

Protocol

# Isolation and Purification of Mast Cells from Murine Colonic Mucosa

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## Abstract

Mast cells (MCs) are immune cells that produce numerous immunological mediators involved in inflammatory and allergic responses. Increased numbers of MCs are observed in chronic inflammatory reactions in organs such as the colon. There, MCs seem to participate in deleterious immune responses and tissue damage, but the detailed mechanisms of their activation are not known, mostly because procedures to obtain MC primary cultures from the colonic mucosa are expensive and time-consuming and present low yield. Here we describe a protocol to obtain MCs from the colonic mucosa (cmMCs) of C57BL/6 mice with high yield, viability and purity. Mucosal colon cells were dispersed by enzymatic digestion, and cmMCs were isolated by Percoll continuous-gradient centrifugation. This method allowed for the purification of  $1,446,667 \pm 112,442$  cell/g of mucosal tissue, with 87.22% viability and 95.16% purity. The mucosal-like phenotype was predominant in isolated cmMCs, characterized by weak toluidine blue staining but strong expression of MC protease-1 (*Mcpt1*). Activation assays showed that freshly isolated cmMCs increased intracellular calcium and showed degranulation in response to ATP or IgE-antigen-dependent FcεRI cross-linking. This highly reproducible technique is cost-effective and requires no specialized equipment. This protocol could be applied in research related to inflammatory bowel disease, colon cancer or food allergies.

**Keywords:** colonic mucosal mast cells; primary cell culture; *Mcpt1* protease gen



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## 1. Introduction

Mast cells (MCs) are long-lived, granular immune cells found in all vascularized tissues of the body, typically residing near blood vessels, nerves, smooth muscle cells, epithelial cells, mucus-producing glands, and hair follicles [1]. Their origin is still under extensive analysis, and data indicate that in adult mice, they are differentiated from a

hematopoietic stem cell in the bone marrow but also can be originated from an embryonic erythroid–myeloid progenitor, giving rise to an MC precursor that circulates in small quantities in the blood [2,3]. MC precursors migrate to tissues where they complete their maturation and acquire tissue-specific characteristics under the influence of locally produced mediators [4].

MCs are abundant in anatomical regions exposed to the environment, including skin, airways, and the gastrointestinal tract [1]. Depending on their anatomical location, they are highly specialized and differ significantly in the expression of receptors, key granular compounds, and enzymes [5]. Historically, mouse MCs have been classified according to their tissue location, receptor expression and the presence of specific proteases in their granules. For example, granules of connective tissue MCs (CTMCs) contain the mMCP-4 chymase, the mMCP-5 elastase, the mMCP-6 and mMCP-7 tryptases, the carboxypeptidase A3 metallopeptidase, heparin and large amounts of histamine. CTMCs are mainly located in the skin, the peritoneal cavity and the submucosa of the intestine [5]. On the other hand, the mucosal or mucous tissue-type MCs (MMCs) possess granules storing the mMCP-1 and -2 chymases, chondroitin sulfate E and low amounts of histamine. MMCs are present in the lungs, the nasal mucosa and the lamina propria close to the epithelium of the intestine [6–8].

The involvement of MCs in the pathogenesis of inflammatory bowel disease (IBD) and food allergies has been demonstrated [9,10]. IBD includes ulcerative colitis and Crohn’s disease, which are characterized by chronic immune dysregulation, recurrent gastrointestinal inflammation and fibrosis, with the colon being one of the most frequently affected tissues [11]. The gastrointestinal phenotype of food allergy, which is very common in children, is normally manifested by colon inflammation [12]. Although these diseases represent a global public health challenge [13,14], the knowledge about the role of MCs in the inflammation of the colon is scarce because the isolation of colonic mucosal MCs (cmMCs) is not an easy task. MC purification protocols from the intestinal or gastric mucosa are scarce and mainly optimized in samples from rats and humans (Table 1), in contrast to the wide variety of protocols for isolating and studying CTMCs or MMCs from other tissues [15–18]. As shown in Table 1, most of the reported protocols for purifying intestinal or colonic MCs that involve enzymatic digestion and Percoll gradient separation result in low yield and MC purity. Then, from the obtained cell suspension, more advanced and costly methodologies are employed to maximize yield and ensure high-purity MC cultures, such as fluorescence-activated cell sorting (FACS) or magnetic bead separation. To simplify the process, some MC lines are useful for certain *in vitro* studies, although they have limitations, such as immaturity in certain physiological aspects, their dependence on growth conditions that fail to replicate the *in vivo* tissue environment, long cell duplication rates, and the need for careful handling during culture to avoid stress [19–21].

**Table 1.** Available techniques for mucosal MC isolation from intestinal mucosa. Only studies in which MC yield, purity or viability were reported are shown.

| Species | Tissue/Source                                                                | Technique of Isolation                                                                                             | Yield, Purity and Viability/Functionality/Limitations                                                                                                                                                                                    | Functionality                                                                       | Limitations                                                                                         | Reference |
|---------|------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|-----------|
| Rat     | Small-intestine mucosa (after <i>Nippostrongylus brasiliensis</i> infection) | Enzymatic digestion                                                                                                | Yield: $70 \times 10^6$ viable cells/small-intestine; MC purity: 11% of the non-epithelial cells.                                                                                                                                        | Histamine secretion in response to anti-IgE and parasite antigen.                   | Requires parasite-infected animals to increase the numbers of MCs in intestinal mucosa.             | [22]      |
| Rat     | Small-intestine mucosa (after <i>Nippostrongylus brasiliensis</i> infection) | Enzymatic digestion, Percoll gradient and sedimentation by unit gravity velocity sedimentation apparatus           | Yield: $1\text{--}2 \times 10^6$ MCs/small-intestine; MC purity: >95%.                                                                                                                                                                   | Histamine secretion in response to parasite antigen.                                | Requires parasite-infected animals to increase MC numbers in intestinal mucosa.                     | [23]      |
| Human   | Colonic mucosa and submucosa                                                 | Mechanical dispersion + enzymatic digestion (collagenase) to obtain cell suspension; subsequent short-term culture | Approximate yield of $4.5 \times 10^5$ MCs per g of tissue; maximum purity of approximately 3.1%. Viability: NA.                                                                                                                         | Histamine secretion and eicosanoid production in response to anti-IgE.              | Low initial purity; requires additional purification steps; relatively low yield.                   | [24]      |
| Human   | Mucosa and submucosa from colon and stomach                                  | Enzymatic digestion of tissue followed by magnetic selection with anti-c-kit (CD117) antibody bound to Dynabeads™  | Before magnetic selection: Yield: $1.4 \times 10^5$ cells/g tissue. MC purity: 16%.<br>After magnetic selection: yield: $0.5\text{--}1.3 \times 10^5$ cells/g of tissue; 90–92% MC purity; 88–89% viability.                             | Histamine secretion in response to anti-IgE, concanavalin A, and calcium ionophore. | High cost. Requires specific antibodies and magnetic separation.                                    | [25]      |
| Human   | Colonic mucosa                                                               | Enzymatic digestion and cell enrichment by MACS™; cultured with SCF and IL-4 to enhance viability and purity       | Before MACS™: Yield: $1\text{--}2.5 \times 10^7$ cells/g mucosal tissue. MC purity: 1–4%.<br>After MACS™: Yield: $0.5\text{--}3 \times 10^6$ MMCs/5 to 10 g of mucosa. MC purity: 85–95% after 1-wk culture; 95–100% after 2-wk culture. | Histamine secretion and eicosanoid production after FcεRI cross-linking.            | Expensive; requires specialized antibodies and equipment. IL-4 incubation changes the MC phenotype. | [26,27]   |

MACS, magnetic cell sorting; wk, week; NA: not available; IL-4, Interleukin 4; SCF, stem cell factor; IgE, immunoglobulin E; FcεRI, high-affinity receptor for IgE.

Here we present the development of a protocol that was optimized to obtain a primary culture of murine MCs isolated from the mucosal layer of the colon dissected by microsurgery. Tissue was enzymatically digested, and MCs were isolated by a Percoll gradient. Our procedure improves the yield of MCs with high purity and good viability per gram of mucosa, exceeding previously reported values in human samples and avoiding expensive methodologies [25–27]. The obtained cmMC suspension was short-term-treated with cromoglycate to stabilize their membrane, and then cells were ready for functional assays. The protocol can be applied to the characterization of cmMC activation under distinct conditions, as well as to preclinical studies aimed at elucidating immunological processes in the colon, such as those involved in inflammatory bowel disease, colorectal cancer, food allergy, interactions with purinergic or microbial mediators, and analyses of the impact of microbiota and diet on MC function.

## 2. Materials and Methods

### 2.1. Overall Study Design

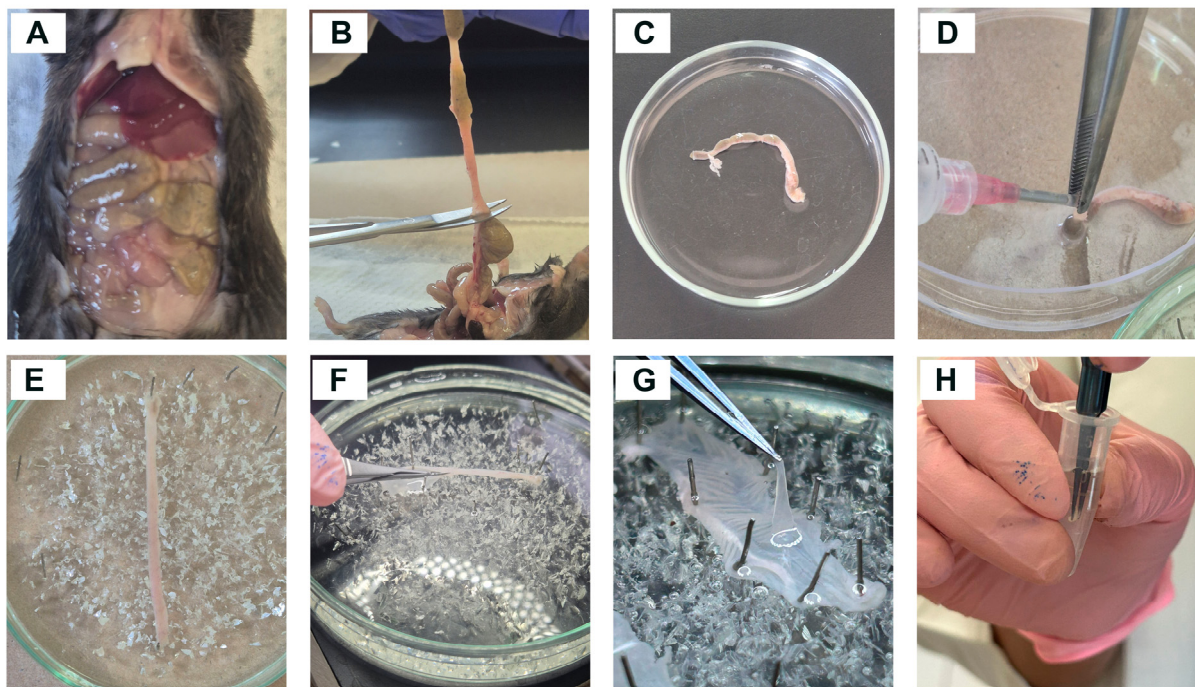
The following protocol was designed to obtain MCs specifically from the mucosal layer of the mouse colon. The objective was to establish methodological conditions for obtaining cmMCs to potentially perform *ex vivo* assays on freshly isolated cells from non-treated mice or murine preclinical models of colonic inflammation while preserving cell viability and functionality. This study focused on the colon, which is a tissue organized in well-defined layers that allows for the dissection and collection of the mucosal layer, separating it from the connective tissue and reducing contamination with other cells. Few protocols exist for obtaining cmMCs and, to our knowledge, none in mice. Furthermore, in our protocol, the collagenase action times were optimized to prevent cell activation or damage, and cell viability was verified at this step by trypan blue exclusion. The Percoll gradient was also optimized, increasing the purity and viability of MCs. To validate reproducibility, the protocol was performed in multiple replicates, obtaining colon samples from male and female C57BL/6 mice. Cell phenotyping was assessed by morphological evaluation, toluidine blue staining and the detection of the specific MMC marker *Mcpt1* by single-cell PCR. Receptor functionality was verified using ATP or IgE plus specific antigen (allergen) as stimuli, and cell activation was assessed by measuring changes in intracellular calcium with the fluorescence reporter calcein or cell degranulation using the Ruthenium Red (RR) dye, respectively. The workflow integrates microsurgical dissection of the colonic mucosal layer, cellular dispersion by collagenase digestion, purification of MCs using a continuous Percoll gradient and cell membrane stabilization by cromoglycate treatment. Then, the phenotypic profiling of the isolated cmMCs was performed, followed by short-term functional assays.

### 2.2. Tissue Sample Collection

The first protocols of MC purification were performed simultaneously in male and female mice to analyze differences in yield between sexes. For functional assays, cmMCs were obtained from female C57BL/6 mice. Male ( $n = 9$ ) and female ( $n = 21$ ) C57BL/6 mice (17–24 g) were maintained in a specialized animal facility, at an ambient temperature of 23–25 °C under 12 h light/12 h dark cycles, with food (Nutricubo Purina, St. Louis, MO, USA) and drinking water provided *ad libitum*. Animal protocols followed the guidelines from the Mexican norm (NOM-062-Z00-1999) and were approved by the Ethics Committee Concerning Animals in Teaching and Research at the Autonomous University of Aguascalientes (CEADI-UAA 5/03/2018).

The procedure to collect colonic mucosa was the following:

- (1) Euthanize the mouse using overdoses of inhaled Isoflurane (SOFLORAN VET, PiSA agropecuaria, Mexico City, Mexico) administered via chamber, and confirm the death by loss of vital signs.
- (2) Atomize the mouse with 70% ethanol on the abdomen to clean the area, and fix it on a dissection bed using pins in a dorsal decubitus position.
- (3) Open the abdominal region using sterile scissors, taking care not to damage the organs.
- (4) Locate the colon, and dissect from the cecum to the distal colon, using sterile scissors.
- (5) Place the colon in pre-cooled 0.9% saline solution, and wash with a 3 mL syringe containing saline solution to remove all feces.
- (6) Place the clean colon in a Sylgard-coated Petri dish containing pre-cooled saline solution, stretch one end of the colon with stainless steel pins, incise it longitudinally, and secure it with additional pins. Use a stereoscopic microscope (Meiji Techno Co., Ltd., Tokyo, Japan) to visualize the layers of the colon.
- (7) Remove the mucosal layer from the submucosa using sterile microsurgical scissors and forceps, and place it in a 1.5 mL microcentrifuge tube containing 1 mL of Locke buffer (140 mM NaCl, 5 mM KCl, 25 mM NaHCO<sub>3</sub>, 5 mM HEPES and 11.1 mM glucose, pH 7.4). Cut the mucosal layer into small pieces with sterile scissors (see Figure 1).



**Figure 1.** Representative photographs of the procedure to dissect the mouse colon and to obtain the mucosal layer. (A) Exposition of the mouse peritoneum. (B) Dissection of the whole colon. (C) Removed colon in a Petri dish with saline solution. (D) Colon lavage to remove fecal matter. (E) The mouse colon on a Sylgard gel matrix secured with pins. (F) Longitudinal incision of the colon. (G) Separation of the mucosa from the submucosa/muscular layer. (H) Collection of the mucosa in a microcentrifuge tube.

### 2.3. Isolation and Purification of MCs from Colonic Mucosa

The following procedure was carried out under sterile conditions within a laminar flow cabinet:

- (1) Transfer the mucosal layer to a 15 mL conical centrifuge tube (polypropylene, RNase-/DNase-free nonpyrogenic) containing 10 mL of Locke buffer + 1 mg/mL of N-acetyl-L-cysteine (Sigma-Aldrich, St. Louis, MO, USA; A7250) + 2 mM EDTA (pre-warmed

at 37 °C), and incubate for 10 min in a shaking incubator at 250 rpm and 37 °C. After incubation, shake the tube gently to loosen the mucus.

- (2) Remove the solution using a micropipette, and transfer the mucosa into a 15 mL conical centrifuge tube containing 10 mL of Locke buffer + 5% FBS (fetal bovine serum) + 5 mM EDTA (pre-warmed at 37 °C). For this procedure, decant a small amount of the pre-warmed solution onto the tube containing the mucosal tissue, and then allow it to flow over the remaining pre-warmed solution in the new tube. Incubate for 20 min in a shaking incubator at 250 rpm at 37 °C to remove epithelial cells.
- (3) Remove the solution using a micropipette, and transfer the mucosal tissue into a 15 mL conical centrifuge tube containing 10 mL of Locke buffer supplemented with 1 mg/mL gelatin (BBL 003680), 1.23 mM MgCl<sub>2</sub>, 10 µL of DNase, and 0.5 mg/mL Collagenase D (Roche 11088866001) (pre-warmed at 37 °C). Repeat the above procedure to transfer the mucosal tissue from one tube to another. Incubate for 15 min in a shaking incubator at 250 rpm at 37 °C.

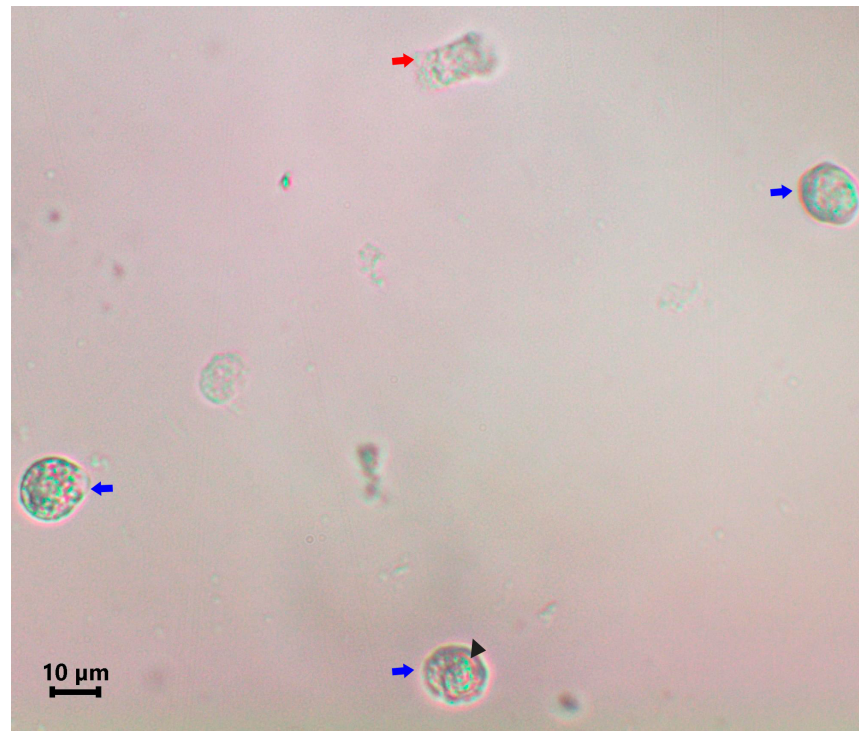
Note: This step is critical. Changes in type and concentration of Collagenase and incubation time can affect cellular viability.

- (4) Disrupt the mucosal tissue using a micropipette for 5 min.
- (5) Centrifuge at  $1293 \times g$  at 4 °C for 5 min, remove the supernatant, and resuspend the mucosal tissue in 3 mL of RPMI medium supplemented with 10% FBS.
- (6) Filter the mucosal tissue through a 70 µm nylon mesh, wash with 7 mL of supplemented RPMI, and centrifuge at  $1293 \times g$  at 4 °C for 5 min. Remove the supernatant with the help of a micropipette, and resuspend the cells in 1 mL of supplemented RPMI.
- (7) Resuspend slowly the cells suspension using the micropipette, and lay on a 20% continuous gradient of Percoll (Cytiva 17089102). Centrifuge at  $1861 \times g$  at 4 °C for 20 min using a free-angle rotor.

Note: Both the type and percentage of the Percoll gradient influence performance and cell viability. The present protocol was established by modifying previously described procedures for the purification of MCs from human colon and rat small-intestine samples [23,27]. In this study, we previously evaluated an 80%/30% Percoll discontinuous gradient and a 35% Percoll continuous gradient that resulted in a yield of 775,000 cells/mouse, 64.5% viability, and 85% MC purity and a yield of 1,000,000 cells/mouse, 68.7% viability and 80% MC purity, respectively, considered suboptimal values.

- (8) Remove the supernatant, resuspend the cell pellet in 12 mL of supplemented RPMI medium, and centrifuge at  $1850 \times g$  at 4 °C for 7 min to wash the cells. Repeat the process to perform two washes.
- (9) Remove the supernatant, and resuspend the cells in 1 mL of supplemented RPMI. Count the number of cells using the Trypan Blue technique in a Neubauer chamber in the four external quadrants, and calculate viability (by counting the number of live and dead MCs) and purity (by counting MCs and epithelial cells).

Note: Isolated cells are identified by their typical morphology under light microscopy (Figure 2). cmMCs are small, with diameters ranging from 5 to 10 µm, and typically exhibit pleomorphic morphology, appearing round or oval. They possess a characteristic unilobed nucleus, which may be centrally or eccentrically located, and their cytoplasm contains numerous secretory granules of variable size. In some cells, the granules are so densely packed that they obscure the nucleus. In contrast, epithelial cells, the sole contaminant cells, are typically columnar, elongated in shape, and measure approximately 20 µm in length.



**Figure 2.** Representative image of cells purified from the colonic mucosa of a C57BL/6J mouse, observed under light microscope at 400× magnification. The cmMCs display a round morphology with a diameter of approximately 10  $\mu\text{m}$  (blue arrow), a characteristic oval mononucleus (arrowhead), and cytoplasm packed with numerous small secretory granules. Epithelial cells (red arrow) are distinguished by their pyramidal shape and a height of about 20  $\mu\text{m}$ .

- (10) To stabilize the cell membranes, incubate  $1\text{--}2 \times 10^6$  cmMCs in 1 mL of supplemented RPMI + 50  $\mu\text{M}$  sodium cromoglycate for 1 h at 4  $^{\circ}\text{C}$ .

#### 2.4. Toluidine Blue Staining

To establish a reference control for toluidine blue-induced metachromasia, peritoneal MCs (pMCs) were isolated and purified from C57BL/6 mice as previously described with slight modifications [28]. Briefly, peritoneal lavage was recovered using supplemented RPMI, and MCs were separated from other peritoneal cells by a 72.5% Percoll continuous gradient, yielding approximately  $3.7 \times 10^5$  pMCs/mouse.

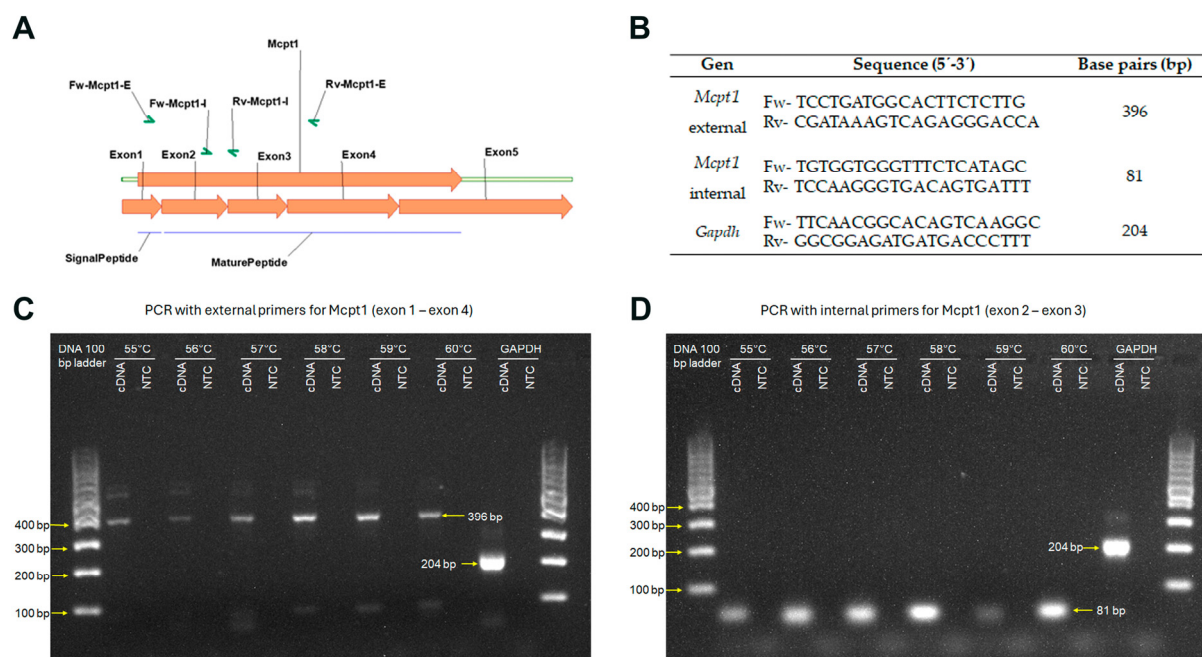
Once cmMCs and pMCs were isolated and purified, the following protocol was implemented:

- (1) Resuspend the cells in 1 mL of supplemented RPMI medium.
- (2) Place an aliquot of 30  $\mu\text{L}$  of the cell suspension onto a glass slide, spread gently using the tip of a micropipette, and allow to air-dry at room temperature.
- (3) Fix the slides with Carnoy's solution (25 mL of glacial acetic acid and 75 mL of absolute ethanol) for 1 h, rinse under running tap water for 5 min, and then wash with distilled water for an additional 5 min.
- (4) Immerse the slides containing cmMCs and pMCs in toluidine blue O solution (0.099 M anhydrous citric acid and 7.39 mM toluidine blue O (SIGMA T-3260, Merck KGaA, Darmstadt, Germany) prepared in 100 mL of 50% ethanol, pH 1) for 24 h and 1 h, respectively.
- (5) Transfer the slides to a destaining solution (0.099 M anhydrous citric acid in 100 mL of 50% ethanol) for 1 min, rinse with running tap water for 5 min, and wash with distilled water for 1 min.

- (6) Allow the slides to dry, and mount the coverslips using a 1:1 PBS/glycerol solution.
- (7) Examine the MC morphology, stain by light microscopy at 40 $\times$ , and take pictures (Motic BA310 microscope (Motic Deutschland GmbH, Wetzlar, Germany) equipped with an OptikamB 10 digital camera (Optika S.R.L., Ponteranica, Italy)).

### 2.5. Nested PCR Standardization to Determine the Expression of Mouse *Mcpt1* by Single-Cell RT-PCR

The *Mcpt1* gene is specifically expressed in MMCs and serves as a reliable phenotypic marker [29]. Outer and inner primers for *Mcpt1* were designed and screened using the software Vector NTI Advance 10 (Invitrogen, Waltham, MA, USA). For primer design, the sequences from the GenBank of mMCP-1 and Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, accession numbers: NM\_008570 and NM\_001289726, respectively) were used (Figure 3). Total RNA was extracted from the isolated and purified cmMCs using the PureLink™ RNA Mini Kit (Invitrogen). Total RNA concentration was measured with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) at 260 nm, and quality was assessed with the relation 260 nm/280 nm > 1.8; then total RNA was used for cDNA synthesis through reverse transcription using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Waltham, MA, USA), following the manufacturer's protocol. cDNA was used to standardize the optimal alignment temperature through endpoint PCR: 1  $\mu$ L of cDNA (30 ng) was added per 10  $\mu$ L of reaction volume containing 5  $\mu$ L of PCR Master mix 2X (Thermo Scientific), along with 0.5  $\mu$ M of each forward and reverse primers, for both outer and inner primer pairs for *Mcpt1* (Figure 3B). The annealing temperature was optimized by testing hybridization temperatures of 55, 56, 57, 58, 59 and 60  $^{\circ}$ C, with 60  $^{\circ}$ C being the most optimal.



**Figure 3.** Primers used in nested PCR. (A) Primers were designed to align with different exons. Outer primers (Fw-Mcpt1-E and Rv-Mcpt1-E) were used to amplify exons 1 and 3 to generate an amplicon of 396 bp (from nucleotide 52 to 447), while inner primers (Fw-Mcpt1-I and Rv-Mcpt1-I) were used to amplify exons 2 and 3 to amplify a segment from position 183 to 263 (81 bp). (B) Table with sequences of internal and external *Mcpt1* oligos used to determine the  $T_m$  and corresponding base pairs. Electrophoresis for the detection of the amplification products of the (C) external and (D) internal primers. NTC, no-template control.

Amplicons were separated using horizontal electrophoresis in a gel of agarose 1.5% with GelRed Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) in TBE buffer (89 mM Tris-borate (pH~8.3), 89 mM boric acid, and 2 mM EDTA) to analyze and identify the PCR fragments size in a MiniBis Pro documentation system (DNR Bio-Imaging Systems, Jerusalem, Israel) (Figure 3C).

To determine the single-cell expression of the *mMcp1* protease gene, follow this procedure:

- (1) Following cmMC incubation with sodium cromoglycate, dispense 80  $\mu$ L of the cell suspension (containing  $8 \times 10^4$  cells) into each well of a 24-well plate. Each well contains a circular coverslip at the bottom, which has been previously coated with sterile 1% poly-L-lysine (Sigma-Aldrich). Incubate the cells for 2 h at 37 °C.
- (2) Place the coverslip with cells in a Patch clamp recording chamber perfused with extracellular solution composed of 150 mM NaCl, 2.8 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, and 6 mM CaCl<sub>2</sub>, at pH 7.2.
- (3) Draw single MCs under visual guidance using an inverted microscope with a 10 $\times$  objective (TMS, Nikon Corporation, Tokyo, Japan), and aspirate into a glass micropipette (tip diameter: 25–100  $\mu$ m) by applying gentle negative pressure. Fabricate micropipettes using a micropipette puller (model PB-7, Narishige Co., Ltd., Tokyo, Japan), and subsequently polish them with a microforge (MF-830, Narishige Co., Ltd., Tokyo, Japan).
- (4) Perform cDNA synthesis directly from single MCs by using the Maxima First Strand cDNA synthesis kit (Cat. No. K1641; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Collect individual MCs into a glass micropipette containing 6  $\mu$ L of reaction mix consisting of 1.2  $\mu$ L of 5 $\times$  reaction mix, 0.6  $\mu$ L of Maxima enzyme mix, and 0.1% *v/v* Triton X-100 (Sigma-Aldrich) with nuclease-free H<sub>2</sub>O added to the final volume. Triton X-100 is used to solubilize the cell membrane.
- (5) Expel the content in the glass micropipette into a PCR tube containing 14  $\mu$ L of the remaining reaction mix (2.8  $\mu$ L of 5 $\times$  reaction mix, 1.4  $\mu$ L of Maxima enzyme mix, 0.1% Triton X-100, and nuclease-free H<sub>2</sub>O), and incubate the reaction for 10 min at 25 °C followed by 15 min at 50 °C. Then, inactivate the reaction by heating to 85 °C for 5 min, and then place tubes on ice.
- (6) Following reverse transcription, perform nested PCR using recombinant Taq DNA polymerase (Invitrogen) and the primers listed in Figure 3B. Use *Gapdh* mRNA expression as an internal expression control. Include as negative controls samples containing fluid collected from the vicinity of the cells, as well as a no-template control.
- (7) The first round of PCR reaction contains 5  $\mu$ L of cDNA, 0.2  $\mu$ M specific outer primers, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 0.5 U Taq DNA polymerase, and 10 $\times$  PCR buffer in a 25  $\mu$ L final volume. Perform nested PCR reactions using 2  $\mu$ L of the first PCR product as the template and the specific inner primers, with the same PCR reagent concentrations.
- (8) PCR conditions: 3 min at 94 °C; 40 amplification cycles, where each cycle includes 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 30 s of elongation at 72 °C; a final extension at 72 °C for 5 min. Visualize the PCR amplification products on 1.5% agarose electrophoresis gel stained with ethidium bromide.

## 2.6. Stimulation of Purified cmMCs by Cellular Membrane Receptor Ligands and Evaluation of Cell Activation

To confirm the functionality of freshly purified cmMCs, the following assays were performed. Cells were activated with ATP, and calcium mobility was recorded using calcein.

On the other hand, IgE-sensitized cells were stimulated by antigen, and cell activation was evaluated by the RR technique.

To activate cmMCs with ATP and analyze intracellular calcium mobility, follow this procedure:

- (1) Following cmMC incubation with sodium cromoglycate, centrifuge the cells at  $241 \times g$  for 3 min at  $4^\circ\text{C}$ , remove the supernatant, and add 1 mL of Tyrode's/BSA buffer (150 mM NaCl, 5 mM KCl, 1 mM  $\text{MgCl}_2$ , 5.6 mM glucose, 20 mM HEPES, and 20% bovine serum albumin, pH = 7.4) and  $1\ \mu\text{g/mL}$  calcein (Sigma-Aldrich C1359) as a fluorescent marker. Incubate for 1 h at  $37^\circ\text{C}$  in an incubator (Prendo INC-40).

Note: Tyrode's/BSA buffer + calcein must be previously warmed to  $37^\circ\text{C}$  for 1 h.

- (2) Centrifuge the cells at  $241 \times g$  for 3 min at  $4^\circ\text{C}$ .
- (3) Remove the supernatant, resuspend the cell pellet in 1 mL of Tyrode's/BSA buffer pre-equilibrated at  $37^\circ\text{C}$ , and distribute equally into 3 tubes: one without stimulus (basal condition), one with the A23187 ionophore at 100 nM (Sigma-Aldrich C7522) (positive control), and one with ATP (100  $\mu\text{M}$ ) (Sigma-Aldrich A7699).
- (4) Take 20  $\mu\text{L}$  from each tube, and place it on a slide. Observe the cells under a fluorescence microscope (ZEISS Axioskop 40 (Carl Zeiss Light Microscopy, Göttingen, Germany) with CoolSNAP-Procf color media cybernetics camera (Roper Scientific, Inc., Tucson, AZ, USA)), and monitor the fluorescence ( $\lambda_{\text{ex}}$ : 488 nm;  $\lambda_{\text{em}}$ : 518 nm) to visualize the fluorescence intensity of the cells at each stage.
- (5) Analyze 10 cells per field from each stage using ImageJ software 1.54p, and plot the fluorescence intensity using Prisma 8 software.

To sensitize cmMCs with IgE and analyze cell activation by antigen, follow this procedure:

- (1) Following cmMC incubation with sodium cromoglycate, centrifuge the cells at  $241 \times g$ , for 3 min at  $4^\circ\text{C}$ .
- (2) Incubate cells with IgE anti-DNP-BSA (Sigma-Aldrich A2831) at 100 ng/mL in supplemented RPMI for 18 h at  $4^\circ\text{C}$ .
- (3) Centrifuge the cells at  $241 \times g$ , for 3 min at  $4^\circ\text{C}$ .
- (4) Remove the supernatant, and add 1800  $\mu\text{L}$  of a 0.005% RR (Sigma-Aldrich 11103-72-3) solution, freshly diluted in phosphate-buffered saline (PBS) from a 0.05% RR stock solution. Prepare the PBS solution as follows: 0.17 M NaCl, 0.002 M KCl, 0.0006 M  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.004 M  $\text{Na}_2\text{HPO}_4$ , and 0.002 M  $\text{KH}_2\text{PO}_4$ .
- (5) Resuspend the cells, and separate 300,000 cells/tube, using 15 mL conic centrifuge tubes. For basal condition, maintain cells in RR solution without stimulus, and for antigen activation, add DNP (81 ng/mL) to the RR solution. Prepare triplicates of each condition.
- (6) Incubate for 30 min at  $37^\circ\text{C}$ .
- (7) Place the tubes on ice to stop the reaction.
- (8) Take a 20  $\mu\text{L}$  aliquot from each experimental condition, load it in a Neubauer chamber, and count activated and non-activated cells under a light microscope (Motic BA310; Motic Deutschland GmbH, Wetzlar, Germany) at  $400\times$  magnification.

Note: The cellular states observed are non-activated cells (absence of stained granules and well-defined cell boundaries) and activated cells (mixture of stained and unstained granules with preserved cell boundaries). Observers are blinded to the experimental condition of the cells being counted.

- (9) Calculate the percentages of each cellular state in each condition.

### 3. Results

#### 3.1. MC Extraction and Purification

With the described protocol, cmMCs were isolated from male and female C57BL/6 mice. The viability, purity, and yield of MCs are shown in Table 2, and no significant sex-related differences were observed in yield and viability. MC purity in the obtained cell suspension was significantly higher in female mice compared with male mice. Integrated data from animals of both sexes showed a yield of  $1,446,667 \pm 112,442$  cell/g of mucosal tissue, with an average viability of  $87.22 \pm 0.76\%$  and a purity of  $95.16 \pm 0.68\%$ .

**Table 2.** Viability, purity and yield of isolated cmMCs from female and male mice.

|         | Viability            | Purity                 | Yield                       |
|---------|----------------------|------------------------|-----------------------------|
| Females | $87.35 \pm 1.029\%$  | $96.11 \pm 0.6421\% *$ | $1,383,333 \pm 133,690$ MCs |
| Males   | $86.92 \pm 0.9282\%$ | $92.95 \pm 1.538\%$    | $1,594,444 \pm 211,221$ MCs |

Data are presented as means  $\pm$  standard error of the mean (SEM), with  $n = 9$  male and 21 female C57BL/6 mice. Group comparisons were statistically determined using two-tailed unpaired Student's *t*-test. \*  $p < 0.05$  versus males.

To confirm protocol reliability under colon inflammation conditions, MCs were also isolated from colonic mucosa of IBD-induced C57BL/6 mice [30]. Values of yield, purity, and viability were  $1,650,000 \pm 93,031$  MCs/g of mucosal tissue,  $95.36 \pm 0.61\%$  and  $88.48 \pm 0.76\%$ ;  $n = 21$  female mice. Using a two-tailed unpaired Student's *t*-test, no significant differences were observed in the three key variables of the MC isolation protocol from colonic mucosa between healthy and IBD mice.

#### 3.2. Toluidine Blue Staining of cmMCs

Toluidine blue staining enabled the confirmation of cmMC morphology from C57BL/6 mice, and their classification based on the proteoglycan type content. The MCs isolated from the mouse colonic mucosa stained light blue, indicating the presence of chondroitin sulfate in their granules. They exhibited a round morphology with an approximate diameter of  $10 \mu\text{m}$  and a characteristic centric or eccentric mononucleus (Figure 4A). In comparison, pMCs stained rapidly and intensely blue to purple with the same toluidine blue composition (Figure 4B), reflecting the high heparin content in their granules. These cells also displayed either a centric or eccentric nucleus but mostly were larger in size, with diameters exceeding  $10 \mu\text{m}$ . It has been reported that mucosal or immature CTMCs require longer staining periods, which can range from 2 to 5 days [31]; however, no differences in cell coloration were observed when the staining time for cmMCs was increased from 1 to 5 days.

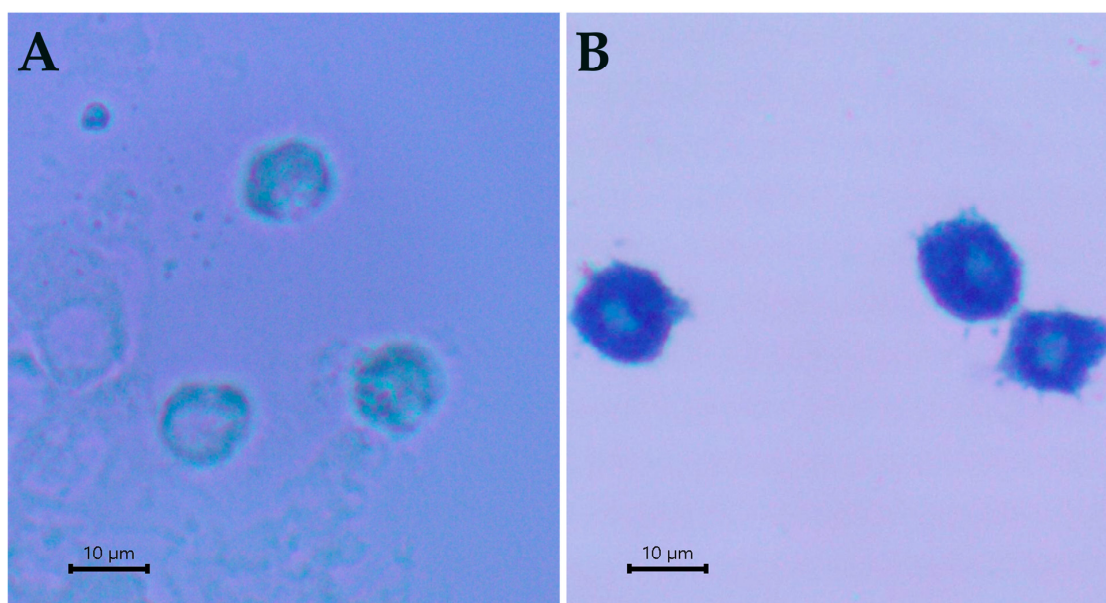
#### 3.3. Single-Cell Gene Expression Analysis of the mMCP-1 Protease in MCs from the Colonic Mucosa

*Mcpt1* mRNA was detected in 73% of *Gapdh*-positive cmMCs (19 out of 26 cells from three mice) (Figure 5), indicating that the majority of cmMCs exhibit a mucosal protease expression profile.

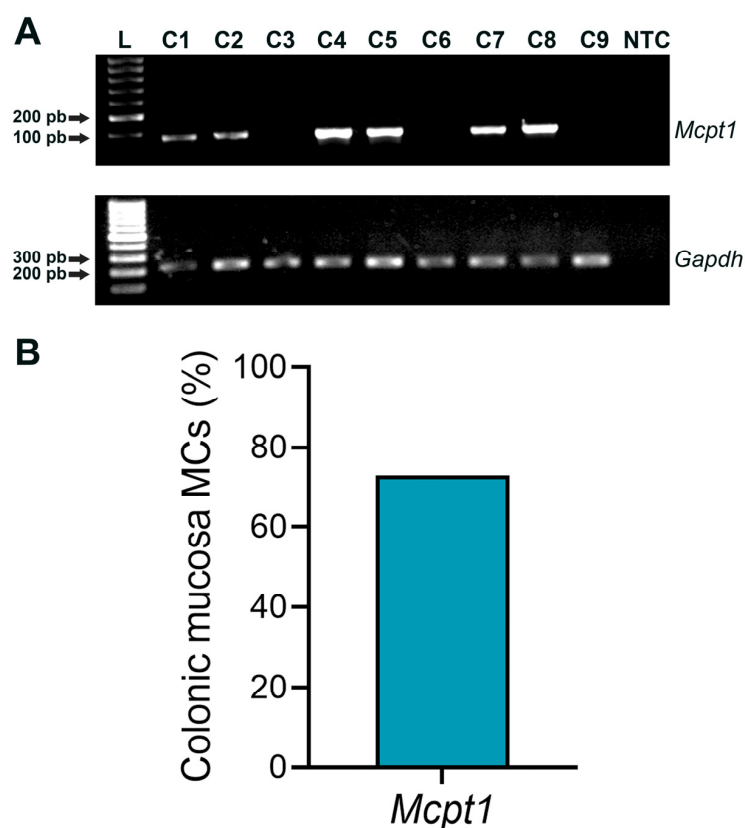
#### 3.4. Activation of cmMCs with ATP and Evaluation of Intracellular Calcium Mobility Using Calcein

The response of cmMCs to ATP ( $100 \mu\text{M}$ ) was evaluated using calcein as a fluorescent marker, evidencing the changes in  $\text{Ca}^{2+}$  intracellular dynamics associated with activation. As shown in Figure 6, under basal conditions (without stimulation), cell fluorescence was low and stable, which indicates the tight homeostatic regulation of intracellular  $\text{Ca}^{2+}$  in MCs in the absence of stimuli [32]. However, cell fluorescence was significantly increased

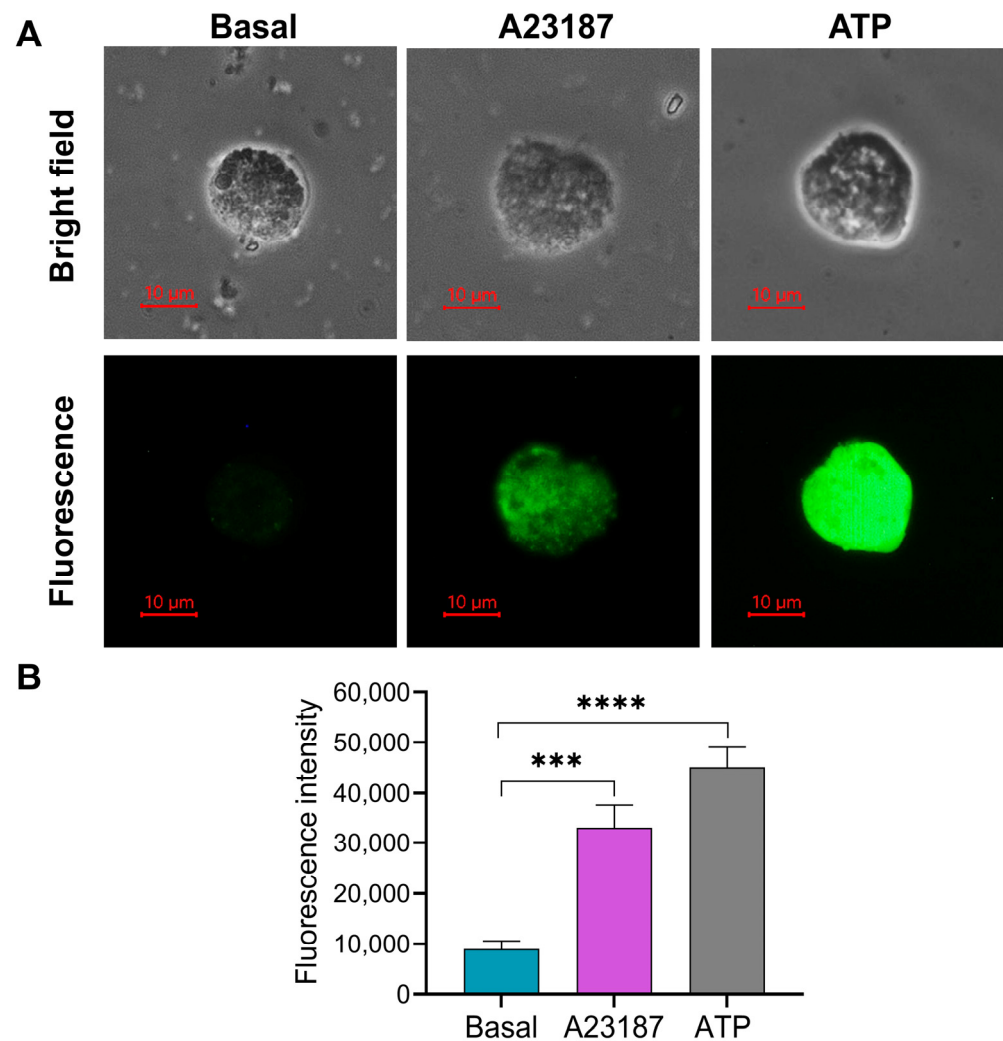
in cmMCs stimulated with ATP (100  $\mu$ M), because of elevated intracellular calcium concentrations (Figure 6). Likewise, the ionophore A23187, a calcium transporter across the plasma membrane, induced a marked rise in fluorescence [33].



**Figure 4.** Toluidine blue stain of cmMCs from C57BL/6J mice, observed under a microscope at 400 $\times$  magnification. (A) Mouse cmMCs stained for 1 day. (B) Mouse pMCs stained for 1 h. Representative cells from at least ten cultures are shown.



**Figure 5.** PCR detection of *Mcpt1* in cmMCs. (A) Representative electrophoresis for the detection of *Mcpt1*-expressing cells: lane L, DNA marker; lanes C1–C9, cmMC samples; lane NTC, no-template control. (B) Percentage of cmMCs positive to the *Mcpt1* protease gene, with  $n = 26$  MCs from colonic mucosa of three mice, each processed in independent isolation protocols.

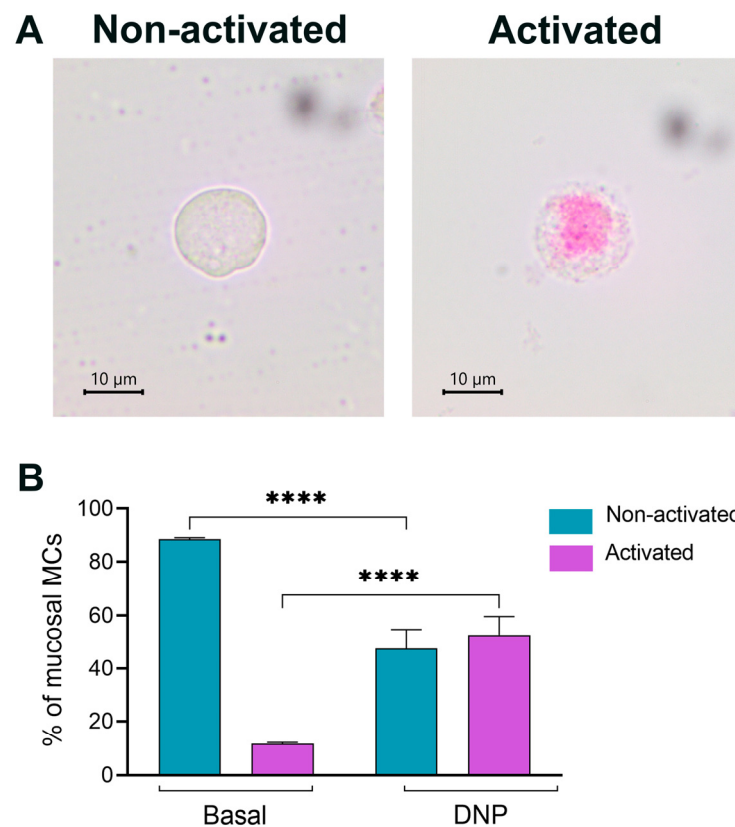


**Figure 6.** Changes in intracellular levels of  $\text{Ca}^{2+}$  of cmMCs in response to ATP. (A) Representative bright-field and fluorescence images of cmMCs under basal conditions or upon stimulation with A23187 (100 nM) and ATP (100  $\mu\text{M}$ ). (B) The mean whole-cell calcein fluorescence intensity (arbitrary units) under each experimental condition. Data are presented as means  $\pm$  standard error of the mean (SEM), with  $n = 30$  MCs from colonic mucosa of three mice, each one processed in independent isolation protocols. Group comparisons were statistically determined using one-way ANOVA and Dunnett's post hoc test. \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

Therefore, these results suggest that isolated cmMCs maintain their functional capacity to respond to stimuli that increase intracellular  $\text{Ca}^{2+}$ , which is a fundamental step in processes such as degranulation, mediator secretion, and inflammatory signaling.

### 3.5. Activation of cmMCs with IgE and Allergen and Evaluation of Cell Activation Using RR Technique

The activation of IgE-sensitized cmMCs in response to a specific antigen was evaluated using RR dye as a marker of cell degranulation, as the dye can only enter cells undergoing exocytosis [28,34]. Inactive MCs presented well-defined cell boundaries and no stained granules, while activated MCs contained a mixture of stained and unstained granules in the cytoplasm (Figure 7A). When cells in each state were counted (Figure 7B), most MCs were in an inactive state under basal conditions. In contrast, stimulation with DNP (antigen) resulted in a significant increase in the proportion of active cells and a concomitant decrease in inactive cells. These results demonstrate the functionality of freshly isolated cmMCs in response to the cross-linking of IgE–Fc $\epsilon$ RI complexes by the antigen.



**Figure 7.** Exocytosis in cmMCs via IgE-FcεRI cross-link by antigen and stain with RR. (A) Representative images of cmMCs in non-activated or activated state; 100× objective. (B) Percentage of non-activated and activated cells under basal or antigen (DNP)-stimulated conditions. Data are presented as means ± standard error of the mean (SEM), with  $n = 5$  independent experiments, each performed with MCs isolated from colonic mucosa of two mice and processed in independent isolation protocols. Group comparisons were statistically determined using one-way ANOVA and Bonferroni's post hoc test. \*\*\*\*  $p < 0.001$ .

#### 4. Discussion

Studies on MCs, particularly those examining activation, signaling pathways, and secretory responses to antigens, allergens or pathogens, frequently rely on cell lines, valued for their maintainability and reproducibility. However, cell lines do not always faithfully preserve the phenotypic and functional characteristics of tissue-resident MCs, particularly those with a mucosal phenotype [35].

Protocols for the isolation and purification of MCs from the colonic mucosa are scarce and exhibit considerable heterogeneity in key performance indicators. In the present study, we optimized a straightforward protocol for the isolation of murine cmMCs based on controlled enzymatic digestion followed by Percoll density-gradient centrifugation. Compared with previously reported intestinal MC isolation protocols, our protocol achieves high yield, purity and viability without requiring antibody-based enrichment, magnetic separation and fluorescence-activated cell sorting. Human colonic biopsy protocols based on enzymatic digestion have reported yields ranging from  $2.9\text{--}8.1 \times 10^5$  to  $2.5 \times 10^7$  cells/g of tissue, although the initial MC purity is low (1–6%), requiring subsequent magnetic or flow cytometric sorting to achieve purities above 90% [25–27,36], usually at the expense of cell recovery and increased cost. Likewise, earlier described rat intestinal protocols either yielded relatively low MC purity after enzymatic digestion alone or required parasite infection together with multiple density separation steps to obtain highly purified MCs [22,23]. In contrast, our protocol consistently yielded  $1.45 \times 10^6$  MCs per g of mucosal tissue with 87%

viability and 95% purity using only enzymatic digestion followed by a single continuous Percoll gradient. These results compare favorably with previously reported rat and human intestinal protocols while substantially simplifying the isolation procedure and eliminating the need for antibody-based cell selection or magnetic separation, reducing experimental cost. Our observation that MC isolation protocols from colonic mucosa yield higher purity in female mice compared with males highlights the relevance of sex-specific biological factors in experimental outcomes. Hormonal influences, particularly estrogen, have been shown to modulate epithelial integrity and immune cell composition [37–39], thereby facilitating more efficient enzymatic digestion and mechanical dissociation in females. In addition, variations in mucosal architecture and baseline inflammatory tone between sexes may alter the susceptibility of colonic tissues to isolation procedures, ultimately affecting the recovery and purity of MC populations [40–42].

We thought that key factors contributing to the improved performance of this protocol were the optimization of Percoll separation and collagenase digestion steps. Unlike discontinuous gradients, in which cells accumulate at predefined interfaces and are exposed to abrupt density transitions, the continuous gradient provides a gradual density distribution that allows MCs to sediment according to their intrinsic buoyant density while reducing mechanical stress during centrifugation [43]. During protocol optimization, discontinuous (80%/30%) and higher-density continuous (35%) Percoll gradients resulted in lower viability and purity than the optimized conditions. We propose that the gentler separation achieved with the 20% continuous gradient reduces contamination by epithelial and stromal cells, while improving the recovery of intact, functional MCs. Besides, dissociation time and enzymes employed for tissue dissociation are critical parameters impacting cell viability, since prolonged exposure to enzymes can compromise cell integrity, functionality, and overall viability [44,45]. Thus, Percoll separation strategy and the optimized collagenase digestion likely explain the high reproducibility and overall performance of the protocol.

Toluidine blue is a common dye used to stain MCs, as it binds to the negatively charged proteoglycans stored in the granules [46]. CTMCs express heparan sulfate and their amount increases as the cells mature [47], while MMCs predominantly present proteoglycans of chondroitin sulfate type, which are less sulfated and present less anionic charge [48]. In this sense, toluidine blue undergoes strong polymerization in CTMC granules, inducing metachromasia (a shift from blue to purple), whereas it aggregates weakly in MMCs, resulting in an orthochromatic stain (blue) [49]. The toluidine blue staining of mouse cmMCs reveals the predominant content of chondroitin sulfate-type proteoglycans and the absence of heparin in their granules, elucidating their mucosal phenotype. Consistent with these observations, 73% of the isolated cmMCs expressed the *Mcpt1* gene, a well-established marker of murine MMCs. The absence of detectable *Mcpt1* transcripts in the remaining 27% of *Gapdh*-positive cells may reflect several, non-mutually exclusive, explanations. First, it likely represents the intrinsic heterogeneity of MCs within the colonic mucosal compartment. Recent studies using *Mrgprb2-cre<sup>tdTomato</sup>* reporter mice have demonstrated that both mucosal and connective tissue-like MCs coexist within the healthy murine colonic mucosa, indicating that these populations are not strictly segregated [50]. This interpretation is further supported by studies showing that bone marrow-derived mast cells (BMMCs) engineered to express MRGPRX2, the human orthologue of *Mrgprb2*, successfully engraft the colonic mucosa, highlighting the phenotypic diversity of intestinal MCs [51]. Another possibility is that *Mcpt1*-negative cmMCs correspond to MC precursors or immature MC residing in the mucosa, which have fewer transcripts of MC-specific proteases and the enzyme responsible for sulfation of heparin than mature MCs [52].

Technical factors should also be considered when interpreting these findings. Although single-cell RT-PCR is a highly sensitive technique, the detection of low-abundance

transcripts may be affected by stochastic variation in mRNA capture, reverse-transcription efficiency, and PCR amplification, potentially resulting in false-negative amplification events. Likewise, transient transcriptional changes induced during collagenase-mediated tissue dissociation cannot be excluded [45]. Therefore, the *Mcpt1*-negative population most likely reflects a combination of biological heterogeneity and technical limitations rather than the presence of non-MCs, as all analyzed cells displayed typical MC morphology. Further studies aimed at deeply phenotyping freshly isolated cmMCs using specific markers of MC subpopulations (e.g., *Mcpt2*, *Mcpt4*, and *Mcpt5*) are needed to understand how cell heterogeneity may influence the functional responses of the obtained MC suspensions and to decipher the potential phenotypic changes during long-term cultures.

Knowledge regarding resident MC populations in the intestinal mucosa remains limited and, in consequence, experimental evidence derived from primary cultures of MMCs is scarce, largely due to the technical challenges associated with their isolation, purification, and in vitro maintenance, as well as their strong dependence on the native tissue microenvironment [7,53]. Consequently, only a small number of studies have employed primary cultures of MCs with a mucosal phenotype, and even fewer have obtained them directly from colonic mucosa. This scarcity persists despite the recognized functional relevance of these cells in maintaining intestinal homeostasis and orchestrating immune responses [54], underscoring the importance of the present protocol.

This work also demonstrates that freshly purified cmMCs are functional and can be activated by different stimuli through plasma membrane receptors. ATP stimulation resulted in a significant increase in intracellular  $\text{Ca}^{2+}$  concentration. These results are consistent with previous findings demonstrating that extracellular ATP activates purinergic receptors, particularly P2X and P2Y in MCs, inducing transient increases in  $\text{Ca}^{2+}$  and modulating their functional activation [55,56]. Purinergic activation by ATP has been reported to trigger both the release of  $\text{Ca}^{2+}$  from intracellular reservoirs and the influx of extracellular  $\text{Ca}^{2+}$ , thereby amplifying intracellular signaling [57,58]. In contrast, antigen stimulation of IgE-sensitized cmMCs resulted in an increase in the proportion of activated cells and a decrease in the proportion of non-activated cells. It has been described that in the absence of antigenic stimuli, MCs remain at rest and do not release preformed mediators, maintaining their secretory machinery without observable changes [59–61]. Nevertheless, multivalent antigens, such as DNP bound to serum albumin, trigger calcium-dependent degranulation [62,63]. RR staining revealed these changes, since a high number of cmMCs presented modifications in the plasma membrane because of exocytosis that facilitates the internalization of the dye into granules. Collectively, these findings are consistent with many reports showing that antigenic stimulation significantly increases the proportion of active MCs compared with basal conditions [64,65], reflecting the functionality of isolated cmMCs. Altogether, these results indicate that the receptors and signaling pathways required for MC activation remain intact after the current isolation procedure and during short-term functional assays. Nevertheless, further analyses are required to evaluate functional stability during long-term cultures.

BMMCs have long been employed in adoptive transfer experiments in mice [51]. Nevertheless, their generation remains a technically demanding, time-consuming, and costly process, and their phenotype has been debated for decades, as they are often characterized as immature MCs with transcriptomic profiles distinct from tissue-resident MCs [5,66]. In this context, the establishment of protocols enabling the isolation and purification of primary MCs from intestinal tissues with high yield and quality, while remaining cost-effective, is critical to ensuring the success of in vivo adoptive transfer approaches. Future studies will be required to validate whether reintroducing the isolated cmMCs into MC-deficient mice can restore the immune response in the intestinal mucosa. Primary MCs

are also broadly used in in vitro sensitization and signal transduction studies [67,68]. The protocol described herein not only enhances the reliability of experimental outcomes but also broadens accessibility by lowering financial barriers, thereby fostering more equitable opportunities for immunological and translational research.

This protocol has some limitations that should be considered. Biological variability among strains or disease severity, may influence the final cell yield. In addition, this protocol was specifically developed to obtain highly viable primary cmMCs for immediate downstream applications and short-term functional assays while preserving their native phenotype. Consequently, it is not intended for long-term culture or in vitro expansion, as the isolated cells are maintained without the exogenous growth factors required to support prolonged survival and proliferation. This design prioritized the study of freshly isolated tissue-resident MCs under conditions that closely resemble their physiological state rather than generating long-term-cultured populations.

Importantly, although the protocol was initially optimized using healthy tissue, it was also successfully validated in inflamed colonic mucosa, where comparable yield, viability, purity, and reproducibility were obtained. These findings support the robustness of the method and its applicability to experimental models of intestinal inflammation, including IBD. The ability to consistently isolate highly viable and purified primary cmMCs from both healthy and inflamed tissues provides a valuable platform for investigating the contribution of MCs to intestinal immune regulation, epithelial barrier dysfunction, and neuroimmune interactions during disease progression. Furthermore, the high purity and viability of the isolated cells make this protocol ideal for downstream molecular applications, including transcriptomic, proteomic, and functional analyses, enabling the characterization of MC phenotype and signaling under physiological and pathological conditions. Finally, as this protocol was specifically designed to obtain freshly isolated primary MCs for short-term functional studies while preserving their native phenotype, conditions for long-term in vitro expansion were not evaluated. However, the isolated cells remained highly viable throughout the experimental time required for functional, molecular, and biochemical assays, making this approach particularly suitable for studies that aim to faithfully reproduce the biology of tissue-resident MCs.

## 5. Conclusions

The present work describes a reproducible method for obtaining a primary culture of MCs specifically isolated from the colonic mucosa of C57BL/6 mice. This approach represents a significant methodological contribution, as it enables the study of MCs that retain characteristics specific to their physiological environment, including the expression of markers associated with the mucosal phenotype and a functional response like that observed in vivo. Furthermore, by focusing on the colonic mucosa, this experimental model is particularly well-suited to studying local immunological processes, such as intestinal inflammation and interactions with purinergic mediators or allergens.

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