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Comparative Analysis of Bone Marrow-derived Mast Cell Differentiation in C57BL/6 and BALB/c Mice

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ABSTRACT

Allergic diseases have increased in the last three decades. Mast cells play a critical role in allergic diseases along with allergen-specific immunoglobulin E (IgE). Following mast cell degranulation elicited by ligation of the IgE-FcεRI receptor complex with allergen, allergic reactions are followed by various symptoms such as vascular hyper-permeability, mucous secretion, itching, sneezing, wheezing, rashes, fever, and anaphylactic shock. Susceptibility or inclination to allergy varies depending on individual genetic traits and living environment, and it has long been believed that such an inclination is determined by an immunologic balance of T helper cell types.

Mouse strains also have different susceptibilities to allergy. Similar to T helper cells and macrophages, it is not known whether mast cells can also be divided into two different types between mouse strains. In this study, we prepared bone marrow-derived mast cells from BALB/c and C57BL/6 mice and examined their cellular properties. Cellular response to IL-3 and the process of mast cell differentiation from bone marrow cells were different on the basis of cell surface marker molecules. BALB/c-derived cells more efficiently exhibited degranulation than did C57BL/6-derived cells following both calcium ionophore and receptor crosslinking.

These functional differences persisted even after a longer cell culture for 8 weeks, suggesting a difference in cell-autonomous characteristics. These results support the concept that mast cells also have different cell types dependent on their genetic background.

Abbreviations: Ab: antibody; BMMC: bone marrow-derived mast cell; DNP: dinitrophenyl; FACS: fluorescence-activated cell sorter; FCS: fetal calf serum; FITC: fluorescein isothiocyanate; FSC: forward scatter; HRP: horseradish peroxidase; HSA: human serum albumin; Ig: immunoglobulin; IL: interleukin; MIP-2: macrophage inflammatory protein-2; MCP: mast cell protease; PE: phycoerythrin; PerCP: Peridinin chlorophyll protein complex; SNP: single nucleotide polymorphisms; SSC: side scatter; Th: T helper; TNF-α: tumor necrosis factor alpha

KEYWORDS

Allergy; degranulation; interleukin-3; mast cell

Introduction

An allergy is generally defined as an unnecessary immune response to a harmless substance, such as pollinosis (hay fever). Allergic diseases have dramatically increased in the last three decades. Mast cells play a central role in immediate-type allergy, and in peripheral tissues, mast cells that hold allergen-specific IgE at FcεRI receptors on their

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surface are waiting to respond to allergens before they enter our bodies. For example, once an allergen such as Japanese cedar pollen in the air or egg antigens in the diet enters our body, especially the lamina propria of the mucous membrane in the respiratory tract or digestive tissues, binds and crosslinks IgE on FcεRI complex, and in turn, mast cells release their granular contents including numerous bioactive substances, so-called chemical mediators (Abraham and St John, 2010; Kalesnikoff and Galli, 2008). As a result, chemical mediators like histamine trigger a sequence of allergy symptoms such as increase in vascular permeability, rash, congestion, nasal mucus production, itching, diarrhea, and, in the most severe case, anaphylaxis and death. A critical step toward conquering allergic diseases is gaining a deep understanding of mast cell functions.

In contrast to allergy, in which mast cells have detrimental effects, mast cells appear to be required for host defense against helminth and bacterial infection. Expulsion of adult *Trichinella spiralis* and *Nippostrongylus brasiliensis* from the intestinal tract is delayed in mast cell-deficient *W/W^v* mice (Crowle and Reed, 1981; Ha et al., 1983; Mitchell et al., 1983). These reports clearly indicate a requirement of mast cells. A molecular mechanism for the expulsion of *T. spiralis* has been attributed to mast cell protease (MCP)-1 release from cytoplasmic granules of mast cells (Knight et al., 2000). MCP-6 contributes to eosinophil recruitment upon *T. spiralis* infection (Shin et al., 2008) as well as neutrophil recruitment upon *Klebsiella pneumoniae* infection (Thakurda et al., 2007). Mast cells also recruit neutrophils via tumor necrosis factor alpha (TNF-α) production in delayed-type hypersensitivity reactions (Biedermann et al., 2000) and indirectly in experimental peritonitis through macrophage inflammatory protein-2 (MIP-2) (Mercer-Jones et al., 1999).

Depending on the genetic background, mice exhibit biased immune responses with a tendency toward a particular direction. It is well known that C57BL/6 mice show type 1 immune responses, whereas BALB/c mice show type 2 immune responses. Although both strains of mice are susceptible to infection with *Leishmania major*, C57BL/6 mice are able to control infection, whereas BALB/c mice are not, because cytokine production by helper T cells and the M-1/M-2 balance of macrophage differentiation are distinct for each strain (Heinzel et al., 1989; Mills et al., 2000). For instances, spleen T cells from C57BL/6 mice produce IFN-γ dominantly, whereas those from BALB/c mice produce IL-4 dominantly in addition to IFN-γ (Mills, 2000; Yagi et al., 2006). C57BL/6 macrophages easily produce NO in response to IFN-γ or LPS, whereas BALB/c macrophages increase arginine metabolism to ornithine by LPS (Mills, 2000). Genetic background influences susceptibility to not only infection but also experimental allergic diseases such as asthma with differential bronchial responses, inflammation, and cytokine production (Gueders et al., 2009). It is not yet known whether mast cells also can be classified into two different functional phenotypes depending on mouse strains such as helper T cells and macrophages.

Mast cells are derived from bone marrow stem cells. Depending on where precursor cells migrate, they differentiate toward mucosal or connective tissue cell types (Kitamura, 1989; Kitamura and Ito, 2005). Even once mast cells are committed to a particular type, if transferred to a different site, they still have the capacity to switch to another type (Kitamura, 1989). Mucosal and connective tissue type mast cells are characterized by the presence or absence of intragranular components such as tryptases and chymases (Pejler et al., 2010). Unlike lymphocytes, which reside in the blood and lymphoid organs, it is hard to collect enough mast cells from peripheral tissues; thus, many studies utilize bone marrow-derived mast cells (BMMCs) to investigate mast cell functions. A few studies

have demonstrated strain differences in BMMC traits such as the amount of granule-associated mediators, cytokines, and synthase (Noguchi et al., 2005; Stevens et al., 1994).

In this study, we used BMBCs from two different strains of mice that exhibit markedly different immune responses to compare their differentiation levels based on cell surface marker molecules and their functions. Thus, we newly found that during cell culture, BMBCs revealed a distinctive differentiation process in a time-dependent fashion, and BMBCs from BALB/c mice exhibited efficient degranulation. Their tyrosine phosphorylation pattern upon receptor stimulation was also different as compared with those of C57BL/6 mice. These differences between BALB/c and C57BL/6 mast cells have never been reported previously. Furthermore, we attempted to examine whether their differences persisted even after further cell differentiation and discussed the possible mechanisms. The results suggest the importance of choice of the mouse strain used for mast cell experiments.

Materials and methods

Animals

BALB/c and C57BL/6 mice were originally obtained from Japan SLC incorporation (Hamamatsu, Japan). All mice were bred in our animal facility, and used at 12–24 weeks of age. They were maintained on a 12-h light/dark cycle at 22–26°C and 40–60% humidity, and water and food were provided *ad libitum*. Animal experiments were approved and conducted according to the guidelines of the animal experiment committee of Nippon Veterinary and Life Science University.

Preparation of bone marrow-derived mast cells

Bone marrow cells were collected from C57BL/6 and BALB/c mice tibias and femurs and cultured for 3 weeks in RPMI 1640 medium supplemented with 10% FCS, 5×10^{-5} M 2-ME, 100 µg/mL of streptomycin, 100 U/mL of penicillin, and 20% of culture supernatant from WEHI-3 cells (myelomonocytic leukemia) that secrete IL-3. WEHI-3 cells were kindly provided from Dr. K. Imanishi (Tokyo Women's Med. Univ., Tokyo, Japan) and cultured in RPMI-1640 supplemented with 10% FCS. The supernatant containing IL-3 was harvested after 5–6 days of a culture period to accumulate IL-3 before the cells became exhausted thoroughly. The IL-3 concentrations were determined in the section of Cytokine assay below. Bone marrow cell culture was replenished every 3 or 4 days, and the cell density was initially at 1×10^7 cells/mL, secondary at 3×10^6 cells/mL, and then 1×10^6 cells/mL. To determine cell number, we always used trypan blue dye to exclude dead cells with blue staining.

Cytokine assay

IL-3 activity in conditioned medium was confirmed in a BaF3 cell line requiring IL-3 for survival. IL-3 concentration in WEHI-3 conditioned medium was 4–7 ng/mL and was determined by Mouse IL-3 ELISA MAX Standard Kit according to the manufacturer's instructions (BioLegend, San Diego, CA, USA).

Antibodies and reagents

For flow cytometric analysis, phycoerythrin (PE)-labeled anti-mouse FcεRI antibody (Ab) (MAR-1), Alexa Fluor® 488 or peridinin chlorophyll protein complex (PerCP)-labeled anti-mouse CD117/c-Kit Ab (2B8), and fluorescein isothiocyanate (FITC)-labeled anti-mouse CD49b Ab (DX5) were all purchased from BioLegend (San Diego, CA, USA). Anti-phosphotyrosine (pY20) Ab (eBioscience; Thermo Fisher Scientific, Waltham, MA, USA), anti-phosphorylated Akt Ab (Cell Signaling Technology, Danvers, MA, USA), anti-phosphorylated ERK1/2 Ab (pT202/pY204, 22A) and anti-ERK1/2 Ab (C-9) (Santa Cruz Biotechnology, Dallas, TX, USA), and mouse monoclonal anti-β-actin Ab (2F3, Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) were used for western blot analysis. Horseradish peroxidase (HRP)-labeled anti-mouse IgG (H + L) Ab and anti-rabbit IgG (H + L) Ab were obtained from Sera Care Life Sciences (Milford, MA, USA).

Flow cytometric analysis

To analyze differentiation of mast cells and their percentages, bone marrow cells periodically underwent two- or three-color Ab staining, such as a combination of PE-labeled anti-FcεRI, PerCP-labeled anti-CD117/c-Kit, and FITC-labeled anti-CD49b (for three colors), or a combination of PE-labeled anti-FcεRI and Alexa Fluor®488-labeled anti-c-Kit or FITC-labeled anti-CD49b (for two colors). To detect IL-3Rα, we used biotin-labeled anti-mouse IL-3 receptor alpha (CD123) Ab (5B11) from BioLegend (San Diego, CA, USA). BMMCs following cell culture for 7–21 days were first rested for 4 h or overnight in the absence or presence of (minimal) IL-3, and then stained for IL-3Rα. The samples were subsequently assessed using the FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA), and the data were analyzed using WinMDI 2.9 free software (The Scripps Research Institute, San Diego, CA, USA). To determine percentages of particular cell populations, we always excluded dead cells with very low forward scatter values.

Degranulation assay

BMMCs were stimulated for degranulation using calcium ionophore A23187 or specific antigen via IgE receptor FcεRI on mast cells. For calcium ionophore stimulation, 5×10^5 BMMCs were incubated with 0.04–4 μM of A23187 for 1 h in 0.2 mL volume per well of a 96-well flat-bottom culture plate. For antigen stimulation, BMMCs were first incubated overnight with 0.05 μg/mL of anti-DNP IgE (Sigma-Aldrich, St. Louis, MO, USA) and stimulated the next day by crosslinking the receptor with 2–50 ng/mL of DNP-HSA antigen for 30 min at 37°C. Next, the supernatants and cell pellets were separately harvested, and cell pellets were solubilized with 200 μL TNE lysis buffer (10 mM Tris-HCl, 1% NP-40, 0.15 M NaCl, 1 mM EDTA), and then 40 μL of supernatants and cell lysate were incubated with 100 μL of 4-nitrophenyl-N-acetyl-β-D-glucosaminide (final concentration 3 mM) for 30 min at 37 °C, and the reaction was stopped with the addition of 200 μL of 0.2 M glycine/NaOH (pH 10.7). Next, absorbance was measured at 405 nm, and degranulation efficiency was calculated as activity value of supernatant $\times$ 100/(activity value of supernatant + activity value of cell lysate).

Western blotting analysis

BMMCs were incubated with anti-DNP-specific Ab (0.05 µg/mL) overnight with 20% conditioned medium containing IL-3. The cells were collected the next day and stimulated with 50 ng/mL of the antigen DNP-HSA at 37 °C in a 1.5 mL microcentrifuge tube containing 5×10^6 cells/0.2 mL, and the reaction was stopped with 0.7 mL cold Hanks' solution with 2 mM EDTA. The stimulated cells were solubilized by incubation for 10 min on ice with a TNE lysis buffer (10 mM Tris [pH 7.5], 0.15 M NaCl, 2 mM EDTA, and 5 mM NaVO₄) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The lysates were cleared by centrifugation to remove the insoluble materials. Protein concentrations of the cell lysates were determined using Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). The cell lysates (6–10 µg protein per sample) were denatured for 5 min at 98 °C in sample buffer, separated by 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane. The membrane was blocked with 3% skim milk in TBST (10 mM Tris [pH 7.5], 0.15 M NaCl, and 0.1% Tween 20) for 1 h to reduce background staining and incubated overnight with a specific primary Ab at an appropriate concentration in TBST containing 2% BSA or skim milk. Following incubation with an HRP-coupled secondary Ab for 1 h, the blots were reacted with a chemiluminescence substrate, ImmunoStar LD (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), according to the manufacturer's instructions, and visualized with the image analyzer LAS 4000 (GE Healthcare Life Sciences, Chicago, IL, USA).

Statistical analysis

The data of two groups were analyzed by a two-tailed non-parametric test (Mann-Whitney U-test). For the degranulation assay, the data were tested with two-way ANOVA with Bonferroni's multiple comparison test. Both analyses were conducted using GraphPad Prism software (La Jolla, CA, USA). Results are presented as mean ± SD.

Results

Cell numbers in bone marrow cell culture for 3 weeks were compared between BALB/c and C57BL/6 mice

To address whether mouse genetic background affects differentiation and properties of mast cells, we attempted to prepare BMMCs from C57BL/6 and BALB/c mouse strains using IL-3-containing medium for 3 weeks. First, we examined a transition in cell number of total bone marrow cells and found that bone marrow cells from C57BL/6 mice were harvested approximately 1.6-fold more than those from BALB/c mice before cell culture (Figure 1a, 92.3×10^6 vs. 56.9×10^6 cells, $p = 0.0051$). However, during the course of in vitro cell differentiation in the presence of IL-3, BALB/c-derived cells varied in cell number (Figure 1b), whereas C57BL/6-derived cells decreased as compared with their initial numbers (Figure 1c). As a result, after 3 weeks in culture, BALB/c cells tended to have better survival compared with C57BL/6 cells (Figure 1d, $p = 0.0033$), indicating a difference in response to IL-3 between these two mouse strains.

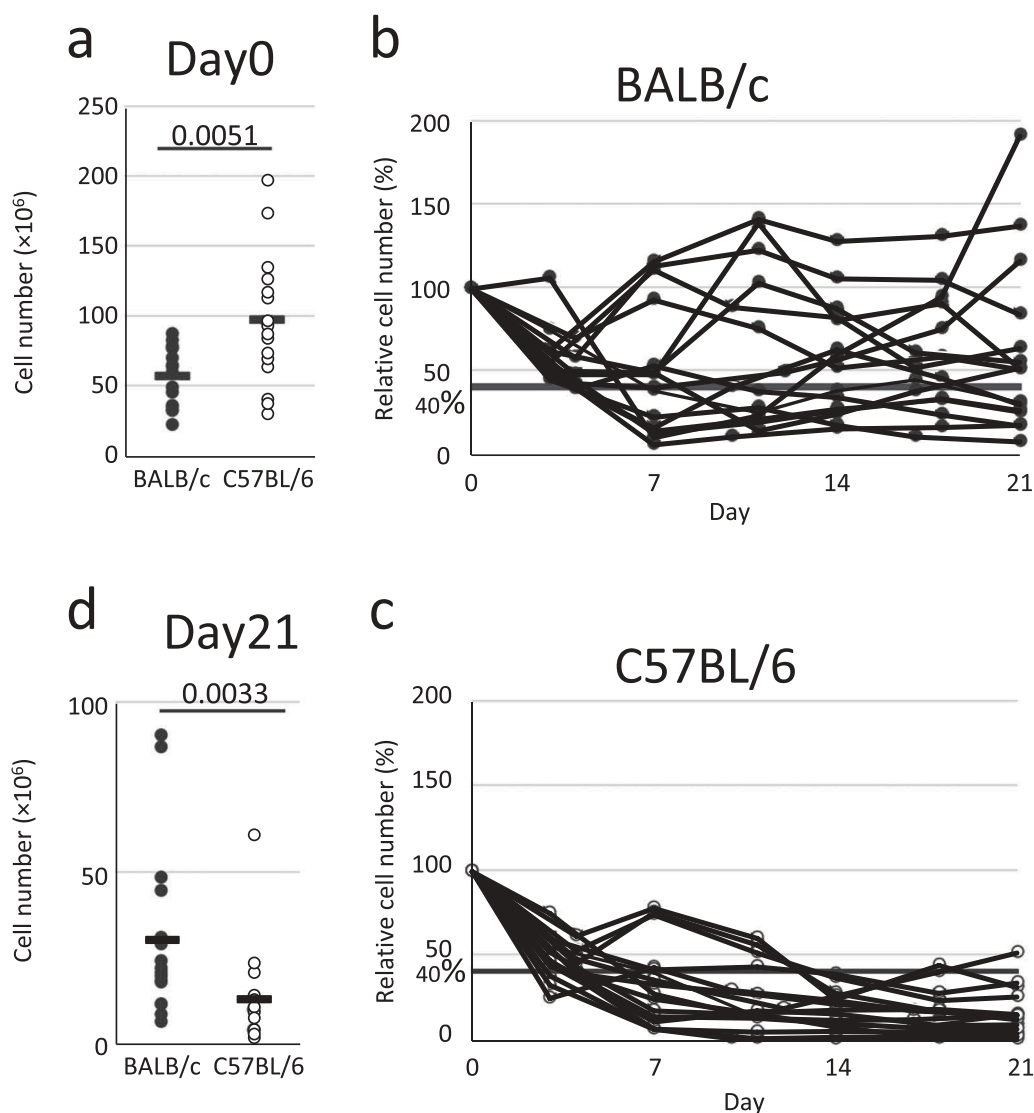


Figure 1. Cell number transition during bone marrow cell culture in the presence of IL-3.

Bone marrow cells were prepared from BALB/c and C57BL/6 mice and cultured in IL-3-containing medium for 3 weeks. Cell numbers were counted every 3 or 4 days during the course of the experiments. Cell numbers prior to (a) and post cell culture (d) are shown, and each dot represents each cell preparation from a single mouse ($n = 16$). The p values were calculated. Transitions in relative cell number compared with the initial values that are set as 100% are also shown, and each line represents each cell culture from a single BALB/c (b) and C57BL/6 (c) mouse. The 40% line is an arbitrary line to roughly distinguish transition patterns of cell numbers between these two mouse strains.

Bone marrow-derived mast cell differentiation was compared between BALB/c and C57BL/6 mice

Next, we examined phenotypes of cell surface molecules during the course of in vitro cell differentiation for 3 weeks. Fc ϵ RI⁺ cells were scarce prior to incubation with IL-3, but they

became apparent on day 3 with small percentages of FcεRI⁺c-Kit⁺ double-positive mast cells (Figure 2a). On days 7–14, percentages of FcεRI⁺c-Kit⁺ mast cells in BALB/c-derived cells appeared to increase to a higher level than in C57BL/6-derived cells (15.3–48.1% vs. 10.0–37.7%), but the differences were not statistically significant in repeated experiments. On day 21, which is a time point often used for mast cell preparation in *in vitro* experiments, FcεRI⁺c-Kit⁺ mast cells from C57BL/6 mice exhibited similar percentages as compared with those from BALB/c mice (Figure 2a, 2b, 73.1% vs. 65.6%). Absolute numbers of FcεRI⁺c-Kit⁺ mast cells harvested on day 21 tended to be higher in BALB/c than in C57BL/6 mice (Figure 2c, $p = 0.0023$), because total bone marrow culture cells were still higher in BALB/c cells, indicating that mast cells from these two mouse strains should undergo comparative analysis.

On the other hand, FcεRI⁺CD49b⁺ cells (basophil-like cells) briefly became apparent at the higher level in C57BL/6 mice compared with BALB/c mice on days 7–14 (Figure 2d, 27.0–34.8% vs. 9.9–27.7%), illustrating a biased potential of C57BL/6 cells for basophil differentiation. As mast cells are often reported as FcεRI⁺c-Kit⁺ or FcεRI⁺CD49b[−] cells, we also monitored c-Kit and CD49b expressions among FcεRI⁺ gated cells in three-color staining (Figure 2e, left). It appeared that c-Kit⁺ cells and CD49b⁺ cells were distinct populations, because they were not distributed continuously (Figure 2e, right), implying that c-Kit⁺ mast cells were not derived from transient basophil differentiation *in vitro*, although c-Kit⁺ mast cells occupied the majority of the cell culture by day 21.

Bone marrow-derived mast cells were compared for FcεRI expression, cell size, and granules

Next, we compared FcεRI expression levels on FcεRI⁺ BMMCs on day 21 and found no significant difference between BALB/c and C57BL/6 mouse strains in repeated experiments (Figure 3a). Moreover, histograms of forward scatter (cell size) and side scatter (intracellular granule content) plots also revealed similar levels (Figure 3b, 3c); hence, bone marrow-derived cells on day 21 were not morphologically different.

Bone marrow-derived mast cells were compared for degranulation capacity by receptor crosslinking and calcium ionophore

For functional analysis of BMMCs, we examined degranulation capacity (β-hexosaminidase) with calcium ionophore A23187, as well as crosslinking of FcεRI with IgE plus specific antigen. As a result, BMMCs elicited degranulation at 0.4 and 4 μM of A23187 (Figure 4a, left), and BALB/c-derived BMMCs tended to exhibit 1.4-fold higher degranulation capacity than those from C57BL/6 mice ($p = 0.0001$ – 0.0025). Conversely, the total amount of β-hexosaminidase activity was 1.6-fold more abundant in C57BL/6-derived BMMCs (Figure 4a, right, $p = 0.0002$). Likewise, upon ligation of FcεRI, BALB/c-derived BMMCs again exhibited 2.0- to 2.5-fold higher degranulation (Figure 4b, left, $p < 0.0001$) at 10–50 ng/mL of DNP-HSA antigen than did C57BL/6-derived BMMCs, irrespective of slightly more abundant total β-hexosaminidase activity in C57BL/6 cells (Figure 4b, right, $p = 0.0003$), indicating higher efficiency of degranulation in BALB/c-derived BMMCs, at least for day 21 preparation.

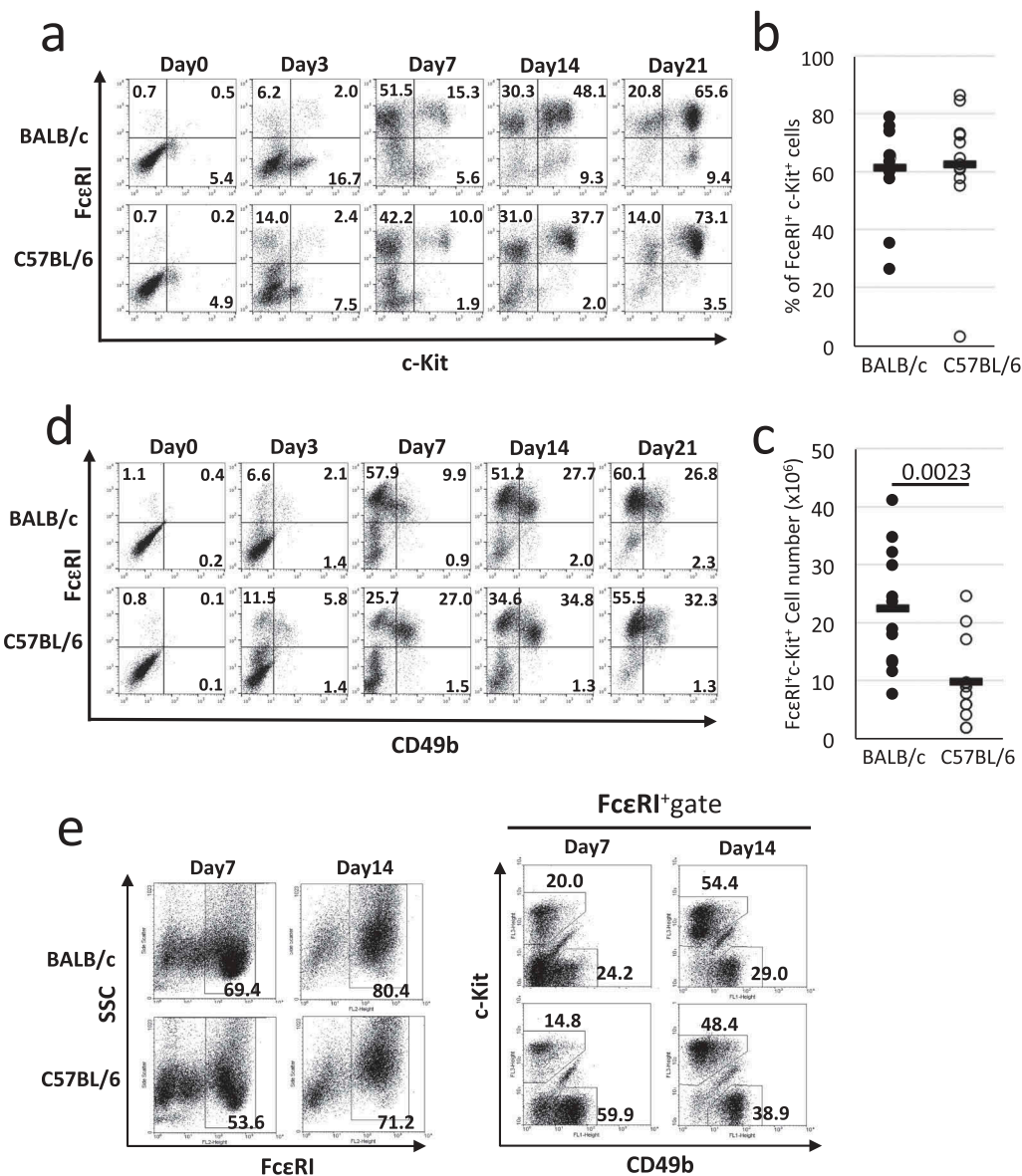


Figure 2. Flow cytometric analysis of cell surface molecules associated with mast cell differentiation. Bone marrow cells were prepared from BALB/c and C57BL/6 mice and cultured in IL-3-containing medium for 3 weeks. Proportions of FcεRI⁺c-Kit⁺ mast cells (a) and FcεRI⁺CD49b⁺ basophil-like cells (d) were periodically monitored. Percentages (b) and absolute numbers (c) of FcεRI⁺c-Kit⁺ mast cells were calculated on day 21 (n = 12). The p values were calculated. (e) Among FcεRI⁺ gated cells (left), c-Kit⁺ and CD49b⁺ cells were plotted on days 7 and 14 (right).

Bone marrow cell-derived mast cells were compared for intracellular signal transduction by receptor crosslinking

Because degranulation capacity was different between the two strains, we examined intracellular signal transduction of BMMCs induced by receptor ligation. BALB/c-derived BMMCs

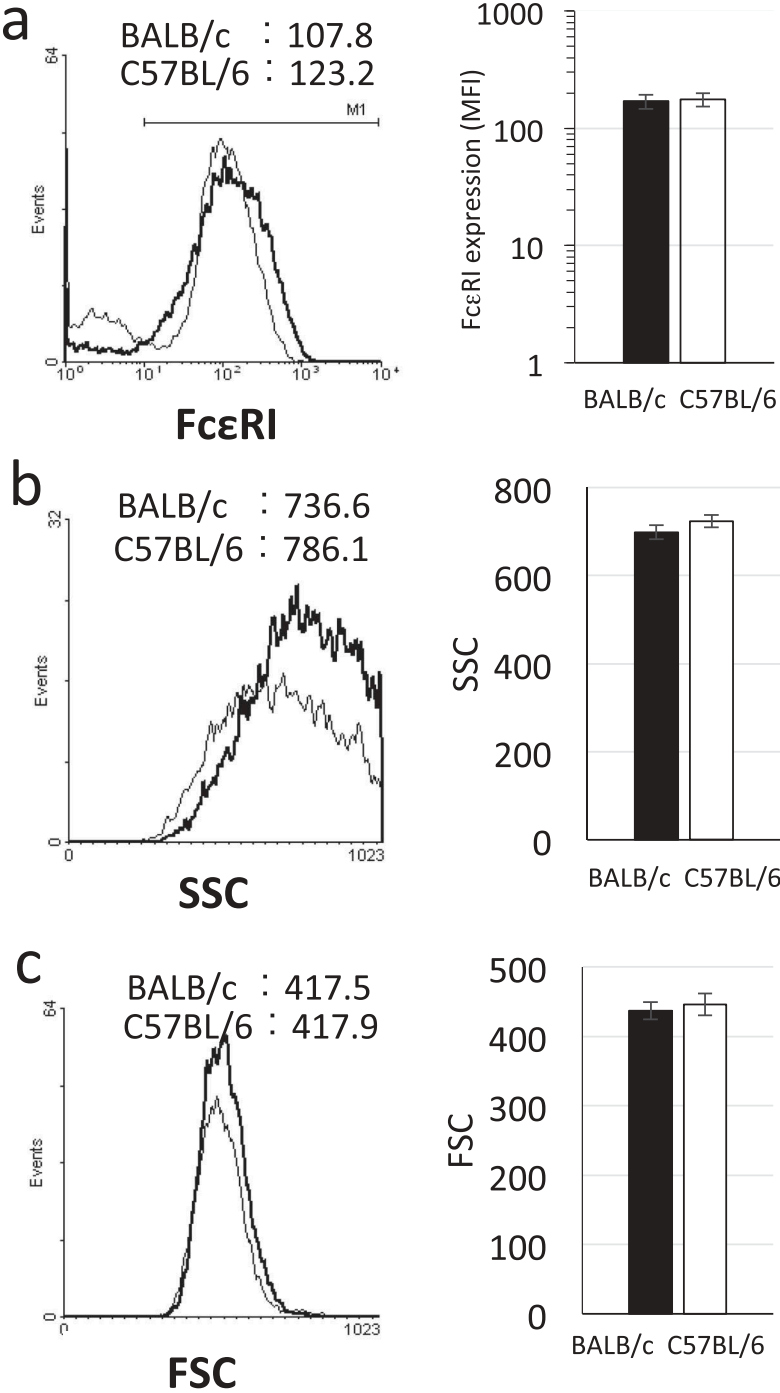


Figure 3. Flow cytometric analysis of FcεRI expression and side and forward scatter. Representative (left panel) and average values (right panel) were compared for FcεRI expression (a), side scatter (SSC) indicating the presence of intracellular granules or complexity (b), and forward scatter (FSC) indicating cell size (c) by flow cytometric analysis (n = 12). On the left panels, the bold lines indicate C57BL/6 cells and the thin lines indicate BALB/c cells. On the right panels, the closed bars show BALB/c cells and the open bars show C57BL/6 cells.

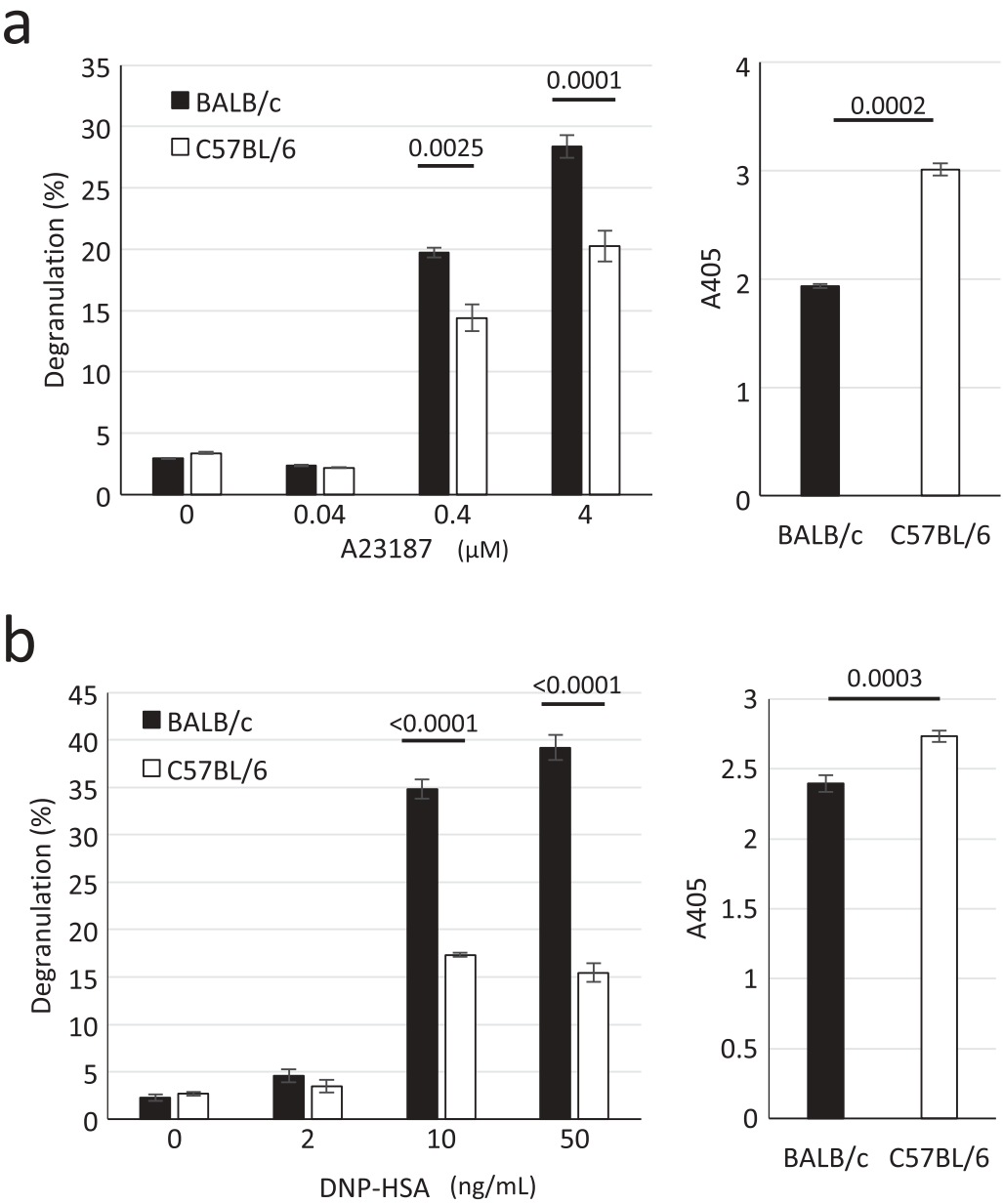


Figure 4. Degranulation of BMMCs following cell culture for 3 weeks. BALB/c (closed bar)-derived and C57BL/6 (open bar)-derived BMMCs were assessed for degranulation capacity following 3 weeks of cell culture as the release of β -hexosaminidase. Cells were stimulated with calcium ionophore A23187 (a) and anti-DNP-specific IgE (SPE-7) and DNP-HSA (b) at the indicated concentrations (left panel). Total β -hexosaminidase activities of the supernatant and the cell pellet were estimated (right panel), and p values were added. These figures are representative of four independent experiments with similar results.

showed a gradual increase in tyrosine phosphorylation at 37, 55, 70, 90, and 120 kDa for 10 min (Figure 5). Conversely, C57BL/6-derived BMMCs elicited transient tyrosine phosphorylation at 5 min with the strongest bands around 70 kDa, after which the phosphorylation levels decreased at 10 min. In addition, serine/threonine kinase Akt showed slightly stronger phosphorylation in BALB/c cells, whereas ERK revealed similar phosphorylation levels in both strains, but C57BL/6 cells again revealed the transient pattern, suggesting that differences in signaling events may reflect differences in degranulation capacity.

Mast cell differentiation during long-term cell culture

Although degranulation and phosphorylation events following 3 weeks of cell culture (Figures 4 and 5) were different between the two mouse strains, this might be attributed to insufficient maturation and/or purity of FcεRI⁺c-Kit⁺ mast cells, which were approximately 60–70% (Figure 2) and might affect functional capacity. Thus, we decided to extend the cell culture period to a maximum of 8 weeks (56 days) for further mast cell differentiation. During this period, unlike the initial 3 weeks, proliferative responses to IL-3 showed the opposite, in that BALB/c-derived cells mostly decreased, whereas C57BL/6-derived cells varied and often increased (Figure 6a, 6b).

The FcεRI⁺c-Kit⁺ mast cell fraction percentages gradually increased in both strains, suggesting further progress in the differentiation level (Figure 7a). In particular, the ratio of C57BL/6-derived mast cells increased as compared with that of BALB/c-derived cells (Figure 7a, 7b, 93.9% vs. 80.2% on day 56, $p = 0.0087$) as well as in the number of mast cells (23.4×10^6 vs. 16.2×10^6 cells on average), although the cell number was not significantly different (Figure 7c). These findings were consistent with a trend in their proliferative responses above.

Long-term cell culture did not alter the degranulation capacity of bone marrow-derived mast cells

Following long-term cell culture, a degranulation assay for mast cells was again conducted using calcium ionophore A23187 as well as antigen receptor crosslinking. As a result, BALB/c-derived cells again exhibited higher degranulation efficiency in both stimulations (Figure 8a, 8b, $p < 0.0001$), irrespective of the lower FcεRI⁺c-Kit⁺ mast cell fraction in BALB/c cells (Figure 7a, 7b). Total β-hexosaminidase activity also tended to be higher in C57BL/6-derived cells (Figure 8, right, $p < 0.0002$). These results indicate a difference in degranulation capacity between the two mouse strains and that long-term cell culture did not alter the degranulation capacity of BMMCs.

Discussion

In this study, using BMMCs from BALB/c and C57BL/6 mice, we found that mast cells also had functional differences between mouse strains such as degranulation capacity and intracellular signal transduction, as well as the cell differentiation process. Our results prompt further questions regarding how such differences between mouse strains are generated, and we discuss them below.

Following bone marrow cell culture for 3 weeks, more BALB/c-derived cells were harvested than C57BL/6-derived cells (Figure 1b–d), suggesting a higher response to IL-3 in BALB/c

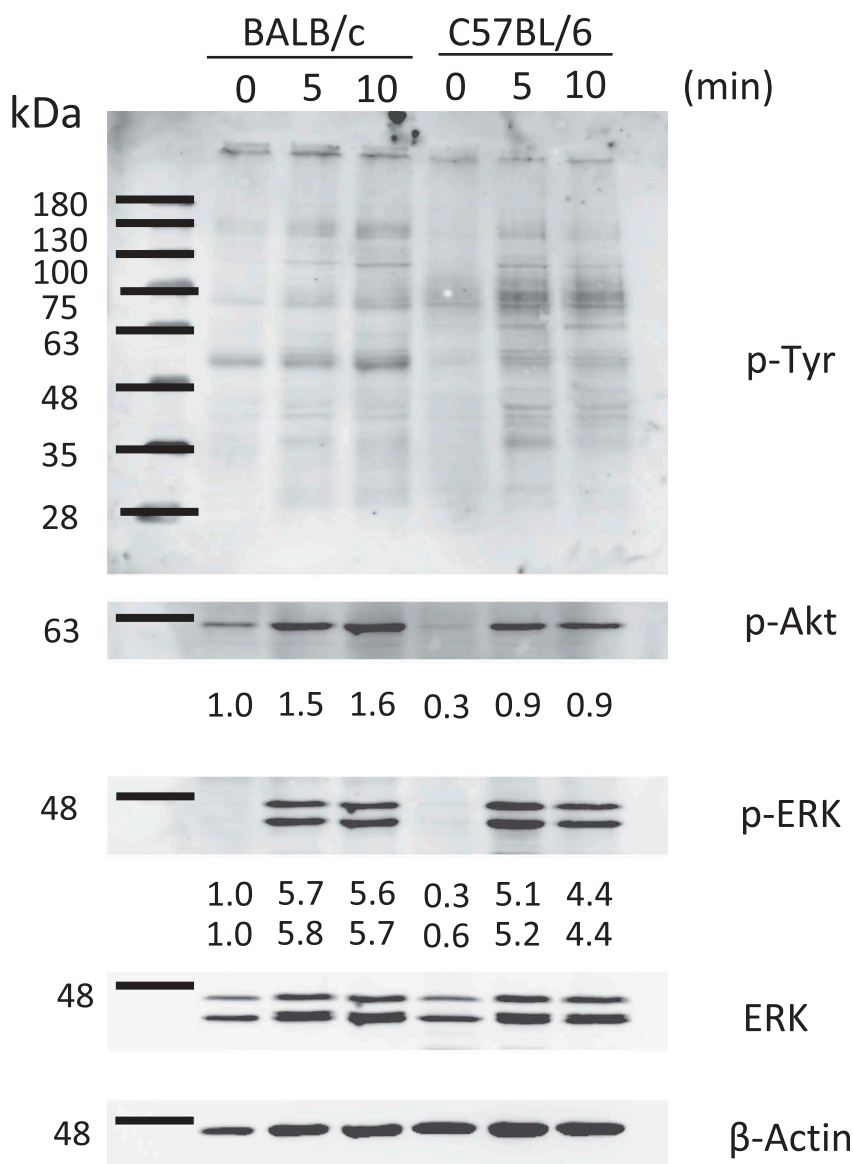


Figure 5. Phosphorylation analysis of BMMCs using western blot following cell culture for 3 weeks. BMMCs following 3 weeks of cell culture were stimulated for degranulation with anti-DNP-specific IgE and DNP-HSA, and their lysates were subjected to western blot analysis using specific antibodies such as anti-phospho-tyrosine, anti-phospho-Akt, anti-phospho-ERK, anti-ERK, and β -Actin. The numbers below the blots were band intensities corrected with β -actin and set as 1.0 for an unstimulated sample of BALB/c-derived BMMCs. These figures are representative of three independent experiments with similar results.

cells. To address the question regarding the difference in sensitivity to IL-3 between mouse strains, we first attempted to compare the IL-3 receptor (IL-3R) expression level on BMMCs but failed to detect using antibody (clone 5B11). We do not know the exact reason for this, but we speculate that it could be ascribed to IL-3 present in the cell culture medium which had already bound and occupied an IL-3R epitope on mast cells for subsequent antibody binding

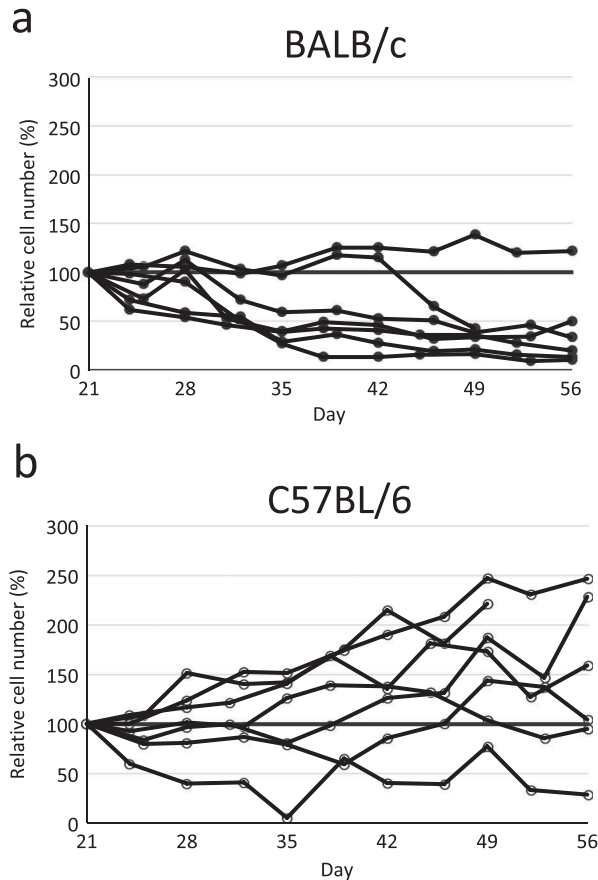


Figure 6. Cell number transition during extended cell culture for 8 weeks in the presence of IL-3. BMMCs that had been cultured for 3 weeks were subjected to long-term incubation for 8 weeks in the presence of IL-3 ($n = 7$). As compared with the initial cell numbers (day 21) that were set as 100%, the relative cell numbers were monitored every 3 or 4 days. Each line represents each cell culture from a single BALB/c (a) and C57BL/6 (b) mouse.

for staining. Thus, we also attempted to rest the cells for 4 h or overnight in the absence or presence of (minimal) IL-3 to release IL-3 from IL-3R or downregulate the IL-3R for recycling leading to expression of empty IL-3R; however, the detection was negligible (data not shown). Furthermore, to clarify the difference in IL-3 sensitivity, we also tried to compare DNA sequences of IL-3R α , IL-3R β , and its signaling molecules, including STAT5a, STAT5b, and JAK2 (Baker et al., 2007), to detect single nucleotide polymorphisms (SNPs) that may affect the sensitivity of intracellular signaling by IL-3 utilizing the Mouse Genome Informatics database. However, many sequences for the BALB/c strain were not available; thus, a mechanism for the difference in sensitivity remains to be explored.

In many reports using BMMCs, bone marrow cells were incubated for 3–4 weeks in the presence of IL-3 (Crowle and Reed, 1981; Levi-Schaffer et al., 1986). Hence, we also used 3 weeks to prepare BMMCs from the BALB/c and C57BL/6 mouse strains and examined the transition in cell populations within the cell culture by FACS analysis. Although Fc ϵ RI $^{+}$ c-Kit $^{+}$ mast cells

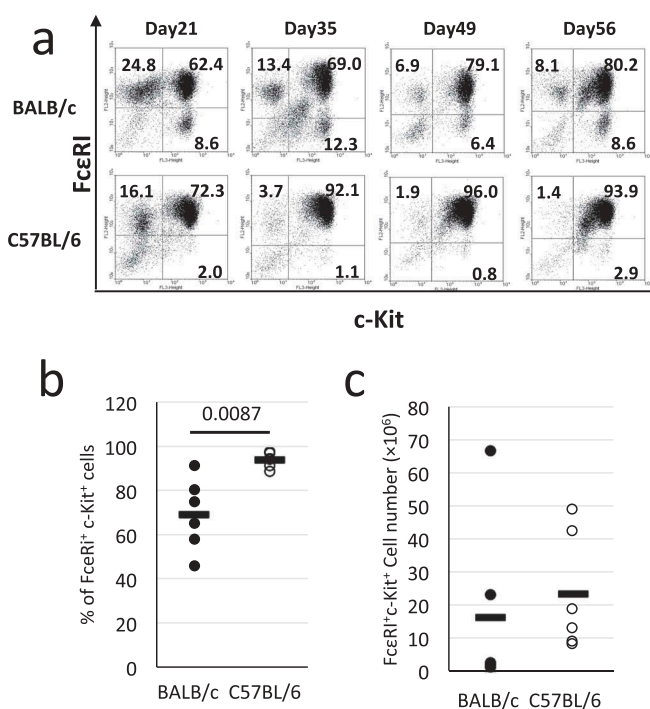


Figure 7. Flow cytometric analysis of cell surface molecules associated with mast cell differentiation during long-term incubation.

BMMCs that had been cultured for 3 weeks were subjected to long-term incubation for total 8 weeks in the presence of IL-3. (a) Proportions of FcεRI⁺c-Kit⁺ mast cells were periodically monitored. (b) Percentages and (c) absolute numbers of FcεRI⁺c-Kit⁺ mast cells were also calculated on day 56 ($n = 6$). The p values were added.

appeared earlier in BALB/c-derived cells on days 7–14, C57BL/6-derived FcεRI⁺c-Kit⁺ mast cells caught up or surpassed the ratio on day 21 (Figure 2a). In the additional 5-week incubation period, C57BL/6 cell dominance became more prominent (Figures 6 and 7). The finding that early appearance of BALB/c mast cells and later dominance of C57BL/6 mast cells was constantly observed in repeated experiments, indicating sequential alteration in the differentiation process, as well as a possible difference in the cell-intrinsic program during differentiation that each cell might possess.

As described above, the proportion of mast cells following cell culture for 3 weeks was 60–70% (Figure 2a), and degranulation capacity was different between mouse strains (Figure 4), raising the question of whether 3 weeks incubation was just on the way to, and not enough for, BMMCs to fully differentiate. Thus, we wonder whether a longer incubation up to a maximum 8 weeks (56 days) might engender further cell maturation and cancel out the differences in the cell populations and functional capacities. Indeed, the FcεRI⁺c-Kit⁺ mast cell population ratio increased in both strains (Figure 7a) as compared with those incubated for 3 weeks. In particular, C57BL/6 cells constituted an almost single population appearing to be terminally differentiated cells (Figure 7a). However, it could not be distinguished whether this rise in the mast cell ratio was attributable to authentic progress in maturation or simply a relative increase in the ratio due to cell death other

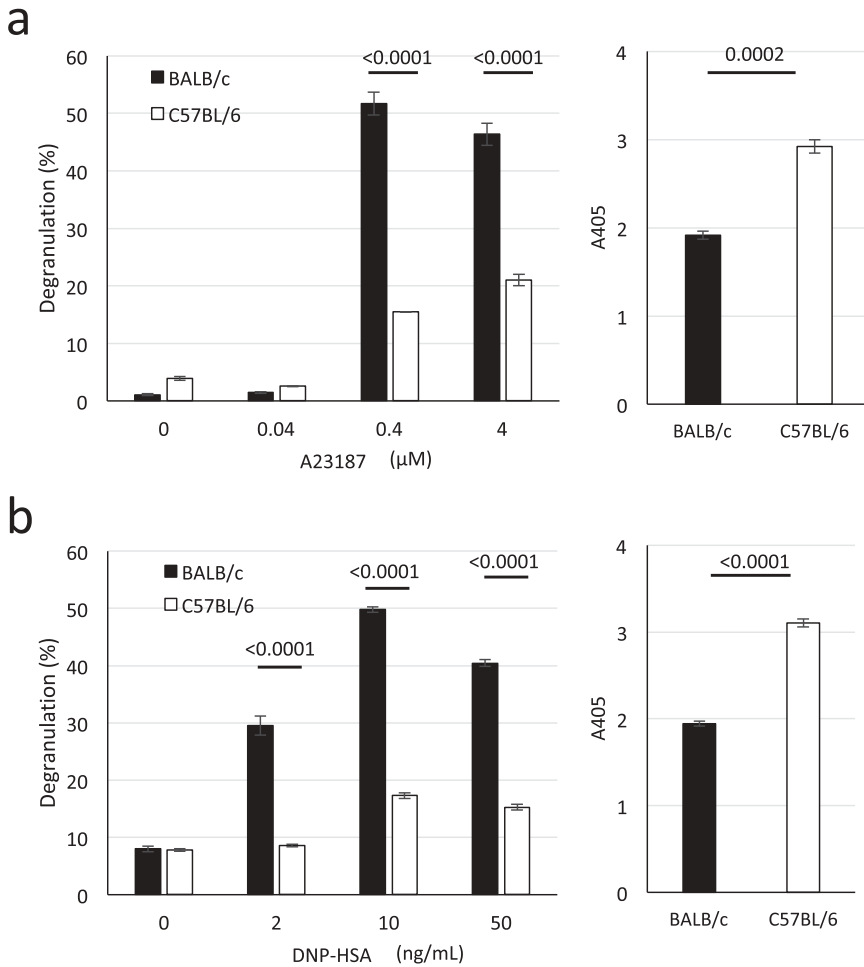


Figure 8. Degranulation of BMMCs following cell culture for 8 weeks.

BALB/c (closed bar)-derived and C57BL/6 (open bar)-derived BMMCs were assessed for degranulation capacity following 8 weeks in cell culture as the release of β -hexosaminidase. Cells were stimulated with calcium ionophore A23187 (a) and anti-DNP-specific IgE and DNP-HSA (b) at the concentrations indicated (left panel). Total β -hexosaminidase activities of the supernatant and the cell pellet were also estimated (right panel), and p values were calculated. These figures are representative of two independent experiments with similar results.

than $\text{Fc}\epsilon\text{RI}^+\text{c-Kit}^+$ mast cells. As a result of our calculation, cell numbers of $\text{Fc}\epsilon\text{RI}^+\text{c-Kit}^+$ -gated mast cells increased in many samples in C57BL/6 cells, although varied (Figure 6), that corroborated the former possibility by ruling out the reduction in cell number of the latter possibility. Thus, it is possible to conclude that differences in degranulation capacity between these mouse strains persist in spite of further cell maturation.

Previously, Noguchi et al. reported that degranulation (β -hexosaminidase) elicited by antigen was higher in C57BL/6-derived cells than in BALB/c-derived cells (Noguchi et al., 2005). However, our result showed the opposite (Figure 4), and we do not yet know the reason for this discrepancy but speculate several possibilities. The first discrepancy was a difference in

the percentage of FcεRI⁺c-Kit⁺ mast cells described as >95% at 4 weeks in Noguchi's report, whereas the percentage was 60–70% at 3 weeks in our study (Figure 2a). They started cell culture using lineage-negative cells that enriched stem cells in the beginning of cell culture, whereas we used whole bone marrow cells. This step probably assisted in increasing the mast cell ratio when harvested. However, in our experiment, we also obtained 93.9% FcεRI⁺c-Kit⁺ mast cells from C57BL/6 mice following 8 weeks of incubation (Figure 7a), but these cells showed a lower degranulation capacity as compared with those from BALB/c mice (Figure 8). The results indicated that the percentage does not always affect degranulation efficiency.

Of the story above, the second discrepancy was another side of the coin, which was a difference in the amount of adherent cells that appeared during the bone marrow cell culture with IL-3. If whole bone marrow cells were used, the amount of adherent cells might be increased, and these cells might produce particular growth factor(s) or ligand molecule(s) such as stem cell factor, affecting cell maturation or other cellular traits (Coleman, 1993; Ito et al., 2012; Lee et al., 2010; Levi-Schaffer et al., 1986).

The third discrepancy was the existence of sublines derived from a mouse strain. C57BL/6 mice in Japan also have at least two sublines including C57BL/6NCrSlc (B6N) and C57BL/6JJmsSlc (B6J), and there are 34 SNPs and 2 indels in the protein coding region of the genome between these two sublines (Simon et al., 2013). However, differences in immune responses against *Listeria* are reported to be limited; thus, it is unlikely that the above SNPs and indels are involved in functional differences in mast cells.

In this study, phosphorylation patterns appeared to be different between BALB/c and C57BL/6 mice (Figure 5). BALB/c cells showed a gradual increase in tyrosine phosphorylation, whereas C57BL/6 cells showed a transient peak at 5 min. Compared with the result of higher degranulation in BALB/c cells (Figure 4), the gradual increase in tyrosine phosphorylation allowed us to consider that it might lead to efficient degranulation, whereas the transient tyrosine phosphorylation in C57BL/6 cells did not.

It is very likely that these tyrosine phosphorylated molecules include well-known protein tyrosine kinases located immediately downstream of FcεRI receptor such as Lyn (56 kDa) and Syk (72 kDa) (Kalesnikoff and Galli, 2008; Nakata et al., 2008). In addition, the reason or mechanism for the transient phosphorylation remains undetermined, but expression of negative regulators in signaling might be increased in C57BL/6 cells following a 3-week culture period with IL-3. The candidates for possible negative-signaling regulators are SOCS family members, tyrosine phosphatases such as Shp1 or Shp2, or others. Multiple bands around 70 kDa that were markedly phosphorylated, especially in C57BL/6 cells, might contain such negative regulators. In addition, Akt phosphorylation was slightly different between mouse strains (Figure 5, middle). A previous report (Takayama et al., 2013) found that inhibition of Akt phosphorylation with a specific inhibitor resulted in reduced degranulation, suggesting a positive role of Akt for mast cell degranulation. Also, we are planning to explore other molecules responsible for the attenuated or transient phosphorylation in C57BL/6 cell signaling.

In conclusion, we have demonstrated that, like T helper cells and macrophages, BMMCs also have distinct properties between mouse strains including cell surface molecule expression, degranulation capacity, and intracellular signaling via FcεRI. Further reasons or mechanisms for the differences in strains observed in the current study remain to be elucidated. Because humans have different inclinations for allergic diseases, mast cells may be partially responsible for these differences. Because of our findings, we are prompted to unravel the big picture of allergy susceptibility in the near future.

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Disclosure statement

We do not have any financial conflict of interest on data in this manuscript.

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