Original Article
Allergy Asthma Immunol Res. 2018 May;10(3):268-277.
https://doi.org/10.4168/aair.2018.10.3.268
pISSN 2092-7355 • eISSN 2092-7363

AAIR
Allergy, Asthma & Immunology Research

Immune Characterization of Bone Marrow-Derived Models of Mucosal and Connective Tissue Mast Cells

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Purpose: It is well appreciated that mast cells (MCs) demonstrate tissue-specific imprinting, with different biochemical and functional properties between connective tissue MCs (CTMCs) and mucosal MCs (MMCs). Although *in vitro* systems have been developed to model these different subsets, there has been limited investigation into the functional characteristics of the 2 major MC subsets. Here, we report the immunologic characterization of 2 MCs subsets developed *in vitro* from bone marrow progenitors modeling MMCs and CTMCs. **Methods:** We grew bone marrow for 4 weeks in the presence of transforming growth factor (TGF)-β, interleukin (IL)-9, IL-3, and stem cell factor (SCF) to generate MMCs, and IL-4, IL-3, and SCF to generate CTMCs. **Results:** CTMCs and MMCs differed in growth rate and protease content, but their immune characteristics were remarkably similar. Both subsets responded to immunoglobulin E (IgE)-mediated activation with signaling, degranulation, and inflammatory cytokine release, although differences between subsets were noted in IL-10. CTMCs and MMCs showed a similar toll-like receptor (TLR) expression profile, dominated by expression of TLR4, TLR6, or both subsets were responsive to lipopolysaccharide (LPS), but not poly(I:C). CTMCs and MMCs express receptors for IL-33 and thymic stromal lymphopoietin (TSLP), and respond to these cytokines alone or with modified activation in response to IgE cross-linking. **Conclusions:** The results of this paper show the immunologic characterization of bone marrow-derived MMCs and CTMCs, providing useful protocols for *in vitro* modeling of MC subsets.

Key Words: Mucosal mast cells; connective tissue mast cells; mast cell subsets

INTRODUCTION

Mast cells (MCs) play a key role in food allergy.[1-3] They also play an important role in host defense and have unique protective activity against toxins and venoms.[4] They are derived from hematopoietic stem cells, which give rise to MC progenitors that circulate in the blood and enter the tissues, where they undergo differentiation and maturation to become mature MCs. The different microenvironments found in tissues modulate the morphology and features of MCs, and therefore specific subpopulations are observed in distinct tissues.[5] Mouse MCs are classified based on their anatomic location into 2 groups, mucosal MCs (MMCs) and connective tissue MCs (CTMCs).[6] In humans, tissue distribution is not as clearly demarcated as in rodents.[7] Most human tissues have a mixed population of MC types that are distinguished on the basis of their protease composition. Tryptase-only MCs are located predominantly in the alveolar wall and gastric mucosa, similar to MMCs in rodents. Chymase-only MCs, or both tryptase- and chymase-positive MCs are located predominantly in the skin and intestinal submucosa like CTMCs in rodents. For all subsets, recent evidence suggests that the expression of their secretory granule proteases is directed by the local tissue in which the cells reside.[8] It is also known that they differ in their amine content as well as in some of their functional properties,[6] but the biological implications of these differences are still poorly understood.

MC knockout mice and MC knockin approaches, as well as other unique humanized mouse models, have been developed to study MC functions *in vivo*.[9,10] There is also a need for reductionist model systems that can be used for mechanistic studies or drug screening. Isolation of tissue MCs from the gastrointestinal tract,[11] skin and lung,[12] or peritoneal cavity[13] have been described, but the low rates of isolated cells as well as the high sample handling make these techniques burdensome and difficult

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Received: August 18, 2017; Revised: December 8, 2017;
Accepted: December 12, 2017
• There are no financial or other issues that might lead to conflict of interest.

to scale up. A number of systems have been developed which allow investigators to readily obtain sufficient quantities of MCs generated from progenitors derived from bone marrow, peripheral blood, or cord blood.[13-15] Using different growth factors that mimic the microenvironments of different tissues enables differentiation of progenitors into different subsets of MCs. Here, we present the characterization of 2 *in vitro* models of MCs derived from bone marrow that mimic mucosal and connective tissue subsets. Moreover, we describe their response to immunoglobulin E (IgE)-dependent and -independent activation.

MATERIALS AND METHODS

Mice

Balb/c and C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA, USA). All animal procedures were approved by the Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai (approval number LA11-00273).

Bone marrow–derived MCs

Bone marrow cells were collected from femurs from 4-to-8-month-old mice and cultured in Dulbecco's Modified Eagle Medium with glucose and L-glutamine, supplemented with 10% fetal bovine serum, penicillin/streptomycin, and sodium pyruvate (all from Gibco™, ThermoFisher Scientific, Waltham, MA, USA) plus 25 ng/mL recombinant murine stem cell factor (SCF) (all cytokines and growth factors were from Peprotech, Rocky Hill, NJ, USA) and 30 ng/mL interleukin (IL)-3. Bone marrow MCs were differentiated into either a MMCs with additional IL-9 at 5 ng/mL and transforming growth factor (TGF)-β at 1 ng/mL or a CTMC with IL-4 at 1 ng/mL. MCs were cultured in 75-cm^2 tissue culture flasks, incubated at 37°C in a humidified incubator under 5% (v/v) CO_2 for a minimum of 4 weeks and up to 8 weeks before they were used for functional assays. Twice a week, the medium was changed by transferring the cell suspension to a 50-mL conical polypropylene centrifuge tube, and centrifuging for 10 minutes at 200×*g*, at room temperature. The culture flasks were changed every time the medium was changed. The maturity and purity of the cells were examined by flow cytometric analysis for the expression of c-Kit (eBioscience, San Diego, CA, USA) and FcεRI (Biolegend, San Diego, CA, USA).

Real-time polymerase chain reaction (RT-PCR)

RT-PCR was performed starting from 1 μg of total RNA, using SuperScript II reverse transcriptase (Invitrogen™, ThermoFisher Scientific). Then, cDNA was amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems™, ThermoFisher Scientific) and run on CFX384 Touch™ real-time PCR detection system (Bio Rad, Hercules, CA, USA), using the primers described in Table 1. β-Actin was used as the housekeeping gene. Relative expression that refers to data normalized to the housekeeping gene or fold increase compared with levels measured in non-activated cells by using ΔΔCT threshold cycle method of calculation was used to represent the data. All amplifications were carried out in triplicate.

Table 1. List of primers for RT-PCR

Gene	Forward sequence 5′-3′	Reverse primer 5′-3′
β-actin	GTGGGCCGCTCTAGGCACCAA	CTCTTTGATGTCACGCACGATTTC
MMCP-1	CAGATGTGGTGGGTTTCTCA	GCTCACATCATGAGCTCCAA
MMCP-2	AGGCCCTACTATTCCTGATGG	ATGTAAGGACGGGAGTGTGG
MMCP-4	GCTACCTGTGGTGGGTTTCT	TCACATCATGAGCTCCAAGG
MMCP-5	CAGGCCCTGGATCAATAAGA	GGCACACAAAACCTGCACTA
MMCP-6	CTATCCAGGGTCAGGCAAGA	GACAGGGGAGACAGAGGACA
MMCP-7	GACCCCAACAAGGTCAGAGT	TGTAGAAGTCGGGGTGTGTG
nMC-CPA	TCCAGGAACCAAAACTCCAC	CATTGAGGCATGGTTTGTG
IL-3	ATAGGGAAGCTCCCAGAACC	TTTCCACGAATTTGGACAGG
IL-4	CTCGTCTGTAGGGCTTCCAA	TCTGCAGCTCCATGAGAACA
IL-6	TTGCCTTCTTGGGACTGATG	ACAGGTCTGTTGGGAGTGGT
IL-10	TGCTATGCTGCCTGCTCTTA	TCATTTCCGATAAGGCTTGG
IL-13	CCATCCCATCCCTACAGAAA	GAAATGTGCTCAAGCTGCTG
IL-17	TCTCTGATGCTGTTGCTGCT	AGTCCTTGGCCTCAGTGTTT
IL-33	ATCGGGTACCAAGCATGAAG	GTCAACAGACGCAGCAAATG
IFN-γ	GCTTTAACAGCAGGCCAGAC	GGAAGCACCAGGTGTCAAGT
TNF-α	CAAAGGGAGAGTGGTCAGGT	GCACCTCAGGGAAGAATCTG
TSLP	GTCACTGCCATGATGAGGTG	CTGGGTCTGAACCCTTTGAC
TLR-1	GGATTTGTCCCACAATGAGC	TATAGGCAGGGCATCAAAGG
TLR-2	CATCGCTTTTTCCCAATCTC	GAAGTCAGCCCAGCAAAATC
TLR-3	TCGGATTCTTGGTTTCAAGG	TTCCCAGACCCAGTCTCTGT
TLR-4	AATGCCCTATTGGATGGAAA	AGGCCCCAGAGTTTTGTTCT
TLR-5	AGTCCTGGAGCCTGTGTTGT	GAGATGAGGCGTCTGGAGAG
TLR-6	TGCCTCCATGAGAGGAACTT	GGGGAGACAGCACAAAGATG
TLR-7	TGCTGTGTGGTTTGTCTGGT	TTGACCTTTGTGTGCTCCTG
TLR-8	TGGCCAGAAGACAAAACAAA	GCCCACCTTTTCCTATCTCC
TLR-9	TGGCTATGCGTACACTGGAG	GTGTGGCTCAGGCTCAGATT
TLR-11	TCCACTTGCATTTCCTCTCC	TTTTTCCCAAGGTCAAGTGC
TLR-12	CCTGCATTGACCACCCTTAG	GCTTCAGGCTCAAGGTATGC
TLR-13	TGTTCCGAGCAACTTTTTCC	CAGAGGGTCAAATTGGTGGT

Flow cytometry

Cells stained with live/dead fixable blue dead cell staining kit (ThermoFisher Scientific) were blocked with anti-CD16/32 antibody (eBioscience) and stained with specified antibodies. For intracellular staining, cells were fixed and permeabilized with fixation/permeabilization working solution (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Antibodies used included those recognizing CD107a, c-kit, phospho-Syk, ST2, CD127 (eBioscience), FcεRI (Biolegend), and thymic stromal lymphopoietin receptor (TSLPR; R&D Systems, Minneapolis, MN, USA). Cells were acquired on a BD LSR Fortessa cytometer (BD Biosciences). Data was analyzed using the Flow-

Jo software (TreeStar Inc., Ashland, OR, USA).

MC staining

MCs were cytospun onto glass slides at 150 rpm for 5 minutes and stained with toluidine blue according to Kovarova [16] or chloroacetate esterase according to Friend *et al.* [17].

MC stimulation

For activation through cross-linking of the IgE receptor, MCs were initially sensitized for 1 hour with 1 μg/mL monoclonal mouse anti-2,4-dinitrophenol (DNP) IgE antibody (Sigma-Aldrich, St. Louis, MO, USA) or 100 ng/mL purified mouse IgE antibody (BD Biosciences) in complete media without exogenous cytokines. After washing by centrifugation at 200×*g* for 10 minutes, MCs were suspended at 1×10^6 cells/mL (5×10^5 cells/mL for N-acetyl-β-D-hexosaminidase release assay) and activated with 1 μg/mL rat anti-mouse IgE antibody (BD Biosciences) or 100 ng/mL DNP-HSA (Sigma-Aldrich). In some experiments, IL-33 or thymic stromal lymphopoietin (TSLP) (both from Peprotech) were added to the culture medium at 100 ng/mL. Lipopolysaccharide (LPS), Poly(I:C) (both from InvivoGen, San Diego, CA, USA) and Compound 48/80 (Sigma-Aldrich), were added to the culture at doses indicated prior to supernatant harvest after 24 hours.

N-acetyl-β-D-hexosaminidase release assay

For detection of the granular enzyme β-hexosaminidase, an enzymatic colorimetric assay was used as previously described.[18] Briefly, after activation of 100 μL of MCs in HEPES degranulation buffer, 50 μL of supernatant was transferred to a 96-well plate and mixed with 100 μL of substrate solution (3.5 mg/mL p-nitrophenyl-N-acetyl-β-D-glucosaminide dissolved in 40 mM citric

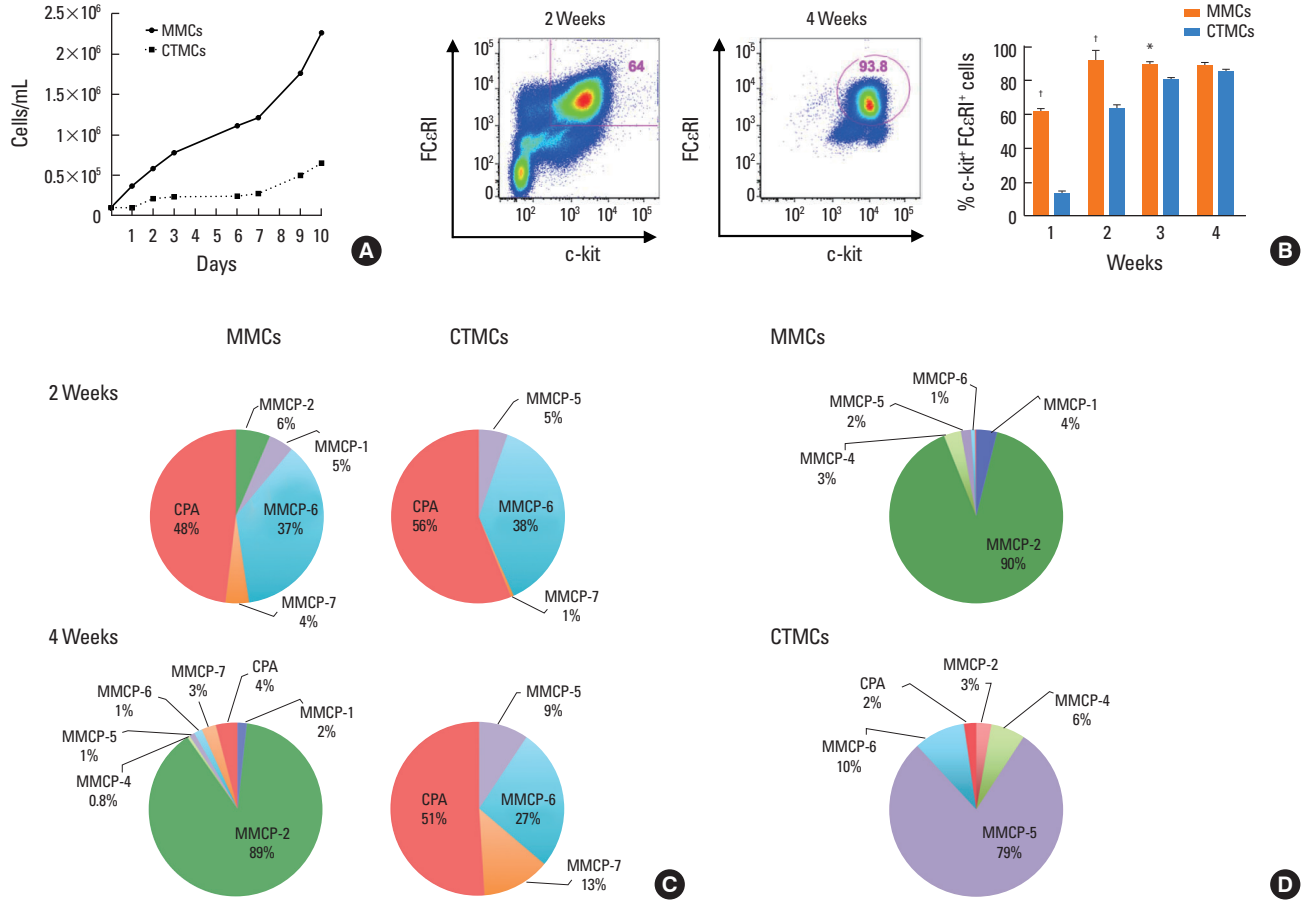

Fig. 1. Maturation and differentiation of MMCs and CTMCs from bone marrow progenitors. (A) Growth rates of MMCs and CTMCs. (B) Representative staining of FcεRI and c-kit expression after 2 or 4 weeks of culture with growth factors (left), and percentage of FcεRI+/c-kit+ cells after 1, 2, 3, or 4 weeks of culture with growth factors. (C) RT-PCR for MMCP-1, MMCP-2, MMCP-4, MMCP-5, MMCP-6, MMCP-7, MMCP-8, and CPA in MMCs and CTMCs after 2 or 4 weeks of culture of bone marrow progenitors from Balb/c mice with growth factors. Relative expression refers to data normalized to the housekeeping gene. Data are expressed has the percentage distribution of the total protease expression. Data represent the mean of 3 different experiments. (D) As in C, but using bone marrow from C57BL/6 mice and analyzed at 4 weeks. MMC, mucosal mast cell; CTMC, connective tissue mast cell; RT-PCR, real-time polymerase chain reaction; MMCP, mouse mast cell protease; CPA, carboxypeptidase. **P*<0.05; †*P*<0.0001.

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acid, pH 4.5). The remaining cells (50 μL) were lysed with 150 μL of 0.1% triton X-100 and the same procedure was done. The mixtures were incubated at 37°C for 90 minutes. After incubation, 100 μL of glycine (400 mM, pH 10.7) was added to each well, and the absorbance at 405 nm was measured using a POLARstar Microplate Reader (BMG LabTech, Ortenberg, Germany). The percentage of β-hexosaminidase release was calculated as a percentage of the total β-hexosaminidase content as follows:

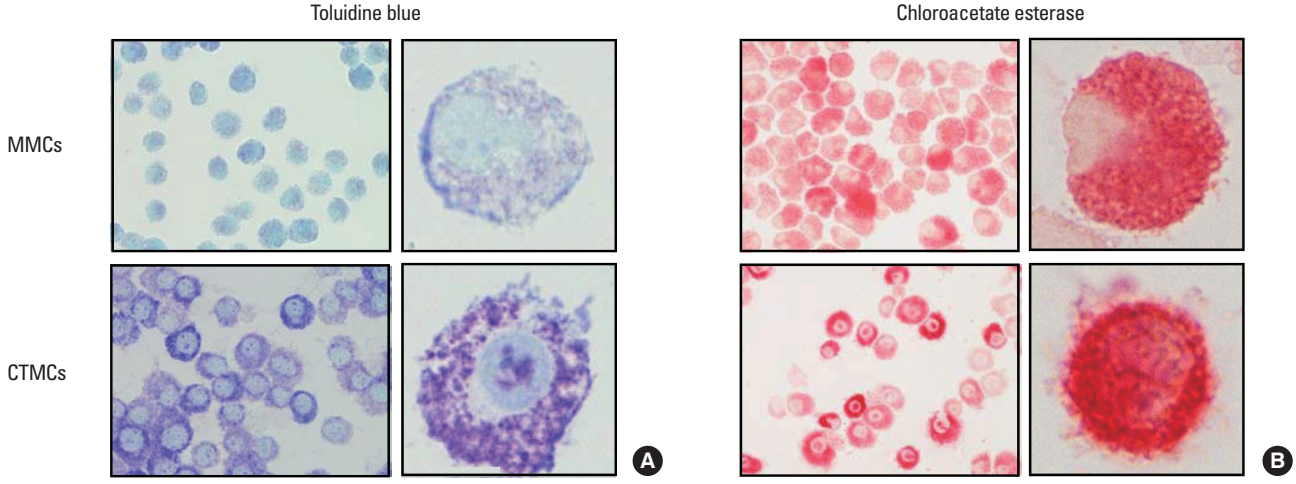

Fig. 2. Metachromatic staining of MMCs and CTMCs with toluidine blue or the chloroacetate esterase dyes. (A) Metachromatic staining with toluidine blue or (B) choroacetate esterase. Cytospins of MCs were prepared at 150 rpm for 5 minutes. Images are representative of 3 samples per condition. Data correspond to bone marrow-derived MCs obtained from Balb/c mice. MMC, mucosal mast cell; CTMC, connective tissue mast cell.

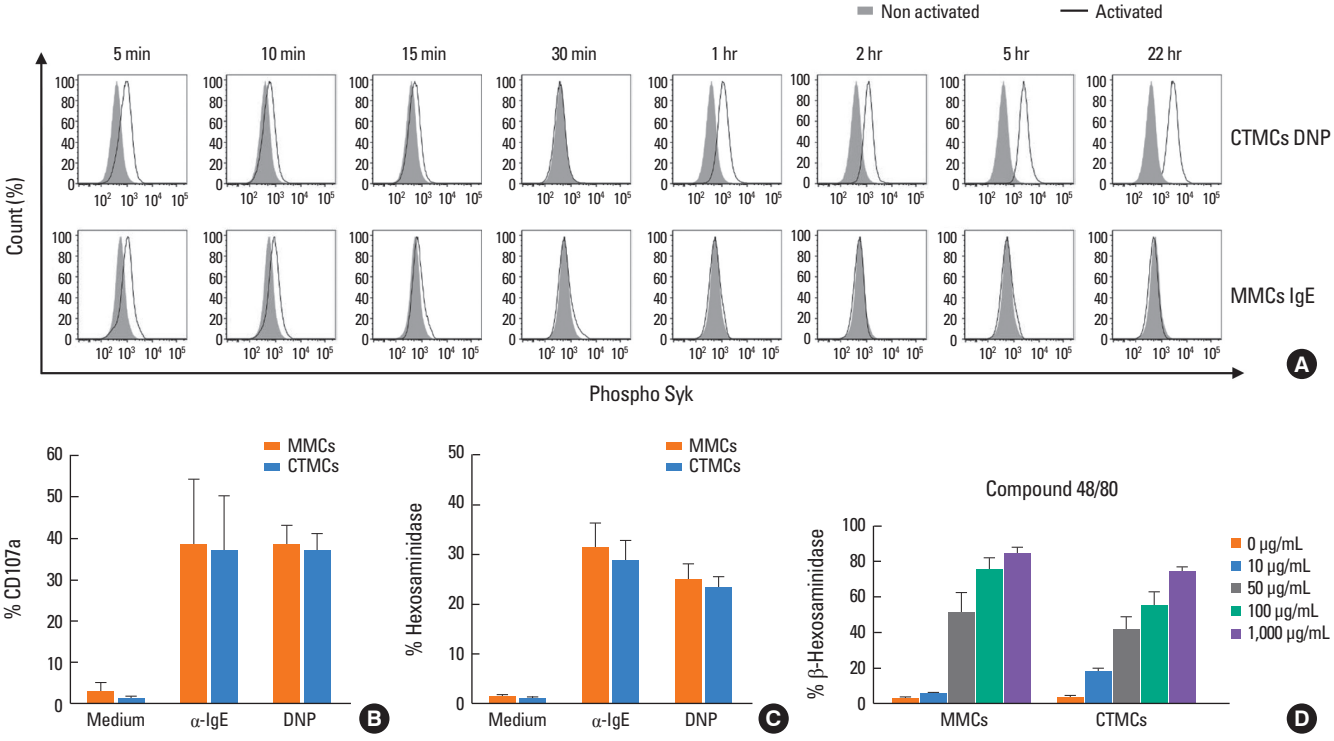

Fig. 3. Activation of MMCs and CTMCs after stimulation through FcεRI. (A) Intracellular expression of phospho-Syk, (B) surface expression of CD107a, and (C) degranulation, measured as percentage of β-hexosaminidase secretion, in sensitized MMCs and CTMCs with mouse IgE or anti-DNP IgE after 30 minutes of activation with α-IgE or DNP-HSA. (D) Percentage of β-hexosaminidase secreted by MMCs and CTMCs after stimulation with compound 48/80. Data correspond to bone marrow-derived MCs obtained from Balb/c mice. MMC, mucosal mast cell; CTMC, connective tissue mast cell; IgE, immunoglobulin E; DNP, 2,4-dinitrophenol; DNP-HAS, dinitrophenylated human serum albumin; MC, mast cell.

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

Enzyme immunoassays

Supernatants of cultured cells were collected 2 or 24 hours after addition of stimuli, IL-4, IL-6, IL-10, IL-13, IL-17, IL-33, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and TSLP were measured by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (eBioscience).

Statistical analysis

All statistical analyses were performed using GraphPad Prism software version 7.0e (GraphPad Software, Inc., La Jolla, CA, USA). Two-tailed Student's *t* test, 1-way analysis of variance (ANOVA) or 2-way ANOVA were used for determining statistical significance ($P<0.05$). Results are expressed as mean±standard error of the mean.

RESULTS

Differentiation and maturation of bone marrow–derived MCs

MMCs from Balb/c mice were differentiated with SCF, IL-3, TGF-β, and IL-9, while CTMCs were differentiated in the presence of SCF, IL-3, and IL-4. We observed a relatively slow growth rate in CTMCs compare with MMCs, probably due to the presence of IL-9 in the media which promotes MCs proliferation (Fig. 1A). Expression of c-kit and FcεRI occurred more rapidly in MMCs with approximately 92% of cells being double positive after 2 weeks of culture, while CTMCs did not reach double expression until the third week (Fig. 1B). At 2 weeks of culture, both subsets showed a similar protease profile, predominantly expressing mouse mast cell protease (MMCP)-6 and carboxypeptidase (CPA) (Fig. 1C). After 4 weeks of culture, the protease profile became distinct and MMCs had a dominant expression of MMCP-2 and also expressed MMCP-1, while CTMCs expressed elevated levels of MMCP-5, MMCP-6, MMCP-7, and CPA. As shown in Fig. 1D, bone marrow from C57BL/6 mice showed a similar skewing of MMCs with dominant expression of MMCP-2 and MMCP-1, while CTMC were more highly skewed toward MMCP-5 expression.

MMCs and CTMCs were stained using toluidine blue or chloroacetate esterase. CTMCs, but not MMCs, demonstrated strong

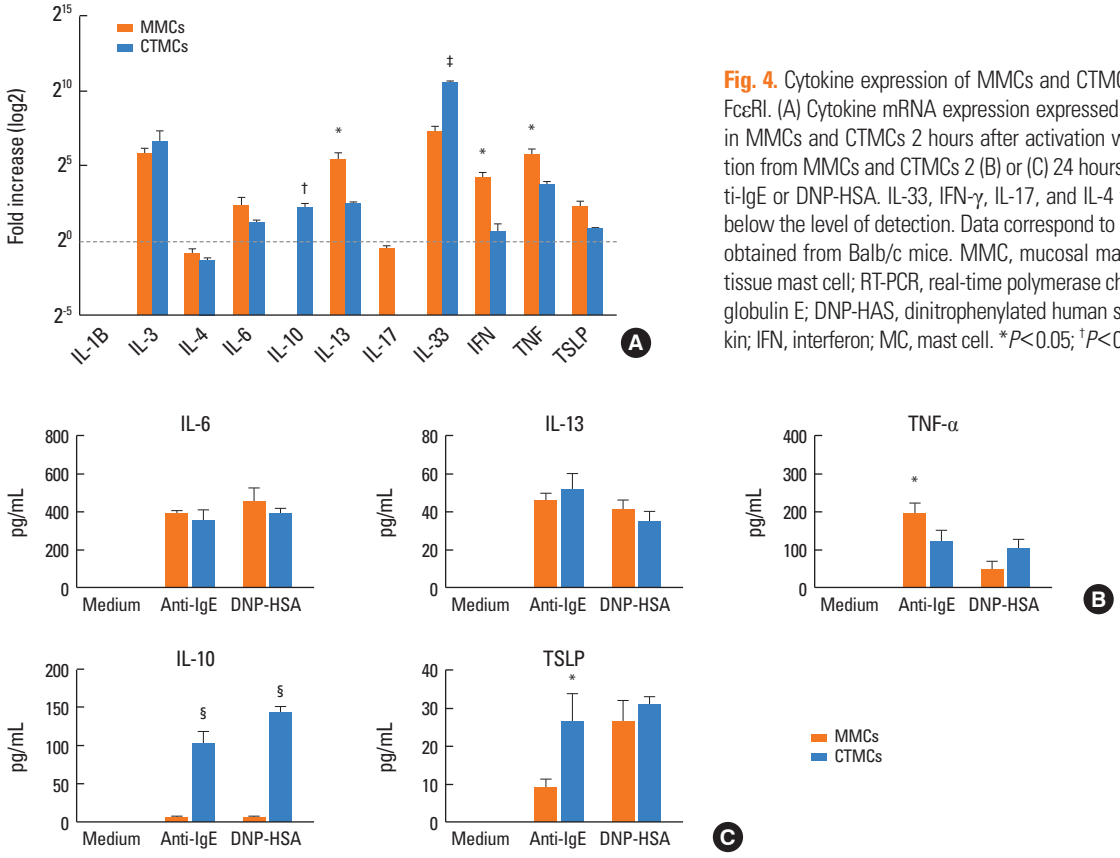

Fig. 4. Cytokine expression of MMCs and CTMCs after activation through FcεRI. (A) Cytokine mRNA expression expressed as fold change by RT-PCR in MMCs and CTMCs 2 hours after activation with α-IgE. Cytokine secretion from MMCs and CTMCs 2 (B) or (C) 24 hours after stimulation with anti-IgE or DNP-HSA. IL-33, IFN-γ, IL-17, and IL-4 were measured, but were below the level of detection. Data correspond to bone marrow derived MCs obtained from Balb/c mice. MMC, mucosal mast cell; CTMC, connective tissue mast cell; RT-PCR, real-time polymerase chain reaction; IgE, immunoglobulin E; DNP-HAS, dinitrophenylated human serum albumin; IL, interleukin; IFN, interferon; MC, mast cell. *$P<0.05$; †$P<0.01$; ‡$P<0.001$; §$P<0.0001$.

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staining with toluidine blue. In contrast, both CTMCs and MMCs could be stained with chloroacetate esterase dye (Fig. 2A and B).

Activation, degranulation, and cytokine production of MMCs and CTMCs

MCs were sensitized either with polyclonal mouse IgE or anti-DNP IgE. Activation of MMCs and CTMCs with anti-IgE or specific antigen (dinitrophenylated human serum albumin [DNP-HSA]) resulted in an increase in intracellular phospho-Syk, surface expression of the granule protein LAMP-1 (CD107), and degranulation (β-hexosaminidase release) (Fig. 3A-C) in a dose-dependent manner (data not shown). When MCs were activated, the levels of phospho-Syk decreased to the levels of non-activated cells after 30 minutes from the addition of stimuli. Surprisingly, when MCs were activated using the system Anti-DNP/DNP-HAS (human serum albumin), but not IgE/anti-IgE, we observed a biphasic increase in phospho-Syk that increased again after 1 hour and was sustained even after 24 hours from initial activation (Fig. 3A). Similar responses to polyclonal or monoclonal activation were observed for MMCs and CTMCs. Compound 48/80 is a polymer that has been used to trigger MC degranulation in an IgE-independent manner and has been described as selective for CTMCs. MMCs and CTMCs degranulated after stimulation with compound 48/80 in a dose-dependent manner (Fig. 3D). CTMC were slightly more sensitive to 48/80, and degranulated at 10 μg/mL, while the threshold was 50 μg/mL for MMCs. However, at higher doses both CTMCs and MMCs responded to 48/80 with degranulation.

We examined cytokine mRNA expression and secretion. Two hours after activation, mRNA expression of IL-3, IL-6, IL-13, IL-33, IFN-γ (in MMCs), TNF-α, and TSLP was increased by IgE cross-linking. The levels of IL-13, IFN-γ, TNF-α, and TSLP were higher in MMCs than in CTMCs (Fig. 4A), while IL-33 expression was higher in CTMCs than in MMCs. Interestingly, IL-10 induction was only detected in CTMCs, but not MMCs. Two hours after activation, MMCs and CTMCs produced and secreted similar levels of IL-6, IL-13, and TNF-α (Fig. 4B). Twenty-four hours after activation, TSLP was detected in both MMCs

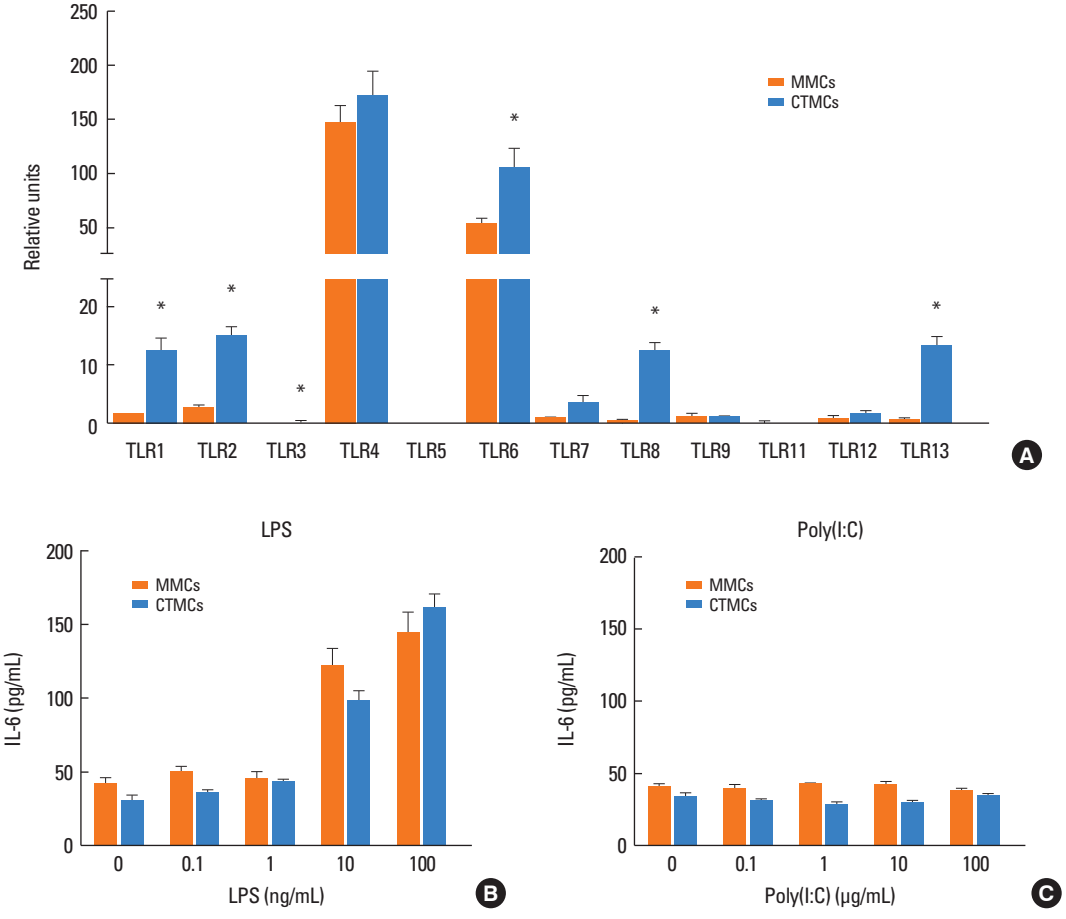

Fig. 5. TLRs on MMCs and CTMCs. (A) TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 expression by MMCs and CTMCs, expressed as relative expression compared to housekeeping gene. IL-6 secretion from MMCs and CTMCs after stimulation with a dose range of LPS (B) or poly(I:C) (C) for 24 hours in the absence of anti-IgE. Data correspond to bone marrow derived MCs obtained from Balb/c mice. TLR, toll-like receptor; MMC, mucosal mast cell; CTMC, connective tissue mast cell; IL, interleukin; LPS, lipopolysaccharide; IgE, immunoglobulin E; MC, mast cell. *$P<0.05$.

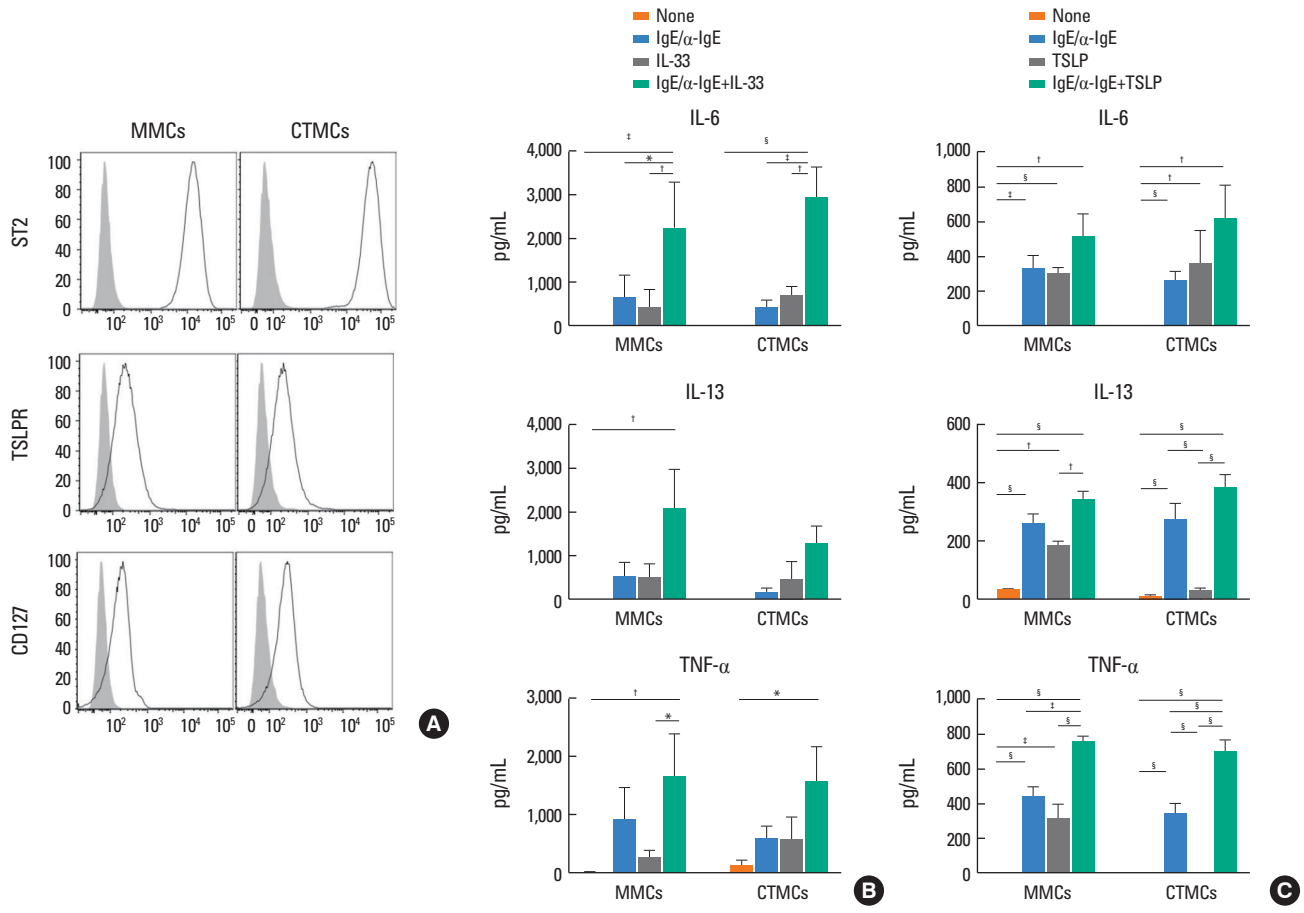

Fig. 6. Effect of IL-33 and TSLP on MMCs and CTMCs. (A) ST2, TSLPR, and CD127 expression by MMCs and CTMCs in the absence of anti-IgE stimulation. IL-6, IL-13, and TNF-α secretion by MMCs and CTMCs after 24 hours of stimulation with 100 ng/mL IL-33 (B) or TSLP (C), alone or in the presence of anti-IgE. Showed data correspond to bone marrow-derived MCs obtained from Balb/c mice. Note the difference in scale between (B) and (C). IL, interleukin; TSLP, thymic stromal lymphopoietin; MMC, mucosal mast cell; CTMC, connective tissue mast cell; TSLPR, thymic stromal lymphopoietin receptor; IgE, immunoglobulin E; TNF, tumor necrosis factor; MC, mast cell. *$P<0.05$; †$P<0.01$; ‡$P<0.001$; §$P<0.0001$.

and CTMCs, and IL-10 was detected in CTMC (Fig. 4C). The cytokines IL-3, IL-4, IL-17, IL-33, and IFN-γ were below the level of detection by ELISA in the supernatants of activated MCs.

Expression of toll-like receptors (TLRs) in MMCs and CTMCs

MCs have been described to express different numbers of TLRs, which can contribute to their host defense function. We examined expression of TLR1-TLR13 by RT-PCR (Fig. 5A). Relative expression of TLRs 4 and 6 dominated, with low level of other TLRs. CTMC had significantly higher levels of TLR1, TLR2, TLR3, TLR6, TLR8, and TLR13 compared to MMCs. Stimulation of CTMCs and MMCs with the TLR4 ligand LPS, but not the TLR3 ligand poly(I:C), resulted in activation shown by IL-6 secretion (Fig. 5B and C). Similar results were obtained from CTMCs and MMCs derived from C57BL/6 mice (not shown).

Effect of IL-33 and TSLP on MMCs and CTMCs

In addition to responding through IgE receptors and TLRs, MCs can also sense and respond to cytokines in the environment. We examined the response of MMCs and CTMCs to IL-33 and TSLP. MMCs and CTMCs express high levels of the IL-33 receptor ST2 and also express 2 components of the TSLPR and the IL-7R alpha chain (CD127) (Fig. 6A). IL-33 increased cytokine production by MMCs and CTMCs, alone or in combination with IgE cross-linking (Fig. 6B). Similarly, TSLP enhanced the production of IL-6, IL-13, and TNF-α at baseline and in combination with IgE cross-linking in MMCs (Fig. 6C). Similar results were obtained for IL-6 and IL-13 secretion by CTMCs after TSLP stimulation. TNF-α secretion by CTMCs was only increased by TSLP when it was applied in combination with IgE cross-linking.

DISCUSSION

In this manuscript, we provide a systematic characterization of the immune profile of bone marrow-derived models of MCs

representing mucosal and connective tissue sites. Our findings are presented as a useful toolkit to have appropriate reductionist model systems in order to study the cell biology of MCs.

MCs derive from mononuclear precursor cells and undergo their final phase of differentiation in tissues under the control of local tissue factors. MCs have been generated *in vitro* from mouse bone marrow, most commonly using recombinant IL-3 (or conditioned media from IL-3 -secreting cell lines). In 1993, Eklund *et al.*[19] described that the combination of SCF and the cytokine IL-9 could induce the expression of MMCP-1 and MMCP-2, 2 proteases associated with MMCs, while IL-4 suppressed the induction of these 2 proteases. Miller *et al.*[20] reported that TGF-β, a cytokine highly expressed in the intestinal microenvironment, also enhanced the expression of MMCP-1. Our goal was to use these approaches described for the generation of CTMCs and MMCs to describe and compare the functional characteristics of these 2 MC subsets. Although it has been described that the sensitivity of the MCs and surface density of FcεRI on MC surface is also influenced by IgE concentration in the culture medium,[21] we did not use it to avoid sensitization of the cells.

Consistent with the literature,[17,22,23] We show that culture of bone marrow cells with SCF, IL-3, and IL-4 for 4 weeks generates cells that express proteases compatible with CTMCs (MMCP-4, MMCP-5, MMCP-6, MMCP-7, and CPA), while growth of bone marrow cells with SCF, IL-3, TGF-β, and IL-9 generates cells that express proteases compatible with MMCs (MMCP-1 and MMCP-2). Moreover, we found MMCP-2 expression to be a good marker for MMCs, as it has previously been confirmed in murine tissues by immunohistochemistry.[5] This was true for both Balb/c and C57BL/6 mice, allowing for the use of widely available genetically modified mice. It should be noted that C57BL/6 mice have a naturally occurring mutation in MMCP-7, and this protease is not a useful biomarker of MC activation for this strain of mouse.[24] In humans, MCs are not classified based on their anatomic location, but the content of MC-specific proteases chymase and tryptase is the main criterion for MC subtypes,[25-27] being the human MCs that only express tryptase more likely MMCs and the human MCs that express chymase and tryptase like CTMCs.[28,29] The yield and rate of growth was substantially greater for cells grown under MMC conditions probably due to the presence of IL-9 in the media, which promotes MC proliferation.[30] We then examined the functional characteristics of MCs grown under these different conditions. We observed that MMCs, but not CTMCs, could be easily stained with toluidine blue dye, as it has previously been described by Broome and Villarreal in 2012.[31] Both *in vitro* systems could be readily activated by similar stimuli, including cross-linking of surface-bound monoclonal and polyclonal IgE as well as non-IgE stimuli, including 48/80, the TLR4 ligand LPS, and cytokines, such as IL-33 and TSLP. We did not observe selectivity of 48/80 for CTMCs using this *in vitro* model system, although CTMCs showed a greater sensitivity to 48/80 than MMCs, as has been described *in vivo*.[32] In addition to activation through IgE and cytokines, MCs are sensors that can respond through pattern recognition receptors to microbial patterns. There are 10 human TLRs (TLR1-TLR10) and 12 mouse TLRs (TLR1-TLR9 and TLR11-TLR13),[33] with no mouse homolog for TLR10.[34] Expression of TLR1-TLR10 with the exception of TLR8 has been identified on human MCs.[35-40] The TLRs appear to be widely expressed by murine MCs, with expression of TLR1-TLR4 and TLR6-TLR9 identified at least at the mRNA level,[41-47] which is very consistent with our *in vitro* systems. We found that CTMCs expressed significantly higher levels of several TLRs (TLR1, TLR2, TLR3, TLR6, TLR8, and TLR13) compared to MMCs. However, MMCs and CTMCs were similarly responsive to LPS stimulation, and TLR4 was similarly highly expressed by the 2 subsets, consistent with previous reports.[48] The role of microbial factors in shaping MC function is of interest as there is growing evidence for an important regulatory influence of the microbiota in pathologies, such as food allergy.[49] Expression of TLR5 has not been demonstrated on murine MCs[41,42,44,45] and we did not detect its expression in either of the 2 *in vitro* MC systems.

Phospho-Syk signaling, degranulation as measured by release of β-hexosaminidase and expression of LAMP-1 (CD107) on the cell surface, and release of the cytokines IL-6, IL-13, and TNF-α were common to both CTMCs and MMCs after stimulation through cross-linking of IgE. The induction of IL-10 was observed at both the mRNA and protein levels after IgE cross-linking in CTMCs, but not MMCs. Therefore, whether MCs promote or suppress regulatory T cell responses may depend on their tissue programming.

An additional mechanism of non-IgE-mediated activation of MCs occurs through cytokines. Epithelial-derived cytokines, including IL-33 and TSLP, play a key role in driving food allergy and atopic dermatitis, as shown in models of sensitization through either the skin or the gastrointestinal tract.[50-54] Our data demonstrated that both MMCs and CTMCs have the capacity to respond to IL-33 and TSLP, and respond with cytokine production in the absence of degranulation. Therefore, the epithelial-derived cytokine environment is another layer of regulation dictating the inflammatory tone of tissue MCs.

In summary, we present a methodological description of *in vitro* modeling of gastrointestinal and skin MCs derived from bone marrow progenitors of mice. We show that the cytokine milieu not only determines the protease content of the MCs, but influences their function through production of tolerogenic and inflammatory cytokines and expression of innate immune receptors. These reductionist model systems are useful experimental tools for mechanistic studies or therapeutic screens in the context of pathologies involving MCs, such as allergy.

ACKNOWLEDGMENTS

This work was supported by grants from National Institute of

Allergy and Infectious Diseases (NIAID; R01 AI093577).

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