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Short Communication

Long-term survival of the mouse ES cell-derived mast cell, MEDMC-BRC6, in mast cell-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice

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Abbreviations: BMMC, bone marrow-derived cultured mast cell; ES cell, embryonic stem cell; FACS, fluorescence activated cell sorting; FcεRI, high affinity receptor for IgE; GFP, green fluorescent protein; IgE, immunoglobulin E; IL-3, interleukin-3; LPS, lipopolysaccharide; MC, mast cell; OVA, ovalbumin; SCF, stem cell factor; TLR, toll-like receptor; TNP, 2, 4, 6-trinitrophenyl.

Abstract

Mast cells (MCs) play pivotal roles in allergic reactions and the host defense against microbial infection through the IgE-dependent and IgE-independent signaling pathways. MC lines that can be analyzed both in vitro and in vivo would be useful for the study of MC-dependent immune responses. Here we investigated the functional characteristics of a mouse embryonic stem cell-derived MC-like cell line, MEDMC-BRC6. The cell line expressed FcεRI and c-Kit and showed degranulation and production of inflammatory cytokines and chemokine, including TNF-α, IL-6 and MCP-1, upon crosslinking FcεRI with IgE. These cytokines and chemokine were also produced by the cell line by stimulation of TLR2 and TLR4. MEDMC-BRC6 survived in the peritoneal cavity and the ear skin for at least 6months after the transfer into genetically compatible MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice, in which systemic anaphylaxis was successfully induced. Thus, MEDMC-BRC6 cells represent a potent tool for investigating the functions of MCs *in vitro* and *in vivo*.

Introduction

Mast cells (MCs) play a central role in IgE-mediated immediate hypersensitivity reactions (1,2). MCs are also involved in the adaptive and innate immune response against pathogens through TLRs (3). MCs originate from hematopoietic stem cells and reside in nearly all vascularized tissues. Previous studies on the *in vitro* analyses of the functions of mouse MCs have mostly been conducted using the rat basophilic RBL-2H3 cell line and bone marrow-derived cultured MCs (BMMCs). However, RBL-2H3 cells carry a constitutively active form of the c-Kit receptor (4), which influences FcεRI-signaling (5). BMMCs have been transferred into an MC-deficient mouse for *in vivo* studies (6). However, BMMCs from genetically engineered animals that exhibit an embryonic lethal phenotype are not available.

Embryonic stem (ES) cells are continuously growing stem cell lines that can be utilized for the generation of lineage-committed cells. MCs were also differentiated from ES cells *in vitro* (7-11). This *in vitro* technique for initiating the differentiation of ES cells into ES-derived MC is a powerful system for investigating the functions of MCs. It was reported that MCs generated from both wild-type and ES cells that are genetically manipulated *in vitro* can survive and display IgE-dependent passive cutaneous anaphylaxis *in vivo* at 3 weeks after intra-dermal injection into MC-deficient $Kit^{W}/Kit^{W\text{-}v}$ mice (9). However, it remains unclear whether ES-derived MCs can be

stably cultured over long periods *in vitro* and whether their properties are maintained during long-term culturing.

MEDMC-BRC6 is an established mouse ES-derived MC-like cell line that expresses FcεRI and c-Kit and requires exogenous cytokines to survive (12). In the present study, we investigated the functional characteristics of MEDMC-BRC6 cells *in vitro* and *in vivo*.

Materials and methods

Cells and reagents

The mouse ES-derived MC-like cell lines, MEDMC-BRC6, were purchased from RIKEN BioResource Center (BRC, Tsukuba, Japan) and were cultured as described previously (12). Briefly, cells were cultured in IMDM (Thermo Fisher Scientific, Waltham, MA, USA) containing the following materials: 15% fetal bovine serum (Sigma, St Louis, MO, USA); 10 μg/ml bovine insulin, 5.5 μg/ml human transferrin, and 5 ng/ml sodium selenite (ITS liquid MEDIA supplement; Sigma); 50 μg/ml ascorbic acid (Sigma); 0.45 mM Monothioglycerol (Wako, Tokyo, Japan); 100 unit/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (PSQ; Sigma). Mouse IL-3 (3 ng/ml; R&D Systems, Minneapolis, MN, USA) and mouse stem cell factor (SCF, 30 ng/ml; R&D Systems) were used for the culturing of MEDMC-BRC6 cells. MEDMC-BRC6 cells were frozen and thawed according to RIKEN BRC's instructions. MEDMC-BRC6 cells that stably expressed GFP were generated using a retroviral vector, pMXs (a kind gift from Dr. Toshio Kitamura, University of Tokyo), as described previously (13). The efficiency of retroviral transduction into MEDMC-BRC6 cells was ~30%. The GFP^+ cells were sorted using a FACSAria cell sorter (BD Biosciences, Mountain View, CA, USA). The BMMCs were prepared by culturing bone

marrow cells with SCF (10 ng/ml) and IL-3 (4 ng/ml) for 4 weeks as described previously (13). The cells were cytocentrifuged at 500 rpm for 5 min (Shandon CytoSpin 3), and stained with Wright (Muto Pure Chemicals Co., Ltd., Tokyo, Japan), or 1.0% alcian blue at pH 2.5 (Wako) followed by 0.1% safranin O (Sigma). All images were acquired using a BZ-X710 microscope (Keyence, Osaka, Japan), and the data were analyzed using a BZ-H3 analyzer (Keyence). TLR2/TLR6-binding diacylated lipopeptide Pam2CSK4 (InvivoGen, San Diego, CA, USA) and TLR4 ligand LPS (Sigma) were used for stimulation of cells.

Quantitative reverse transcription PCR analysis

Total RNA was isolated from cell pellets (TRIzol, Thermo Fischer Scientific, Waltham, MA, USA) and used as a template for reverse transcription reactions (High-Capacity cDNA RT Kit, Applied Biosystems, Foster City, CA, USA). Quantitative PCR analysis of mouse MC proteases (MCPs) was performed by using an ABI7500 sequence detector (Applied Biosystems) and Power SYBR Green Master Mix (Applied Biosystems). The primers were:

Mmcp-1 (forward,5′-ttcccttgcctggtccct-3′; reverse, 5′-gttttcccccagccagct-3′)

Mmcp-2 (forward, 5′-cattgcctagttcctctgacttca-3′; reverse, 5′-cctgttttccccccatccag-3′)

Mmcp-4 (forward, 5′-cttctgactttatcaagccggg-3′; reverse, 5′-cactccagttcgcccccc-3′)

Mmcp-5 (forward, 5′-ctgcagtggcttcctgataa-3′; reverse, 5′-ggaattgcttttccacctca-3′)

Mmcp-6 (forward, 5′-gcccagccaatcagcg-3′; reverse, 5′-ccagggccacttactctcaga-3′)

Mmcp-7 (forward, 5′-catgcagcccccggt-3′; reverse, 5′-ttcccatgtgcctcctgtc-3′)

Mmcp-8 (forward, 5′-ttcctcgggtattcaccagaa-3′; reverse, 5′-gggttgttgcaggagtttcatt-3′)

Mmcp-9 (forward, 5′-gggtggcccatggtattgta-3′; reverse, 5′-cgggtgaagattgcaggg-3′)

Mmc-cpa (forward, 5′-gctacacattcaaactgcctcct-3′; reverse, 5′-gagagagcatccgtggcaa-3′)

Gapdh (forward, 5′-tggtgaaggtcggtgtgaac-3′; reverse, 5′-atgaaggggtcgttgatggc-3′)

All experiments were performed in triplicate.

Cytometric bead array analysis

The concentrations of inflammatory cytokines (TNF and IL-6) and chemokine (MCP-1) were measured by a cytometric bead array (CBA) analysis according to the manufacturer's instructions. Briefly, the culture supernatants (10 μl) from stimulated cells (4-5 $\times 10^5$ cells in 100-200 μl) were mixed with 10 μL of mixed captured beads from mouse Flex Set Cytometric Bead Array (Becton Dickinson, San Jose, CA, USA, Cat. No. 51-9005324, 51-9005236, 51-9005252), then detected with PE detection reagents (Becton Dickinson, Cat. No. 51-9004161, 51-9004153, 51-9004296). Flow

cytometric analysis was performed using the BD LSR Fortessa cell analyzer (BD Biosciences) and CBA analysis FCAP software (Becton Dickinson).

Flow cytometry

The cells were stained with mAbs as indicated. Cells were treated with anti-CD16/32 mAb (2.4G2; TONBO Biosciences, San Diego, CA, USA) to avoid binding to FcγR on ice for 10 min prior to incubation with the indicated combination of antibodies. Monoclonal antibodies against mouse c-Kit (2B8) and TLR2 (6C2) were purchased from BD Biosciences. Anti-mouse TLR4 (SA15-21) and CD11b (M1/70) were purchased from BioLegend. Anti-mouse FcεRIα (MAR-1) was purchased from eBiosciences. Anti-mouse CD45.2 (104) was purchased from TONBO Biosciences. Stained cells were acquired on a BD LSR Fortessa cell analyzer and were analyzed using the FlowJo software program (Tree Star, Inc., Ashland, OR, USA).

Degranulation assay

Degranulation was evaluated by measuring the release of β-hexosaminidase and by a flow cytometric analysis of the cell-surface exposure of CD107a (LAMP-1). LAMP-1 is an intracellular protein found on granule membranes that becomes exposed

on MC surface upon degranulation (14). The cells were sensitized with the indicated concentration of IgE anti-2,4,6-trinitrophenyl (anti-TNP, C38-2; BD Biosciences) in culture medium for 15 h at 37°C and were then stimulated with the indicated concentration of TNP_4-conjugated ovalbumin (OVA) in 100 μl HEPES–Tyrode's buffer (pH 7.4) at 37°C. Cells that were stimulated with ionomycin (1 μg/ml; Sigma) for 30 min were used as a positive control of degranulation. Culture supernatants or cells were collected at 30 or 10 minutes after the addition of the antigen, respectively. The culture supernatants were analyzed to detect the release of β-hexosaminidase and the percent degranulation was calculated as described previously (13). Cells were stained with anti-CD107a mAb (1D4B; BD Biosciences) and were analyzed by flow cytometry.

Reconstitution of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice with MEDMC-BRC6 cells

MC–deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice on the C57BL/6 background and C57BL/6 mice were purchased from RIKEN BRC and Clea Japan, respectively. *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were reconstituted with 2×10^7 of GFP-expressing MEDMC-BRC6 cells or 10^7 of BMMCs by intravenous injection. Three to 6 months after the injection, mice were analyzed for reconstitution of MC in the peritoneal cavity and ear pinnae by flow cytometry and the stomach by histology. Mouse ear pinnae were treated and stained as described

previously (15,16). Briefly, ears were treated with enzyme mixture containing collagenase II and DNase I (400 U/ml and 50 U/ml, respectively; Worthington Biochemical, Lakewood, NJ, USA) for 1 h at 37°C with shaking, then stained with indicatedmAbs. Peritoneal MCs and ear skin MCs were positive for both FcεRI and c-Kit. To analyze tissue MC in the stomach, 3 μm paraffin sections of fixed tissues with 4% paraformaldehyde were stained with 0.05% toluidine blue O (Chroma-Gesellschaft, Schmid & Co., Stuttgart, Germany) or with 1 % alcian blue, pH2.5 (Wako) in 3% acetic acid for 30 minutes followed, after washing, by 0.1% safranin O (sigma) for 5 minutes.

Passive systemic anaphylaxis

Kit$^{W\text{-}sh/W\text{-}sh}$ mice that had been intravenously injected with 2×10^7 cells of GFP-expressing MEDMC-BRC6 cells 6 months previously were sensitized with an intravenous injection of mouse IgE anti-TNP (20 μg; C38-2; BD Biosciences). Twenty-four hours later, the mice were challenged with an intravenous injection of TNP_6-OVA (1 mg). After the antigen challenge, the rectal temperatures were measured every 5 min for 60 min using a digital thermometer (Shibaura Electronics, Tokyo, Japan). The mice used in all of the experiments were 8 to 13-week-old males and had been bred in the specific pathogen-free facilities at the University of Tsukuba. All

animal experiments in this study were carried out humanely, and all efforts were made to minimize suffering after receiving approval from the Animal Ethics Committee of the Laboratory Animal Resource Center, University of Tsukuba (Permit Number: 16–281), and in accordance with Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the Jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

Statistical analysis

The results were expressed as mean ± SEM. The values were analyzed by parametric ANOVA. P values of <0.05 were considered to indicate statistical significance.

Results and discussion

The FcεRI-mediated activation of signaling in MEDMC-BRC6 cells

MEDMC-BRC6 cell was generated by culturing C57BL/6-derived ES cells (BRC6) on feeder cells (OP9 cells) with cytokines (SCF, EPO, and IL-3) (12). MEDMC-BRC6 cells exhibit mast cell (MC)-like phenotypes because they harbor basic granules (Fig. 1A) and express high-affinity receptor for IgE, FcεRI and c-Kit on their cell surface (Fig. 1B) (12). However, the number and size of granules in the cytoplasm of MEDMC-BRC6 cells were smaller and the expression level of FcεRI and c-Kit was lower than those of BMMCs (Fig. 1A), suggesting that MEDMC-BRC6 cells were immature compared with BMMCs. Similarly to bone BMMCs, MEDMC-BRC6 cells require both IL-3 and SCF for their survival and proliferation (12). However, their functions remain unclear. To characterize the phenotype of MEDMC-BRC6 cells, we stained MEDMC-BRC6 cells and BMMCs with alcian blue followed by safranin O. Mucosal MCs (MMCs) are preferentially stained with alcian blue due to the predominance of chondroitin sulphate. In contrast, safranin O rather than alcian blue preferentially stains connective tissue MCs (CTMC) due to the predominance of heparin (17). While BMMCs were positive for alcian blue, but not safranin O, MEDMC-BRC6 cells were positive for both alcian blue and safranin O (Fig. 1C), suggesting that

MEDMC-BRC6 had both characteristics of MMCs and CTMCs. Classification of CTMC and MMC can be determined by the expression of mouse MC secretory granule proteases (mMCPs) (18). To further phenotypically characterize MEDMC-BRC6 cells, we examined the relative gene expression levels of mMCPs in MEDMC-BRC6 and BMMC by quantitative reverse transcription PCR analyses. MEDMC-BRC6 cells expressed lower levels of mMCP-1, -2, -4, -5, -6, -7, -8, -9, and MC–carboxypeptidase A (MC-CPA) than did BMMCs (Fig. 1D), consistent with the results of morphological and phenotypical analyses (Fig. 1A), suggesting again that MEDMC-BRC6 cells were immature compared with BMMCs.

To clarify whether MEDMC-BRC6 cells can transduce signals via FcεRI, the cells were sensitized with an anti-TNP IgE antibody and then stimulated with various concentrations of TNP-conjugated OVA. Stimulation of MEDMC-BRC6 cells as well as BMMCs sensitized with an anti-TNP IgE with the TNP-conjugated OVA antigen induced degranulation, as determined by CD107a expression and β-hexosaminidase release (Fig. 2A and B). These results indicated that although the degranulation was less in MEDMC-BRC6 cells compared with BMMCs, FcεRI mediated an activating signal for degranulation in MEDMC-BRC6 cells. Next, we measured the production of proinflammatory cytokines and chemokine, including TNF-α, IL-6, and MCP-1 at 24 h

after antigen stimulation using a CBA. In accordance with the results of the degranulation experiment, MEDMC-BRC6 cells secreted inflammatory cytokines and chemokines upon crosslinking FcεRI although the amounts of secreted cytokines and chemokines were less in MEDMC-BRC6 cells compared with BMMCs (Fig. 2C). Together, these results suggest that although the strength of signal through FcεRI in MEDMC-BRC6 cells seemed to be lower than that in BMMCs, MEDMC-BRC6 cells can be utilized to investigate the FcεRI-mediated signaling pathway in MCs *in vitro*.

TLR-mediated signaling in MEDMC-BRC6 cells

MCs are also activated through TLR-mediated signaling (19) and play an important role in the host defense against pathogens (20). Next, we examined the expression of TLR2 and TLR4 on MEDMC-BRC6 cells by flow cytometry and found that they expressed both TLRs (Fig. 3A and B). The cells were then stimulated with TLR2–TLR6-binding diacylated polypeptides, Pam2CSK4 or the TLR4 ligand, LPS. MEDMC-BRC6 cells, as well as BMMCs secreted inflammatory cytokines, including TNF-α, IL-6, and MCP-1 in response to stimulation with Pam2CSK4 (Fig. 3C) or LPS (Fig. 3D). Although, the expression of TLR2 on BMMCs was hardly detected by flow cytometry, the production of cytokines was induced in a dose-dependent manner of

Pam2CSK4. Nonetheless, these results indicate that MEDMC-BRC6 cells can be used to investigate the signaling pathways mediated by TLR2 and TLR4 in MCs *in vitro.*

MEDMC-BRC6 cells survive in MC-deficient mice

MEDMC-BRC6 cells are differentiated from mouse ES cells with a C57BL/6 background and require exogenous IL-3 and SCF for their survival (12). Based on these observations, we hypothesized that—similarly to BMMCs—MEDMC-BRC6 cells may survive in MC-deficient mice with a C57BL/6 background without exhibiting tumorigenicity *in vivo*. To achieve this, MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice with a C57BL/6 background was intravenously injected with 2×10^7 MEDMC-BRC6 cells, which had been retrovirally transduced with GFP. $Kit^{W\text{-}sh/W\text{-}sh}$ mice transplanted with 10^7 of BMMCs were generated as a control. We analyzed the MCs in the peritoneal cavity fluid (PEC) and ear skin by flow cytometry and found that in C57BL/6 mice, 1–3% of the $CD11b^-$ cells in the PEC and 4–7% of the $CD45^+$ $CD11b^-$ cells in the ear skin were $Fc\varepsilon RI^+c\text{-}Kit^+$ MCs (Fig. 4A and B). In accordance with previous reports, no $Fc\varepsilon RI^+c\text{-}Kit^+$ cells were detected in the PEC or ear skin of $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Fig. 4A and B) (6). However, at 24 weeks after the transfer of MEDMC-BRC6 cells into $Kit^{W\text{-}sh/W\text{-}sh}$ mice, we detected $Fc\varepsilon RI^+c\text{-}Kit^+$ cells in the PEC (Fig. 4A). Similarly to BMMCs,

MEDMC-BRC6 cells in the peritoneal cavity of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were detected at 12 weeks after the transfer. Previous study demonstrated that intravenous injection of BMMCs did not result in reconstitution of MC in the ear skin of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (6). Consistently, we also did not detect MC in the ear skin of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice with intravenous injection of BMMC at 12 and 24 weeks after the transfer (data not shown). However, at 24 weeks after the transfer of MEDMC-BRC6 cells into *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, we could detect FcεRI^{+}c-Kit^{+} cells in the ear skin (Fig. 4B), which might be dependent on the characteristic of MEDMC-BRC6 cells with the safranin O-positive phenotype that is also observed in CTMC locating primarily in the skin. Therefore, we addressed whether MEDMC-BRC6 cells with the characteristic of the safranin O-positive phenotype survive in the stomach, where MC shows the MMC phenotype positive for alcian blue but negative for safranin O in wild–type mice (Fig 4D). While *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that received BMMCs intravenously showed the recruitment of MCs positive for alcian blue, but not safranin O, into the stomach at 12 weeks after the transfer, MCs observed in the stomach of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that received MEDMC-BRC6 cells showed the safranin O, but not alcian blue, -positive phenotype (Fig. 4D). This result suggests that MCs in the stomach of MEDMC-BRC6-transferred mice exhibit the CTMC-like phenotype. No tumors were observed in those mice (data not shown). These results

indicate that MEDMC-BRC6 cells can survive for at least 6 months without tumorigenicity *in vivo*.

MEDMC-BRC6 cells compensate for the function of MCs in MC-deficient mice

Since MEDMC-BRC6 cells were adapted *in vivo*, we next assessed whether MEDMC-BRC6 cells in $Kit^{W\text{-}sh/W\text{-}sh}$ mice could orchestrate typical IgE-dependent and MC-dependent passive systemic anaphylaxis. $Kit^{W\text{-}sh/W\text{-}sh}$ mice before and 6 months after the transfer with GFP-expressing MEDMC-BRC6 cells as well as wild type–mice were passively sensitized with IgE antibodies against TNP and then challenged with an intravenous injection of TNP-conjugated OVA (TNP-OVA). We measured the rectal temperature, an indicator of passive systemic anaphylaxis, every 5 min after the antigen challenge. The IgE-sensitized wild–type mice showed a progressive decrease in rectal temperature within 20 min after the antigen challenge, whereas the $Kit^{W\text{-}sh/W\text{-}sh}$ mice did not show a decrease in rectal temperature (Fig. 4E). This finding was consistent with previous observations (13). As we expected, $Kit^{W\text{-}sh/W\text{-}sh}$ mice transferred with GFP-expressing MEDMC-BRC6 cells showed a progressive decrease in rectal temperature at 24 weeks after the transfer (Fig. 4E). These results indicate that MEDMC-BRC6 cells can be utilized for the systemic analysis of the FcεRI-mediated

functions of MCs *in vivo*.

In the present study, we demonstrated that gene-manipulated MEDMC-BRC6 cells survive and compensate for the IgE-dependent functions of MCs in MC-deficient mice without displaying tumorigenicity. These properties were stable and were observed continuously for >1 year after the induction of their differentiation from ES cells. Moreover, the doubling time of MEDMC-BRC6 cells was 17 ± 0.2 h, which was 3 to 5-fold faster than that of BMMCs. The efficiency of retroviral transduction into MEDMC-BRC6 cells was ~30%. Thus, large numbers of genetically manipulated MCs can be ready for use in a short period, and their physiological role can be investigated systemically *in vivo*. Importantly, intravenous injection of MEDMC-BRC6 cells, but not BMMCs, preferentially adapt to the skin. Thus, MEDMC-BRC6 cells may be a useful material to investigate the function of MC, particularly in the skin, *in vivo* as well as *in vitro*.

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Author contributions

S.S. did all of the experiments and analyzed the data; T.H. and Y.N. contributed materials tools; S.T.–H. designed experiments, analyzed the data and wrote the manuscript; and A.S. supervised the overall project. All authors contributed to the critical review of the manuscript.

Conflicts of Interest statement:

The authors declare no conflicts of interest.

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Figure legends

Fig 1. The phenotypic analyses of MEDMC-BRC6 cells

Cytospins of MEDMC-BRC6 cells and BMMCs were stained with Wright (A) or with alcian blue followed by safranin O (C). (B) Flow cytometric analysis of cell-surface expressions for FcεRI and c-Kit. The data are shown as 5% probability contour plots. (D) Quantitative reverse transcription PCR analysis of MCPs and CPA. The relative gene expression levels were determined in triplicate. GAPDH, glyceraldehyde 3-phosphate dehydrogenase (used as an internal control for normalization). The figures are representative results of two independent experiments.

Fig 2. FcεRI-mediated signaling in MEDMC-BRC6 cells

(A) MEDMC-BRC6 cells and BMMCs were sensitized with the anti-TNP IgE mAb, and then challenged with the indicated concentrations of TNP_4-conjugated OVA antigen. (A) The cells were stained with mAbs against CD107a and c-Kit at 10 min after antigen stimulation and were analyzed by flow cytometry (n = 3). The proportion of $CD107a^+$ $c\text{-}Kit^+$ cells is shown. (B) The culture supernatants were collected from at 30 min after the antigen challenge and β-hexosaminidase activity was determined (n = 3). (C) The culture supernatants were collected from (A) at 24 h after antigen challenge, and

cytokines and chemokines production was analyzed by CBA (n = 3). The data represent the results of three independent experiments (mean ± SD). ** $P < 0.01$, *** $P < 0.001$.

Fig 3. TLR-mediated signaling in MEDMC-BRC6 cells

Flow cytometric analyses of the expression of TLR2 (A) and TLR4 (B) on MEDMC-BRC6 cells and BMMCs. The cells were stimulated with Pam2CSK4 (C) or LPS (D) for 24 h, and then the culture supernatants were analyzed for cytokine production by CBA (n = 3). The data represent the results of three independent experiments (mean ± SD). *** $P < 0.001$.

Fig 4. MEDMC-BRC6 cells survive for 24 weeks and compensate for the function of MCs in MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice

Kit$^{W\text{-}sh/W\text{-}sh}$ mice were transplanted with 2 ×10^7 of GFP-expressing MEDMC-BRC6 cells or 10^7 of BMCMs. Flow cytometric analyses of FcεRI$^+$c-Kit$^+$ MCs in the PEC (A) and the ear skin (B) were analyzed at 12 or 24 weeks after the transplantation. The numbers indicate the proportion of FcεRI$^+$c-Kit$^+$ MCs in CD11b$^-$ cells (A) and CD45$^+$ CD11b$^-$ cells (B). The data are shown as 5% probability contour plots. Tissues from stomachs were stained with toluidine blue (C) or with alcian blue followed by safranin O (D) at

12 weeks after the transplantation. Original magnification: ×400. Arrowheads indicate the MCs (E) The rectal temperatures of C57BL/6 mice (n = 2/group) and $Kit^{W\text{-}sh/W\text{-}sh}$ mice transplanted with (MEDMC-BRC6 → $Kit^{W\text{-}sh/W\text{-}sh}$; n = 3/group) or without MEDMC-BRC6 cells ($Kit^{W\text{-}sh/W\text{-}sh}$ mice; n = 3/group). At 24 weeks after the transfer, the mice were sensitized with mouse IgE anti-TNP (20 μg), then challenged with TNP_6-OVA (1 mg). The data represent the results of two independent experiments (mean ± SEM). * $P < 0.05$ as compared with values for $Kit^{W\text{-}sh/W\text{-}sh}$ mice by ANOVA.

Figure 1. The morphology of MEDMC-BRC6 cells

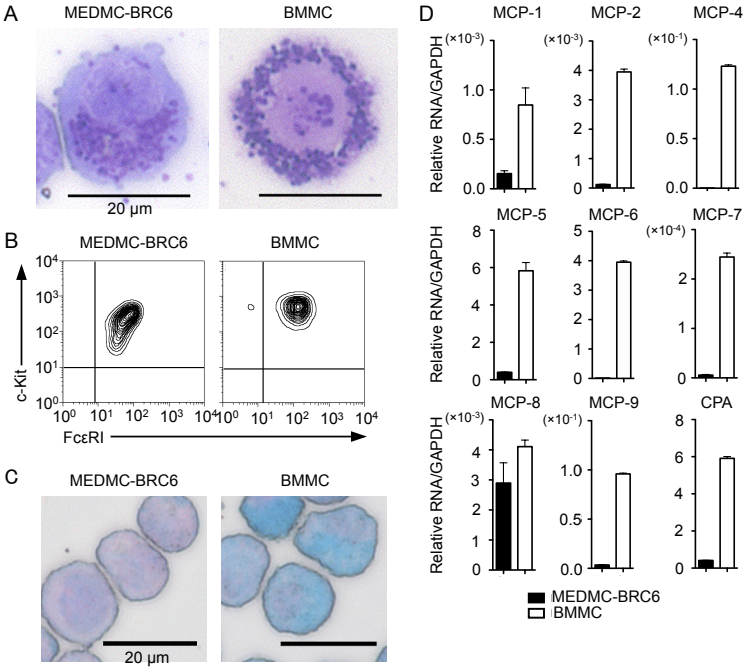

Figure 2. FcεRI-mediated signaling in MEDMC-BRC6 cells.

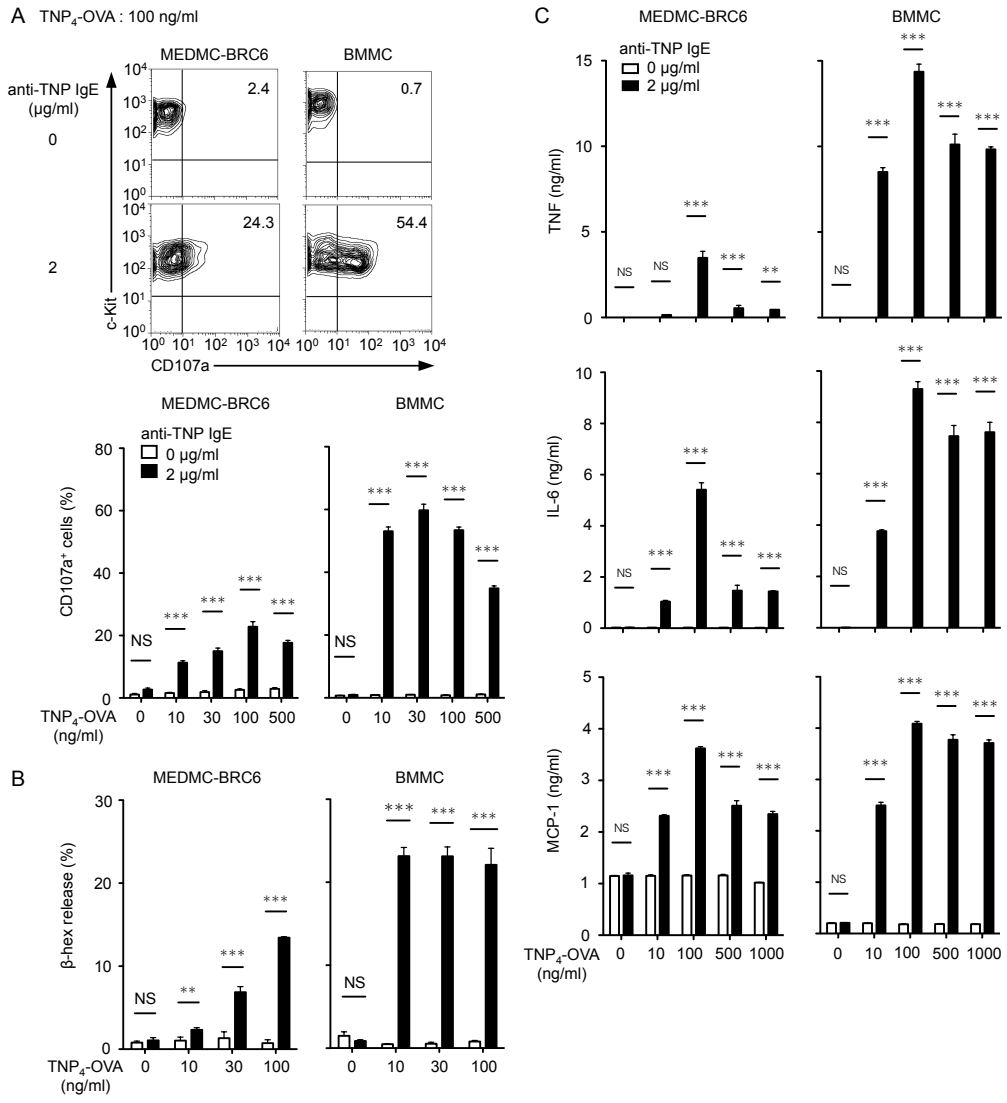

Figure 3. TLR-mediated signaling in MEDMC-BRC6 cells.

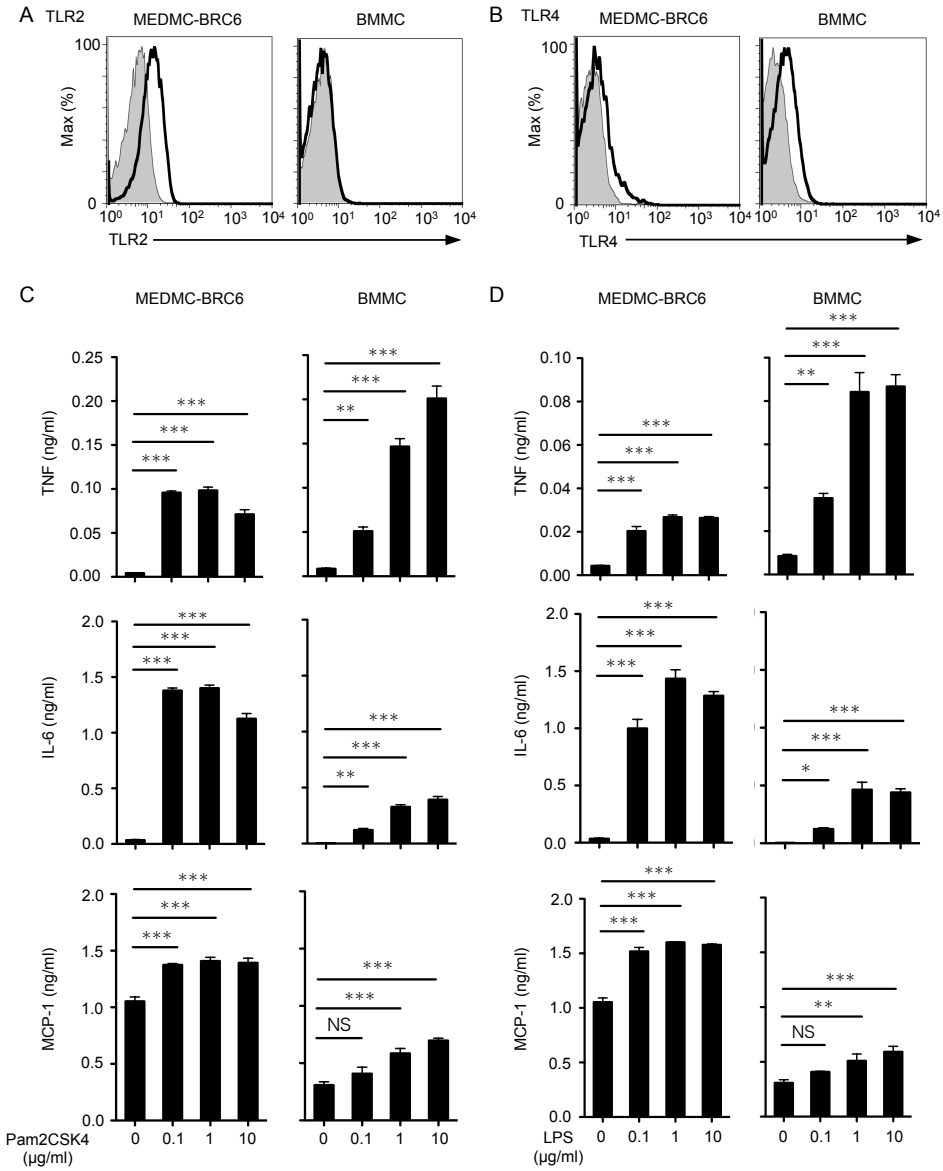

Figure 4. MEDMC-BRC6 cells survive and compensate for the functions of MCs in MC-deficient mice.