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# The effects of age, origin, and biological sex on rodent mast cell (BMMC and MC/9) and basophil (RBL-2H3) cell phenotype and function[1]

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Running title: A comparison of BMMC, MC/9 and RBL-2H3 phenotypes and function

**Abstract:**

Mast cells initiate allergic inflammatory immune responses and play a role in disease by releasing various inflammatory and immunomodulatory mediators. Several mast cells lines and primary cultured cells have been used as mast cell models with inconsistent results among research groups. Bone marrow-derived mast cells (BMMC) cultured from mouse bone marrow progenitor cells are often used as a representative model of mucosal mast cell behaviour, however their reported phenotype is variable due to inconsistent culture protocols. The RBL-2H3 cell line is a rat basophilic histamine-releasing cell line that has some characteristics of both mast cells and basophils but is not a true representation of either cell. The murine mast cell line MC/9 is IL-3-dependent but has incomplete mast cell characteristics. In this study, we have compared the response of BMMC (male and female derived from C57BL/6 mice), two sources of RBL-2H3 (purchased directly from ATCC and a lab curated culture), and MC/9 (ATCC) at several critical stages to some common stimuli (IgE/Ag, A23187) and analyzed mast cell morphology, expression level of common mast cell markers (CD117 and FcεRI), protease expression, and function (growth kinetics, viability, ROS production, degranulation, cytokine release and signaling). The objective of this study was to provide insight into the effects of culture conditions, biological sex and age on variability among reported phenotypes and, to determine optimal conditions for activation of these cells. Our data show that factors that are often overlooked such as source, age and biological sex of mast cells play an integral role in phenotypic outcomes and may account for the reported variability in their function.

**Introduction:**

Histamine-releasing cells such as mast cells are the central effector cells and immunomodulators in a variety of different diseases, including allergic inflammation. As such, cell lines that closely mimic mast cells have been used as models to test anti-inflammatory drugs for clinical use. Although many of these mast cell models have features of mast cells, they are also lacking in key mast cell biomarkers. *In vivo*, mast cells are heterogenous cells that originate in the bone marrow as progenitors and differentiate in peripheral tissues. Fully mature mast cells are best characterized by their large size (8-20 μm) and electron dense granules that store proinflammatory mediators such as histamine, β-hexosaminidase, tryptase and chymase.[1,2] In rodents, mast cells fall into two major phenotypic subtypes: connective tissue mast cells (CTMC) and mucosal mast cells (MMC) based mainly on their responsiveness to cationic secretagogues and their expression of mast cell-specific proteases.[3] CTMC such as *in vivo*-derived peritoneal mast cells (PMC) degranulate in response to basic secretagogues (ex: substance P, compound 48/80) and express both tryptases and chymases (Mcpt2, Mcpt4, Mcpt5 and Mcpt6).[45] MMC are not activated by basic secretagogues and mainly express tryptases Mcpt1 and Mcpt2.[6,7] Both mast cell subtypes express the high affinity immunoglobulin receptor (FcεRI), stem cell factor receptor (CD117), G protein-coupled receptors (GPCR), immunoglobulin receptors and innate immune receptors (ex: toll-like receptors) that can be activated by binding to their respective ligands. Within seconds of activation, mast cells degranulate and release these pre-formed mediators, along with reactive oxygen species (ROS). Within hours of stimulation, it is possible to measure the release of leukotrienes and prostaglandins and *de novo* synthesized protein mediators

such as cytokines and chemokines. In order for a mast cell model to be a suitable surrogate for *in vivo* mast cells, it must possess these morphological characteristics and consistently and measurably simulate all of these responses. Currently, there are three main models of rodent mast cells that are used in mast cell biology research.

Bone marrow-derived mast cells (BMMC) are cultured from CD34+ progenitor cells obtained from mouse bone marrow in the presence of recombinant mouse interleukin-3 (IL-3), or conditioned media from WEHI cells for 4 weeks at which point approximately 99% of the cells have differentiated into FcεRI$^{+}$/CD1117$^{+}$ mast cells. As a primary cell culture, BMMC have been described as a MMC model as they are not activated by basic secretagogues[8] however, BMMC highly express Mcpt5 and Mcpt6 and have been reported to have very low to undetectable expression of MMC specific proteases Mcpt1 and Mcpt2[9] which suggests they possess characteristics that do not fully depict either CTMC or MMC. It is also reported that BMMC can upregulate expression of MMC proteases when cultured with SCF, TGF-β or IL-9 [10] further proving the phenotypic fluidity of this cell type under different culture conditions. Although BMMC are an attractive mast cell model, it has been anecdotally suggested that they are phenotypically unstable after several weeks in culture and it is not possible to cryopreserve them in their fully differentiated state. It is also unknown whether the sex of the source bone marrow influences their responses.

The rat basophilic cell line (RBL-2H3) is often described as a MMC model mainly because it expresses FcεRI and is not activated by basic secretagogues, but it is, in fact, a

basophil cell line that was as originally isolated from rats that developed basophilic leukaemia upon treatment with [2-(a-chlor-b-isopropylamine)ethylnaphthalene].[11] Much like *in vivo*-derived basophils, RBL-2H3 express FcεRI and can be activated by IgE-antigen complexes to release inflammatory mediators such as histamine, β-hexosaminidase, and tumor necrosis factor alpha (TNF).[12] Unlike primary basophils, RBL-2H3 adhere to tissue culture plastic, proliferate quickly, are morphologically homogenous, and do not express the basophil specific CD123.[13] Although RBL-2H3 are reported to express CD117[14] surface protein expression has not been confirmed. Unlike mast cells, RBL-2H3 do not express any of the eight rat tryptase genes (*Tpsb2, Tpsab1, Tpsg1, Prss34, Gzmk, Gzma, Prss29, Prss41*), and do not release tryptase upon stimulation.[15] Furthermore, data using RBL-2H3 are highly variable among research groups with some reporting robust degranulation only at early passage numbers while other groups report no effect of passage number on RBL-2H3 responses.[16–18]

The cloned mouse mast cell line (MC/9) was derived from the fetal liver of a (B6 X A/J) F1 mouse and express FcεRI and CD117 and require IL-3 to proliferate.[19] Like BMMC and RBL-2H3, MC/9 have been described as a MMC [20] model but also have phenotypic dichotomy since they possess some characteristics of CTMC. MC/9 highly express the CTMC chymases Mcpt5 and Mcpt6[21] but do not activate in response to basic secretagogues. While MC/9 cells are widely used in the literature as a MMC model, data reported is inconsistent, with varying culture protocols and degranulation data often standardized, and it is uncertain how their responses compare to RBL-2H3 and BMMC under tightly controlled experimental conditions.

In this study, we characterized the quintessential mast cell characteristics of BMMC, RBL-2H3 and MC/9 and found that each lacked important morphological and/or phenotypical mast cell features to varying degrees. We also compared the response of BMMC, RBL-2H3 and MC/9 to an FcεRI-dependent (IgE/Ag) and FcεRI-independent (A23187) stimulus to better understand the mediator release and signaling differences of these cells. Finally, we show the distinct effects of culture conditions, age, origin, and sex of these cell types on phenotypic outcomes. Our data has important implication for the use of these cells in research applications, especially when extrapolating to *in vivo* mast cell functions.

## MATERIALS AND METHODS

### BMMC culture

BMMC were isolated from femurs of male (BMMC-M) or female (BMMC-F) C57BL/6 mice and cultured in RMPI media supplemented with 10% heat inactivated FBS (Cytiva., Waltham. MA, USA), 4 mM L-glutamine (Gibco, Waltham, MA, USA)), 50 uM β-mercaptoethanol (Sigma, St. Louis, MO, USA), 1mM sodium pyruvate (Gibco), 100 uM non-essential amino acids (Gibco), penicillin (100 U/ml), and streptomycin (100 µg/ml), 25mM HEPES (Gibco) and, 30 ng/ml murine IL-3 (Peprotech, Cranbury, NJ, USA) and maintained at 5% $CO_2$ and 37°C for four weeks until differentiated. Cells were maintained at a density of 0.25 x $10^6$ cells/ml and fed twice per week.

**RBL-2H3 culture**

Two sources of RBL-2H3 cells, RBL-2H3-a (purchased directly from ATTC, Manassas, VA, USA) and RBL-2H3-b (obtained from a long-term culture at the University of Alberta) were used in these analyses. Both RBL-2H3 sources were obtained as cryopreserved samples and cultured in DMEM high glucose (Gibco) supplemented with heat inactivated 10% FBS (Gibco), 1 mM sodium pyruvate (Gibco), 100 U/mL penicillin streptomycin (Gibco), and maintained at 5% $CO_2$ and 37°C. The cells were split at a ratio of 1:20 twice per week once they had reached 70% confluency. Passage numbers of both sources of cells were matched.

**MC/9 Culture**

MC/9 were obtained from ATCC and cultured in RPMI-1640 supplemented with 10% heat inactivated FBS, 4 mM L-Glutamine (Gibco), 50 µM β-mercaptoethanol (Sigma), 1 mM sodium pyruvate (Gibco), 100 µM non-essential amino acids (Gibco), penicillin (100 U/mL), and streptomycin (100 µg/mL), 25 mM HEPES (Gibco) and, 30 ng/ml murine IL-3 (Peprotech) and maintained at 5% $CO_2$ at 37°C. Cells were maintained at a density of 0.25 x $10^6$ cells/ml and fed twice per week.

**Toluidine Blue Staining**

Cells were centrifuged onto glass slides at 800 x *g* for 5 min (Shandon Cytospin, ThermoFisher). The slides were then fixed with Mota's fixative (12 mM lead acetate in 2% acetic acid (v/v) and, 50 % (v/v) ethanol)) for 30 min in a humidified chamber,

washed in mq$H_2O$, stained with Toluidine blue (2mM in 30% (v/v) ethanol, pH < 1)) for 20 minutes, washed in mq$H_2O$, and allowed to dry for 15 min at room temperature. Coverslips were mounted using 30 µl of Permount (Fisher) and images were taken on Zeiss AX10 microscope at 200x magnification (Zeiss, Oberkochen, Germany).

**Flow Cytometry**

Cells were washed in PBS and blocked in 1 mL of 3% BSA-PBS on ice for 30 min, then labeled with 50 ng/ml or 250 ng/ml mouse FcεRI-APC (eBiosciences, San Diego, CA, USA) and mouse CD117-PE (eBiosciences) or 50 ng/ml or 250 ng/ml isotype controls rat IgG-PE and armenian hamster IgG-APC (eBiociences) for BMMC and MC/9 or RBL-2H3 respectively for 1 hr on ice in the dark. The cells were then washed in 0.1% BSA-PBS and analyzed on a BD Fortessa X-20 instrument (BD Biosciences, Franklin Lakes, NJ, USA). Data analysis was performed using FlowJo v.10.6.2 software (FlowJo, Ashland, OR, USA).

**Proliferation curves**

One hundred thousand cells were seeded in a 24-well plate and incubated for 0-96 hr. At each timepoint the cells were stained with trypan blue (Gibco) and the trypan negative cells were counted in a haemocytometer to determine viable cell number. The number of cells per ml was plotted versus time and significant change in cell number was determined relative to time zero.

**Histamine assay**

One million cells were lysed in 600 µL of 0.1% Triton X-100 (Fisher) in 10 mM HEPES buffer without BSA (10mM HEPES, 137 mM NaCl, 2.7mM KCl, 0.4 mM $Na_2HPO_4 \cdot 7H_2O$, 5.6 mM glucose, 1.8 mM $CaCl_2 \cdot 2H_2O$, 1.3 mM $MgSO_4 \cdot 7H_2O$) and 60 µL of lysate was added to a 96-well black microplate that included 60 µl/well of a standard curve of histamine (7.8 – 500 ng/ml) in 10 mM HEPES buffer without BSA. Twelve microlitres of 1 M NaOH was added to each well followed by the addition of 2 µl of a 10 mg/ml o-phtalaldehyde (OPT) solution in methanol and a 4-minute incubation at room temperature. Finally, 6 µl of 3 M HCl was added to each well to stop the reaction and the plate was read in a fluorescent microplate reader (Varioskan Lux, ThermoFisher) at 360/450 ex/em.

**Degranulation assay**

Cells stimulated with IgE/Ag were sensitized overnight with 500 ng/ml IgE (SPE-7, Sigma) at a density of $1x10^6$ cells/ml. The cells were washed with 10 mM HEPES buffer pH 7.4 (10mM HEPES, 137 mM NaCl, 2.7mM KCl, 0.4 mM $Na_2HPO_4 \cdot 7H_2O$, 5.6 mM glucose, 1.8 mM $CaCl_2 \cdot 2H_2O$, 1.3 mM $MgSO_4 \cdot 7H_2O$, 0.4% (w/v) bovine serum albumin (BSA)) and stimulated with DNP-BSA (0.1-1000 ng/ml (Invitrogen, Waltham, MA., USA)) for 15-60 at 37$^o$C. Cells stimulated with A23187 were stimulated with 0.1-10 mM A23187 (Sigma) for 30-120 min at 37$^o$C. Cell pellets were separated from cell supernatants and the cell pellets were lysed with 0.1% TritonX-100 for 10 min. β-hexosaminidase activity in the supernatants and cell lysates was quantified by adding p-nitrophenyl N-acetyl-β-D-glucosamide (100 mM, Sigma) in 0.1 M sodium citrate buffer pH 4.5 (40 mM citric acid, 20 mM $Na_2HPO_4 \cdot 7H_2O$) for 60 min and incubating at 37$^o$C.

Hydrolysis of p-nitrophenyl N-acetyl-β-D-glucosamide was quantified by reading absorbance at $A_{405}$ on a microplate reader (Varioskan Lux, Thermofisher, Waltham, MA, USA) with $A_{570}$ as a reference. The percentage of β-hexosaminidase release was calculated as a percent of total content according to the formula: [supernatant/(supernatant+pellet)] x 100.

**Measurement of ROS generation**

One million cells were incubated with 20 µM DCFDA (Sigma) in DMEM without phenol red (Gibco) for 40 minutes in 5% $CO_2$ at 37°C. The cells were washed once with DMEM without phenol red and resuspended in DMEM without phenol red. Fifty thousand stained cells/well were added to a 96-well plate containing 10 µl of stimulant (A23187 or DNP-BSA) and incubated in 5% $CO_2$ at 37°C. Fluorescence was measured at 485/535 (ex/em) on a microplate reader and the concentration of ROS was determined using a TBHP standard curve. Results are presented as the concentration of DCFDA deacetylated to DCF (µM) which is proportional to ROS release.

**Cytokine enzyme-linked immunosorbent assays (ELISAs)**

One million BMMC or MC/9 cells were seeded in each well of a 24-well and, $0.5 \times 10^6$ RBL-2H3 were seeded in each well of a 6-well plate and treated with 1µM A23187 for 18 hr or sensitized for 18 hr with 500 ng/ml of IgE followed by stimulation with 10 ng/ml DNP-BSA for 18 hr. Cell culture medium was removed and centrifuged at 200 x *g* for 5 min and cell free supernatant was collected. The cytokines and chemokines released in

the supernatants were quantified using commercial ELISA kits (Invitrogen) and data is corrected to $1x10^6$ cells/ml.

**Mesoscale analysis of mediator release**

BMMC, RBL-2H3 or MC/9 were activated with A23187 (1 μM) for 18 hr or sensitized with IgE (500 ng/ml) for 18 hr, then activated with DNP-BSA (10 ng/ml) for 18 hr and supernatants were isolated by centrifugation at 200 x *g* for 5 minutes. Supernatants were analyzed using MSD U-plex plates following manufacturer instruction (Mesoscale, Rockville, MD, USA), and fluorescence was read on an MSD Discovery Microplate Reader (Mesoscale).

**Western Blotting**

Five hundred thousand cells were seeded in each well of a 24-well plate and treated with 1 μM A23187 or 10 ng/ml of DNP-BSA for 0-30min. Before each time point elapsed, the cells were centrifuged for 2 min at 200 x *g*, the supernatant was discarded, the cells were resuspended in 40 μl of Laemmlli buffer (50mM Tris pH 6.8, 1% SDS, 9% glycerol, 1.8% β-mercaptoethanol, and 0.002% bromophenol blue) and immediately boiled at 100°C for 15 min. Proteins were separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were blocked for 1 hr at room temperature with 5% skim milk in TBS-T, washed with TBS-T three times, incubated with primary antibodies in 3% BSA TBS-T (total-SYK (Abcam, Cambridge, UK), and phospho-SYK (Y348, Abcam), or total-ERK (Cell Signalling, Danvers, MA, USA) and phospho-ERK 1&2 (Sigma) for 18 hr, washed again three times with TBS-T and

incubated with secondary antibodies in 3% BSA TBS-T; goat-anti mouse 680 (LiCOR, Lincoln, NE, USA) and goat-anti rabbit 800 (LiCOR) and then wash three more times with TBS-T. Fluorescence was analyzed and quantified on a LiCOR Odyssey DLx (LiCOR).

**qPCR analysis of protease expression**

Samples were harvested from five-week-old BMMC and MC/9 and, RBL-2H3 at passage 14 collected from three independent cultures. Four million cells were centrifuged at 300 x *g* for 5 minutes, the supernatant was removed, and the cells were lysed in TRIzol (Invitrogen). RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) that employed on-column DNase (Qiagen) digestion. The purity and concentration of the RNA was determined using the Nanodrop One (ThermoFisher) and cDNA was synthesized using 1000 ng of total RNA by utilizing the High-capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA). qPCR was performed with Fast SYBR Green master mix (Applied Biosystems), 20ng of cDNA, gene specific IDT oligonucleotide primers as described in Table 1 and a StepOnePlus real time PCR machine (Applied Biosystems). The data was analyzed relative to average gene expression obtained from two independent sources of bone marrow cells less than one week post isolation derived from C57 BL/6 mice for BMMC an MC/9 analysis, and relative to Tbsp2 gene expression for RBL-2H3.

**Table 1**

List of introns spanning oligonucleotide primers used in this study.

| Gene ID | Gene name | Abbreviation | Forward primer | Reverse primer |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| NM_008570.1 | Mouse Mast cell protease 1 | Mcpt1 | gcacttctcttgccttctgg | taaggacgggagtgtggtct |
| NM_008571.1 | Mouse Mast cell protease 2 | Mcpt2 | gcacttcttttgccttctgg | taaggacgggagtgtggttt |
| NM_010779.2 | Mouse Mast cell protease 4 | Mcpt4 | gcacttctcttgccttctgg | atgtaagggcgagaatgtgg |
| NM_010780.3 | Mouse Chymase 1 (Cma1) | Mcpt5 | tgcatcttcttactcttcatctgc | cgtgcctccaatgatctctc |
| NM_010781.3 | Mouse Tryptase beta 2 (Tpsb2) | Mcpt6 | gctggggcgacattgata | tgggaaccttcacttgcttc |
| NM_031187.4 | Mouse Tryptase alpha/ beta 1(Tpsab1) | Mcpt7 | tcctcactgtgtccaaatgc | ccttctcgtgtcatagctgga |
| NM_008572.1 | Moue Mast cell protease 8 | Mcpt8 | ggatgttcctgctcctggt | tggggtttggactctgtacc |
| NM_007753.2 | Mouse Carboxypeptidase A3 (Cpa3) | CPA3 | gctattaattccttatggctacacatt | gtggcaatccttgcaacttt |
| NM_008084.3 | Mouse Glyceraldegyde-3-phosphate dehydrogenase | GapDH | gggttcctataaatacggactgc | ccattttgtctacgggacga |
| NM_017145.2 | Rat Mast cell protease 1 | rMcpt1 | gctgtggtgggtttctcata | ctccaagggtgactgtgattt |
| NM_172044.1 | Rat Mast cell protease 2 | rMcpt2 | ctgcacattgtaaaggaagagaaa | gaagattgggaacggagttgta |
| NM_001170466.2 | Rat Mast cell protease 3 | rMcpt3 | catcatgttactgaagcttgaagag | caggcttgataaagtcagagga |
| NM_019321.2 | Rat Mast cell protease 4 | rMcpt4 | catgttactgaagcttgaacagaaa | cacattgtcccaggcttgata |
| NM_013092.2 | Rat Chymase 1 (Cma1) | rCma1 | catgttactgaagttgaaggagaaag | gtgtcagaggctggttcatt |
| NM_019180.2 | Rat Tryptase beta 2 (Tpsb2) | rTpsb2 | tgatagtgacgagcctctcc | tagaggccagtgtggtactt |
| NM_019322.2 | Rat Tryptase alpha/ beta 1(Tpsab1) | rTpsab1 | gccctgctgaaactcactaa | ggtggcaggcttacatcat |
| NM_019300.1 | Rat Carboxypeptidase A3 (Cpa3) | rCpa3 | tatcaggcagccaagagttatg | tctccacattcgatcctttgtc |
| NM_021598.3 | Rat Mast cell protease 8 | rMcpt8 | tctcacaccctgacttctacat | agggagacagtgtggacat |
| NM_017008.4 | Rat Glyceraldegyde-3-phosphate dehydrogenase | rGapDH | gggtgtgaaccacgagaaata | ctgtggtcatgagcccttc |

**Statistical analysis**

Experiments were conducted from at least three independent cultures of cells or with MC obtained from at least three separate mice and values represent mean of n=3-6 $\pm$ standard error of the mean. P values were determined by one-way ANOVA (between groups) or Student t test.

## RESULTS

### Proliferation, morphology, and histamine content

To compare the phenotypes of BMMC-F, RBL-2H3-b and MC/9, cells were stained with the commonly used dye, toluidine blue. Toluidine blue is an acidophilic metachromatic dye that selectively binds to proteoglycans and heparin contained within mast cell granules and was the dye used by Paul Ehrlich in 1877 when he first described mast cells.[22] Metachromatic staining of mast cells with toluidine blue is due to the binding of toluidine blue to heparin, a heteroglycan rich in half-sulfate esters stored in their granules, which produces a slightly purple colour, and this approach has been used as a hallmark test of mast cell granularity ever since. Toluidine blue staining of BMMC-F indicates large cells (15-20 µm) with the expected blue nucleus and purple cytoplasm, indicating the presence of heparin-rich granules (Fig. 1A). Toluidine blue staining of RBL-2H3-b showed smaller cells (~10-15 µm) with a blue nucleus but very few purple-coloured granules (Fig. 1D). Of the three cell types, MC/9 appeared the least "mast cell-like" with poor toluidine staining of both nucleus and cytoplasm and relatively small (~10-15 µm) compared to BMMC-F and RBL-2H3-b (Fig. 1G).

Flow cytometric analysis of FcεRI and CD117 expression is often used to define mast cell populations in mixed cell populations and is also used as a measure of mast cell maturity. At the optimal age of <5 weeks, BMMC-F expressed high levels of both FcεRI and CD117 (Fig. 1B) with more than 95% of BMMC-F expressing both receptors at 4 weeks of culture. A small subpopulation of cells expressed CD117 but not FcεRI (4.06% in lower right quadrant), possibly representing mast cell progenitor cells that have yet to differentiate and perhaps representing a proliferative pool that sustains these cultures for

the subsequent weeks. At < passage 11, RBL-2H3-b, in keeping with their basophil-like origins, do not express CD117 and very low levels (~27%) of FcεRI (Fig. 1E). At < 5 - weeks post-thaw, MC/9 appeared as two distinct populations of cells, approximately 26% of the cells expressed FcεRI and approximately 59% expressed both FcεRI and CD117 (Fig. 1H).

After 4 weeks in culture, BMMC-F do not divide rapidly and even after 96 hr, cell numbers only increase slightly (Fig. 1C). After less than 11 passages, RBL-2H3-b proliferate very rapidly with a doubling time of approximately 26 hr (Fig. 1F). MC/9 that are older than 5 weeks in culture divide even more rapidly than RBL-2H3 with a doubling time of about 24 hr (Fig. 1I).

BMMC-F contained the least amount of histamine and MC/9 contained approximately 4 times more histamine than BMMC. RBL-2H3-b contained the most histamine than either BMMC or MC/9 (Supplementary Fig. 1)

**Reactive oxygen species (ROS) production in response to A23187 and IgE/antigen (IgE/Ag)**

Although all three cell types have been reported to release some ROS, it is unknown whether all three cell types can produce ROS in response to A23187 and IgE/Ag. Therefore, a direct comparison of relative quantities of ROS produced by each cell type in response to A23187 or IgE/Ag was performed (Fig. 2). BMMC-F at 4 - 5 weeks in culture produced the highest amount of ROS amongst all cell types with significant amounts of ROS produced when they were stimulated with IgE/Ag (Fig. 2B) and high amounts of ROS when activated with 1 μM of A23187 (Fig. 2A). The amount of ROS

released from BMMC when stimulated with IgE/Ag plateaued at 10 ng/ml DNP-BSA and peaked at 1 µM A23187. When stimulated with either A23187 or IgE/Ag, RBL-2H3 produced very low amounts of ROS which were barely detectable using this assay (Fig. 2C and D). MC/9 produced detectable levels of ROS when stimulated with both A23187 and IgE/Ag that peaked at 10 µM A23187 (Fig. 2E) and 100-1000 ng/ml DNP-BSA (Fig. 2F).

**Degranulation in response to A23187 and IgE/Ag over a range of concentration and time points**

Degranulation is often used as a measure of mast cell activation but even the earliest measures of this process indicated that this is an extremely dynamic process that occurs rapidly and within a very small window of antigen/ionophore concentration. In addition, it has been suggested anecdotally that MC/9 do not degranulate whereas BMMC are the best model of mast cell degranulation due to their rapid and robust response to both IgE/Ag and A23187 compared to RBL-2H3. To test this hypothesis, the degranulation of BMMC-F, RBL-2H3-b and MC/9 were compared at different concentrations of stimulus and at different time points. A23187 activated BMMC-F to degranulate reaching maximum response of approximately 31-48% degranulation with 1 µM of A23187 after 45minutes (Fig. 3A and 3B). RBL-2H3-b degranulated to a maximum 38% when activated with 1 µM A23187 for 45 minutes and similarly to BMMC, activation with higher concentrations of A23187 did not correspond to an increase in degranulation (Fig. 3E and 3F). BMMC degranulated the most robustly in response to IgE/Ag with a maximum release of approximately 57% when stimulated with

10 ng/ml DNP-BSA for 90 minutes. RBL-2H3 response to IgE/Ag was much lower than BMMC, with maximum degranulation of approximately 20% when the cells were stimulated with 10 ng/mL of DNP-BSA (Fig. 3G) and even after 90 min of stimulation, this response did not increase (Fig. 3H). MC/9 did not degranulate to either A23187 or IgE/Ag at any concentration, even after 60 and 120 min of stimulation, respectively.

**Comparative protease expression profiles**

Mast cells are often characterized by their expression of proteases, namely chymases and tryptases. To assess the protease expression of BMMC-F, RBL-2H3-a and MC/9 we performed a qPCR analysis of the commonly expressed mouse (*Mcpt1, Mcpt2, Mcpt4, Mcpt5, Mcpt6, Mcpt7, Mcpt8 and CPA3*) and rat (*Mcpt1, Mcpt2, Mcpt4, Mcpt8, Tpsb1, Tpsb2, CMA1 and CPA3*) chymases and tryptases. BMMC highly expressed *Mcpt5*, *Mcpt6* and *CPA3*, and very low levels of *Mcpt1* and *Mcpt2*, (Fig. 4A). MC/9 expressed high amounts of *Mcpt6* and *CPA3*, modestly expressed *Mcpt5* and *Mcpt7* and, showed low expression of *Mcpt1* and *Mcpt2* (Fig. 4B) Finally, RBL-2H3 modestly expressed MMC specific tryptases *Mcpt1* and *Mcpt2* and, highly expressed CTMC specific tryptase *CPA3* (Fig. 4C).

**A23187 and IgE/Ag mediated cytokine and chemokine production**

To compare BMMC-F, RBL-2H3-b and MC/9 production of chemokines and cytokines, the cells were stimulated with A23187 or IgE/Ag and the supernatants were analyzed using a mouse-specific multiplex analysis (Supplementary Fig. 2). This preliminary screen suggested that BMMC-F produced several mediators in response to A23187

activation (IL-1β, IL-4, IL-6, IL-13, IP-10/CXCL10, CCL2, MMP-9 and RANTES) but did not produce measurable amounts of IL-10. Activation of BMMC-F with IgE/Ag produced virtually no mediators, apart from IL-6 and CCL2. Most notably, BMMC-F were the only cell type to produce high amounts of RANTES. MC/9 produced many mediators when activated with A23187 (IL-1β, IL-4, IL-6, IL-10, IL-13, IP-10, CCL2, MMP-9) and were the only cell type to produce IL-10. Like BMMC, IgE/Ag stimulated MC/9 to produce IL-6 and CCL2. None of the cytokines analyzed in this screen were detected in RBL-2H3-b supernatants, suggesting that this mouse-specific approach is not appropriate for RBL-2H3-b, which are of rat origin.

To further confirm the production of mediators by these rodent mast cell models, ELISA analysis was utilized (Fig. 5). RBL-2H3-a was chosen for this analysis due to its more robust activation in response to both A23187 and IgE/Ag. BMMC-F, RBL-2H3-a and MC/9 all produced TNF when activated with A23187 or IgE/Ag, with A23187-activated MC/9 producing the most (Fig. 5A). All three cell types produced relatively large quantities of CCL2, however, RBL-2H3-a and MC/9 produced large amounts of basal CCL2 in unstimulated cells, while BMMC-F only produced CCL2 in response to A23187 or IgE/Ag activation. (Fig. 5B). Only BMMC-F and MC/9 produced IL-6 upon activation with either A23187 or IgE/Ag and RBL-2H3-a did not produce any detectable IL-6 (Fig. 5C). Similarly, both BMMC-F and MC/9 produced IL-4 when activated with A23187 but RBL-2H3-a did not produce IL-4 (Fig. 5D). BMMC and MC/9 also produced IL-13 when they were stimulated with A23187 but relatively small amounts when they were activated with IgE/Ag, however, MC/9 produced significant basal

amounts of IL-13 in unstimulated cells. RBL-2H3-a also produced basal IL-13 in unstimulated cells however, IL-13 release did not increase in response to either A23187 or IgE/Ag (Fig. 5E).

**SYK and ERK Signaling**

To compare signaling between the different cell types, we analyzed phosphorylation of SYK (pSYK), a membrane proximal signaling event activated by crosslinking of FcεRI, and phosphorylation of ERK (pERK), a more distal signaling event that occurs in response to FcεRI crosslinking but can also be activated by other stimuli (Fig. 6). When activated with IgE/Ag, BMMC-F rapidly phosphorylates both SYK and ERK, with maximum activation of both molecules already at 5 min post-activation (Fig. 6A, D, E and F) and sustained pERK signal until at least 30 min post-activation. RBL-2H3-a show a similarly fast response to antigen activation, but activation is sustained for 30 minutes and, pERK diminishes quickly and is almost completely dephosphorylated 30 min post-activation (Fig. 6B, D, E and F). MC/9 show the weakest pSYK response with a similar activation time of 5 minutes that rapidly declines, but the most robust pERK response of all cell types at 5 minutes that steadily declines (Fig. 5 C, D, E and F).

**Effects of Age on degranulation, TNF production and biomarker expression**

Another anecdotal observation that we made throughout our analyses was that older cultures of these cell types appeared to respond differently than their younger counterparts. As a result, the activation of young (more than 4 weeks but less than 5 weeks in culture) and old (greater than 9 weeks in culture) BMMC-F were compared. Old

BMMC-F degranulated similarly to young BMMC-F when they were activated by A23187, but old BMMC degranulated significantly less when activated with IgE/Ag (Fig. 7A). Similarly, old BMMC-F released significantly less TNF when activated with either A23187 or IgE/Ag (Fig. 7B). Paradoxically, older BMMC expressed more FcεRI and CD117 (Fig. 7C) in terms of mean fluorescent intensity (MFI) but not in terms of percent positive cells – since the percent of cells positive for FcεRI decreased approximately 7.5% with age (compare Fig. 1B and 7C) Higher passages of RBL-2H3-b degranulate normally when activated with A23187 but degranulate significantly less when activated with IgE/Ag (Fig. 7D), produced higher amounts of TNF in response to A23187 compared to young RBL-2H3-b (Fig. 7E) and again FcεRI expression increased significantly (Fig. 7F). By comparison, MC/9 have the most stable phenotype since they do not degranulate no matter their age (Fig. 7G), their expression of FcεRI and CD117 increased with age (Fig. 7I), and they released significantly more TNF when treated with either stimulus at more than 9 weeks post thaw (Fig. 7H).

**RBL-2H3 subcultures have different responses**

Throughout the analyses above, two different sources of RBL-2H3 were used: one that was purchased from ATCC (RBL-2H3-a) and another that was obtained from a long-term culture that had been maintained by collaborators at the University of Alberta for more than 20 years (RBL-2H3-b). We noticed a difference in their responses to A23187 and IgE/Ag that was passage number dependent. Routine mycoplasma checks were performed on all cell types, and all cell cultures were mycoplasma-free (Supplementary Fig. 3). Throughout the study, we have indicated the subculture and age of the RBL-2H3 cells used

to generate the data. However, to compare the two subcultures more accurately, we simultaneously cultured them at the same passage number and activated with A23187 or IgE/Ag side-by-side in the same analysis and assessed degranulation and TNF release at passage number > 11 (Fig. 8A and B). When activated with A23187, both subcultures degranulated similarly however, when activated with IgE/Ag, RBL-2H3-a degranulated more robustly (~44%) compared to RBL-2H3-b (~8%). Similarly, when activated with IgE/Ag, RBL-2H3-a produced significantly more TNF compared to RBL-2H3-b which did not produce any detectable TNF (Fig. 8B). This data shows that RBL-2H3-a are much more responsive to both A23187 and IgE/Ag at passage numbers higher than 11. These data directed us to analyze differences in FcεRI expression at < passage 11 and > passage 11 in both subtypes. Our data show that both RBL-2H3 subtypes lack CD117 surface expression and increase FcεRI expression past passage 11 (Fig. 8C and 8E). We also investigated the effects of passage number on histamine content of RBL-2H3-a and RBL-2H3-b and found that the histamine content of RBL-2H3-b was initially higher relative to RBL-2H3-a but significantly decreased past passage 11 while RBL-2H3-a histamine content remained stable throughout the time of our analysis (Fig. 8D and 8F).

**Effects of sex on signalling, biomarker expression, degranulation, and mediator production,**

The influence of sex on mast cell function has not been adequately addressed, particularly in terms of signaling, biomarker expression and mediator release. BMMC cultured from male (BMMC-M) and female (BMMC-F) C57BL/6 mice exhibited notable differences in SYK phosphorylation with BMMC-M maintaining a more robust phosphorylation for up

to 15 minutes, while female derived BMMCs steadily decline 5 minutes post activation (Fig. 9A, B and C). Both sexes of BMMC activated with A23187 or IgE/Ag degranulated similarly, although BMMC-M appeared to degranulate at a slightly higher rate in response to A23187 (Fig. 9F). Both BMMC-M and BMMC-F expressed similar levels of FcεRI and CD117 with cells being ~95% positive for both at 4 week of age, and both BMMC sexes significantly declined in expression of FcεRI and CD117 at 15 weeks of age (Fig. 9H). Analysis of mediator release showed that BMMC-M release higher levels of IL-4 and IL-6 when stimulated with A23187 and higher levels of TNF, IL-6 CCL2, and IL-13 when stimulated with IgE/Ag relative to BMMC-F (Fig. 9 G, I, J, K, and L).

**DISCUSSION**

Although BMMC, RBL-2H3 and MC/9 have been used in hundreds of published studies over the past few decades, their responses to stimuli and release of mediators has been very diverse. Additionally, the influence of age and biological sex on BMMC behaviour has not been adequately analyzed. Our data indicates five important novel observations; (1) BMMC and RBL-2H3-b cultures decrease in their responses to IgE/Ag as they age, (2) RBL-2H3 and MC/9, despite being cell lines, require several weeks in culture to fully differentiate after removal from cryopreservation (3) RBL-2H3 cultured in different laboratories acquire slightly different phenotypes, (4) the sex of the donor bone marrow has a small, but measurable, effect on the responses of BMMC, and (5) BMMC and MC/9 more accurately represent a MMC model than RBL-2H3. Our data also confirms an observation made several times over the past few decades: RBL-2H3 are an imprecise model of mast cell function[23] and should not be referred to as a “mast cell line”.

Our first observation is that BMMC and RBL-2H3-b cultures decrease in their responses to IgE/Ag as they age. It has been anecdotally observed by several labs that BMMC "age out" over time and no longer respond well to IgE and antigen stimulation. For this reason, many labs use these cultures within 4-8 weeks of culture, although many studies do not clearly state the age of their BMMC cultures in their methods section. We have observed that as BMMC age, they lose their responsiveness to antigen (degranulation and TNF release) and A23187 (TNF release) and that this decrease in responsiveness becomes significant after about 9 weeks in culture. This change is not associated with a decrease in FcεRI expression since FcεRI appears to increase slightly, in terms of MFI, with culture (Fig. 7C). A similar effect is observed for RBL-2H3-b, which decrease in their degranulation response when activated with antigen after 11 passages but retain their degranulation response to A23187. Interestingly, their production of TNF in response to A23187 increases after passage 11 as does their expression of FcεRI (Fig. 7D, E and F). MC/9 increase their production of TNF in response to A23187 and antigen stimulation after 9 weeks in culture (Fig. 7H) and this is associated with an increase in FcεRI expression. These data suggest that prolonged culture causes increases in FcεRI expression but, in the context of RBL-2H3-b, perhaps a decrease in the signaling machinery that controls release after FcεRI crosslinking. Certainly, the granule content of the cells does not appear to be affected (or may in fact be increased) since the BMMC and RBL-2H3 degranulation response to A23187 is not affected by age. It could be hypothesized that prolonged exposure to IL-3 or serum factors may cause this increase in FcεRI expression, but since this increase in FcεRI also

occurs in RBL-2H3 it is more likely that serum factors from FBS are mediating this effect.

Our second observation is that RBL-2H3 cultured in different laboratories acquire slightly different phenotypes. We compared RBL-2H3-a that were purchased directly from ATCC and RBL-2H3-b which are a long-standing culture at our university that have been passed between laboratories and the exact origin and date of acquisition of these cells is no longer known. The RBL-2H3-a cell culture remains fairly stable with age, whereas the RBL-2H3-b showed decreased responses to antigen as described above. Interestingly, the RBL-2H3-b cells contained more histamine at the start of culture than the RBL-2H3-a, but this decreased for both cells after 11 passages albeit more significantly in RBL-2H3-b (Fig. 8D and F). Both cells showed very low expression of FcεRI below 11 passages and no detectable expression of CD117, and both cells showed a slight increase in FcεRI and maintained no detectable expression of CD117, after 11 passages. The RBL-2H3-b cells express slightly more FcεRI than RBL-2H3-a at the start of culture (passage 4). This low expression of FcεRI was surprising but could be due to the use of mouse-specific antibodies, and although these antibodies have been routinely used by others to characterize FcεRI in RBL-2H3 [24,25] they may not have the same specificity for the rat-derived RBL-2H3 as they do for the BMMC and MC/9. Overall, the RBL-2H3-b appear to become less stable in terms of their responsiveness to antigen within a small number of passages.

Our third observation was that the sex of the donor bone marrow has a small, but measurable, effect on the responses of BMMC. BMMC-M appeared to sustain SYK phosphorylation for longer (Fig. 9B), degranulate slightly more when stimulated with

A23187 (Fig. 9F) and release more TNF when stimulated with antigen (Fig 9G). FcεRI and CD117 expression in BMMC-F and BMMC-M appeared similar at 5 weeks in culture, but it appeared that BMMC-M lost FcεRI expression, more readily than BMMC-F after 15 weeks in culture (Fig. 9H). The most pronounced differences between BMMC-M and BMMC-F appeared in the amount of cytokines produced upon antigen stimulation since BMMC-M appeared to make significantly more IL-6, CCL2 and IL-13 compared to BMMC-F (Fig. 9I, J, K and L).

Thus, our data suggest that an often under-reported and overlooked factor, the genetic sex of the donor bone marrow, may play an integral role in the phenotypic outcomes of BMMC. Most investigators use either male or female mice as a source of bone marrow or pool the bone marrow when generating their mast cell cultures. Some studies do not mention the genetic sex of their animal source – a feature common to most cell line research as well.[26,27] Our data suggests that although the differences are modest, there are some reproducible differences in terms of activation of male and female BMMC – which supports observations made in culture models of other primary cell types.[28–30] Since BMMC are cultured over several weeks, it is surprising that these differences persist for so long *ex vivo*. There may be several reasons for this persistence of phenotype, even after weeks in culture: (1) these cells are cultured in phenol red which has been shown to bind to estrogen receptors with a weak (Kd= $2 \times 10^{-5}$ M) but biologically significant affinity;[31,32] (2) the presence of sex hormones from fetal bovine serum (FBS) in the culture media;[33,34] (3) there are several sex-specific epigenetic DNA methylation modifications that can persist throughout differentiation, even contributing to the senescent clock;[35] and (4) genetic sex can influence several biological processes,

including extracellular vesicle production[36] which may influence response to activation. Understanding the influence of biological sex on mast cell function is crucial but the sex of these cells is rarely examined and lightly considered.

We conclude that BMMC are perhaps the best in vitro MC model in the present study since the cells are approximately 99% FcεRI$^{+}$/CD1117$^{+}$, display metachromatic granularity with toluidine blue staining, express proteases typical of CTMC, contain histamine, and are activated when their FcεRI receptors are crosslinked with antigen. However, although BMMC are a convenient MC model, they are phenotypically unstable after several weeks in culture such that they lose FcεRI expression and their responsiveness to IgE/Ag and A23187 diminishes over time. RBL-2H3 are the least accurate model of mast cells and although they are often used in research applications to represent mast cell responses, they should not be referred to as a mast cell line but a basophil cell line. Although they contain the most histamine of all the cell lines tested in our study (Suppl. Fig. 1), and stain metachromatically with toluidine blue, RBL-2H3 express only minimal mast cell proteases (Mcpt1 and Mcpt2 and CPA3) and do not express CD117. Additionally, RBL-2H3 produce minimal amounts of ROS when activated with A23187 or antigen (Fig. 2) and thus are not a very good model for ROS-related research.

The MC/9 are a more accurate model of mast cell phenotype since they express FcεRI, CD117, mast cell-specific proteases (Mcpt 1, 2, 5, 6, 7 and CPA3) and substantial amounts of histamine. Yet they also do not produce substantial amounts of ROS when activated with either A23187 or antigen (Fig. 2), do not degranulate (Fig. 3) and do not stain with toluidine blue, suggesting the absence of proteoglycan-containing granules.

This is likely why MC/9 do not release preformed mediators such as β-hexosaminidase when stimulated with either A23187 or antigen. Since MC/9 proliferate even more rapidly than RBL-2H3 (Fig. 1), they may be a useful model in situations where extremely large amounts of cells are required for analysis such as proteomic or metabolomics analyses. Certainly, MC/9 activate SYK and ERK signaling pathways similarly to BMMC when stimulated with A23187 (Fig. 6). MC/9 also produce significant amounts of various proinflammatory mediators (Suppl. Fig. 2 and Fig. 5), which makes them an appropriate model in which large amounts of these cytokines are required – for example, in coculture analyses. MC/9 produce very significant amounts of IL-1β, IL-10, IL-13 and IP-10. However, since BMMC do not produce these cytokines (or produce very small amounts), it is questionable whether MC/9 are an appropriate model of mast cell cytokine production. Overall, MC/9 are not an ideal model of mast cell phenotype or function, but they maintain a relatively stable phenotype and proliferate quickly which may be required in some experiments.

In conclusion, our data provides a novel and comprehensive comparison of BMMC, RBL-2H3 and MC/9 as models of mast cells and insight into the importance of commonly unreported factors such as origin, sex, and age and, serves to identify potential causes of phenotypic variability amongst research groups.

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## Legends to Figures

Figure 1.

Figure 2.

Figure 3

Figure 4.

Figure 5.

Figure 6.