

Double expression of CD34 and CD117 on bone marrow progenitors is a hallmark of the development of functional mast cell of *Callithrix jacchus* (common marmoset)

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Abstract

Mast cells (MCs) are developed from hematopoietic progenitor cells and play an important role in inflammation. Study of the kinetics of development and accumulation of primate MC *in vivo* is crucial for the control of human inflammatory diseases, as evolution of the immune system is quite rapid and inflammation including MC response is considered to be different between mouse and human. In the present study, we examined the development of MC from hematopoietic progenitors of *Callithrix jacchus* (common marmoset), an experimental animal of nonhuman primates. Bone marrow cells were fractionated for the expression of CD34 and CD117 by cell sorting. MCs were developed *in vitro* or by transplanting the cells to NOD/SCID/IL-2 γ c knockout (NOG) mice. *In vitro* culture of CD34⁺CD117⁺ (double positive, DP) cells with stem cell factor could generate high-affinity Fc epsilon receptor (Fc ϵ R)-expressing CD117⁺ cells with typical granules. The developed MC released β -hexosaminidase and produced leukotriene C₄ after the stimulation of Fc ϵ RI. Transplantation of DP cells gave rise to a marked expansion of CD34⁺CD45⁺CD117⁺Fc ϵ R⁺ cells in NOG mice. They expressed transcripts encoding chymase 1 and tryptase β . Differentiation of CD34⁺CD117⁺ cells to MCs was relatively limited compared with the DP cells, similarly to human MCs. These results suggest that this marmoset system provides a good model for human MC development.

Keywords: hematopoietic progenitor cells, immunodeficient mouse, mast cells, Nonhuman primate.

Introduction

Allergic inflammation is associated with various human diseases including pollinosis and asthma. Molecular mechanisms involved in the onset and progression of allergic inflammation have been extensively studied at both cellular

and tissue levels (1,2). In parallel, intense efforts have been made to develop animal models using mammals such as rodents, expecting to mimic or reflect the human pathology of allergic inflammation (3–6). However, rodents (mouse) and

primates (human) are evolutionarily distant from each other, and they exhibit many differing features in their immune and allergic reactions (7). Especially, the engagement of cytokines and their receptors differs between humans and mice. For example, T_H17 , a subset of T helper cells, is closely involved in allergy. IL-6, IL-21 and TGF- β are necessary for mouse T_H17 cell differentiation, whereas IL-1, IL-6 and IL-23 are important in the case of human T_H17 cells (8). Furthermore, gene configurations that encode allergy-suppressive signaling molecules such as Siglec and leukocyte Ig-like receptors/Ig-like transcripts are substantially different between mice and humans (9).

As a cellular element responsible for the induction of allergic inflammation, mast cells (MCs) are still one of the most important players, although it has become clear that a very complicated mechanism is involved in the establishment of chronic inflammation. Mechanisms of signal transduction in allergic reaction have been analyzed using *in vitro* cultures of human and mouse MCs (10,11). MCs can be activated by various stimuli such as high-affinity Fc epsilon receptor I (Fc ϵ RI) cross-linking, TLR-based recognition of bacterial components, complements and substance P (12–14). Upon stimulation, MCs release enzyme-containing granules and inflammatory mediators. Studies with MC-deficient and MC knockin mice have clearly revealed that MCs are involved in the initiation of IgE-dependent immediate hypersensitivity.

The development of MC lineage in mouse is becoming clear (15). MCs are derived from hematopoietic stem cells. The MC progenitor and basophil progenitor are the same lineage, and MCs and basophil develop from the same precursor, basophil/mast cell progenitor. However, the pathway is yet to be identified in human although there is a possibility that MCs and monocytes are the same lineage.

While it is difficult to clarify the lineage of MCs in human, a comparison with monkeys might allow us to speculate because they can be expected to carry immune potentials more similar to those of humans. One monkey species that has recently attracted researchers' attention is *Callithrix jacchus*, conventionally called the common marmoset (16). This is a new world monkey, whose colonies have been established by planned breeding and maintenance in Japan for 50 years. A draft genome sequence of the common marmoset is available at UCSC Genome Browser (WUGSC 3.2, <http://genome.ucsc.edu/cgi-bin/hgGateway>), and transgenic marmoset individuals carrying the transgene through generations have been generated successfully (17). Furthermore, in analyses of 30 immunity-related genes, amino acid sequences are 86% identical in average between orthologs of human and common marmoset, whereas identities are 60% between orthologs of mouse and human (18). Thus, based on technological and genetic advances, the likelihood of the common marmoset being a candidate experimental animal mimicking human immunity can be explored. For this purpose, analysis of common marmoset cells responsible for allergic inflammation is of major importance.

One trial to examine the development of cell lineage is the xenotransplantation of cells in immunodeficient mice. Hematopoietic progenitors prepared from bone marrow, granulocyte-colony stimulating factor-mobilized peripheral blood or cord blood of humans have been transferred into

immune-deficient mice such as NOD/SCID/IL-2 γ c knockout (NOG) mice (19–21). Then, in these mice, T and B lymphocytes of human origin have been successfully regenerated to certain extents, but immune reactions to antigen exposure have been very limited. In reconstituted mice, formation of a germinal center is not evident, and production of antigen-specific antibodies is very poor (20,22,23). Under these circumstances, it was striking to witness the successful development of human MCs in NOG mice transplanted with hematopoietic progenitors (24).

Recently, we reported the development of several lines of monoclonal antibodies (mAbs) that were directed specifically to antigens present on blood cells of the common marmoset (25). These mAbs did not show cross-reactivity to homologous antigens of mouse or human cells, indicating the necessity to establish such marmoset-specific antibodies. The anti-marmoset mAbs that we reported included anti-CD4 and anti-CD8 for T lymphocytes and anti-CD34 and anti-CD117 for hematopoietic and MC progenitors. Based on the marker phenotype of MCs, it is reasonable to employ anti-CD34 and anti-CD117 for analysis of MCs, in addition to progenitors. An aim of the present study was to identify progenitors of MCs in bone marrow of the common marmoset and assay their differentiation potentials to MC lineage both *in vitro* and *in vivo*. The similarity of marmoset MCs with those of humans is discussed.

Methods

Animals

Common marmosets were obtained from CLEA Japan (Tokyo, Japan) and kept at the Central Institute for Experimental Animals (CIEA, Kawasaki, Japan) during the experiments. Experiments using common marmosets were approved by the Institutional Committee for Animal Care and Use and performed at CIEA according to the institutional guidelines. Marmoset age (1–4 years) and sex were arbitrary. NOD/Shi-scid, IL-2 γ c-null (NOD/SCID/ γ c-null; NOG) mice were provided by CIEA and kept under specific pathogen-free conditions. Experiments using mice were approved by the Institutional Committee for Animal Care and Use and performed at Tokai University following the University guidelines.

Preparation of marmoset cells

Spleens were taken from marmosets and cells were released from tissues. After red blood cells were lysed with low osmotic buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NH_4Cl), they were suspended in RPMI 1640 medium (Nissui, Tokyo, Japan) containing 10% (v/v) heat-inactivated fetal calf serum (FCS; SAFC Biosciences, Tokyo, Japan). Bone marrow cells of marmosets were collected into a heparinized tube and centrifuged on Lymphocelepar (IBL Co., Takasaki, Japan) at 2000 r.p.m. for 30 min. Mononuclear cells were collected and remaining erythrocytes were lysed.

MC-inducing culture

In the first experimental series, marmoset bone marrow cells were used directly without fractionation. To induce MC differentiation, cells were cultured in RPMI 1640 medium under

various cytokine conditions, such as in the presence of 10 ng/ml of recombinant mouse stem cell factor (rmSCF), 10 ng/ml of rmSCF + 5 ng/ml of recombinant mouse IL-3 or 10 ng/ml of rmSCF + 10 ng/ml of recombinant mouse IL-6. Cytokines were purchased from Peprotech (Rocky Hill, NJ). Every other day, nonadherent cells were collected and resuspended in fresh medium containing fresh cytokines as reported previously (26). Cell culture was continued for 4 weeks. In the second series, marmoset bone marrow cells were fractionated, and each fraction was assayed for MC differentiation activity. Cells were incubated with mixtures of phycoerythrin (PE)-labeled anti-marmoset CD34, allophycocyanin (APC)-labeled anti-marmoset CD117 (Oriental Yeast Co. Ltd., Tokyo, Japan), Peridinin chlorophyll protein-cyanine5.5 (Per-CP-Cy5.5)-labeled anti-human CD3 mAb (SP34-2, BD Pharmingen, San Diego, CA) and FITC-labeled anti-human CD20 mAb (B-Ly1, Dako, Glostrup, Denmark), and the CD3⁺CD20⁺ fractions were sorted into CD34⁺CD117⁺, CD34⁺CD117⁻, CD34⁻CD117⁺ and CD34⁻CD117⁻ fractions using FACS Aria (Becton Dickinson, Franklin Lakes, NJ). Then, each cell fraction was cultured in RPMI1640 supplemented with 10% (v/v) FCS, 10 ng/ml of rmSCF, 50 μ M of 2-mercaptoethanol, 100 μ M of nonessential amino acids (Gibco/Invitrogen, Carlsbad, CA), 1 mM of sodium pyruvate, 100 U/ml of benzyl penicillin potassium and 100 μ g/ml of streptomycin sulfate. Medium was changed as above, and cell culture was continued for 4 weeks.

Flow cytometry

Cells were incubated with appropriately diluted, fluorescence-labeled primary mAb for 15 min at 4°C and washed with 1% (w/v) BSA-containing PBS. In some cases, cells were reincubated with labeled secondary antibody. The mAbs used were as follows: anti-human CD3-Per-CP-Cy5.5 (SP34-2), anti-human CD20 (B-Ly1), streptavidin-PE and APC-labeled streptavidin were purchased from BD Pharmingen; anti-human CD14-FITC (61D3), human IgE (HE-1) and anti-human IgE (BE5) were purchased from e-Bioscience, BioPorto Diagnostics (Gentofte, Denmark) and Abcam Japan (Tokyo, Japan), respectively. To detect surface expression of Fc ϵ RI, marmoset-derived MC cultures (2×10^5) were incubated with 5 μ g/ml of human IgE overnight. Cells were washed and further incubated with FITC-conjugated mouse anti-human IgE mAb on ice for 20 min. Stained cells were analyzed on FACSCalibur (Becton Dickinson).

Immunoblotting

The human IgE-sensitized cells were washed twice with PBS and then stimulated with 10 μ g/ml rabbit anti-human IgE polyclonal antibody for the indicated times. The stimulated cells were washed twice with ice-cold PBS and lysed for 30 min on ice in a lysis buffer (Tris-buffered saline containing 1% NP-40, 2 mM phenylmethylsulfonylfluoride, 10 μ g/ml aprotinin, 2 μ g/ml leupeptin and pepstatin A, 50 mM NaF and 1 mM sodium orthovanadate). The lysates were centrifuged for 15 min at $15\,000 \times g$. The samples were then boiled, separated on a 12% SDS-PAGE gel and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated with a primary antibody and an appropriate secondary horseradish peroxidase-conjugated antibody. The signals were

detected by enhanced chemiluminescence (GE Healthcare Biosciences, Buckinghamshire, UK).

Assay of degranulation and leukotriene C_4 production

IgE-sensitized and unsensitized cells were washed twice with PBS and then stimulated with rabbit anti-human IgE polyclonal antibody at the indicated concentrations for 30 min. Degranulation was determined by β -hexosaminidase release, as described previously (6). The percentage of β -hexosaminidase release was calculated as follows: (supernatant optical density of cells)/(total cell lysate optical density of cells) $\times$ 100. The leukotriene C_4 (LTC₄) production was analyzed with an LTC₄ EIA kit (Cayman Chemical, Ann Arbor, MI).

Measurement of $[Ca^{2+}]_i$ mobilization

The IgE-sensitized and unsensitized cells were washed twice with PBS and then loaded with 4 μ M Fluo3-AM (Dojindo, Kumamoto, Japan) for 30 min. The cells were resuspended in $1 \times$ Tyrode's buffer (126 mM NaCl, 4 mM KCl, 641 μ M KH₂PO₄, 10 mM HEPES, 5.6 mM D-glucose, 1 mM CaCl₂, 600 μ M MgCl₂ and 0.1% BSA) and stimulated with rabbit anti-human IgE polyclonal antibody. Changes in dye fluorescence upon addition of stimuli were monitored at 200-ms intervals by a flow cytometer (FACSCalibur, BD Biosciences).

Histological analyses

Cells in suspension were placed on glass slides pretreated with 3-aminopropylethoxysilane (Digene, Beltsville, MD), incubated in 5% (v/v) CO₂ in air at 37°C in a humidified chamber for 30–40 min and centrifuged at 100 g for 15 min using Cytospin 3 (Shandon, Pittsburgh, PA). Cells settled onto glass surfaces were fixed with 99.8% pure methanol. In case of tissues such as spleen, they were fixed with 20%-buffered formalin and embedded in paraffin. A paraffin block was microsectioned, deparaffinized and postfixed. Cells or tissue sections on glass slides were conventionally stained with hematoxylin-eosin. In order to stain metachromatic granules specifically present in MCs, acidic toluidine blue (pH 1.0), alcian blue (pH 2.5) or 0.1% safranin O solution was used (Merck, Darmstadt, Germany). Immunohistochemistry was performed using Bond-max automatic slide stainer (Leica Microsystems, Tokyo, Japan) and associated Bond Refine Polymer Detection Kit. Primary antibody used was anti-human-HLA clone EMR8-5 (Hokudo, Sapporo, Japan). Sections were examined by microscope DP71 (Olympus, Tokyo, Japan).

Confocal immunoscopy

Marmoset cells stained with APC-labeled anti-CD14, CD34 and CD117 MaAb as above were treated with 4% paraformaldehyde and mounted on glass slides by cytospin (500 r.p.m. $\times$ 10 min) method. Cells were washed, retreated with 4% paraformaldehyde and analyzed by confocal laser microscope (META 510, Carl Zeiss, Hertfordshire, UK).

Reverse transcription-polymerase chain reaction

RNA was extracted from cells by RNeasy Mini Kit (Qiagen, Germantown, MD). RNA (50 ng) was reverse transcribed, and

Table 1. Primers for RT-PCR

Animal	Genes	Forward strand primer	Reverse strand primer
Marmoset	chymase 1	5'-TCTATAACAGT CACCTTGG-3'	5'-AGCTTCACCTCTT GCAGAGT-3'
	tryptase β	5'-AAGCAGGTGA AGGTCCCAT-3'	5'-TGATAGTCCAG GCCGGTT-3'
	HPRT	5'-TGACCAGTCA ACAGGGGAC-3'	5'-GCTCTACTAAGC AGATGGC-3'
Mouse	β -actin	5'-TCTCCCCAAG TTAGGTTTGTGTC-3'	5'-ATCATGTTTGAGA CCTTCAACAC-3'

generated cDNA was amplified using primers and OneStep reverse transcription-polymerase chain reaction (RT-PCR) kit (Qiagen). Reverse transcription was at 50°C for 30 min, polymerase activation at 95°C for 15 min with 33 cycles of PCR, each cycle consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. PCR products were subjected to agarose gel electrophoresis. The primers used are summarized in Table 1.

Transplantation of marmoset hematopoietic cells into NOG mice

CD34⁺CD117⁺ and CD34⁺CD117⁺ cells were purified from marmoset bone marrow by cell sorter as described above. Purity was more than 98% based on flow cytometry. Nine-week-old NOG mice were irradiated with 2.5Gy X-ray prior to transplantation, and marmoset cells (6.7×10^5 for CD34⁺CD117⁺ cells and 1.3×10^6 for CD34⁺CD117⁺ cells) were injected into mice intravenously. Four weeks after transplantation, peripheral blood was collected via orbit under inhalation anesthesia. Mononuclear cells were prepared and analyzed by flow cytometry.

Miscellaneous

Student's *t*-test was performed, and data were expressed as mean $\pm$ SD.

Results

Bone marrow-derived CD34⁺CD117⁺ cells can differentiate into MCs in vitro

In humans, MC progenitors are present in bone marrow. We first examined whether MC progenitors also existed in bone marrow of the common marmoset by culturing bone marrow cells *in vitro*. Mouse stem cell factor (SCF), IL-3 and/or IL-6 were added in combination to the medium as indicated in Fig. 1A. Mouse SCF was used because it can induce differentiation of human MCs more efficiently than human SCF, when hematopoietic progenitors from human cord blood are cultured *in vitro* (24). After culture was continued for 4 weeks, the cell surface expression of high-affinity IgE receptors (Fc ϵ RI) was monitored as a MC marker. This was done by incubating cells with human IgE and subsequently with FITC-labeled anti-human IgE. As seen by flow cytometry (Fig. 1A), approximately 15% of cells in the culture showed phenotypes of SSC^{lo} Fc ϵ RI⁺, and similar percentages were observed irrespective of the cytokine combinations added.

Thus, it became clear that the addition of SCF alone was sufficient for *in vitro* differentiation of MCs from marmoset bone marrow cells. As mouse IL-3 did not affect the development of marmoset MCs, we cut IL-3 in the following culture.

We previously reported that CFU-mix activity was most abundantly detected in the CD34⁺CD117⁺ fraction prepared from bone marrow cells of the common marmoset (25). We, therefore, fractionated bone marrow cells using anti-marmoset CD34 and anti-marmoset CD117 mAbs and FACS sorting. Supplementary Figure S1 (left panel), available at *International Immunology* Online, shows fractionation of CD34⁺CD117⁺ (double positive, DP), CD34⁺CD117⁺ (single positive, SP) and CD34⁺CD117⁺ (double negative, DN). They did not include any CD3⁺ or CD20⁺ cells (Supplementary Figure S1, available at *International Immunology* Online, right panels). Each fraction was cultured in the presence of mouse SCF for 4 weeks and processed for flow cytometry (Fig. 1B). Again, MC differentiation was monitored by the extent of Fc ϵ RI expression detected by human IgE and FITC-labeled anti-human IgE. Culture of DP cells produced 13% of SSC^{lo}Fc ϵ RI⁺ fraction, whereas that of SP cells produced only 8.4% of the corresponding fraction. Histograms in Fig. 1C indicate the fold increase of cell numbers after 4-week culture. Cell numbers in culture were increased by 330-fold in the DP fraction, 40-fold in the SP fraction and none in the DN fraction. The results shown in Fig. 1B and C indicate that the DP fraction likely contains a larger number of MC progenitors or mostly competent MC progenitors compared with the SP fraction.

During differentiation, MCs initiate the expression of Fc ϵ RI and simultaneously maintain CD117 expression. DP cells cultured for 4 weeks were processed for flow cytometry of Fc ϵ RI and CD117 (Fig. 1D). The Fc ϵ RI⁺ fraction occupied 19% of the culture, and 6.7% of cells were Fc ϵ RI⁺CD117⁺. The staining characteristics of Fc ϵ RI⁺CD117⁺ suggest that the cells represent authentic MCs. Confocal microscopy showed the Fc ϵ RI⁺CD117⁺ cells as rather small, granule-rich cells, distinct from larger CD117 SP cells, which were CD34⁺ progenitors still remaining in the culture (data not shown). The results in Fig. 1 thus indicate that the culture of marmoset bone marrow cells in the presence of mouse SCF can induce differentiation of MCs and that the CD34⁺CD117⁺ fraction contains MC progenitors in greater abundance compared with other fractions.

Function of in vitro-developed MCs

Next we examined whether the developed Fc ϵ R⁺ cells were functional MCs. We used the cells cultured for 10 weeks in the presence of mouse SCF, as most of the cells became Fc ϵ R⁺ cells (Fig. 2A). At first we examined whether Fc ϵ RI stimulation induces activation of signaling molecules. Cultured cells were sensitized with human IgE and stimulated by cross-linking the Fc ϵ R-bound IgE molecule with anti-human IgE antibody. We observed the upregulation of tyrosine phosphorylation level of spleen tyrosine kinase (Syk) (Fig. 2B). Calcium mobilization was also observed, as shown in Fig. 2C. The release of β -hexosaminidase by MCs was enhanced significantly by the cross-linking (Fig. 2D, upper panel). LTC₄ was significantly produced by MCs in a dose-dependent manner (Fig. 2D, lower panel). These results suggest that the developed MCs were functional MCs.

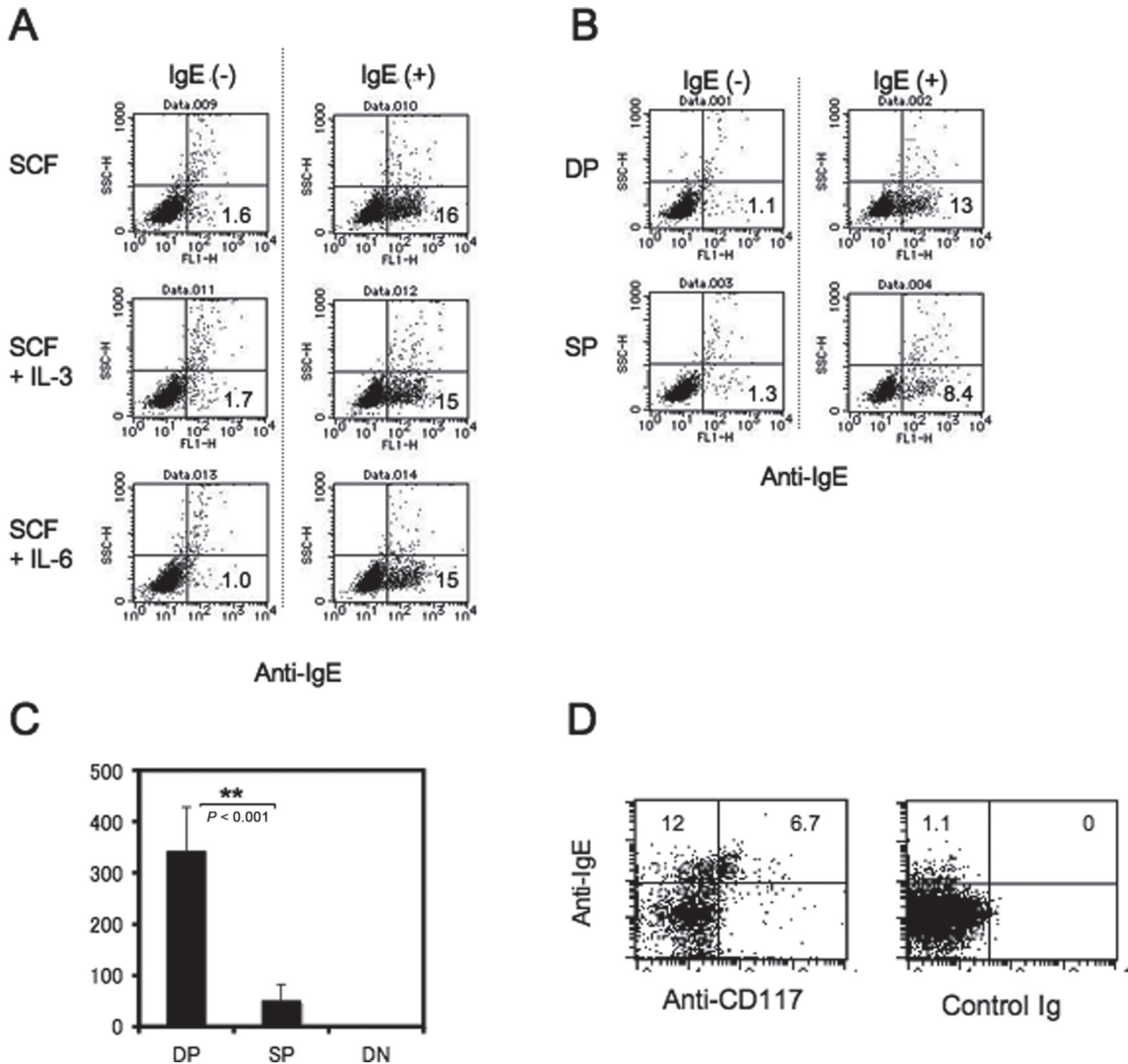


Fig. 1. *In vitro* differentiation of MCs from marmoset bone marrow cells. (A) Effects of cytokines on IgE receptor expression. Bone marrow cells of common marmoset were subjected to *in vitro* culture in the presence of mouse SCF, IL-3 and IL-6 as indicated. After 4 weeks of culture, human IgE was added or was not added to the medium, and incubation continued overnight. The cells were processed for flow cytometry using FITC-labeled anti-human IgE mAb, and profiles of SSC and FITC intensity are shown for each culture condition. The numbers indicate the percentages of cells in the SSC⁺FcεR⁺ quadrant. (B) IgE receptor expression in cultures of MC progenitors. Bone marrow cells of common marmoset were stained with anti-marmoset CD117-FITC and anti-marmoset CD34-APC, and the CD117⁺CD34⁺ (DP) and CD117⁺CD34⁻ (SP) fractions were isolated by flow cytometer. The DP and SP cells were cultured in the presence of mouse SCF for 4 weeks, incubated with or without human IgE overnight and processed for flow cytometry as in (A). (C) Expansion of cell numbers after culture of MC progenitors. DP, SP and CD117⁺CD34⁻ (DN) cells were isolated and cultured as in (B). Independent cultures were done in triplicate for each of DP, SP and DN. Folds in cell number increase are shown as mean ± SD. Statistically significant difference was detected between DP and SP ($P < 0.01$). (D) CD117 expression in cultures of MC progenitors. DP cells as cultured in (B) were processed for flow cytometry using anti-human IgE-FITC and anti-marmoset CD117-APC (left) or control IgG-FITC and control IgG-APC (right).

Development of marmoset MCs in transplanted NOG mice

Next, we transplanted the marmoset bone marrow cell fractions into immunodeficient NOG mice, and the possible existence of a MC population in the leukocyte fraction *in vivo* was examined. The monocyte-gated fraction was examined for CD34 and CD117 expressions (Fig. 3A). A substantial

population of CD34⁺CD117⁺ cells was detected in splenocytes (15% in DP-NOG and 13% in SP-NOG). Because the transplanted cells were originally CD34⁺CD117⁺ in the case of DP, the emergence of CD34⁺CD117⁺ cells suggests their differentiation toward MC lineage in NOG mice. The expression of FcεR was further examined by staining the CB-NOG spleen

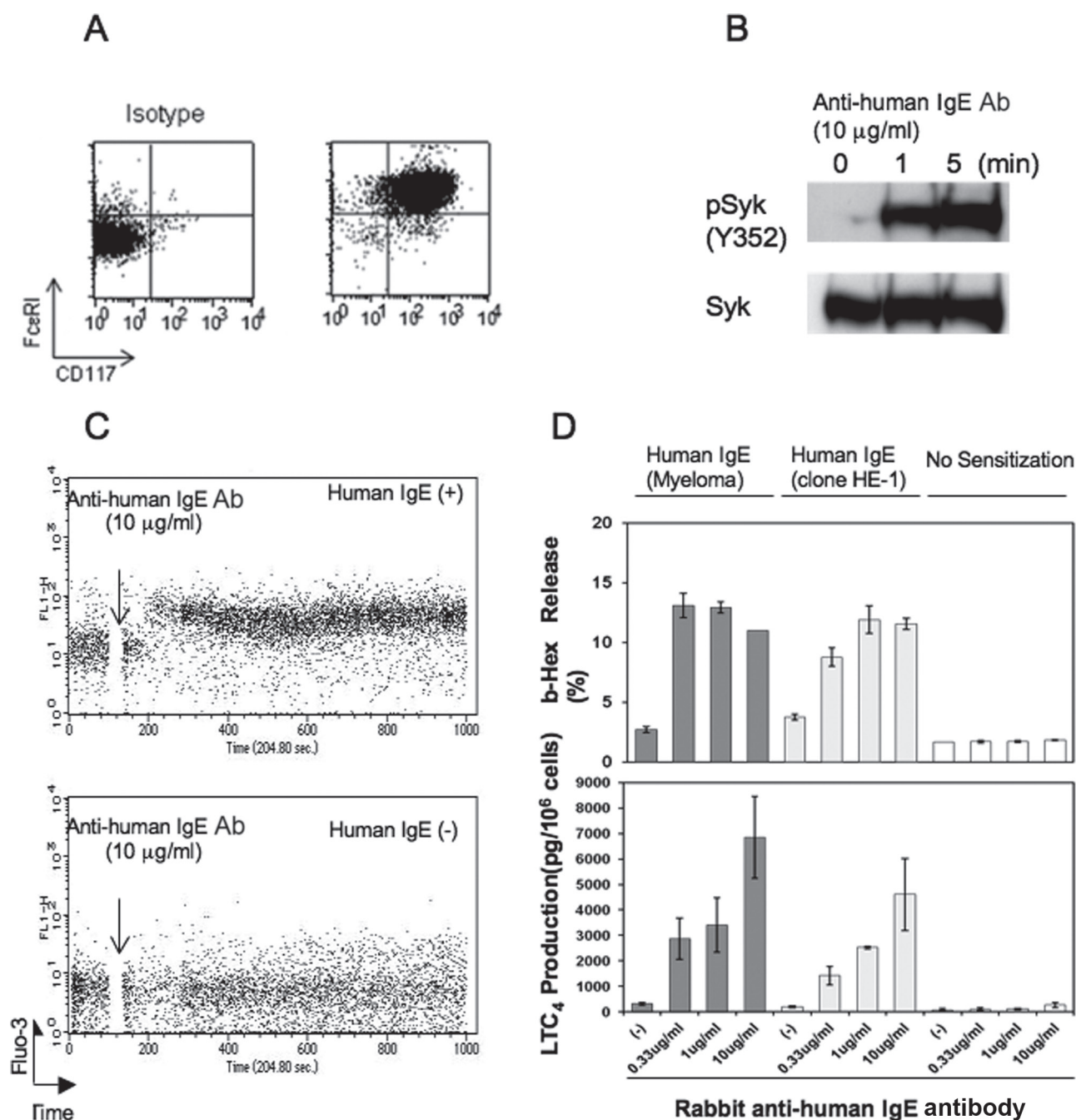


Fig. 2. Functional MCs developed *in vitro*. (A) The DP cells were cultured in the presence of mouse SCF for 10 weeks, incubated with hybridoma-derived human IgE (HE-1) overnight and processed for flow cytometry as described in Methods. (B) Marmoset MCs sensitized with human IgE (HE-1) were stimulated with rabbit anti-human IgE polyclonal antibody (10 µg/ml) for the indicated times. The total cell lysates were analyzed by immunoblotting with anti-phospho Syk (Tyr352) antibody. The blot was reprobed with anti-non-phospho Syk antibody. (C) Calcium mobilization in the human IgE (HE-1)-sensitized and unsensitized MCs. Changes in Fluo3 fluorescence were determined by employing flow cytometry after loading of rabbit anti-human IgE polyclonal antibody (10 µg/ml). (D) Marmoset MCs can degranulate and release LTC₄ in response to FcεRI stimulation. Marmoset MCs were sensitized with myeloma-derived human IgE or hybridoma-derived human IgE (HE-1) overnight. The IgE-sensitized MCs were stimulated with rabbit anti-human IgE polyclonal antibody at the indicated concentrations for 30 min. Degranulation response was determined by β-hexosaminidase release (upper panel). LTC₄ production was analyzed with an LTC₄ EIA kit (bottom panel).

cells with both anti-CD117 mAb and human IgE. Almost all of CD117⁺ cells expressed FcεR, confirming that the cells are MCs (Fig. 3A, right panel). In addition, because marmoset spleen itself contained only a small (3.4%) population of

CD34-CD117⁺, NOG mice appear to provide favorable conditions for progenitors to expand and differentiate into MCs.

As shown in Fig. 3B, the percentage of CD34-CD117⁺ population in spleens was higher in DP-NOG mice (15% on

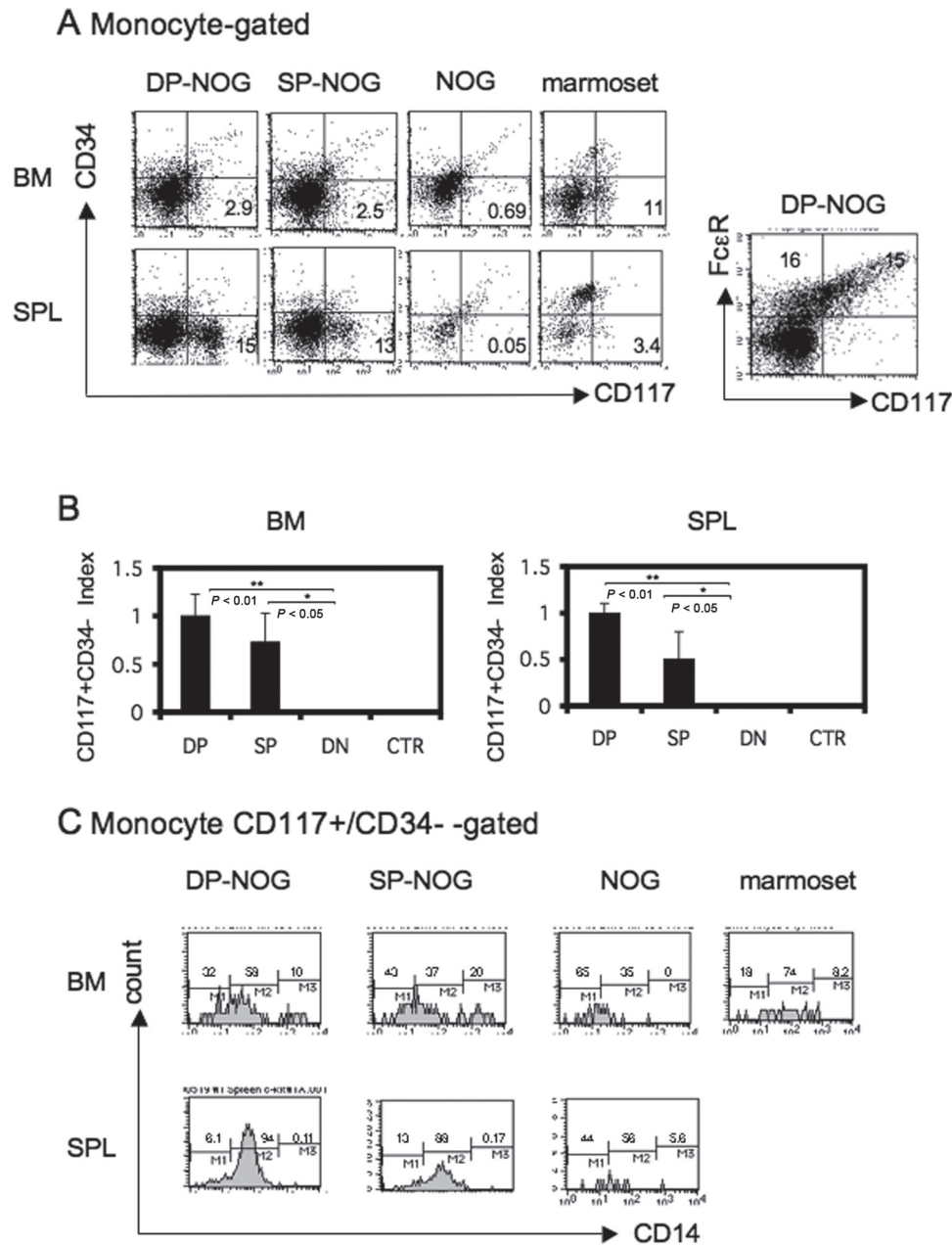
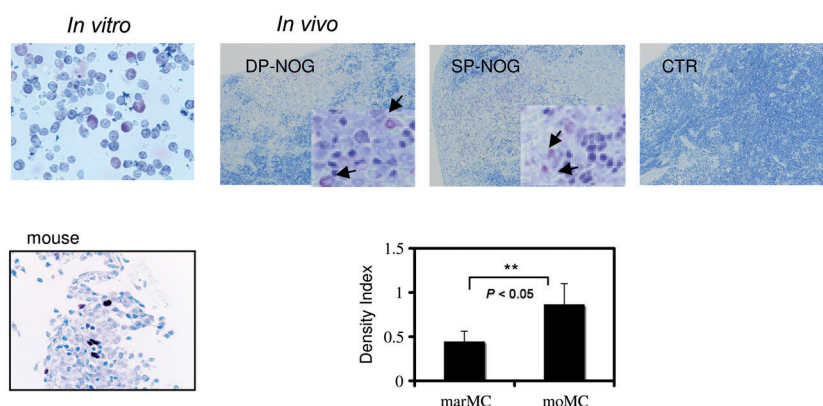


Fig. 3. Expression of MC markers in marmoset cells that were developed in NOG mice. CD117⁺CD34⁺ (DP) and CD117⁺CD34⁻ (SP) cells were prepared from marmoset bone marrow and transplanted into NOG mice. After 4 weeks, bone marrow cells (BM) and splenocytes (SPL) were taken from mice and processed for flow cytometry with anti-marmoset CD45, anti-marmoset CD34, anti-marmoset CD117, anti-human CD14 mAbs and human IgE/anti-human IgE mAbs. (A) The monocyte-gated fractions are displayed for the CD34 and CD117 expression (left panels). Cells from nontransplanted NOG mice and those from marmoset served as controls. The numbers indicate the percentages of CD34⁺CD117⁺ subfraction. Right panel shows the DP-NOG spleen cells stained with CD117 and human IgE. (B) Mean $\pm$ SD were calculated and expressed as index for the percentages of CD34⁺CD117⁺ subfractions from three independent experiments ($n = 3$) as in (A). Control (CTR) means cells from nontransplanted NOG mice. Statistically significant differences were detected between DP and SP in spleen ($P < 0.05$). (C) The monocyte/CD45⁺CD117⁺CD34⁻-gated fractions are displayed for their CD14 expressions. The numbers indicate the percentages of CD14^{lo}, CD14^{int} and CD14^{hi} subfractions, respectively.

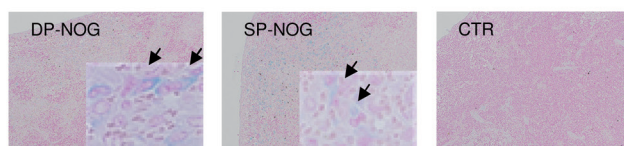
average, equalized to index 1.0) compared with SP-NOG mice (7% on average, equalized to index 0.5). This 2-fold difference was statistically significant ($P < 0.05$). The cell number of CD34⁺CD117⁺ population in NOG spleen showed around 1×10^5 to 2×10^5 in DP-NOG and 0.5×10^5 in DP-NOG and the pattern was very similar to that of the cell ratio (Supplementary

Figure S2 is available at *International Immunology* Online). In the case of bone marrow, percentages of CD34⁺CD117⁺ population were as low as 2–3%, but DP-NOG mice generated a slightly higher level of this population compared with SP-NOG mice. This suggests that the DP fraction likely contains more MC progenitors than the SP fraction, a notion

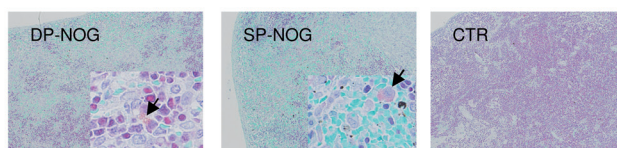
A Toluidine blue



B Alcian blue



C Safranin O



D Immunostaining

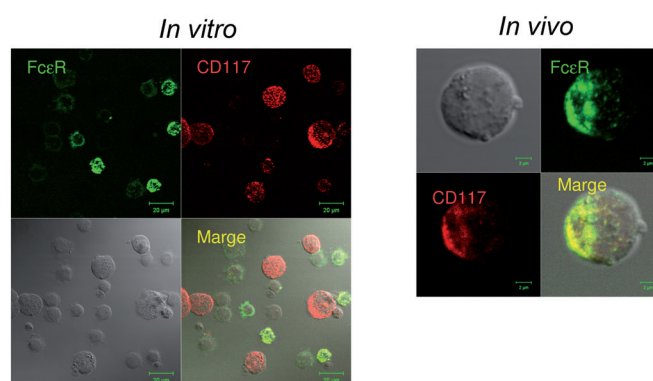


Fig. 4. Histochemical analyses of marmoset cells expanded *in vitro* and in transplanted NOG mice. (A) Toluidine blue staining of cells. Top left: CD117⁺CD34⁺ (DP) cells prepared from marmoset bone marrow were cultured *in vitro* in the presence of mouse SCF for 4 weeks, fixed and stained with toluidine blue. Top middle: CD117⁺CD34⁺ (DP) and CD117⁺CD34⁻ (SP) fractions of marmoset bone marrow cells were transplanted into NOG mice. After 4 weeks, spleens were taken and their sections were stained. Arrowheads indicate the metachromatically stained cells. Top right: Control (CTR) means a spleen from nontransplanted mouse. Bottom left: A section of mouse spleen was stained. Bottom right: Histograms represent the relative densities of metachromatically stained granules in MCs detected in spleens of marmoset cell-transplanted (marMC; $n = 69$) or nontransplanted NOG (moMC; $n = 8$) mice. A statistically significant difference was detected between the two samples ($P < 0.05$). (B) Alcian blue and (C) safranin O staining of spleen sections prepared as in (A). Arrowheads indicate metachromatically stained cells. (D) Confocal microscopy of *in vitro*- and *in vivo*-developed mast cells. BM cells cultured with SCF for 4 weeks (left panels) and sorted CD117 SP cells from DP-transplanted NOG spleen were stained for human FcεR (Alexa488-labeled) and CD117 (PE-labeled). FcεR (Green) and CD117 (Red) double expression is shown in small granule-positive cells. Most CD117 SP cells were small human IgE-positive cells and included granules in the cytoplasm.

in line with the results obtained by *in vitro* culture experiments (Fig. 1). In addition, MC differentiation was observed more prominently in spleen than in bone marrow of NOG mice.

To further characterize the nature of MCs, the monocyte/CD34⁺CD117⁺-gated fraction was then examined for the expression of CD14 (Fig. 3C). CD14 expression is known to be absent in progenitors, be induced in myeloid-monocyte lineage *in vitro* and then decline again to a background level in mature MCs in humans (27). In bone marrow, and more clearly in splenocytes, a CD14^{int} subfraction was detected. Thus, in transplanted NOG mice, the DP and SP fractions of marmoset bone marrow cells tended to differentiate into a MC lineage at least up to an immature stage.

Histochemical staining revealed development of marmoset MCs in transplanted NOG mice

Heparin-containing granules present in the cytoplasm of MCs were stained metachromatically by toluidine blue dye. Marmoset bone marrow cells cultured *in vitro* in the presence of SCF were stained by this dye (Fig. 4A, top). In addition to normochromatically stained cells (blue), metachromatically stained cells (red-purple) were identified. This observation further supports the concept of the differentiation of MCs from marmoset bone marrow cells. Histological sections of spleens were prepared from marmoset cell-transplanted NOG mice and processed for toluidine blue staining (Fig. 4A, middle). In both DP- and SP-transplanted spleens, metachromatically stained cells were detected, indicating the development of MCs *in vivo* as well.

We noticed that the extent of metachromatic staining was relatively mild in DP-transplanted spleen MCs ($n = 69$; Fig. 4A, middle) compared with that in nontransplanted spleen MCs ($n = 8$; Fig. 4A, bottom). Then, we measured the density of dye deposition on granules by densitometer, calculating it as density index. MCs of mouse origin were significantly heavily stained compared with marmoset MCs ($P < 0.05$). The differential staining patterns by toluidine blue might have reflected some qualitative/quantitative differences in the contents of heparin-containing granules in MCs of these two species.

Developing MCs are known to be distributed into distinct parts of the body and further differentiate into tissue-residing and/or mucosa-residing subtypes. To distinguish the subtypes of MCs developing in transplanted NOG mice, spleen sections were stained by another set of dyes (Fig. 4B and 4C). In both DP- and SP-transplanted spleens, cells were also metachromatically stained not only by alcian blue but also by safranin O. These results suggest that a substantial portion of marmoset MCs developing in transplanted NOG spleens might correspond to the tissue type (characterized by metachromasy for toluidine blue, alcian blue and safranin O) but does not exclude the possible coexistence of mucosa-type MCs (characterized by metachromasy for toluidine blue and alcian blue but not for safranin O).

Finally, we purified the spleen CD117SP cells and stained them with FcεR. Confocal microscopy revealed that the CD117SP cells were FcεR-positive and includes many granules, suggesting them to be MCs (Fig. 4D, right panels). Compared with *in vitro*-developed cell populations (4 weeks) in which CD117⁺FcεR⁻ progenitor cells were observed, FcεR-expressing small cells simultaneously expressed CD117 although the level was not high (Fig. 4D, left panels). FcεRI cross-linking with avidine-conjugated secondary antibody induced degranulation of the cells.

Marmoset MCs developed in NOG spleen is functional

Chymase and tryptase are signature proteases of MCs. Chymase 1 is expressed only in tissue-type MCs, whereas tryptase β2 expression is detected in both tissue and mucosa types of MCs. Using information available from a draft genome of *C. jacchus* from the UCSC genome browser, primers were designed to detect chymase 1 and tryptase β2 transcripts. Authenticity of PCR-amplified sequences using these primers was confirmed (data not shown).

We prepared RNAs from *in vitro* cultures and *in vivo*-transplanted spleens and processed them for RT-PCR analyses (Fig. 5A). Both chymase 1 and tryptase β2 transcripts were detected in the cultures of DP and SP marmoset bone marrow cells although the band density of tryptase β2 was

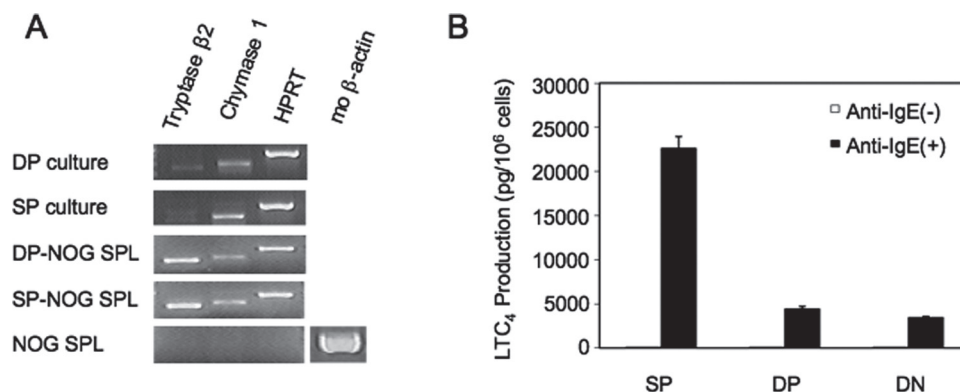


Fig. 5. Functional analysis of CB-NOG-derived MCs. (A) RT-PCR analyses of transcripts encoding MC. RNA was prepared from the indicated samples including the cultures of CD117⁺CD34⁺ (DP) or CD117⁺CD34⁻ (SP) cells, spleens of DP- or SP-transplanted or nontransplanted NOG mice or bone marrow or spleens of common marmoset. Transcripts of MC proteases were examined. Hypoxanthine phosphoribosyl transferase (HPRT) served as a control. (B) The SP (MC), DP and DN fractions were sorted out and IgE sensitized overnight. After that they were stimulated with rabbit anti-human IgE polyclonal antibody at 10 µg/ml for 30 min. LTC₄ production was analyzed with an LTC₄ EIA kit ($n = 3$).

relatively low. In addition, both transcripts were also observed in spleens of DP- and SP-transplanted NOG mice, whereas corresponding transcripts were not found at all in nontransplanted NOG mice, indicating the origin of chymase 1 and tryptase $\beta 2$ transcripts to be from transplanted and expanding marmoset cells. These results support the notion that the CD117⁺ cells found in *in vitro* cultures and *in vivo*-transplanted spleens indeed represented MCs and also that at least a portion of the population contained tissue-type MCs.

Next, *in vivo*-developed MCs were sorted out, sensitized with human IgE and stimulated by cross-linking the Fc ϵ R-bound IgE molecule with anti-human IgE antibody. LTC₄ was significantly produced by CD117SP compared with other fractions (Fig. 5B). These results suggest that CB-NOG-developed MCs are at least partially functional MCs.

Discussion

The initial trial to identify hematopoietic progenitors in the common marmoset was reported by Izawa *et al.* in 2004 (28). They developed an anti-marmoset CD34 mAb, isolated CD34⁺ cells from marmoset bone marrow and transplanted them into immunodeficient NOD-SCID mice. Four weeks after transplantation, CD11b⁺CD14⁺ cells appeared in the peripheral blood of mice, and their origin was judged to be marmoset because of their cross-reactivity to anti-human CD11b and CD14 mAbs (29). This result demonstrated the differentiation potential of marmoset CD34⁺ cells to myeloid lineage. Subsequently, our group established anti-marmoset CD117 and CD45 mAbs and fractionated marmoset bone marrow cells into four sub-fractions based on the expression of CD34 and CD117 (25). We showed that the CD34⁺CD117⁺ DP fraction contained differentiation activity to multiple hematopoietic lineages in colony-forming assays. In the present study, we extended the analysis of marmoset hematopoietic progenitors to MC differentiation by utilizing anti-marmoset-specific mAbs.

According to extensive studies on hematopoiesis in humans, CD34⁺CD117^{lo} cells turn out to represent hematopoietic stem cells (30–34), whereas CD34⁺CD117^{hi} cells contain myeloid progenitor activity that can develop lineages of MCs and monocytes (32). Upon differentiation, mature MCs exhibit the following characteristics in surface marker expression: (i) CD34[−]CD117⁺ (NB, mature monocytes are CD34[−]CD117[−]), (ii) Fc ϵ RI⁺ (NB, basophils are Fc ϵ RI⁺CD117[−]), (iii) CD45⁺ (NB, all leukocyte lineages including MCs are CD45⁺) and (iv) CD13[−]CD14[−] (NB, hematopoietic stem cells are also CD13[−]CD14[−], but myeloid progenitors including MC progenitors become CD13^{int}CD14^{int}). Then, mature MCs return to CD13[−]CD14[−] again, whereas mature monocytes transit to CD13^{hi}CD14^{hi}. We prepared DP cells from marmoset bone marrow and subjected them to both *in vitro* culture in the presence of SCF and *in vivo* transplantation assays into NOG mice. *In vitro* we could detect the appearance of cells with CD34[−]CD117⁺ and Fc ϵ RI⁺ (thus capable of binding human IgE), and *in vivo* we demonstrated the emergence of cells with CD34[−]CD117⁺. These cells also expressed CD45 (data not shown). These are indeed indications of authentic MC differentiation. In the developed MCs, Syk was tyrosine phosphorylated and the Ca²⁺ influx was observed after the Fc ϵ R cross-linking. They released LTC₄ and β -hexosaminidase simultaneously. The results

confirmed that the MCs were functional MCs. A particularly important finding was that the differentiation activity to MC lineage was detected at a significantly higher rate in the DP fraction compared with the SP fraction (no such activity in the DN fraction). This tendency was true for both *in vitro* and *in vivo* assays. The exact differentiation pathway along the MC lineage is not known in terms of the DP and SP fractions (15,35). It is also not clear whether the CD34[−]CD117⁺CD45⁺CD14^{int} fraction detected *in vivo* corresponds to myeloid progenitors and/or immature MCs. In any case, because we could demonstrate differentiation of the DP fraction to MC lineage, we can label the progenitor cells with green fluorescent protein (GFP), transplant them into syngenic marmoset and monitor the pathway of MC development *in vivo*. For this purpose, marmoset cells might be useful as a good model or control to mimic human MCs.

In mice (and humans), mature MCs are categorized into tissue and mucosa types, depending on their site of residence (36). The two types can be distinguished from each other by histochemical staining patterns with dyes and by differential expression patterns of proteases. Firstly, tissue-type MCs contain heparin-positive granules and are stained metachromatically by toluidine blue, alcian blue and safranin O. On the other hand, mucosa-type MCs, which possess chondroitin sulfate-positive granules, are stained metachromatically by toluidine blue and alcian blue but not safranin O. To be noted as a complicated situation is the mucosa type of human MCs, which are reported to contain both chondroitin sulfate and heparin. In our analyses of marmoset cell-transplanted NOG mice, we could find metachromatically stained MCs. They were stained by all three dyes, suggesting the differentiated MCs to be a tissue type. Secondly, tissue-type MCs express both tryptase and chymase, whereas mucosa-type MCs express only tryptase. We could detect both transcripts of tryptase $\beta 2$ and chymase 1 in *in vitro*-cultured and *in vivo*-transplanted marmoset cells. This again indicates the existence of tissue-type MCs. Thus, although identification of mucosa-type MCs remains ambiguous, DP cells from marmoset bone marrow were shown to have the potential to differentiate at least into tissue-type MCs based on dye-staining and protease expression profiles. It must be noted that the density of toluidine blue-stained granules was significantly lower in marmoset MCs (suggesting a smaller amount of heparin content) compared with those in mouse MCs.

In spleen sections of marmoset and NOG mice, morphologically diagnosed MCs are detected only scarcely (data not shown). Also, in mononuclear cells of peripheral blood and splenocytes prepared from marmoset or NOG mice, a transcript encoding *IL-4* is barely detected (25). In contrast, we found substantial numbers of differentiated MCs and an enhanced level of *IL-4* transcript in spleens of NOG mice transplanted with marmoset DP cells (data not shown). Also, in the case of spleens of NOG mice transplanted with human hematopoietic progenitors, development of tissue-type MCs has been reported (24). Therefore, xenotransplantation (transfer of marmoset DP cells or human hematopoietic progenitors into mouse) might somehow provide an environment favorable for MC differentiation in spleens of NOG mice. As a subject for future research, candidate molecule(s) involved in such an environmental modification must be explored. Particularly interesting might be the possible contribution of cytokines including *IL-4*.

It remains to be determined to what extent marmoset MCs developing in NOG mice can mimic human MCs. Models of type I allergy such as pollinosis have been established in rodents but not in nonhuman primates. As the common marmoset immune system is more akin to human than mouse, it would be better to establish an allergy model of nonhuman primate like the common marmoset. Expansion and characterization of marmoset MCs in transplanted NOG mice may contribute to a novel model for understanding the pathogenesis of human allergic diseases.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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