supernatant. For each mouse, combine the sample pellets in 4 mL of cold (4–10 °C) PMC Medium and transfer the cell suspension to a 25 cm^2 culture flask. Add the growth factors IL-3 and SCF to the final concentrations 10 ng/mL and 30 ng/mL, respectively. Place the flask containing the cells in an incubator (37 °C and 5% CO_2) and incubate for ~48 h until the next procedure.

2. **PMC culture**
    1. Day 2 (~48 h after isolation): Medium change
        1. To remove the supernatant and non-adherent cells (erythrocytes and dead cells), aspirate the medium from the flask by placing a Pasteur pipette tip at the edge of the flask and holding the flask almost horizontally. Add 4 mL of pre-warmed PMC Medium per mouse. Add the growth factors IL-3 (10 ng/mL) and SCF (30 ng/mL). Place the flask containing the cells in an incubator (37 °C and 5% CO_2).
    2. Day 9: Cell splitting

1. Transfer the cell suspension from the cell culture flask into a 50 mL plastic centrifuge tube. Wash the flask three times with 10 mL pre-warmed (37 °C) Dulbecco's phosphate-buffered saline (DPBS), collecting the cell suspension with the detached cells in the same 50 mL plastic centrifuge tube. Count the cell concentration by a hemocytometer, and calculate the total number of the cells.
2. Centrifuge the cell suspension at ~300 x *g* for 5 min and aspirate the supernatant. Recover the pellet in pre-warmed PMC Medium (37 °C) to obtain the cell concentration 1 x 10^6 cells/mL. Transfer the cell suspension to a new 25 cm^2 culture flask. Discard the old flask with fibroblasts adhering to the bottom.
3. Add the growth factors IL-3 (10 ng/mL) and SCF (30 ng/mL), and place the flask containing the cells in an incubator (37 °C and 5% CO_2).

3. Days 12–15: Cell measurements
   1. If any experiments with stimulation of FcεRI receptors are planned, pretreat the cells overnight with 300 ng/mL of IgE anti-DNP in standard culture medium. Proceed with step 2 or step 3.

2. Fluorometric Intracellular Free Calcium Concentration Measurement in PMCs

1. Prior to the measurements, prepare the materials listed in **Table 2**. Also, prepare an Imaging Setup consisting of: Inverted Fluorescence Microscope; Objective: 40X/1.3 Oil; Fura 2 Filter Set; CCD Camera; Excitation Light Source; Imaging Acquisition Program; Gravity fed solution application system.

1	PMC (12–15 days old) 1 x 10^6 cells/mL
2	Concanavalin A
3	Fura-2 AM
4	Pluronic F-127 20% solution
5	Cover slips glasses round; ø 25 mm; No. 1
6	DNP-HSA (dinitrophenol-human serum albumin) stock solution
7	Anti-DNP-Antibody (IgE) stock solution
8	Flat bottom plate (12 wells)
9	Compound 48/80 stock solution
10	Cover Well Imaging Chambers
11	Hemocytometer
12	Transfer pipettes (20–200 µL)

Table 2: Materials for Step 2.

2. **Fura-2 imaging preparation**
   1. Perform a cell count using a hemocytometer and adjust the cell concentration to 1 x 10^6 cells/mL. Split the PMCs colonies by gently pipetting (3–5 times) the cell suspension with a 1 mL pipette tip.
   2. Prepare a Ca^{2+}-containing HEPES buffered salt solution (Ca^{2+}-HBSS) composed of 135 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, and 12 mM glucose. Adjust the pH to 7.4 with 3 M NaOH.
   3. Prepare concanavalin A coated slides by pipetting 1 drop of concanavalin A solution (0.1 mg/mL in H_2O) on each 25 mm round cover glass under a sterile hood. Leave the drops on the cover slips for at least 1 min, then remove most of the solution with a pipette and let the cover slips air-dry for 45 min. Carefully press the concanavalin A treated cover slips on the rubber imaging chamber (see **Table of Materials**) until they attach to obtain microscopy imaging chambers.
      NOTE: Poly-L-Lysine coated slides can be used as an alternative.
   4. Dissolve the fluorescent Ca^{2+} dye Fura-2 AM (1 mM) in dimethyl sulfoxide (DMSO). Store this solution protected from light.
   5. Dilute the Fura-2 AM stock solution in Ca^{2+}-HBSS to a final concentration of 5 µM in order to prepare the "loading buffer". Supplement with 5 µL/mL of 20% pluronic F-127 in DMSO.
   6. Mix 500 µL of the "loading buffer" with 500 µL of the PMC cell suspension. Pipette the mixture into the imaging chamber and incubate the cells for 20–30 min at room temperature in the dark.
   7. Set up the gravity fed application system with different solutions according to the stimulation protocol. Supplement syringe 1 with 40 mL of Ca^{2+}-HBSS solution, syringe 2 with 40 mL of Ca^{2+}-HBSS solution containing 100 ng/mL DNP, syringe 3 with 40 mL of Ca^{2+}-HBSS solution containing 50 µg/mL Compound 48/80, syringe 4 with 40 mL of nominally Ca^{2+}-free-HBSS solution, and syringe 5 with 40 mL of nominally Ca^{2+}-free-HBSS solution containing 2 µM Thapsigargin.
   8. Prepare a 40X high-aperture oil immersion objective by placing a small drop of immersion oil on it.
   9. Mount the application system on the stage of the inverted microscope. Put the imaging chamber with the loaded cells in the application system and secure it to prevent movement during recording. Turn on the transmitted light and focus the cells.
   10. Rinse the cells three times with Ca^{2+}-HBSS buffer using the gravity-fed application system.
   11. Incubate the cells in Ca^{2+}-HBSS for 5 min for Fura-2 AM de-esterification.
   12. Rinse the cells one more time before starting the imaging, to remove any potentially leaked fluorescent dye.

3. **Cell imaging**

1. Start the imaging acquisition program in the physiological acquisition mode. Open the setup window, and adjust the focus and the optimal acquisition time (which should be the same for excitation with 340 nm and 380 nm and typically ranges between 50–150 ms).
   NOTE: Minimize the exposure of the Fura-2 loaded cell to any light to avoid photobleaching of the dye.
2. Image a reference picture, and mark the cells as regions of interest (ROI). Mark the last ROI in an area where no cells are present and define it as the background.
3. Complete the setup and start the measurement with a 5 s acquisition rate.
   NOTE: The cells will be alternately illuminated with the excitation light of 340 nm and 380 nm, and the emitted fluorescence (>510 nm) will be collected using the CCD camera. The images of fluorescence signals F340 nm and F380 nm as well as the ratio (F340 nm/F380 nm) will be displayed in three windows. The charts of time course of individual ROIs fluorescence intensity mean values will be also continuously displayed.
4. Add solutions at the proper cycle number according the design of the experiment:
   1. To measure antigen-induced elevation of $[Ca^{2+}]_i$, as illustrated in **Figure 2A, 2B**, apply the syringe 2 solution after cycle 20, and stop recording after cycle 150.
   2. To measure Compound 48/80-induced elevation of $[Ca^{2+}]_i$, as illustrated in **Figure 2C**, apply the syringe 3 solution after cycle 20, and stop recording after cycle 150.
   3. To measure the elevation of $[Ca^{2+}]_i$ evoked by SOCE, as illustrated in **Figure 2D**, apply the syringe 4 solution after cycle 10, apply the syringe 5 solution after cycle 70, apply the syringe 4 solution after cycle 120, apply the syringe 1 solution after cycle 150, and stop recording after cycle 220.
5. After finishing the measurement, convert the results into a ratio table containing the measured intensities for both excitation wavelengths with and without background correction, as well as the ratio values with and without background correction for every ROI and every measurement time point. In the acquisition program, consecutively press the buttons: "start cutter", "mark all", "convert all", "physiology measurements", "Ok, "Ok". Save the files as tables and image stacks for off-line analysis.

3. Beta-hexosaminidase Release Measurement in PMCs

1	PMC (12–15 days old) 1 x 10^6 cells/mL
2	Anti-DNP-Antibody (IgE) stock solution
3	DNP-HSA stock solution
4	Compound 48/80 stock solution
5	Ionomycin
6	96-Well plate, v-shaped bottom
7	96-Well plates, flat bottom
8	Plastic centrifuge tubes (15 mL)
9	Serological pipettes
10	Cell incubator (37 °C and 5% CO_2)
11	Bench Centrifuge
12	Microtiter plate reader for optical density measurements
13	Multichannel pipette (20–200 µL)
14	Hemocytometer

Table 3: Materials for Step 3.

1. Prior to the measurements, prepare the materials listed in **Table 3**.
   NOTE: β-hexosaminidase released from MCs after their stimulation hydrolyzes p-nitrophenyl-acetyl-D-glucosamine (pNAG) into p-nitrophenol and N-acetyl-D-glucosamine. The amount of β-hexosaminidase in the sample is proportional to the amount of p-nitrophenol that will be formed. In a high pH environment of "Stop Solution", p-nitrophenol exists as fully deprotonated p-nitrophenolat and can be detected by its light absorbance at 405 nm.
2. Prepare Tyrode's solution containing 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5.6 mM glucose, and BSA 0.1%. Adjust the pH to 7.4 with 3 M NaOH.
3. Prepare the lysis solution by adding a 1% volume of Triton X-100 to the Tyrode's solution.
4. Prepare the stop solution composed of 200 mM glycine and adjust the pH to 10.7 with NaOH.
5. Prepare the pNAG solution (4-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside) solution by dissolving pNAG in 0.4 M citric acid to a final concentration of 4 mM.
6. Calculate the amount of the cells needed for the assay (perform the assay in duplicate). Use 200,000 PMCs per well. Use 2 wells as negative controls (Tyrode's solution without any secretagogues such as DNP or compound 48/80) and 2 wells as positive controls (Tyrode's solution with 10 µM Ionomycin) for every genotype.
7. Transfer the cells into a 15-mL plastic centrifuge tube, adjust the volume to 15 mL with Tyrode's solution, and centrifuge at 200 x *g* for 4 min. Remove the supernatant with a Pasteur pipette and resuspend the cells in Tyrode's solution to 2 x 10^6 cells/mL.
8. Transfer 100 µL of the cell suspension per well to a 96-well V-bottom well plate. Add 25 µL of either stimulation or control solution to each well (according to the pipetting scheme illustrated in **Figure 3A**). Incubate the cells for 45 min at 37 °C and 5% CO_2.

9. Stop the reaction by placing the 96-well plate on ice for 5 min. Then, centrifuge the plate at 4 °C for 4 min at 120 x *g*. Transfer 120 µL of the supernatant using a multichannel pipette to a flat-bottom 96-well plate and place it on ice. Carefully avoid touching the cell pellets, but completely aspirate the supernatant.
10. Add 125 µL of lysis buffer to the cell pellets. Incubate the cell pellets for 5 min at room temperature and resuspend them after the incubation by repeated pipetting (approximately 5 times).
11. Pipette 25 µL of the pNAG solution (4 mM) in the required wells of a new flat-bottom 96-well plate. Add 25 µL from each supernatant to the prepared pNAG solution as well as 25 µL of each cell lysate.
12. Incubate the reaction batches for 1 h at 37 °C. After the incubation, pipette 150 µL of stop solution to each well to stop the reaction.
13. Analyze the plate with a microplate reader using a dual-wavelength setting at 405 nm with reference 630 nm for automatic background subtraction. In the acquisition program, tick the box "Reference" and in the "Absorbance" program element menu, select "630 nm" from the drop-down list. If the optical density of the samples is too high, prepare an appropriate dilution of the probe before remeasuring.
14. Calculate the percentage of β-hexosaminidase release according to the following equation:

$$Release\ [\%] = 100\% \times \left(\frac{A[supernatant]}{A[supernatant] + A[lysate]}\right)$$

where Release [%] is the percent of specific beta-hexosaminidase release, A[supernatant] is the background corrected absorption of the supernatant, and A[lysate] is the background corrected absorption of the corresponding cell lysate.

Representative Results

PMCs were isolated from 3 wild type mice (C57BL/6N) by intraperitoneal lavage and further cultured in RPMI medium supplemented with 20% FCS and 1% Pen-Strep in the presence of growth factors (IL-3 at 10 ng/mL and SCF at 30 ng/mL) under sterile humidified conditions at 37 °C and 5% CO_2. The medium was changed on days 2 and 9 after the cell isolation. The cell morphology was analyzed using transmission light DIC imaging (see **Figure 1A, B**). The cell contrast and granularity demonstrated good cell viability. After 2 weeks of culturing under these conditions, a homogeneous population of 1.5 x 10^7 cells was obtained. Flow cytometry analysis of the cells co-stained with anti-IgE antibody conjugated with FITC and Anti-c-Kit antibody conjugated with PE revealed 98.6% of FITC positive and 99.8% PE positive cells (**Figure 1C**). 98.5% of the cultured cells were double positive for FITC and PE (**Figure 1C**), demonstrating typical features of mature MCs.

For further functional analysis of the obtained cells, fluorometric $[Ca^{2+}]_i$ measurements using Fura-2 fluorescent indicator were performed. Fura-2 loaded cells were alternately illuminated with 340 nm and 380 nm excitation light every 5 s and fluorescence >510 nm was registered using a CCD camera. The ratio of fluorescence (F340/F380) was constantly monitored. Application of DNP (100 ng/mL) to PMC incubated overnight with 300 ng/mL of IgE anti-DNP evoked a pronounced elevation of the $[Ca^{2+}]_i$ level, which slowly decayed to its half maximum over several minutes (**Figure 2A, B**). Variability of cell responses was relatively low (**Figure 2A, B**). Stimulation of MRGPRB2 receptors with 50 µg/mL of Compound 48/80 with the same time protocol also significantly increased $[Ca^{2+}]_i$ in Fura-2-loaded PMCs with a very similar mean amplitude of the response (**Figure 2C**). For the analysis of the SOCE in PMCs, a "re-addition" protocol was applied: 10 cycles after the beginning of the measurement, external Ca^{2+} was removed, and during cycles 70–120, Thapsigargin (2 µM), an inhibitor of endoplasmic reticulum ATPase, was applied for the depletion of intracellular Ca^{2+} stores and for maximal activation of SOCE. The transient $[Ca^{2+}]_i$ elevation reflected the Ca^{2+} release from the intracellular Ca^{2+} stores in a nominally Ca^{2+}-free bath solution (**Figure 2D**). Restoring the external Ca^{2+} concentration to 2 mM after cycle 150 resulted in a prominent increase of $[Ca^{2+}]_i$ level due to the Ca^{2+} influx through the SOCE channels (**Figure 2D**). This component of the Ca^{2+} response was strongly inhibited in the presence of 10 µM GSK 7975A, known as a CRAC blocker, whereas the Ca^{2+} release from the intracellular Ca^{2+} stores remained unchanged (**Figure 2D**).

In the next step, we tested the ability of the isolated PMCs to undergo degranulation upon crosslinking of the FcεRI high affinity receptors or stimulation of the MRGPRB2 receptors. For this purpose, a ß-hexosaminidase release assay was performed. The cells were added to a V-bottom 96-well plate (2 x 10^5 cells/well) and stimulated according to the scheme illustrated in **Figure 3A** for 45 min at 37 °C. The plate was centrifuged and after removal of the supernatant, the pellets were lysed. The ß-hexosaminidase content of individual supernatants and pellet lysates was analyzed by their reactions with pNAG during a 1 h incubation at 37 °C. The amount of the reaction products was detected colorimetrically and the percentage of the released ß-hexosaminidase was calculated (see **Figure 3B**). The spontaneous release was very low (1%), whereas the responses to strong degranulation stimuli such as 10 µM Ionomycin and Compound 48/80 at 50 µg/mL were over 40% and 60%, respectively. Degranulation responses to DNP stimulation were dose-dependent over the concentration range of 10–300 ng/mL. Together, these data clearly indicate a good functional state of the isolated PMCs.

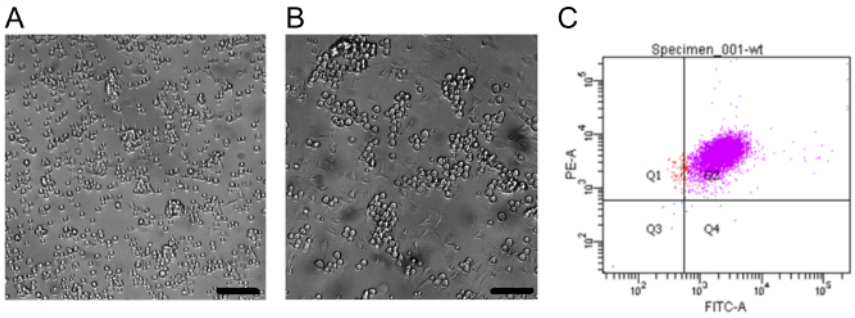

Figure 1: Characterization of primary cultured murine wild type PMCs performed using DIC microscopy and flow cytometry analysis. (**A**, **B**) Representative images obtained using 20X plasDIC objective of PMCs cultured in 25 cm^2 plastic flasks 1 h after cell isolation by intraperitoneal lavage (**A**) and on day 9 of culturing (**B**). Scale bars in the right lower corner indicate 100 µm. (**C**) Flow cytometry analysis of PMCs (cultured 12 days according to the above described protocol) co-stained with anti-IgE antibody conjugated with Fluorescein Isothiocyanate (FITC) and Anti-c-kit antibody conjugated with Phycoerythrin (PE). The cell density plot is split into four quadrants: Q1, PE above autofluorescence level/FITC below autofluorescence level; Q2, PE above autofluorescence level/FITC above autofluorescence level; Q3, PE below autofluorescence level/FITC below autofluorescence level; Q4, PE below autofluorescence level/FITC above autofluorescence level. Please click here to view a larger version of this figure.

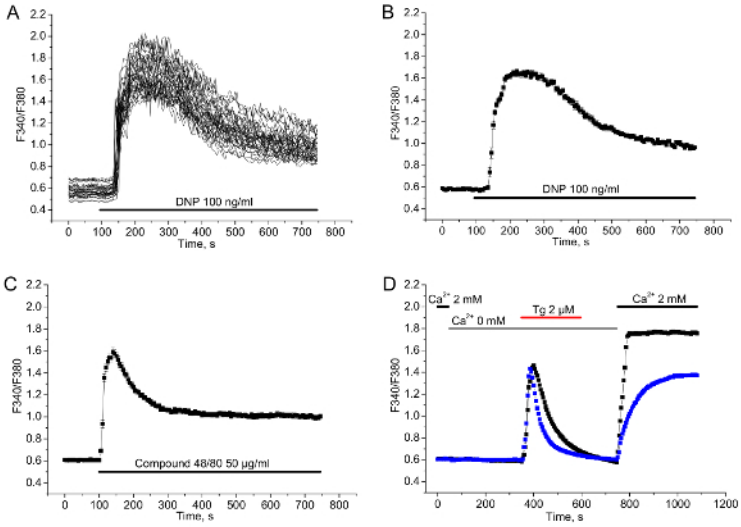

Figure 2: Recording of $[Ca^{2+}]_i$ in Fura-2 loaded PMCs isolated from wild type mice. $[Ca^{2+}]_i$ presented as fluorescence F340/F380 ratio. (**A**) Representative traces of the $[Ca^{2+}]_i$ time course of individual PMCs (n = 38) stimulated with DNP (100 ng/mL). (**B**) Time course of DNP (100 ng/mL)-evoked $[Ca^{2+}]_i$ elevation. Every data point indicates mean values obtained from 38 individual cells illustrated in panel A. The PMCs were pretreated overnight with 300 ng/mL of IgE anti-DNP. (**C**) Time course of the mean values of Compound 48/80-induced $[Ca^{2+}]_i$ elevation in PMCs (n = 60). (**D**) Time course of the mean values of $[Ca^{2+}]_i$ changes during intracellular Ca^{2+}-store depletion induced by application of 2 µM Thapsigargin (Tg) in nominally Ca^{2+}-free solution, followed by store operated calcium influx after re-addition of 2 mM Ca^{2+} in bath solution in the control group (n = 44) of the PMCs (black squares) and in the presence of 10 µM of GSK 7975A in the bath solution (blue squares) (n = 41). In B, C, and D, the error bars show the standard error of the mean. Please click here to view a larger version of this figure.

A

	1	2
A	Spontan	Spontan
B	IM 10 µM	IM 10 µM
C	DNP 10 ng/mL	DNP 10 ng/mL
D	DNP 30 ng/mL	DNP 30 ng/mL
E	DNP 100 ng/mL	DNP 100 ng/mL
F	DNP 300 ng/mL	DNP 300 ng/mL
G	Cp 48/80 50 µg/mL	Cp 48/80 50 µg/mL

B

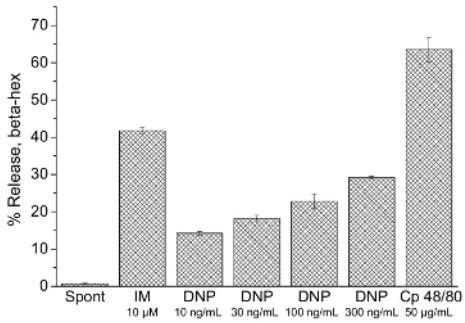

Figure 3: Beta-hexosaminidase release colorimetric multi-well measurements performed with PMCs isolated from wild type mice. (**A**) Stimulation scheme of 96-well plate pipetting for performing the beta-hexosaminidase assay; results are presented in panel B. "Spontan" corresponds to pipetting of Tyrode's Solution without the addition of any secretagogues; IM = Ionomycin; Cp 48/80 = Compound 48/80. (**B**) Bar graph illustrating percentage of ß-hexosaminidase release under basal conditions (Spont), after stimulation with Ionomycin (IM), dinitrophenol-human serum albumin (DNP) and Compound 48/80 (Cp 48/80) with indicated concentrations. The assay was performed in duplicate; error bars show the standard error of the mean. Please click here to view a larger version of this figure.

Discussion

MC models can be successfully used to study MC degranulation, chemotaxis, adhesion, as well as to elucidate intracellular signaling transduction pathways involved in MC activation. Some researchers use human MCs models, such as immortalized lines (LAD2 and HMC1) or human MCs derived from cord blood progenitor cells (CD133+) and peripheral blood progenitor cells (CD34+). Others prefer rodent MC models, such as immortalized (rat basophilic leukemia RBL-2H3 MC line) or primary cultured (mouse bone-marrow-derived MCs BMMCs, PMCs) models. Murine primary MCs can be used to analyze MC function in numerous "knock-out" mouse models, which are the gold standard approach to the exploration of physiological functions of particular genes and encoded proteins. The deficit of pharmacological tools for sufficient selectivity and potency makes this method particularly valuable for the investigation of mechanisms of signal transduction by MC activation[18]. Murine PMCs are of the connective tissue phenotype that exhibits a mature morphology and high granularity. They do not only respond well to antigen crosslinking of FcεRI like BMMCs, but are also activated by agonists of several additional receptors such as endothelin 1 or compound 48. However, the yield of the PMCs is typically significantly lower as compared to that of BMMCs and is usually not sufficient to perform a Western blot or another technique that requires a large number of cells. Several techniques have been described for the isolation and purification of PMCs. For example, Percoll gradient centrifugation was reported to yield a high purity of the cells[19]; however, the number of cells obtained with this technique is typically relatively low. In the protocol described here, the most critical step is the aspiration of the cell suspension from the peritoneal cavity. Avoiding blood contamination of the sample and aspirating the maximal amount of medium possible are essential to obtain a high number of PMCs of high purity. With some mouse strains, a maximal PMC number is only obtained by a shortened (11–12 days) culturing period. Leaving these cells in the culture conditions for a longer time leads only to a reduction of the cell number.

In the present study, we describe a simple protocol suitable for the isolation of mature MCs that makes it possible to obtain a highly pure MC population from the mouse peritoneal cavity. The obtained cells are characterized by a high homogeneity and exhibit typical MC features, such as the expression of specific marker receptors (KIT and FcεRI). These cells clearly exhibit the MC characteristic to degranulate and increase their $[Ca^{2+}]_i$ in response to FcεRI and MRGPR activation.

MC signaling is tightly related to the $[Ca^{2+}]_i$ level determined by Ca^{2+} release from intracellular stores and Ca^{2+} entry via plasma membrane channels. $[Ca^{2+}]_i$ elevation activates a complex of intracellular signaling pathways that trigger MC degranulation. As in other non-excitable cells, the main Ca^{2+} influx pathway in MCs is the SOCE activated by the depletion of the Ca^{2+}-stores. Identification of the channels involved in this signaling pathway is crucial for understanding the regulation of MC function. For the identification of the molecular constituents of the SOCE pathway, the microfluorometric technique utilizing fluorescent Ca^{2+} indicators and the "patch-clamp" electrophysiological approach have played a prominent role. However, elevation of $[Ca^{2+}]_i$ level is the sum of Ca^{2+} release and SOCE. To discriminate these two phases of Ca^{2+} mobilization, the so-called "Ca^{2+} re-addition" protocol (for review see Bird *et al.* 2008[20]) was previously developed. In this protocol, the external salt solution is switched from one containing normal $[Ca^{2+}]_o$ (1–2 mM) to a nominally Ca^{2+}-free solution. Under these conditions the cells are treated with thapsigargin to empty Ca^{2+} stores and thus fully activate SOCE. In the absence of extracellular Ca^{2+}, thapsigargin treatment evokes the transient $[Ca^{2+}]_i$ elevation, reflecting a release of Ca^{2+} ions from intracellular Ca^{2+} stores. The re-addition of extracellular Ca^{2+} leads to an increase of $[Ca^{2+}]_i$ representing the SOCE. In this article, we present the successful utilization of this approach for the characterization of SOCE in MCs. To monitor $[Ca^{2+}]_i$ changes, Fura-2 was used as a calcium indicator. In its acetoxymethyl form, Fura-2 can easily be loaded in PMCs. The use of this ratiometric dye allows the comparison of not only amplitudes of $[Ca^{2+}]_i$ elevations, but also differences in basal $[Ca^{2+}]_i$ levels, which is particularly important for analyzing wild type/knockout cells. Compared with genetically encoded fluorescent dyes, one of the main disadvantages of the Fura-2 is its high accumulation in different cellular organelles and thereby contamination of the cytoplasmic fluorescence.

The Fura-2 imaging requires a dual excitation technique. Typically, it is technically much easier to use than the dual emission technique that requires not only the usage of a triggered light source but additionally the involvement of a filter wheel or an image splitter in the emission light path. The described protocol can be used for Ca^{2+} imaging of other non-excitable cells.

A key feature of MCs is their high content of the electron-dense secretory granules, which are filled with large amounts of mediators and immunomodulatory substances. MC activation leads to a rapid release of preformed granule compounds. Several approaches to analyze MC degranulation *in vitro* are available, including the detection of radiolabeled serotonin, histamine detection using antibodies (ELISA), and

the detection of an increase of plasma membrane surface using electrophysiological "patch-clamp" cell capacitance measurements. In the present paper, we describe a practical application of the assay using multi-well colorimetric detection of *in vitro* released β-hexosaminidase. This assay is based on the ability of β-hexosaminidase to hydrolyze terminal *N*-acetyl-D-hexosamine residues in *N*-acetyl-β-D-hexosaminides[21]. β-hexosaminidase is co-released with histamine but also with other mediators such as Cathepsin D or TNF and can thus be used as a correlate for degranulation from multiple types of secretory vesicles in MCs[12,22].

In the described technique, PMCs were stimulated at 37 °C with different secretagogues, followed by an estimation of supernatants as well as pellets of β-hexosaminidase contents by their reactions with β-hexosaminidase substrate. As the substrate, 4-Nitrophenyl N-acetyl-β-D-glucosaminide was used. It was hydrolyzed to N-acetyl-D-glucosamine and *p*-nitrophenol. The amount of *p*-nitrophenol was colorimetrically quantified by the light absorbance at 405 nm. In some modifications of the ß-hexosaminidase release assay, 4-methylumbelliferil-N-acetyl-*beta*-glucosamine is used as a substrate. After enzymatic hydrolysis, the substance generates a fluorometrically detectable compound. We expressed the extent of degranulation as a percentage of the release, normalized to the total content. This allowed us to avoid the variability introduced by differing cell numbers and their granular content between various cell preparations. We did not subtract a spontaneous release (observed in the absence of MCs secretagogues) from net stimulated degranulation, since a spontaneous release was separately reported due to its importance as an indicator of MC viability. To avoid high variability in the assay results, it is important to separate very carefully the supernatants and pellets after centrifugation of the stimulated cells. It is also essential to avoid the formation of any bubbles in the multi-well plates prior to performing the colorimetric measurements. A significant limitation of the described method is that the result is represented not as an absolute value, but as a percentage of the released mediator.

In contrast to methods of direct detection of released serotonin or histamine, the reported assay is much more cost-effective and requires no specialized equipment. In addition, the described technique is highly reproducible, does not need to be performed in a radioactive laboratory, and does not require the purchase of expensive antibodies. Therefore, it could be an excellent alternative to more costly and difficult techniques.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors would like to acknowledge Julia Geminn for technical assistance in mast cell preparation and culturing. This work was supported by The Transregional Collaborative Research Centre (TR-SFB)152.

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