Common marmoset CD117⁺ hematopoietic cells possess multipotency

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Abstract

Analysis of the hematopoiesis of non-human primates is important to clarify the evolution of primate-specific hematopoiesis and immune regulation. However, the engraftment and development of the primate hematopoietic system are well-documented only in humans and are not clear in non-human primates. *Callithrix jacchus* (common marmoset, CM) is a New World monkey with a high rate of pregnancy and small size that lives in closed colonies. As stem cell factor (SCF) is an essential molecule for hematopoietic stem cell development in mice and humans, we focused on CD117, the SCF receptor, and examined whether CD117-expressing cells possess the hematopoietic stem/progenitor cell characteristics of newborn marmoset-derived hematopoietic cells that can develop into T cells and B cells. When CD117⁺ cell fractions of the bone marrow were transplanted into immunodeficient NOD (non-obese diabetic)/Shi-scid, common yc-null (NOG) mice, these cells engrafted efficiently in the bone marrow and spleens of the NOG mice. The CD117⁺ cells developed into myeloid lineage cells, CD20⁺ B cells and CD3⁺ T cells, which could express CM cytokines in vivo. The development of B cells did not precede that of T cells. The development of CD8⁺ T cells was dominant in NOG mice. The engraftment was comparable for both CD117⁺CD34⁺ cells and CD117⁺CD34⁻ cells. These results suggest that the CD117⁺ cell fraction can differentiate into all three cell lineages, and the development of marmoset immunity in the xenogeneic environment follows diverse developmental pathways compared with human immunity.

Keywords: common yc-null mice, lymphoid lineage, NOD/Shi-scid, xeno-transplantation

Introduction

Although primate hematopoiesis has been thoroughly investigated for humans, hematopoiesis in non-human primates (NHPs) has not been clarified. While NHPs are expected to possess hematopoietic systems more similar to humans than to mice, unique characteristics may have evolved. *Callithrix jacchus*, conventionally called the common marmoset (CM) (¹, ²), is a New World monkey for which colonies have been established via planned breeding and maintenance in Japan for 50 years. Transgenic CM individuals carrying the green fluorescein protein transgene through multiple generations have been successfully generated (³). The MHC gene structure of CMs was previously determined (⁴, ⁵). Furthermore, the
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Amino acid sequences of immune molecules are 86% identical on average between human and CM orthologs, whereas the identities are 60% identical between mouse and human orthologs (6). This phylogenetic situation and recent technological advances may increase the likelihood of the CM being a candidate experimental animal for studying the primate hematopoietic system. For this purpose, identification of CM hematopoietic stem cells (HSCs) is essential. While human HSCs possess a CD34+CD117+ phenotype and mouse HSCs possess a CD34+CD117+ phenotype, the expression of CD34 and CD117 must be determined for CM HSCs (7–12).

To analyze human hematopoiesis, a repopulation assay using a humanized mouse has been developed and improved (13–15). In this system, HSCs or progenitors of human HSCs have been transferred into immunodeficient mice, such as NOD (non-obese diabetic)/Shi-scid, common γc-null (NOD/SCID)/γc-null; NOG) mice (16–19). Then, in these mice, T cells and B cells of human origin have been successfully regenerated to a certain extent. Currently, the xenotransplantation system using immunodeficient mice is popular for analyzing the development of blood cells in experimental animals (15).

Previous studies by Izawa et al. and our group have reported the repopulation ability of CM bone marrow (BM) cells into the myeloid lineage (20–22). Izawa et al. developed an anti-marmoset CD34 mAb, isolated CD34+ cells from CM BM, and transplanted them into immunodeficient NOD-SCID mice (20). The mice developed CD11b+CD14+ cells cross-reacting to antihuman CD11b and CD14 mAbs (23). However, the authors could not detect lymphoid cells, partially because they used NOD-SCID mice, which cannot develop human T cells. Subsequently, our group established an anti-marmoset CD117 mAb and showed that the CD34+CD117+ fraction contained differentiation activity for mast cells (MCs) using NOG mice, which is an improved immunodeficient mouse line to achieve higher engraftment and to develop human T cells (22). However, both Izawa et al. and our group could not develop a CM lymphoid lineage in these previous systems.

Thus, the aims of the present study were to determine whether CM hematopoietic cells that express CD117 can develop into the lymphoid lineage and to clarify their potential for differentiation into T cells and B cells.

Methods

Animals

CM animals were obtained from CLEA Japan (Tokyo, Japan) and kept at the Central Institute for Experimental Animals (CIEA, Kawasaki, Japan) or Hamamatsu Medical University during the experiments. Experiments using CMs were approved by the Institutional Committee for Animal Care and Use and performed at CIEA or Hamamatsu Medical University according to institutional guidelines. The CMs were aged 0–4 years, and the sex was arbitrary. With regard to newborn individuals, newborn pups that were actively rejected or neglected by their parents were provided from the laboratory animal facility at Hamamatsu University School of Medicine and used in the experiments according to the institutional guidelines as reported previously (24). Two newborn animals, eight adult (3–9 year old) animals, four young (1–2 year old) animals and four cord blood (CB) samples were used for the experiments. All animals were unrelated healthy animals. This study adhered to the legal requirements of Japan and to the American Society of Primatologist Principles for the Ethical Treatment of Non-Human Primates.

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 university guidelines.

Preparation of human and marmoset cells

Human umbilical CB was obtained from full-term, healthy newborns immediately after vaginal delivery. Informed consent was obtained according to the institutional guidelines, and this work was approved by the Tokai University Human Research Committee. Mononuclear cells (MNCs) were separated by Ficoll-Paque gradient centrifugation. CD34+ cells were purified from MNCs using a two-step magnetic bead sorting method (Miltenyi Biotec, Gladbach, Germany), and the purity was >95%. Marmoset CB cells were collected from the cord of the placenta just after the delivery of the baby with heparin. MNCs were separated in Lymphocephal (IBL Co., Takasaki, Japan) at 670 × g for 30 min. MNCs were collected, and the remaining erythrocytes were lysed. With regard to the marmoset tissue cells, spleens were removed from marmosets, and cells were isolated from tissues. After red blood cells were lysed with low osmotic buffer (20 mM Tris–HCl, pH 7.4, 0.15 M NH4Cl), they were suspended in RPMI1640 medium (Nissui, Tokyo, Japan) containing 10% (v/v) heat-inactivated FCS (SAFC Biosciences, Tokyo, Japan). BM cells of CMs were collected into heparinized tubes and centrifuged in Lymphocephal. MNCs were collected, and the remaining erythrocytes were lysed.

Flow cytometry

Cells were incubated with appropriately diluted, fluorescence-labeled primary mAbs for 15 min at 4°C and washed with 1% (w/v) BSA-containing PBS. In some cases, the cells were re-incubated with a labeled secondary antibody. The mAbs used were as follows: anti-marmoset CD117-APC (allophycocyanin), anti-marmoset CD34-PE (phycoerythrin) and anti-marmoset CD4-Alexa488 (Oriental Yeast Co., Tokyo, Japan); antihuman CD3-PerCP-Cy5.5 (SP34-2), antihuman CD20 (B-Ly1), antihuman CD8-FITC (HI18a), streptavidin-PE and streptavidin-APC (BD Biosciences, Franklin Lakes, NJ, USA) and antihuman CD14-FITC (61D3), human IgE (HE1) and antihuman IgE (BE5) purchased from e-Bioscience, BioPorto Diagnostics (Gentofte, Denmark) and Abcam Japan (Tokyo, Japan), respectively. An anti-marmoset CD45 mAb was prepared as reported previously (25).

Histological analyses

Tissues were fixed with 20%-buffered formalin and embedded in paraffin. A paraffin block was micro-sectioned, deparaffinized and post-fixed. Cells or tissue sections on glass slides were conventionally stained with hematoxylin–eosin. The primary antibody was the antihuman-HLA clone EMR8-5 (Hokudo, Sapporo, Japan). Sections were examined using a DP71 microscope (Olympus, Tokyo, Japan).
Reverse transcription–polymerase chain reaction (RT–PCR)

RNA was extracted from cells using the RNaseasy Mini Kit (Qiagen, Germantown, MD, USA). RNA (50 ng) was reverse transcribed, and the generated cDNA was amplified using primers and a OneStep RT–PCR kit (Qiagen). Reverse transcription was performed at 50°C for 30 min and 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 and human hematopoietic cells into NOG mice

Lineagenegative [Lin(−)] cells were prepared via the depletion of CD3+/CD20+/CD14+ cells with magnetic bead sorting (AutoMACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Afterwards, Lin(−), CD34+CD117− (DN, double-negative), CD34+CD117+ (DP, double-positive) and CD34+CD117+ (SP, single-positive) cells were stained as described above and purified from BM cultures (2 × 10^6) derived MC cultures (2 × 10^6) and CD117+ cells with multipotency (beta-Actin is a mouse internal control).

Confocal microscopy

To detect the surface expression of FcεRI, marmoset-derived MC cultures (2 × 10^6) were incubated with 5 μg ml⁻¹ of human IgE overnight. The cells were washed and further incubated with an FITC-conjugated mouse antihuman IgE mAb on ice for 20 min. CM cells stained with APC-labeled anti-CD14, CD34 and CD117 mAbs as above were placed on glass slides pre-treated with 3-aminopropyl etheroxysilane (Digene, Beltsville, MD, USA), incubated in a 5% (v/v) CO₂ atmosphere at 37°C in a humidified chamber for 30–40 min, and centrifuged at 100 × g for 15 min using a Cytospin 3 (Shandon, Pittsburgh, PA, USA). The cells were washed, re-treated with 4% paraformaldehyde and analyzed by confocal laser microscopy (META 510, Carl Zeiss, Hertfordshire, UK).

Colony-forming assay using bone marrow and cord blood-derived mononuclear cells

Cells were collected, washed and plated at 2 × 10⁴ cells in 1 ml of methylcellulose-containing medium (Methocult GF-H4435: Stem Cell Technologies, Vancouver, Canada) in a 35-mm dish with human stem cell factor (SCF) (10 ng ml⁻¹), IL-3 (10 ng ml⁻¹), Epo (2 U ml⁻¹) and G-CSF (10 ng ml⁻¹) and cultured at 37°C in a 5% CO₂ atmosphere. After 14 days of culture, types and numbers of hematopoietic colonies (colony-forming units) were counted according to the standard criteria. Samples from one animal were processed for the assay in triplicate, and eight different animals were used (M1-M4 for CB, M5-M8 for BM of adult animals mentioned above).

Table 1. RT–PCR primers

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward strand primer</th>
<th>Reverse strand primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>5'-AGTACAGCATGACGCTGC-3'</td>
<td>5'-GCTTGTACAGAAGGTCTACCC-3'</td>
</tr>
<tr>
<td>IL-4</td>
<td>5'-TGTCCAGGGACAAATGCAG-3'</td>
<td>5'-CATGATCGTTCCTAGCTTTC-3'</td>
</tr>
<tr>
<td>IL-5</td>
<td>5'-GCCCAGGCAACGCAGACAGGCTGCAG-3'</td>
<td>5'-AACCTTCCGTGCAACAAACCCGTTAGTC-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-AGAATCTCCTTCTGGGATCGCC-3'</td>
<td>5'-TCCTGGAGGCAATATGAGTC-3'</td>
</tr>
<tr>
<td>IL-10</td>
<td>5'-GGTTACCTGTGGTTCAGGCT-3'</td>
<td>5'-ATGTACAGCATGCTGACGCT-3'</td>
</tr>
<tr>
<td>IL-17A</td>
<td>5'-CTCTGGAGGAGACATCTG-3'</td>
<td>5'-CTGAGAAGGCGCCTCTGGCT-3'</td>
</tr>
<tr>
<td>IL-17F</td>
<td>5'-CCAGGACGATCCGCGGA-3'</td>
<td>5'-CACATTGGCCACCGCTGCTTCAGTC-3'</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-CTGTTCTGACGCTCCAGACACT-3'</td>
<td>5'-GCATAGTCCAAAGTGACTGCGCCGACT-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-GATGCGAAGGCGCTGATGAGGCAG-3'</td>
<td>5'-CCATCTGAGGCGACAGCAACCA-3'</td>
</tr>
<tr>
<td>CD11c</td>
<td>5'-CACAGTGTCTGACGAGGT-3'</td>
<td>5'-GCATAGTCCAAAGTGACTGCGCCGACT-3'</td>
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<tr>
<td>CD13</td>
<td>5'-GATACAGGAGGCGCATGAC-3'</td>
<td>5'-CCATCTGAGGCGACAGCAACCA-3'</td>
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<tr>
<td>CD14</td>
<td>5'-ATCCATTGTGCATACGCTC-3'</td>
<td>5'-GCATAGTCCAAAGTGACTGCGCCGACT-3'</td>
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<tr>
<td>HPRT</td>
<td>5'-TGACGAGTACGAGGAGC-3'</td>
<td>5'-ATCATGTTTGGACAGCTTACAAC-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-TCTCCCAAGATGTTGCTTC-3'</td>
<td>5'-ATCATGTTTGGACAGCTTACAAC-3'</td>
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</table>

HPRT, hypoxanthine-guanine phosphoribosyltransferase.

*β-Actin is a mouse internal control.
Table 2. List of marmoset WBC-transplanted NOG mice

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Tx No.</th>
<th>Tx period</th>
<th>BM HCD45(%)</th>
<th>Lymph gate</th>
<th>Mono gate</th>
</tr>
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<tbody>
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<td>CTR</td>
<td>0</td>
<td>3 W</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>Lin(−)</td>
<td>1.5 × 10⁵</td>
<td>3 W</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.61</td>
</tr>
<tr>
<td>Lin(−)</td>
<td>1.5 × 10⁵</td>
<td>3 W</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.61</td>
</tr>
<tr>
<td>CD34SP</td>
<td>4.6 × 10⁴</td>
<td>3 W</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.61</td>
</tr>
<tr>
<td>DN</td>
<td>1.0 × 10⁵</td>
<td>3 W</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.61</td>
</tr>
<tr>
<td>DN</td>
<td>1.0 × 10⁵</td>
<td>3 W</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.61</td>
</tr>
<tr>
<td>DN</td>
<td>1.0 × 10⁵</td>
<td>3 W</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.61</td>
</tr>
<tr>
<td>DP</td>
<td>1.0 × 10⁴</td>
<td>2 W</td>
<td>15.2</td>
<td>58.2</td>
<td>6.21</td>
</tr>
<tr>
<td>DP</td>
<td>1.6 × 10⁶</td>
<td>3 W</td>
<td>5.83</td>
<td>74.5</td>
<td>6.21</td>
</tr>
<tr>
<td>DP</td>
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<td>3 W</td>
<td>10.3</td>
<td>69.1</td>
<td>6.21</td>
</tr>
<tr>
<td>DP</td>
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<td>5 W</td>
<td>3.64</td>
<td>34.8</td>
<td>6.21</td>
</tr>
<tr>
<td>DP</td>
<td>1.0 × 10⁷</td>
<td>8 W</td>
<td>13.1</td>
<td>12.8</td>
<td>6.21</td>
</tr>
<tr>
<td>DP</td>
<td>1.0 × 10⁷</td>
<td>8 W</td>
<td>5.15</td>
<td>17.3</td>
<td>6.21</td>
</tr>
<tr>
<td>DP</td>
<td>1.0 × 10⁷</td>
<td>12 W</td>
<td>0.99</td>
<td>3.8</td>
<td>6.21</td>
</tr>
<tr>
<td>SP</td>
<td>1.7 × 10⁵</td>
<td>3 W</td>
<td>2.13</td>
<td>60.5</td>
<td>6.21</td>
</tr>
<tr>
<td>SP</td>
<td>1.7 × 10⁵</td>
<td>3 W</td>
<td>12.4</td>
<td>73.8</td>
<td>6.21</td>
</tr>
<tr>
<td>SP</td>
<td>1.0 × 10⁵</td>
<td>3 W</td>
<td>7.01</td>
<td>78.3</td>
<td>6.21</td>
</tr>
</tbody>
</table>

Tx, transplantation; WBC, white blood cell.

Statistical analyses

Statistical analysis was performed using a two-tailed Student’s t-test, and the data are expressed as the mean ± SD. Significant differences are indicated with asterisks: *P < 0.05.

Results

Engraftment of CD117⁺ CM cells in NOG mice

In humans, CD34 is an HSC marker, while in mice, CD117 is an HSC marker. Thus, we focused on the repopulation ability of CD117⁺ cells with and without CD34 expression. First, we attempted to transplant whole BM MNCs from adult CMs. However, the BM MNCs could not repopulate in NOG mice up to 5 × 10⁶ per head (data not shown). Therefore, we fractionated the BM cells into Lin(−), CD34⁺CD117⁺ (DP), CD34⁺CD117⁺ CD117 SP) CD34⁺CD117⁻ (CD34 SP) and CD34⁺CD117⁻ (DN) cells using anti-marmoset CD34 and anti-marmoset CD117 mAbs and FACS sorting, as reported previously (21) (Supplementary Figure 1). Then, Lin(−), DP, CD117 SP, CD34 SP and DN fractions were injected intravenously into irradiated NOG mice. As shown in Table 2, cells, Lin(−) CD34 SP cells and DN cells did not engraft in NOG mice, when the PB MNCs were examined for CD45 expression. Moreover, as in most CM BMs, the CD34 SP cell numbers were very low, and it was very difficult to administer 1 × 10⁸ cells into one mouse. The mouse lymphoid organs and PB were collected, and the detected engraftment was confirmed. Thus, we concentrated on analyzing CD117⁺ cells in further experiments.

To assess short-term HSCs (ST-HSCs), BM cells and splenocytes were prepared from NOG mice 3 weeks after the transplantation of DN (negative control), DP and SP CM cells. The CM CD45⁺ cells were detected by flow cytometry. We set monocyte and lymphocyte gates for the preparation of CM cells and splenocytes on the basis of forward scatter (FSC) and side scatter (SSC; Supplementary Figure 2). Figure 1A shows the CD45 expression of monocyte-gated fractions. CM MNCs have high auto-fluorescence, and false-positive cells appear substantially more frequently than in human cells.

In contrast, when a lymphocyte gate was employed as shown in Fig. 1(B), only a small CD45⁺ population was detected, specifically 10–13% among BM cells and 7% among splenocytes. Therefore, the development of CM CD117⁺ cells in NOG mice early after transplantation was far more prominent in the direction of the myeloid lineage compared with the lymphoid lineage during this period, which is similar to the results observed for the transplantation of human and mouse HSCs into immunodeficient mice.

Next, we calculated the positive cells by subtracting the autofluorescence⁻ cells. As a result, BM cells and splenocytes contained similar levels of the CD45⁺ fraction when DP and SP cells were transplanted into NOG mice. BM or spleens of non-transplanted NOG mice exhibited no CD45⁺ cells. Figure 1C shows the mean percentages of the CD45⁺ fraction. Both DP and CD117 SP (but not DN) BM cells generated similar levels of CD45⁺ leukocytes in the BM and spleens of transplanted NOG mice.

Expansion of class-I⁺ marmoset cells in NOG mice

Unfortunately, a marmoset-specific CD45 antibody is not available for immunohistochemistry (IHC); therefore, we used an antihuman MHC antibody to recognize CM-derived cells. Spleens were collected 3 weeks after transplantation, and paraffin-embedded thin sections were processed for IHC staining with an anti-HLA-class I mAb recognizing a common epitope present in the heavy chains of HLA-A, -B and -C but not mouse MHC. As shown in Fig. 2, almost all cells from CM spleens were stained with the antibody, showing that this antibody reacted with the CM MHC. Positively stained cells were found in the spleens of DP- or SP-transplanted NOG mice but not in non-transplanted NOG mice. Thus, DP and SP BM cells of CM could engraft in the spleens of NOG mice.

At higher magnification, the CM cells diffusely dispersed around the blood vessels in the mouse spleen, and no B-cell follicles or germinal centers were observed, as reported in human HSC-transplanted NOG mice (17). These results suggest that CM CD117⁺ cells can engraft in NOG mice irrespective of CD45 expression, although the tissue structure is barely reconstituted when the purified cells are transplanted.

Differentiation of marmoset CD117⁺ cells into myeloid lineage cells

We then examined whether CM DP progenitors could differentiate into various myeloid cells in addition to the MCs that we reported previously (22). We examined the expression of CD13, CD14 and CD11c because these are markers for myeloid cells other than MC myeloid markers. Because CM-specific antibodies were not available or their specificity has not been validated, we used RT–PCR for the analysis. We designed primers for transcripts of these genes based on cDNA and genome databases and used them in RT–PCR analyses (Table 1, Fig. 3A). The DP and SP cells sorted from CM BM and splenocytes of non-transplanted NOG mice did...
not express any of these three transcripts, suggesting that the myeloid cells were not contaminated in the transplanted cells. After transplantation of CD117+ cells into NOG mice, transcripts of CD11c, CD13 and CD14 were clearly detected. As we reported previously, CM MCs were observed in the mouse spleen (Fig. 3B) (22). These results indicate that the DP and SP progenitors of CM were capable of developing into cells of myeloid lineages.

**Fig. 1.** Expansion of CD45+ marmoset cells in transplanted NOG mice. CD117+CD34+ (DP), CD117+CD34− (SP) and CD117−CD34− (DN) cells were isolated from marmoset bone marrow (BM) and transplanted into NOG mice intravenously. After 3 weeks, BM cells as well as splenocytes (SPL) were collected from the mice and processed for flow cytometry with an anti-marmoset CD45 mAb. (A) The monocyte-gated fraction as defined by SSC and FSC (see Supplementary Figure 2) is displayed for the CD45 fluorescence intensity. Cells from non-transplanted NOG mice were used as a negative control, whereas cells from CMs were used as a positive control. In the marmoset spleen, a fraction in the control sample exhibited auto-fluorescence. (B) The mean ± SD was calculated for the percentages of the CD45+ sub-fractions from more than three independent experiments as in (A). NOG indicates cells from non-transplanted NOG mice. (C) Experiments were performed as in (A), and the lymphocyte-gated fractions were analyzed. The data are shown as the mean ± SD. Significant differences were not detected between DP and SP (P < 0.05). Filled lines represent the negative control stained with isotype control, and the solid lines represent anti-CD45 antibody-stained cells. The number shown in each panel is an index calculated as the margin of the percentages of the cell fractions stained and un-stained cells gated for FL2CD45-hi and FSC-hi.

**Differentiation of marmoset CD117+ cells into lymphoid cells**

We next examined whether T cells and B cells developed from CM CD117+ cells and compared with human HSC development in NOG mice. Because there were very few adult CD117−CD34− cells, only one NOG mouse was transplanted with the cells, and CM cell engraftment was not detected (Table 2), whereas adult CD117+ cell fractions...
achieved the reconstitution of CM cells, as we reported previously (22). However, they did not maintain CM cells until 12 weeks after transplantation in vivo, and lymphoid cells were not observed. Because human CB HSCs possess a higher reconstitution ability compared with adult BM HSCs (17), we speculated that newborn HSCs possess higher multipotency. Therefore, we used newborn CM BM cells for further xenotransplantation. Both SP and DP cell-transplanted NOG mice were analyzed at 8 and 12 weeks after transplantation. After 12 weeks, the engraftment was compared. In this newborn system, both SP-NOG and DP-NOG showed CM cell engraftment in both BM and spleen, although the percentage of human CD45 cells in BM (CB-NOG in Fig. 4A) was not significantly higher than that of older marmosets (CM in Fig. 4A). CD3 and CD19 mRNA was already detected 8 weeks after transplantation in DP- and SP-NOG spleen cells (Fig. 4B), while a few lymphocytes were detected by flow cytometry. After 12 weeks, the surface expression of both markers on the lymphoid cells was evident (Fig. 4C and D). Developed CD3+ cells contained CD8+ T cells, but CD4+ T cells were not detected in these mice (Table 3). As reported previously, when human HSCs were transplanted into NOG mice, the B-cell ratio was higher in the mice during the early period (16, 17, 19). Moreover, both CD4+ and CD8+ T cells were detected in the mice. Thus, the cellularity of reconstituted lymphoid cells was different between CMs and humans. The PB MNCs of CMs contained equal amounts of CD4 and CD8 T cells, while that of humans contained substantially higher amounts of CD4 T cells, suggesting that CD8 T-cell-dominant differentiation in NOG mice reflects a unique characteristic of the CM hematopoietic immune system (Supplementary Figure 3). When TCR repertoire analysis was employed (24), the CD3+ cells contained detectable Vβ9-1 and Vβ24, the most frequent Vα and Vβ in CM peripheral lymphoid organs, which exist equally in CD4 T cells and CD8 T cells in the thymus, indicating the existence of functional TCR-expressing T cells (R. Suzuki, personal communication). No thymic T cells were observed in the NOG mice, indicating the extra-thymic differentiation of the CD8+ cells.

We also examined the expression of cytokines in the differentiated CM cells to determine whether the lymphocytes have any bias regarding cytokine production. Primers specific for CM cytokine transcripts were set and used for RT–PCR analyses (Table 1). The sorted DP cells of CM and the splenocytes of non-transplanted NOG mice did not express detectable amounts of cytokine transcripts, except for transforming growth factor (TGF)-β in DP cells (Fig. 5). The DP and SP cells cultured in vitro expressed large amounts of TGF-β and small amounts of IL-4 and IL-17F. Interestingly, the transcripts of T,2, T,17 and Treg-related cytokines, including IL-4, IL-5, IL-6, IL-10, IL-17A, IL-17F, IFN-gamma, TGF-beta and tumour necrosis factor TNF-alpha, were detected in the DP-NOG and SP-NOG spleens 8 weeks after transplantation. Of note, cells of the CD4 lineage were not detected in the spleens of NOG mice. Therefore, it is possible that myeloid cells, B cells and CD8+ T cells might be producing these various cytokine transcripts in the CM CD117+ cell-transplanted NOG spleen.

Collectively, newborn CD117+ cells can differentiate into lymphoid cells in the NOG environment. B cells and CD8+ T cells were involved in the differentiated lymphoid cells. The cells express T-cell cytokines, suggesting that the immune system is at least partially functional for cytokine production.

Maintenance of newborn marmoset CD117+ cells
To evaluate the characteristics of newborn CD117+ cells, we examined the ratio of CD117+ cells and their colony-forming ability (CFA) in CB MNCs of four independent newborn marmosets (Supplementary Figure 4). The results showed that all CBs contained CD117+ cells, and the ratio was different among the individuals. High CFA was evident in the samples that contained high ratios of CD117+ cells (M2). These included erythroid and myeloid colonies, but monocyte and megakaryocyte lineages were not detected.

Next, we examined whether aged BM MNCs contained CD117+ cells and CFA. Similar levels of CD117+ cells were found among 5-year-old BM MNC fractions, while 9-year-old marmoset BM contained fewer CD117+ cells. These BM cells possessed comparable CFA to the erythroid and myeloid

Fig. 2. Expansion of transplanted marmoset cells in the spleens of NOG mice. CD117+-CD34+ (DP), CD117+-CD34− (SP) and CD117+-CD34− (DN) fractions were isolated from the BM of the CM and injected intravenously into NOG mice as mentioned in Fig. 1. Spleens were collected after 3 weeks, and paraffin-embedded thin sections were processed for immunohistochemical staining with an antihuman HLA class I mAb. Deposition of brown dye indicates positive staining. Spleens from non-transplanted NOG mice and the CM served as negative and positive controls, respectively. Representative data for each transplanted mouse are shown. The ×400 data show that the positive cells are dispersed from the blood vessels of NOG spleens.
new NOG mice, they could not be engrafted in the second group of NOG mice (Table 4).

Collectively, CD117+ cells of newborn BM had the ability to repopulate, but their LT-HST characteristics could not be evaluated with this system.

**Discussion**

The initial trial to identify hematopoietic progenitors in the CM was reported by Izawa et al. in 2004 (20). The results of this study demonstrated the differentiation potential of CM CD34+ cells into the myeloid lineage. Subsequently, our group established an anti-marmoset CD117 mAb and showed that the CD117+ fraction contained differentiation activity for the erythroid and myeloid lineages in colony-forming assays and developed into CD45+ leukocytes, including MCs, in transplanted NOG mice. However, the development of CM BM cells into the lymphoid lineage has not been achieved. In the present study, we extended the analysis of CM hematopoietic progenitors to lymphocyte differentiation by utilizing newborn BM CD117+ cells.

On the basis of extensive studies of hematopoiesis in humans, CD34+CD117+ cells represent HSCs (11, 12), whereas CD34+CD117hi cells contain myeloid progenitor activity that can develop lineages of MCs and monocytes (9, 26, 27). In CMs, while the development of myeloid lineage cells was also observed with CD34+CD117hi cells, CD34+CD117+ cells could not achieve engraftment themselves (Table 2). This evidence suggests that in CMs, CD34 is not a definitive marker of CM HSCs. In humans and rhesus macaques, Lin(−) CD34+ cells are conveniently used as HSCs. Although most human and NHP CD34+ cells express CD117 (28, 29), CD117 is not an HSC marker, as the expression is maintained in myeloid cells such as MCs (30). In our results, a large portion of the CD117hi cells were also CD34+ cells, which have hematopoietic characteristics. On the other hand, mouse HSCs express no or very low levels of CD34. Thus, although CD34 SP cells cannot repopulate, and CD117 SP cells can also repopulate, their characteristics may not be more similar to mouse. This may represent an intermediate between mice and humans.

In our results, only newborn BM CD117+ cells developed into lymphoid cells (Fig. 4). The reason may be the BM aging of HSCs in adult CM BM or the difficulty in purifying stem cells from the BM matrix that aggregates in adult BM (data not shown). As BM MNCs had a higher CFA for the myeloid lineage compared with CB CD117+ cells (29), CD117 is not an HSC marker, as the expression is maintained in myeloid cells such as MCs (30). In our results, a large portion of the CD117hi cells were also CD34+ cells, which have hematopoietic characteristics. On the other hand, mouse HSCs express no or very low levels of CD34. Thus, although CD34 SP cells cannot repopulate, and CD117 SP cells can also repopulate, their characteristics may not be more similar to mouse. This may represent an intermediate between mice and humans.

Apart from MC development, we also observed additional remarkable phenomena in NOG mice transplanted with CM CD117+ cells. First was the appearance of CD13+, CD14+ and/or CD11c+ cells, whose expression was verified at the transcriptional level through RT–PCR analysis. It is probable that these cells correspond to mature myeloid cells, including

**Fig. 3.** Myeloid lineage cells were developed from marmoset BM cell fractions. CD117hiCD34+ (DP) and CD117loCD34hi (SP) cells were prepared from marmoset bone marrow BM and transplanted into NOG mice. After 4 weeks, BM cells as well as splenocytes (SPL) were collected from mice and purified based on CD34 and CD117 expression. Cells were processed for RT–PCR or confocal microscopy. (A) RNA was prepared from the indicated samples including cultures of CD117hiCD34+ (DP) or CD117loCD34hi (SP) cells, spleens of DP- or SP-transplanted or non-transplanted NOG mice, or bone marrow or spleens of CM. Transcripts of myeloid lineage-markers were examined. Internal controls: hypoxanthine-guanine phosphoribosyltransferase (HPRT) served as a control for CM, β-actin served as a control for NOG mice. (B) MCs developed in NOG mice. The monocyte-gated fractions were sorted out and analyzed by confocal microscopy after CD117 and FceR staining.

lineages. These results demonstrate that CFA was present in both newborn and adult hematopoietic cells of the CM, and their differentiation ability was somewhat decreased among CB hematopoietic stem/progenitor cells in terms of myeloid and erythroid cells. Younger marmosets tended to possess more CD117+ cells in the BM, but the CD117+ cell ratio was not strictly correlated with the colony number.

As lymphocytes developed in the NOG mice transplanted with newborn BM cells, we examined whether the transplanted mouse BM still contained transplantable stem cells. As a result, DP cells were detected in the BM of transplanted NOG mice 12 weeks after newborn BM transplantation (Table 3), irrespective of the CD34 expression of transplanted CD117+ cells (Fig. 6). However, when the BM cells of these transplanted NOG mice were prepared and transplanted into
Table 3. NOG-derived marmoset cells transplanted with newborn marmoset CD117+ cells

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Tx no.</th>
<th>Tx period</th>
<th>BM CD45 (%)</th>
<th>SPL cell no.</th>
<th>CD3+ (%)</th>
<th>CD8+ (%)</th>
<th>CD4+ (%)</th>
<th>DN</th>
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<tr>
<td>Newborn DP</td>
<td>1.35 x 10^6</td>
<td>4 W</td>
<td>0.193</td>
<td>0.928</td>
<td>1.0 x 10^7</td>
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<td>&lt;0.01</td>
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<td>1.0 x 10^6</td>
<td>5 W</td>
<td>33.7</td>
<td>45.7</td>
<td>4 x 10^6</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>4.93</td>
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<tr>
<td>Newborn DP</td>
<td>1.0 x 10^6</td>
<td>8 W</td>
<td>54.7</td>
<td>71.9</td>
<td>5.0 x 10^7</td>
<td>2.04</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Newborn DP</td>
<td>1.35 x 10^6</td>
<td>12 W</td>
<td>12.4</td>
<td>9.21</td>
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<tr>
<td>Newborn DP</td>
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<td>1.8</td>
<td>4.44</td>
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<td>6.34</td>
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<td>0.237</td>
<td>0.775</td>
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<td>0.551</td>
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<td>5 W</td>
<td>0.196</td>
<td>9.18</td>
<td>9 x 10^7</td>
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<td>39</td>
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Fig. 4. Lymphoid lineage cells were developed from newborn CD117+ marmoset BM cell fractions. CD117+CD34+ (DP) and CD117+CD34- (SP) cells were prepared from marmoset BM. Human CD34+ cells were prepared from CB. These cell fractions were transplanted into NOG mice. After 8 and 12 weeks, splenocytes (SPL) were taken from mice and analyzed for lymphocyte marker expression by RT–PCR or flow cytometry. (A) The average CD45+ lymphoid gate cell numbers from two mice (12 weeks) are shown in the figure. As the number of mice was only two, statistical analysis could not be performed. The mean % of the SPL from CM (n = 4, young adults), NOG (n = 3) and NOG mice transplanted with human CB HSCs (CB-NOG) (n = 9) are also shown. (B) RNA was prepared from the spleens of DP- or SP-transplanted NOG mice and assayed for mRNA expression. Transcripts of lymphoid lineage markers were examined. (C) CD20 and CD3 expression was analyzed for DP-NOG, SP-NOG and CB-NOG SPL. NOG, non-transplanted NOG spleen. (D) CD3+ T cells were analyzed for CD4 and CD8 expression. CD45+ lymphoid-gated cells were stained for CD3, CD4 and CD8. The percentages of the CD45-gated cell fractions further gated in the squares are shown in each panel.
monocytes (26, 27). It is most likely that the transplanted CM DP cells are capable of differentiating not only into MCs but also into other myeloid cells. Furthermore, these cells appeared to expand and massively infiltrate into the spleen (and liver, data not shown), as judged by staining with an anti-human HLA class I antibody. Second, the expression of various cytokines was detected at the transcriptional level (Fig. 4). The types of cells transcribing these cytokines could not be identified due to the lack of anti-marmoset cytokine monoclonal antibodies. Considering the differentiation profiles of the CD117+ cells described above, MCs and/or other myeloid cells might be the source of these cytokine transcripts. Because human and NHPs share many common characteristics with regard to the immune system, CM cytokine-producing cells might mimic the human cells (32–34).

Most importantly, newborn CM CD117+ cells developed into CD20+ cells and CD3+ cells in NOG mice (Table 3 and Fig. 4). In human HSC-transplanted NOG mice, B cells are dominant in the BM and periphery 2 months after the transplantation (17). CD3+ T cells appear in the periphery several weeks after B cells appear (35). Contrary to this phenomenon, in our study, CM T cells appeared simultaneously with B cells, and the amount of B cells was substantially lower than in humanized mice. Moreover, DN T cells dominated in the early period (4–5 weeks), and CD8+ T cells were dominant in the late period (8–12 weeks) among the CD3+ cells. While a similar ratio of CD4+ T cells and CD8+ T cells is observed in the PB of wild-type CMs (21), these results indicate that CD8+ cells are prone to developing in CM under lymphopenic conditions. CD4+ T cells were not observed in the thymus and periphery of this mouse system. We observed neither mouse nor marmoset thymocytes in the mice. Thus, marmoset CD8+ T cells might have developed extrathymically. Although we could not clarify the underlying reason, marmoset helper T-cell development in the thymus might require extra factors other than those from mice. This may be a unique characteristic of the CM or other NHPs. Otherwise, the mouse environment cannot develop conventional CD4+ T cells of CMs.

On the basis of the results indicating the development of CM T cells and B cells in NOG mice, CD117+ cells are multipotent because they can develop into erythroid cells (colony assay) (21), myeloid cells (colony assay and xeno-transplantation assay) (22) and lymphoid cells (xeno-transplantation assay). However, they could not maintain their repopulation ability after the secondary transplantation. While human CD34+ cells can engraft after a second transplantation in NOG mice (19). The NOG environment for the hematopoiesis of CM is different from humans and might be incompatible for long-term HSC repopulation.

In this study, we first demonstrated the development of CM lymphoid lineage cells in immunodeficient mice. This is an important finding because detailed marmoset immunity should be clarified not only for the sake of determining the evolution of immunology but also to evaluate this primate as an experimental animal for the development of immune-related molecular targeting reagents for human beings.

**Supplementary data**

Supplementary data are available at *International Immunology* Online.

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**Fig. 5.** The mRNA expression of marmoset cytokines. The mRNA expression of cytokines (IL-5, IL-4, IL-10, TGF-β, IL-6, TNF-α, IFN-γ, IL-2, IL-17F and IL-17A; internal controls: hypoxanthine-guanine phosphoribosyltransferase served as a control for CM, β-actin served as a control for NOG mice) was examined by semi-quantitative RT–PCR using DP-NOG and SP-NOG spleen cells (SPL) 8 and 12 weeks after transplantation, CM whole BM cells, whole SPL, purified DP cells, purified SP cells from CM BM and non-treated NOG SPL.

**Fig. 6.** Self-renewal of HSCs. DP-NOG and SP-NOG BM cells were prepared 4 weeks after transplantation and analyzed by flow cytometry for CD34 and CD117 expression.
The BM MNCs of DP-NOG and SP-NOG were re-transplanted into NOG mice. Tx cell no.: number of MNCs transplanted into NOG mice. Mouse #1 died before analysis. None of the mice with secondary transplantation demonstrated the engraftment of marmoset cells.

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**References**


express low levels of Kit protein are enriched for long-term marrow-engrafting cells. Blood 87:4136.


