research in recent years has greatly contributed to our understanding of the importance of basophils, further research on the function of basophils will be more forthcoming in the future. As for the development of new treatments, basophils can now be viewed as essential components of immune responses and potential therapeutic targets.

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Antiproliferative Effect of Cucurbitacin B Extracted from *Trichosanthes cucumerina* L. on Human Cancer Cell Lines

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ABSTRACT

Objective: To determine the antiproliferative effect of cucurbitacin B extracted from *Trichosanthes cucumerina* L. on human cancer cell lines.

Methods: Two human lung non-small cell (adenocarcinoma) cancer cell lines i.e., LK87, and QG95, two human colon adenocarcinoma cell lines i.e., HCT15, and HT29, including one renal cancer cell line, A498, and one pancreatic cancer cell line, NOR-P, were used in this study. The viability of cells was assessed by using WST-8 which is based on detection of LDH released from damaged cells and reacts with WST-8 to form a yellow color. Cells were treated with the compound at various concentration from 1 through 100 μg/ml.

Results: The ED50 values (effective doses that are required for 50% inhibition growth of tumor cells) of the compound on human cancer cell lines ranged from approximately 69 μg/ml in HCT15 cells up to 231 μg/ml in QG95 cells. The inhibition of proliferation of this compound on these human cancer cell lines was observed to be in a dose dependent manner.

Conclusion: It could be concluded from this observation that this compound has a modest direct toxic effect to these cell lines with the highest toxic effect on human colon cancer cells.

Keywords: *Trichosanthes cucumerina*, Buap-Khom, cucurbitacin B, human cancer cell lines

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Trichosanthes cucumerina L., named in Thai "Buap- Khom", is a plant in the Cucurbitaceae family which is commonly found in Southeast Asia and Australia. From several reports, plants in this family are composed of many genera accounting for 110 genera and about 640 species which are mostly woody or herbaceous with climbing or trailing stems bearing tendrils and often arising from a tuberous rootstock.[1,2] In Thailand, this plant wildly grows along the river in some provinces such as Kanjanaburi. For Thai folk medicine, some of them have been used for the properties of antihelmintic, antidiabetic, and antiinflammatory effects except for *Trichosanthes cucumerina* which is inedible because of the bitter taste inducing nausea and vomiting symptoms.[3] In addition, in other countries, such as India, these special group of plants especially their seeds and fruit have been prescribed to treat various diseases i.e., infections and malignancies.[4] Several investigations have demonstrated the striking cytotoxic activities of these familial plants isolated compounds, cucurbitacins which are the major component, against several human cancer cell lines such as breast and lung cancer cells.[5-7]

In this study, cucurbitacin B was isolated from the juice of the fruit of *Trichosanthes cucumerina* and purified. This compound was tested against several human lung, colon, pancreatic, and renal cancer cell lines which have not previously been investigated.

MATERIALS AND METHODS

Plant extraction process

30 kg of fresh fruit of *Trichosanthes cucumerina* were collected from Kanjanaburi Province. They were immediately pressed and filtered to get the juice. After

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getting the juice (5 liters), it was shaken in ether in the separatory funnel and 1 gram of crystalline mixture was obtained. The crystalline mixture was purified by reversed phase column (RP-18) with the solvent system of water and acetonitril (55:45).[8] The major component which comprises cucurbitacin B (mixtured with dihydrocucurbitacin B) was gained from this extraction and purification procedure.

Extract preparation

For preparation, 0.0015 gm of the compound was dissolved in 50 μl of 95% ethanol and added up with 950 μl media to get the 1 ml stock. For all the experiment, the stock was diluted with media to get the concentrations at 10, 50, 100, 500, and 1,000 μg/ml. For the final concentrations from 1-100 μg/ml, 10 μl of each prepared diluted compound was added to each well in 96 well plates.

Cell lines and culture

Two human lung non-small cell (adenocarcinoma) cancer cell lines i.e., LK87, and QG95, two human colon adenocarcinoma cell lines i.e., HCT15, and HT29, one renal cancer cell line, A498, and one pancreatic cancer cell line, NOR-P, were used in this study. Human lung cancer cell lines were kindly provided by Dr.Koichi Takayama and Dr.Hiroyuki Inoue, Research Institute of Diseases of the Chest, Kyushu University, Japan. The other cell lines were kindly provided by Dr.Kenzaburo Tani, Medical Institute of Bioregulation, Kyushu University, Japan. All human lung and colon cancer cells were maintained in RPMI1640 (Nacalai Tesque, Kyoto, Japan) except for HT29 colon cancer cell line which was maintained in McCoy's 5A (Gibco, USA). Human renal carcinoma cell line was maintained in MEMalpha (Gibco, USA) and pancreatic cancer cell line was maintained in DMEM (Nacalai Tesque, Kyoto, Japan). All medium were supplemented with 10% fetal bovine serum (Japan Bioserum, Japan) and 1% antibiotic plus antimycotic agent (Nacalai Tesque, Kyoto, Japan). All cell lines were incubated at 37°C with 5% CO_2 and humidified atmosphere.

Cell viability assay

Viability of cells was assessed by using WST-8 (Nacalai Tesque, Kyoto, Japan) asssay (2-2(methoxy-4-nitrophenyl)3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt), which is based on detection of LDH releasing from damaged cells and reacts with WST-8 to form a yellow color.[9] In brief, cells were seeded at a density of $1x10^4$ cells/well in 96 well plates and treated with the compound at various concentrations from 1 through 100 μg/ml. Doxorubicin was used as a positive control. After a 48-hour incubation, 10 μl of WST-8 was added to each well and incubated at 37°C with a 5% CO_2 incubator for an additional 4 hours. The absorbance at 450 nm. of the dissolved solution was measured by using an Elisa plate reader (ThermoLabsystem, Japan). Data was calculated by using the formula as followed[10]:

Cell death (%) = [(control O.D.-sample O.D.)/ control O.D.] x100

Cell viability = 100-(% Cell death)

Statistic analysis

All experiments were performed in triplicate with three experiments. Data were expressed as the mean ± standard deviation. The R square equation was used to calculate the ED50 value. A P-value less than 0.05 were considered statistically significant.

RESULTS

Antiproliferative effects of compound on human cancer cell lines.

The ED50 values (effective doses that are required for 50% inhibition growth of tumor cells) of the compound on human cancer cell lines were summarized in Table 1 ranging from approximately 69 μg/ml in HCT15 cells up to 231 μg/ml in QG95 cells.

The antiproliferative effect, expressed as cell viability, of colon cancer cell lines, HCT15 and HT29, renal cancer cell, A498, and pancreatic cancer cell, NOR-P, showed the modest inhibition by the compound with the ED50 values at 69.391 ± 18.382, 106.431 ± 20.756, 105.912 ± 3.057, and 87.396 ± 1.950 μg/ml respectively as shown in Fig. 1A and 1C. The compound inhi-bited growth of lung cancer cells, LK87 and QG95, in the modest to low activity with the ED50 values at 99.517 ± 6.116 and 231.830 ± 11.182 μg/ml respectively as illu strated in Fig. 1B. All inhibition of cancer cells by the compound on the proliferation of these human cancer cell lines was observed to be in a dose dependent manner.

DISCUSSION

This investigation of the antiproliferative effects of cucurbitacin B was performed on several human cancer cell lines which have never been previously reported. The biological inhibitory effect of the compound depends on not only healthy or malignant cells but also the difference in the type of cell lines. Comparing in all these cancer cells, this compound has a modest to low activity on human lung cancer cell lines, whereas the inhibitory activity of the compound on other cell lines was in a modest pattern. Therefore, it could be concluded from this observation that this compound has a modest direct toxic effect to these cell lines.

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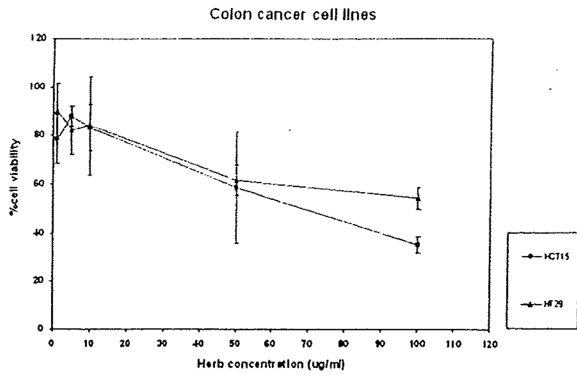
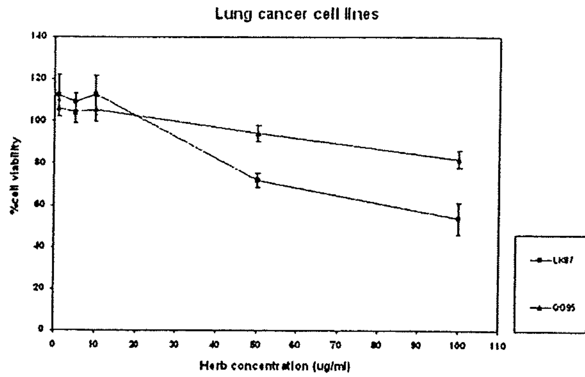
TABLE 1. ED50 values of human cancer cell lines were demonstrated as mean ± standard deviation.

Type of cancer cell line	ED50 value (mean SD)		
Colon cancer: HCT15	69.391	±	18.382 μg/ml
HT29	106.431	±	20.756 μg/ml
Renal cancer: A498	105.912	±	3.057 μg/ml
Pancreatic cancer: NOR-P	87.396	±	1.950 μg/ml
Lung cancer: LK87	99.517	±	6.116 μg/ml
QG95	231.830	±	11.182 μg/ml



1A



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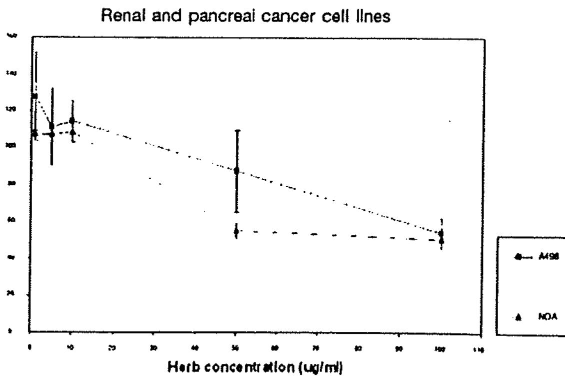

1C

Fig 1. Proliferative inhibition of cucurbitacin B on two colon cancer cell lines, two lung cancer cell lines, one renal cancer cell line and one pancreatic cancer cell line. The cells were seeded at $1x10^4$ cell/well in 96 well plates for 48 hours. HCT15 and HT29 colon cancer cell lines (1A), LK87 and QG95 lung cancer cell lines (1B) A498 renal cancer cell line and NOR-P pancreatic cancer cell line (1C) at different concentrations of 1, 5, 10, 50, 100 and 150 µg/ml for 48 hours. The cell viabilities were determined by MTT assay.

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Experimental Hematology

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Development of monoclonal antibodies for analyzing immune and hematopoietic systems of common marmoset

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Objective. **Common marmosets are considered experimental animals of primates useful for medical research. We developed several monoclonal antibodies (mAbs) directed to CD molecules to gain initial insight into the immune and hematopoietic systems of this organism, and analyzed the basic cellularity and characters of marmoset lymphocytes.**

Materials and Methods. **Anti-marmoset CD antigen mAbs were prepared using marmoset antigen-expressing transfectants and used for flow cytometric analyses and cell fractionation. Expression of T-cell−related cytokine gene transcripts was examined in response to T-cell receptor stimulation by reverse transcription polymerase chain reaction analyses. Hematopoietic progenitor activities of marmoset bone marrow cells were examined in fractionated cells by mAbs against CD117 (c-kit) and CD34.**

Results. **CD4 and CD8 expression profiles in T-cell subsets of marmoset were essentially similar to those in mouse and human. CD4$^+$ and CD8$^+$ subsets were isolated from marmoset spleens. Detected transcripts after stimulation of T cells included Th1-, Th2-, and Th17-related cytokines in CD4$^+$ cells and cytotoxic proteases in CD8$^+$ cells, respectively. Colony-forming abilities were detected mainly in CD117 (c-kit)$^+$ cells, irrespective of CD34 expression.**

Conclusions. **Marmoset immune system was basically similar to human and mouse systems.** **© 2009 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Mice are the most commonly used experimental animals as an alternative for humans, and many disease models have been generated using these animals. However, because of the evolutional distance between the two species, there are remarkable differences recognized in their genetic and physiological functions [1–5]. In particular, humans and mice exhibit differences in various aspects of immunity, such as T-cell subset differentiation and cytokine signal transduction. For example, for Th17 differentiation, both interleukin (IL)-1 and IL-6 are necessary in humans, whereas transforming growth factor−β (TGF-β) and IL-6 are important in mice. Thus, mice are not always versatile as a human alternative, despite their ease in gene manipulation.

One of the approaches for overcoming this species specificity is to establish a human immune system in immunodeficient mice. For generating such humanized mice, severe combine immunodeficient (SCID), nonobese diabetic (NOD)-SCID, RAG2-null, and NOD/shi-SCID/IL-2Rg^{null} (NOG) mice have been used as recipients because they each lack their own immunity, to varying degrees, because of gene mutation/deficiency [6–8]. On the other hand, human cord blood cells, lymphoid tissues/cells, or leukemic cells have been engrafted as donors into such mice [9–11]. Reconstitution of the human immune system in mice has been successful to varying extents, depending on both the type of recipient mouse and donor cells. In a combination

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of human cord blood cells and NOG mice, T and B cells of human origin emerge in peripheral blood, but the produced antigen-specific human antibody is mainly immunoglobulin (Ig) M but not IgG [12–16]. This suggests that the interaction of T and B cells does not occur properly in these xenografted mice [17]. Thus, reconstitution of human immunity is not necessarily complete. Furthermore, use of human tissues may involve an ethical issue, precluding the possibility of preclinical use.

The common marmoset, a new world monkey, and humans are in the same evolutional entity of anthropoidea. The common marmoset is the only experimental animal among primates that has been artificially bred and maintained as a closed colony for >20 years. This species was identified as one of the most useful primates because of its size, availability, and widespread use in biomedical research [18,19]. Disease models of neurological disorders and human-specific virus infections have been established with this animal [20–24]. In particular, models of autoimmune diseases, such as experimental allergic encephalomyelitis, which resembles clinical and pathological features of human multiple sclerosis, compared to other animal models including other monkeys, have been generated and found to be useful for drug screening and evaluation [22,25,26]. Thus, compared to mice, common marmosets appear to emerge as a closer alternative to humans. Recently, we succeeded in the development of green fluorescent protein (GFP)–transgenic marmoset [27]. This might open another approach to human immunity in addition to the aforementioned human-to-mouse xenograft.

However, our knowledge of the immune system of the common marmoset is still not enough to establish it as an experimental animal and use as an alternative for human immunity and its disorders. One major obstacle to overcome initially is the lack of various fundamental molecular resources necessary for marmoset research. This led us to cloning and determining the primary structures of 30 of the most representative immune system–related genes of the marmoset [28]. In addition to the gene resources, antibodies directed toward immune-system–related molecules also have to be developed. Although anti-human antibodies have been screened for their cross-reactivity with marmoset immune cells, it is not certain whether cross-reacting antibodies, if any, authentically recognize the corresponding marmoset antigens [29,30].

In this study, we established several anti-marmoset monoclonal antibodies (mAbs) that were directed against CD4, CD8, CD25, CD45, and CD117 (c-kit). By using these mAbs, we have preliminarily characterized T-cell subsets and hematopoietic progenitors of the common marmoset, and we also analyzed expression profiles of various cytokine genes in T-cell subsets. The present findings are expected to serve as a basis for further development of the common marmoset as a useful experimental animal concerning primate immunology.

Materials and methods

Animals

Common marmosets were obtained through CLEA Japan (Tokyo, Japan) and kept at the Central Institute for Experimental Animals (Kawasaki, Japan) during the experiments. Experiments using common marmosets were approved by the Institutional Committee for Animal Care and Use and performed at Central Institute for Experimental Animals according to institutional guidelines. The marmoset age (1 to 4 years old) and sex were arbitrary. In some cases, marmosets were injected subcutaneously with 10 mg/kg/d of human granulocyte colony-stimulating factor (G-CSF; Neutrogin, Chugai Co. Ltd, Tokyo, Japan) for 5 successive days. Female 5- to 6-week-old BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan) and kept in specific pathogen-free conditions in the animal facility at Juntendo University School of Medicine (Tokyo, Japan). NOD/Shi-scid, common gc-null (NOD/SCID/γc-null; NOG) mice were provided from the Central Institute for Experimental Animals (Kawasaki, Japan). Experiments using mice were performed following the guidelines set by the university.

Reverse transcription polymerase chain reaction, cDNA cloning, and sequence analyses

RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). RNA at 50 ng was reverse-transcribed to cDNA and amplified using the primers and OneStep RT-PCR kit (Qiagen). Reverse transcription polymerase chain reaction (RT-PCR) amplification was performed under the following conditions: reverse transcription was at 50°C for 30 minutes, polymerase activation was at 95°C for 15 minutes with 33 cycles of PCR, each cycle consisting of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. PCR products were subjected to agarose gel electrophoresis. Primers used for PCR were as follows: for *IL-2*, 5′-ATGTACAGCATGCAGCTC GC-3′ and 5′-GCTTTGACAGAAGGCTATCC-3′; for *IL-4*, 5′-TGTCCACGGACACAAGTGCGA-3′ and 5′-CATGATCGTCTTT AGCCTTTCC-3′; for *IL-5*, 5′-GCCAAAGGCAAACGCAGAACG TTTCAGAGC-3′ and 5′-AATCTTTGGCTGCAACAAACCAGTT TAGTC-3′; for *IL-6*, 5′-ATGAACTCCTTCTCCACAAGCGC-3′ and 5′-GAAGAGCCCTCAGGCTGGACTG-3′; for *IL-10*, 5′-GGT TACCTGGGTTGCCAAGCCT-3′ and 5′-CTTCTATGTAGTTGA TGAAGATGTC-3′; for *IL-17A*, 5′-CTCCTGGGAAGACCTCAT TG-3′ and 5′-CAGACGGATATCTCTCAGGG-3′; for *IL-17F*, 5′-CA AAGCAAGCATCCAGCGCA-3′ and 5′-CATTGGGCCTGTACAA CTTCTG-3′; for *IFN-γ*, 5′-CTGTTACTGCCAGGACCCAT-3′ and 5′-CGTCTGACTCCTTCTTCGCTT-3′; for *TGF-β*, 5′-GCCCTG GACACCAACTACTGC-3′ and 5′-GTCGCATTTGCAGGAGCGC AC-3′; for *TNF-α*, 5′-GAGTGACAAGCCTGTAGCCCATGTT GTAGCA-3′ and 5′-GCAATGATCCCAAAGTAGACCTGCCCAG ACT-3′; for *granzyme B*, 5′-ATATGAGGCCAAGCCCCACT-3′ and 5′-TCTCCAGCTGCAGTAGCATA-3′; for *perforin-1*, 5′-GGCC TGTGAGGAGAAGAAA-3′ and 5′-GCCCATCAGGTACTGAC TCA-3′; for *HPRT*, 5′-TGACCAGTCAACAGGGGAC-3′ and 5′-GC TCTACTAAGCAGATGGC-3′. The procedure of cDNA cloning was the same as reported by us previously [28]. The primers used were as follows: for *granzyme B*, 5′-CCAAGAGCTAAAAGAGAGTAAG GGGGAAAC-3′ and 5′-AGCGGGGGCTTAGTTTGCTTCCTGTA GTTA-3′; for *perforin 1*, 5′-GTGTAGCCGCTTCTCTATACGGGA

TTCCAG-3′ and 5′-TTCAGTCCAAGCATACTGGTCCTTTC-CAAG-3′.

The tool for sequence homology search was BLAST [31], whereas that for multiple sequence alignment was CLUSTALW.

Construction of cDNA expression vectors

cDNA sequences of marmoset genes used in this study were based on AF452616 as *CD4*, DQ189217 as *CD8a*, DQ520834 as *CD25*, AB097501 as *CD34*, and AB097502 as *CD117*. RNA was extracted from cells by Isogen (Nippon Gene Co. Ltd., Tokyo, Japan) and reverse-transcribed to cDNA by Superscript (Invitrogen, Carlsbad, CA, USA). A portion of cDNA sequence corresponding to an extracellular domain of the protein was amplified by a PCR method, using cDNA as a template and AccuPrime Pfx DNA Polymerase (Invitrogen, Tokyo, Japan). The set of forward and reverse primers used were as follows: for *IL-2*, 5′-ATGTACAG-CATGCAGCTCGC-3′ and 5′-GCTTTGACAGAAGGCTATCC-3′; for *CD4*, 5′-CCACCATGAATGGGGGAATCCCTTTC-3′ and 5′-CACCGGGGGAGACCATGTGGGCA-3′; for *CD8α*, 5′-CCAC CATGGCCTCGCCAGTGACCGC-3′ and 5′-ACAGGCGAAGT CCAGCCCC-3′; for *CD25*, 5′-CCACCATGGATTCACAACTG CTGATG-3′ and 5′-CTTCGTCGTAAGTATGAATGTCTCC-3′; for *CD34*, 5′-ATGCTGGTCCGCAGGGGC-3′ and 5′-TCGGGAA TAACTCTGGTGGCTTGC-3′; for *CD45*, 5′-CCACCATG-TATTTGTGGCTTAAACTGCT-3′ and 5′-TGATTGAAATTTAC-TAACTGGGTG-3′; for *CD117*, 5′-CCACCATGAGA GGCGCTCGTGGCG-3′ and 5′-GAACAGGGTGTGGGGCTG-GATTTGC-3′. Each PCR product was inserted into the *Eco*RV site of pCXGFP-1 vector (GenBank, Accession No. AB281497) in order to express an extracellular domain of interest as a fusion protein with an enhanced EGFP.

Preparation of cDNA-transfected cells

pCXGFP-1 vectors expressing marmoset cDNAs were transfected into Chinese hamster ovary (CHO) cells by electroporation using Gene Pulser (BioRad, Tokyo, Japan) according to manufacturer's instructions. After G418 selection at 1 mg/mL, the GFP$^+$ cells were isolated by fluorescence-activated cell sorter.

Production of mAbs

We adopted the hydrodynamic immunization procedure reported by Song et al. [32]. Briefly, 25 mg pCX-GFP plasmid DNA was suspended in 2 mL saline and injected intravenously into BALB/c mice. Mice were immunized three to six times, and serum antibody titers were checked by flow cytometric analyses using cDNA-transfected cells as a source of antigen. Four days after a final boost, mice were sacrificed and splenocytes were fused with the mouse myeloma cell line P3U1 according to a standard procedure. Positive clones were identified by flow cytometry, isolated, expanded, and stocked.

Preparation of human blood cells

Human umbilical cord blood was obtained from full-term healthy newborns immediately after vaginal delivery. Informed consent was obtained from individual mothers according to the institutional guidelines, and the work was approved by the Tokai University Human Research Committee. Peripheral blood was collected from healthy human volunteers. Mononuclear cells were isolated from cord and peripheral blood by Ficoll-Paque (GE Healthcare Biosciences, Uppsala, Sweden) gradient centrifugation as reported previously [15].

Preparation and stimulation of marmoset lymphocytes

Thymi, intestinal lymph modes, or spleens were taken from marmosets and cells were released from the tissues. After red blood cells were lysed osmotically, cells were suspended in RPMI-1640 medium containing 10% fetal calf serum. Peripheral blood or bone marrow cells were collected from marmosets with heparin and centrifuged on Lymphocepal (IBL Co., Takasaki, Japan) at 2,000 rpm for 30 minutes. Mononuclear cells were collected and the remaining erythrocytes were lysed.

To stimulate T cells, the cells were cultured in medium supplemented with anti-human CD3 (SP34-2; BD Pharmingen, San Diego, CA, USA) and anti-human CD28 (CD28.2; eBioscience, San Diego, CA, USA) or TSST-1 (1 mg/mL; Toxin Tec. Co., Sarasota, FL, USA) for 24 hours. Then, the cells were harvested, incubated with anti-marmoset CD4 or CD8 mAb (prepared in this study) or anti-human CD20 mAb (B-Ly1, Dako, Glostrup, Denmark) at 4°C for 15 minutes, washed, and reincubated with anti-mouse IgG–magnetic cell sorting (MACS) micro beads for 15 minutes. After a final wash, CD4-, CD8- or CD20-positive cells were purified using a MACS system (Miltenyi Biotec, Gladbach, Germany).

Colony-forming assay using bone marrow–derived mononuclear cells

Cells were recovered from bone marrow of the common marmoset, and a mononuclear fraction was isolated using Lymphoprep (Axis-Shield, Oslo, Norway). Then cells were sorted into CD34$^+$CD117$^+$, CD34$^+$CD117$^-$, CD34$^-$CD117$^+$, and CD34$^-$CD117$^-$ fractions using FACS Aria (Becton Dickinson, Franklin Lakes, NJ, USA). Anti-marmoset CD34 mAb was reported previously [29], and anti-marmoset CD117 mAb was prepared in the present study. The sorted cells in each group were plated at 2×10^2 cells in 1 mL 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), IL-3 (10 ng/mL), human erythropoietin (Epo) (2 U/mL), and human G-CSF (10 ng/mL) and cultured at 37°C in a 5% CO_2 atmosphere. After 14 days of culture, types and numbers of hematopoietic colonies (colony-forming units [CFU]) were counted according to standard criteria. Samples from one animal were processed for the assay in triplicate, and two different animals were used (experiments 1 and 2).

Transplantation of hematopoietic progenitors into NOG mice

CD34$^+$CD117$^+$ and CD34$^-$CD117$^+$ cells were purified from bone marrow of the common marmoset by cell sorter as described here. Purity was >98% according to flow cytometry. Nine-week-old NOG mice were irradiated with 2.5 Gy x-ray prior to transplantation, and the marmoset cells (6.7×10^5 for CD34$^+$CD117$^+$ cells/ 1.3×10^6 for CD34$^-$CD117$^+$ cells) were transplanted intravenously. Four weeks after transplantation, peripheral blood was collected via orbit under inhalation anesthesia. Mononuclear cells were prepared and reconstitution efficiency was calculated by the ratio of marmoset CD45$^+$ cells.

Flow cytometric analysis

Cells were incubated with labeled primary mAb for 15 minutes at 4°C and washed with 1% (w/v) bovine serum albumin-containing phosphate-buffered saline. In some cases, cells were reincubated with labeled secondary antibody. The flow cytometer was FACSCalibur

(Becton Dickinson). The mAbs used were as follows: purchased from BD Pharmingen were anti-human CD3-peridinin chlorophyll Cy5.5 (PerCP) (SP34-2), anti-human CD4-allophycocyanin (APC) (SK3), anti-human CD8–fluorescein isothiocyanate (FITC) (HIT8a), anti-human CD20 (B-Ly1), anti-human CD25-phycoerythrin (PE) (M-A251), anti-human CD34 (581), anti-human CD45-APC (HI30), anti-human CD117-APC (YB5.B8), streptavidine-PE and streptavidine-APC. Purchased from eBioscience were anti-mouse CD4-APC (GK1.5), anti-mouse CD8-FITC (53.67), anti-mouse CD45.1-APC (30-F11), anti-mouse CD25-PE (PC61) and anti-mouse CD34 (RAM34). Anti-mouse CD117-APC (3C1) was from CALTAG Laboratories (Burlingame, CA, USA), and anti-mouse IgM-PE and anti-mouse IgG-PE were from Jackson ImmunoResearch Inc. (West Grove, PA, USA). We previously obtained anti-marmoset CD8 and CD45 mAbs by a different approach from that taken in this study [33]. These two mAbs were used as controls in the present study.

Results

cDNA expression systems and generation of mAbs

In the previous study, we cloned and sequenced 37 cDNA of immune-related genes obtained from marmoset spleen cells. Among them, we selected CD4, CD8, CD25, CD45, and CD117 as the targets to develop mAbs against marmoset proteins in this study. In humans and mice, CD4, CD8, and CD25 are representative surface molecules of T lymphocytes, CD117, and CD34 are hallmarks of hematopoietic progenitors, and CD45 represents a pan-leukocyte antigen.

To express a marmoset-derived cDNA, a pCXGFP1 vector was used (Fig. 1A). A cDNA portion encoding an extracellular domain of marmoset protein of interest to us was inserted into the *EcoR*V cloning site. From a recombinant, a marmoset cDNA was expressed as a fusion protein with the transmembrane domain of human MICA and cytoplasmically located enhanced GFP. Transcription was driven by CAG promoter. This recombinant was injected into mice as a DNA vaccine to sensitize the animals. In parallel, for the purpose of screening antibody production, a source of antigen was prepared by transfecting this expression plasmid into CHO cells. The cells expressing a fusion protein were selected by incubating the culture in the presence of G418. The plasmid harbored the neomycin-resistant gene.

By conventional experimental procedures, we could isolate the following six mAb clones (a subclass of Ig indicated). They were the clone Mar4-33 producing anti-CD4 mAb (IgG1), the clone Mar8-10 producing anti-CD8 mAb (IgG1), the clone Mar25-3 producing anti-CD25 mAb (IgG1), the clone Mar45-14 producing anti-CD45 mAb (IgG1), the clone MA24 producing anti-CD34 mAb (IgM), and the clone Mar117-22 producing anti-CD117 mAb (IgG1). Each mAb, including the previously described clone MA24, that produces anti-CD34 IgM mAb [29] was labeled with fluorochrome.

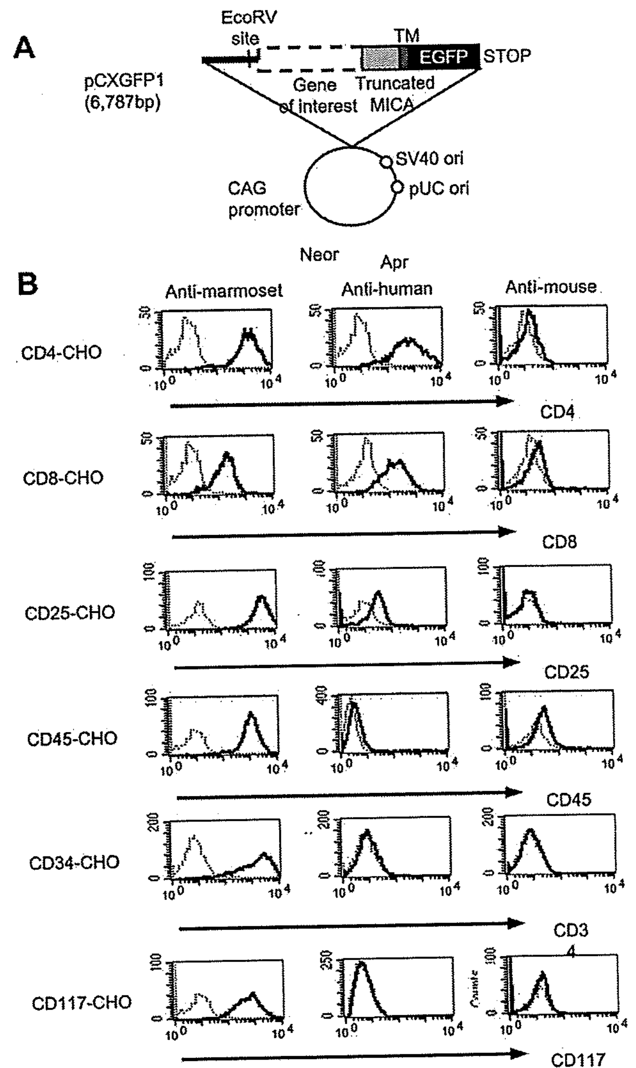

Figure 1. Reactivity of anti-marmoset, anti-human, and anti-mouse monoclonal antibodies (mAbs) against marmoset proteins expressed on CHO cells. **(A)** Schematic illustration of cDNA-expressing pCXGFP-1 vector. The *EcoR*V site is a cloning site of cDNA whose expression is driven by a CAG promoter. A gene of interest is expressed as a fusion protein with a transmembrane (TM) domain of human MICA and an intracytoplasmically located enhanced green fluorescent protein (EGFP) protein. The neomycin-resistance gene (Neor) is a drug selection marker. **(B)** Flow cytometric analyses of marmoset cDNA-expressing CHO cells stained by anti-marmoset, anti-human and anti-mouse mAbs.

Characterization of anti-marmoset mAbs

Figure 1B illustrates flow cytometric profiles of marmoset cDNA-transfected CHO cells stained by the newly prepared 6 mAbs and commercial mAbs. As can be seen in the left columns, each of the generated anti-marmoset mAb reacted with the respective antigen on CHO cells to a substantial degree of fluorescence intensity. This confirmed the authenticity of each mAb to recognize the respective antigen.

Cross-reactivity of anti-human mAb was examined in parallel (middle columns) using the cDNA-transfected CHO cells. An anti-human CD4 mAb (SK3) cross-reacted with marmoset CD4 as efficiently as anti-marmoset CD4 mAb. An anti-human CD8 mAb (HIT8α) gave a similar result. On the other hand, an anti-human CD25 mAb (M-A251) weakly cross-reacted with marmoset CD25, whereas anti-human CD45 (HI30), anti-human CD34 (581), and anti-human CD117 (YB5.B8) mAbs did not cross-react at all with the corresponding marmoset antigens. None of the anti-mouse mAbs showed any cross-reactivity with marmoset antigens (right columns).

Detection of marmoset T lymphocytes by anti-marmoset CD4 and CD8 mAbs

As shown in Figure 1B, the anti-marmoset mAbs reacted with the marmoset antigen that was overexpressed from transfected cDNA. Then, we investigated the reactivity of the same mAbs toward endogenously expressed marmoset antigens. As for the cells endogenously expressing these antigens, marmoset spleen cells were examined for reactivity of mAbs against marmoset CD4 and CD8 by two-color flow cytometric analysis. In the left panel of Figure 2A, marmoset splenocytes were stained by anti-marmoset CD4 and anti-human CD4 mAbs. The existence of double-positive cells in the upper right quadrant indicates the recognition of the same molecule by the two antibodies. A similar result was obtained when anti-marmoset CD8 and anti-human CD8 mAbs were used (see right panel of Fig. 2A).

CD3 is a pan-T lineage marker. We confirmed that an anti-human CD3ε mAb (SP34-2) recognized marmoset CD3ε-transfected cells (data not shown). In fact, marmoset splenocytes were stained with anti-human CD3ε and anti-marmoset CD4, or anti-human CD3ε and anti-marmoset CD8 mAbs, respectively (Fig. 2B). The CD3ε$^+$ T lymphocytes were divided into CD4$^+$ and CD8$^+$ cells. The combination of anti-human CD3ε and anti-human CD4 mAbs, or that of anti-human CD3ε and anti-human CD8 mAbs, gave the same result. It must be noted that anti-marmoset CD4 and anti-marmoset CD8 mAbs did not react with human peripheral blood mononuclear cells (Fig. 2C), indicating that anti-marmoset mAbs do not recognize the corresponding human antigens.

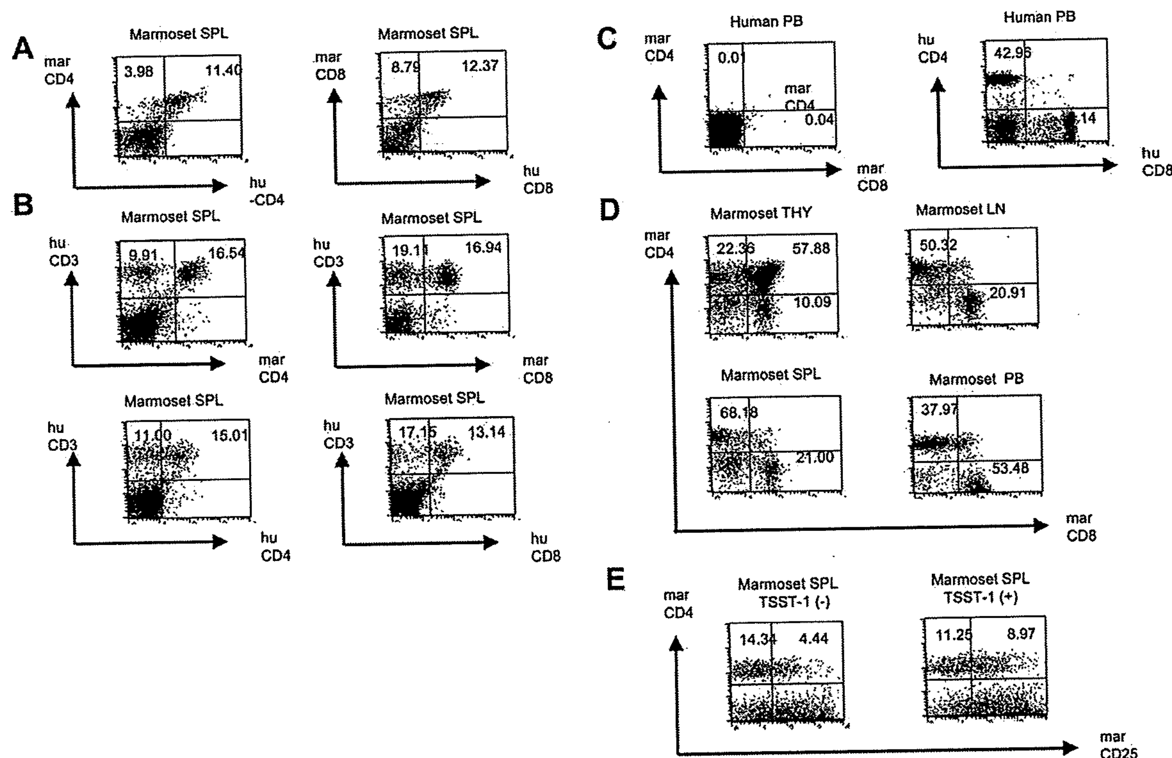

Figure 2. Characterization of anti-marmoset CD4, CD8, and CD25 monoclonal antibodies (mAbs), and identification of marmoset CD4$^+$ and CD8$^+$ T lymphocytes. (**A, B**) Marmoset splenocytes (SPL) were stained by the indicated mAbs, and their flow cytometric patterns are displayed. The mAbs used were anti-marmoset CD4, anti-marmoset CD8, anti-human CD4, anti-human CD8 and anti-human CD3ε. (**C**) Flow cytometry of human peripheral blood (PB) mononuclear cells. The mAbs were used as indicated. (**D**) Flow cytometry of marmoset-derived thymocytes (THY), SPL, lymph node cells (LN), and PB mononuclear cells. Anti-marmoset CD4 and CD8 mAbs were used as indicated. (**E**) Flow cytometry of marmoset SPL stained with anti-marmoset CD4 and anti-marmoset CD25 mAbs. Freshly isolated splenocytes (left) and the cells treated with TSST-1 in vitro (right) were analyzed.

Table 1. Comparison of Callithrix jacchus–derived CD34 and c-kit with human and mouse orthologs

	Identical aa residues (%)		
Protein	Marmoset vs human	Marmoset vs mouse	Human vs mouse
CD34	80	60	63
c-kit	94	81	82

Amino acid sequences of CD34 and c-kit from common marmoset (BAD04017.1 and BAD04018.1, respectively) were compared by BLAST homology search with human and mouse orthologs (human CD34, NP_001020280.1; human c-kit, NP_000213.1; mouse CD34, NP_598415.1; mouse c-kit, NP_066922.1) in three way pair-wise comparisons. The numbers indicate the percentages of identical aa residues between the two proteins compared.

These results confirmed that anti-marmoset CD4 and CD8 mAbs indeed recognize endogenous molecules. Then we also examined the distribution of T-cell subsets in other marmoset lymphoid tissues using these two mAbs (Fig. 2D). In marmoset thymi, the four subpopulations detected were $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$, whereas only the two mature subsets $CD4^+CD8^-$ and $CD4^-CD8^+$ were detected in marmoset peripheral lymphoid tissues, such as spleen, lymph node, and peripheral blood. The profiles of these T-cell subsets were indistinguishable from those seen in human and mouse lymphoid tissues.

CD25 has been identified as an activation marker expressed on T cells in mice and humans. Then, mAb against marmoset CD25 was used to determine whether CD25 is expressed on marmoset splenocytes pre- and/or poststimulated with TSST-1 superantigen derived from *Staphylococcus aureus*, which can strongly stimulate $CD4^+$ T cells via T cell receptor (TCR)-β chain. The results showed an increase of CD25-expressing cells in $CD4^+$ cells (23–44%) after TSST-1 stimulation, indicating that CD25 is induced to express via T-cell activation in marmosets as well as in humans. A relative proportion of $CD25^+$ cells without CD4 expression in both pre– and post–TSST-1 stimulation was observed (Fig. 2E), and these $CD4^-CD25^+$ cells may belong to non–T cells, such as B cells and dendritic cells (DCs), as will be discussed later.

Cytokine gene expression profiles in marmoset T lymphocytes

Because the existence of $CD4^+$ and $CD8^+$ fractions was confirmed in lymphoid tissues, we then examined the cytokine expression profiles in marmoset T lymphocytes. To purify $CD4^+$ and $CD8^+$ cells from marmoset spleens, biotin-labeled anti-marmoset mAbs were used. As a control, B lymphocytes were isolated using anti-human CD20 mAb, because a transfectant expressing marmoset CD20 was recognized by anti-human CD20 (data not shown). The spleen cells were stimulated in vitro by anti-human CD3ε mAb, anti-human CD3ε, and anti-human CD28 mAbs, or superantigen TSST-1. After that, CD4 T cells were purified and RNA was prepared from the cells for semi-quantitative RT-PCR analyses (Fig. 3). Primers were designed based on published sequences of marmoset cDNAs.

In splenic $CD4^+$ cells (Fig. 3A), transcripts of *IL-4, IL-5, IL-6, IL-10, IL-17A, IL-17F*, and *IFN-γ* were barely or only slightly detected prior to stimulation, but were induced substantially by T-cell stimulation. *IL-2* induction was observed only after stimulation by TSST-1. In contrast, a relatively high amount of transcripts was detected for *TGF-β* and *TNF-α* prior to stimulation, and their expression persisted after stimulation. All of the $CD4^+$cells freshly isolated from five marmosets' peripheral blood showed a similar transcription profile to those in spleen cells (Fig. 3B).

$CD8^+$ splenic T cells were also examined (Fig. 3C). *IL-2* transcript was not detected before stimulation, but was substantially induced by TCR-mediated stimulations. Expression of *granzyme B* and *IFN-γ* was moderately

Table 2. Colony formation of marmoset bone marrow progenitor cells

Group[a]	CFU-mix[b]	CFU-G[b]	CFU-GM[b]	CFU-M[b]	Mega[b]	GFU-E/CFU-E[b]
Experiment 1						
$CD34^+$/c-kit^+	2 ± 1.4	6.3 ± 1.7	5.3 ± 1.9	0.3 ± 0.5	1 ± 1.4	2 ± 0.8
$CD34^+$/c-kit^-	0 ± 0	1.3 ± 0.5	0.7 ± 0.9	2 ± 0	1 ± 0	1 ± 0.8
$CD34^-$/c-kit^+	1.3 ± 0.5	5.3 ± 1.2	3.0 ± 0.8	0.3 ± 0.5	0 ± 0	4.0 ± 1.6
$CD34^-$/c-kit^-	0.7 ± 0.9	2 ± 0.8	1 ± 0.8	0 ± 0	1 ± 0	0.7 ± 0.9
Experiment 2						
$CD34^+$/c-kit^+	4.3 ± 2.1	12 ± 2.9	12 ± 1.6	1.7 ± 0.5	0.3 ± 5	6 ± 0.8
$CD34^+$/c-kit^-	0 ± 0	0.3 ± 0.5	0 ± 0	2 ± 0	0 ± 0	0 ± 0
$CD34^-$/c-kit^+	1.0 ± 0.8	8.7 ± 1.7	9.0 ± 3.7	5.0 ± 0	0.3 ± 0.5	6.7 ± 1.9
$CD34^-$/c-kit^-	0 ± 0	0.3 ± 0.5	0.7 ± 0.5	4 ± 1.4	0 ± 0	0 ± 0

Data are expressed as mean ± standard deviation.

[a]Marmoset bone marrow cells were sorted to four fractions according to CD34 and c-kit expressions and cultured in methylcellulose-containing medium for 2 weeks.

[b]Colony-forming unit (CFU)-mix, colony-forming unit granulocyte (CFU-G), colony-forming unit granulocyte-macrophage (CFU-GM), colony-forming unit macrophage (CFU-M), megakaryocyte (Mega)/colony-forming unit erythroid (GFU/CFU-E)/2 × 10^2 sorted bone marrow cells are shown. Colonies in triplicate were counted by microscopy. One marmoset per experiment was used.

detected before stimulation, and was significantly upregulated by stimulation. In contrast, *perforin-1* expression was sufficiently detected without stimulation and did not show any enhancement with stimulation.

Overall, the expression profiles of various cytokine genes seen in marmoset T cells were generally similar to those observed in humans and mice.

A primary structure of marmoset granzyme B gene

In the previously described plasmid construction and RT-PCR analyses, we utilized marmoset cDNA sequences that had mostly been reported in our previous study [28]. Exceptions were *perforin 1* and *granzyme B*. The nucleotide and deduced amino acid sequences of these two genes were determined in this study and were registered at National Center for Biotechnology Information (NCBI) as EU918127 and EU918128, respectively.

An interesting feature was observed in the primary sequence of *granzyme B* transcript (Fig. 4). Near the carboxy terminus of open reading flame, an additional sequence of 317 nt was inserted, which was not found in the corresponding region of human and chimpanzee *granzyme B* (see the sequence indicated by blue in (Fig. 4A), and note that Hs, Pt, and Cj represent human, chimpanzee and the common marmoset, respectively). This insertion caused a shift in the reading frame (see the location of termination codon indicated by red). Thus, the number of amino acid residues in the predicted marmoset polypeptide became longer by 23 aa at its carboxy terminus compared to human and chimpanzee granzyme B proteins (Fig. 4B). We cloned the genomic DNA from marmoset peripheral blood cells, and confirmed the insertion of the sequence in question into the exactly corresponding site of *granzyme B* gene (data not shown). Interestingly, the BLAST tool revealed a close homology of the inserted sequence to the

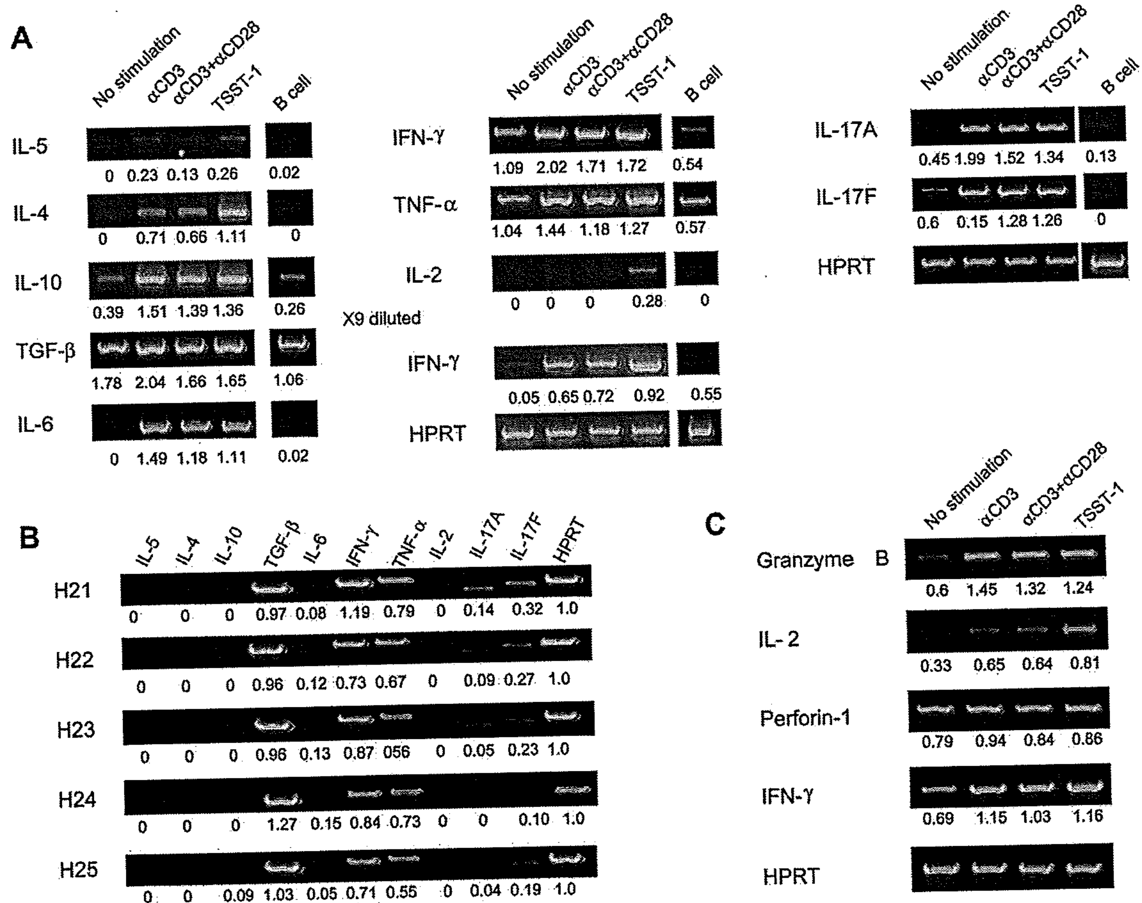

Figure 3. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analyses of marmoset cytokine gene transcripts. CD4$^+$ (**A**) and CD8$^+$ cells (**C**) were isolated from marmoset spleens and stimulated in vitro with anti-CD3, anti-CD3/anti-CD28 or TSST-1, respectively. CD4$^+$ cells were prepared from peripheral blood (PB) of normal marmosets (**B**). H21 to H25 represent individual marmoset numbers. RNA was prepared from the cells and processed for RT-PCR. B lymphocytes were isolated from marmoset spleens using anti-human CD20 monoclonal antibody (mAb), and its RNA was used in parallel. The amplified fragments were quantified and shown as indexes calculated using the formula (cytokine/HPRT). HPRT = hypoxanthine-guanine phosphoribosyltransferase; IFN = interferon; IL = interleukin; TGF = transforming growth factor; TNF = tumor necrosis factor.

A

```
Hs  CCACGAGCCTGCACCAAAGTCTCAAGCTTTGTACACTGGATAAAGAAAACCATGAAACG-
Pt  CCACGAGCCTGCACCAAAGTCTCAAGCTTTGTACACTGGATAAAGAAAACCATGAAACG-
Cj  CCACGGGCCTTCACCAAAGTCTCAAGCTTTGTACACTGGATAAAGAAAACCATGAGGCCG
    *****.**** *********************************************..*

Hs  ------------------------CTACTAAGTACAGGAAGCAAACTAAG----------
Pt  ------------------------CCACTAAGTACAGGAAGCAAACTAAG----------
Cj  GGGACGATGGCTTACACCTGTAATCCCAGGACTTTGGGAGGCCGAGGCAGGTGGATCACG
                            * .. .***: .***.**..*   .**

Hs  ----------------CCCCCGCTG-------TAATGAAACACCTTCTCTGGAGCCAAGT
Pt  ----------------CCCCCGCTG-------TGATGAAACACCTTCTCTGGAGCCAAG-
Cj  AGGTCAAGAGTTTGAGTCCAGCCTGGCTAAGATGTTGAAACTCCACCTCAGTTAAAAATA
                   **.    ***         *.:******:**: ***:* :...**

Hs  CCAGATTTACACTGGGAG------------------------------------------
Pt  CCAGATTTACACTGGGAG------------------------------------------
Cj  CAAAAATTTGCCAGGCATGGTGGCATATACCTGTAATCCCAGCTATTCAGGAGGCTGAGG
    *.*.*:**: .*:** *

Hs  -------------------------------------------AGGTGCCAGCAACTGA
Pt  -------------------------------------------AGGTGCCAGCAACTGA
Cj  CAGAAGAACCACTTGAATCCAGGAGGCAGAGGTTGCAGTGAGCCAAGATCATGCCACTGT
                                               *.*: *.:**.****:

Hs  ATAAATACCT-----------CTTAGCTGAGTGGAAAAAAAAAAAAAAAAAAAAAAAAAA
Pt  ATAAATACCT-----------CTTAGCTGAGTGGAAAA----------------------
Cj  ACTCCAGCCTGGGTGACAGAGCAAGACTCCATGTAAAAAAAAAAAAAAAAAAAAAAAAAA
    * :..:.***           *::..** ..** ****

Hs  AAAAAA----------------------------
Pt  ----------------------------------
Cj  GCGAAAGGAAAAGAAAACCAGGAAATGCCACTAA
```

B

```
Hs  ----------------------------------MQPILLLLAFLLLPRADAGEIIGGHE
Pt  MKSLSLLHLFPLPRAKREQGENNSSSNQGSLPEKMQPILLLLAFLLLPRADAGEIIGGHE
Cj  ----------------------------------MQPILLLLAFLLLPRADAGEIIGGHE
                                      **************************

Hs  AKPHSRPYMAYLNIWDQKSLKRCGGFLIQDDFVLTAAHCWGSSINVTLGAHNIKEQEPTQ
Pt  AKPHSRPYMAYLNIWDQKTLKRCGGFLIREDFVLTAAHCWGSSINVTLGAHNIKEQEPT
Cj  AKPHSHPYMAYLAIWDQESLKRCGGFLVREDFVLTAAHCWGSSINVTLGAHNIKKQERTQ
    *****:****** ****::********:::*************************:** **

Hs  QFIPVKRPIPHPAYNPKNFSNDIMLLQLERKAKRTRAVQPLRLPSNKAQVKPGQTCSVAG
Pt  QFIPVKRPIPHPAYNPKNYSNDIMLLQLERKAKRTRAVQPLRLPSNKAQVKPGQVCSVAG
Cj  QSNPVRRTFCHPDYNPENFSSDIMLLQLERKAKRTTAVQPLRLPSSKAQVKPGQVCSVAG
    * :**:*.: ** ***:*:*.************** *********.********.*****

Hs  WGQTAPLGKHSHTLQEVKMTVQEDRKCESDLRHYYDSTIELCVGDPEIKKTSFKGDSGGP
Pt  WGQTAPLGKHSHTLQEVKMTVQEDRKCESDLRHYYDSTIELCVGDPEIKKTSFKGDSGGP
Cj  WGRTTPNGTYSHTLQAVNLTVQEDRKCESCLRNYYDSTVELCVGDPEIKKASFKGDSGGP
    **:*:*:*.:***** *::********** **:*****:***********:*********

Hs  LVCNKVAQGIVSYGRNNGMPPRACTKVSSFVHWIKKTMKRY-------------------
Pt  LVCNKVAQGIVSYGRNNGMPPRACTKVSSFVHWIKKTMKRH-------------------
Cj  LVCNKVAQGIVSHGPRNGKSPRAFTKVSSFVHWIKKTMRPGTMAYTCNPRTLGGRGRWIT
    ************:* .** .*** **************:

Hs  ----
Pt  ----
Cj  RSRV
```

C

```
Query: An Alu-like sequence in the Cj-derived granzyme B gene
Subject: gnl|alu|M21005_HSAL002524 (Alu-J), Length=347
Score=244bits (132), Expect=4e-67, Identities=232/280 (82%), Gaps=7/280 (2%)

Query    4    GGCCGGGGACGATGGCTTACACCTGTAATCCCAGGACTTTGGGAGGCCGAGGCAGGTGGA  63
              ||||||| ||| ||||| ||  ||||||||||| |||||| ||||||||||||||||||
Subject  1    GGCCGGGCACGGTGGCTCACGGCTGTAATCCCAGCACTTTGAGAGGCCGAGGCAGGTGGA  60

Query    64   TCACGAGGTCAAGAGTTTGAGTCCAGCCTGGCTAAGATGTTGAAACTCCA-C-CT-C--A  118
              ||| ||||||| |||||   || |||||||||| || ||| |||||| ||| | || |  |
Subject  61   TCATGAGGTCAGGAGTTCAAGACCAGCCTGGCCAATATGGTGAAACCCCATCTCTACTAA  120

Query    119  GTTAAAAATACAAAAATTTGCCAGGCATGGTGGCATATACCTGTAATCCCAGCTATTCAG  178
                ||||||| |||||| ||||||| ||||||     |  |||||| ||||||||| ||||
Subject  121  AAAAAAAATACCAAAATTAGCCAGGCGTGGTGGTGCACGCCTGTAGTCCCAGCTACTCAG  180

Query    179  GAGGCTGAGGCAGAAGAACCACTTGAATC-CAGGAGGCAGAGGTTGCAGTGAGCCAAGAT  237
               ||||||||||| |||||| ||| || |   |||||| ||||||||||||||||||||
Subject  181  TAGGCTGAGGCAGGAGAACCCCTTTAA-CGTGGGAGGCGGAGGTTGCAGTGAGCCAAGAT  239

Query    238  CATGCCACTGTACTCCAGCCTGGGTGACAGAGCAAGACTC  277
              |||||||||| |||||||| |||| |||||||   ||||||
Subject  240  CATGCCACTGCACTCCAGCGTGGGCGACAGAGTGAGACTC  279
```

Figure 4. Insertion of *Alu*-like sequence into the marmoset *granzyme B* gene. **(A)** Alignment of *granzyme B* transcripts from human (Hs, NM_004131.3), chimpanzee (Pt, XM_509879.2) and common marmoset (Cj, EU918128.1). The 3′ portions of each transcript were aligned using the CLUSTALW tool. The common marmoset sequence indicated by bold, which does not have a counterpart in the transcripts from human and chimpanzee, is the Alu sequence. The termination codons of open reading flame (ORF) are indicated by underlines. Asterisks indicate identical residues. **(B)** Alignment of amino acid sequences of granzyme B proteins from human (NP_004122), chimpanzee (XP_509879), and common marmoset (EU918128). **(C)** Alignment of common marmoset-derived 317 nucleotide sequence (indicated by bold in panel A) with the human-derived *Alu-J* element.

human *Alu-J* element (Fig. 4C). Thus, the *Alu*-insertion into the ORF of *granzyme B* gene appears to be a feature unique to the common marmoset.

Detection of hematopoietic progenitor activity in marmoset bone marrow

CD117 and CD34 are the representative markers of murine and human hematopoietic progenitors, respectively. An extent of sequence conservation among the orthologous proteins of human, the common marmoset and mouse origin was examined by BLAST homology search (Table 1). The numbers therein represent the percentages of identical amino acid residues in three-way pair-wise comparisons. It can be seen that CD34 is relatively divergent among the three species, whereas CD117 is better conserved.

We then examined the activity of hematopoietic progenitors in marmoset bone marrow. Marrow cells were stained with anti-marmoset CD117 and CD34 mAbs (Fig. 5A). Four distinct fractions, namely $CD117^{+}CD34^{+}$, $CD117^{+}CD34^{-}$, $CD117^{-}CD34^{+}$, and $CD117^{-}CD34^{-}$, were detected. These anti-marmoset mAbs did not detect a positive population in human cord blood cells due to the lack of cross-reactivity with human antigens.

Using these mAbs, the four fractions described here were isolated from marmoset bone marrow cells by flow cytometry and assayed for their colony-forming activity in vitro. Human recombinant cytokines were added to the culture. Figure 5B shows the morphological appearance of burst-forming unit erythroid (BFU-E), colony-forming unit granulocyte (CFU-G), colony-forming unit macrophage (CFU-M), and colony-forming unit granulocyte-macrophage (CFU-GM) in the isolated $CD117^{+}CD34^{+}$ cells. At a level of microscopic resolution, these marmoset-derived colonies were indistinguishable from those of human and mouse. The

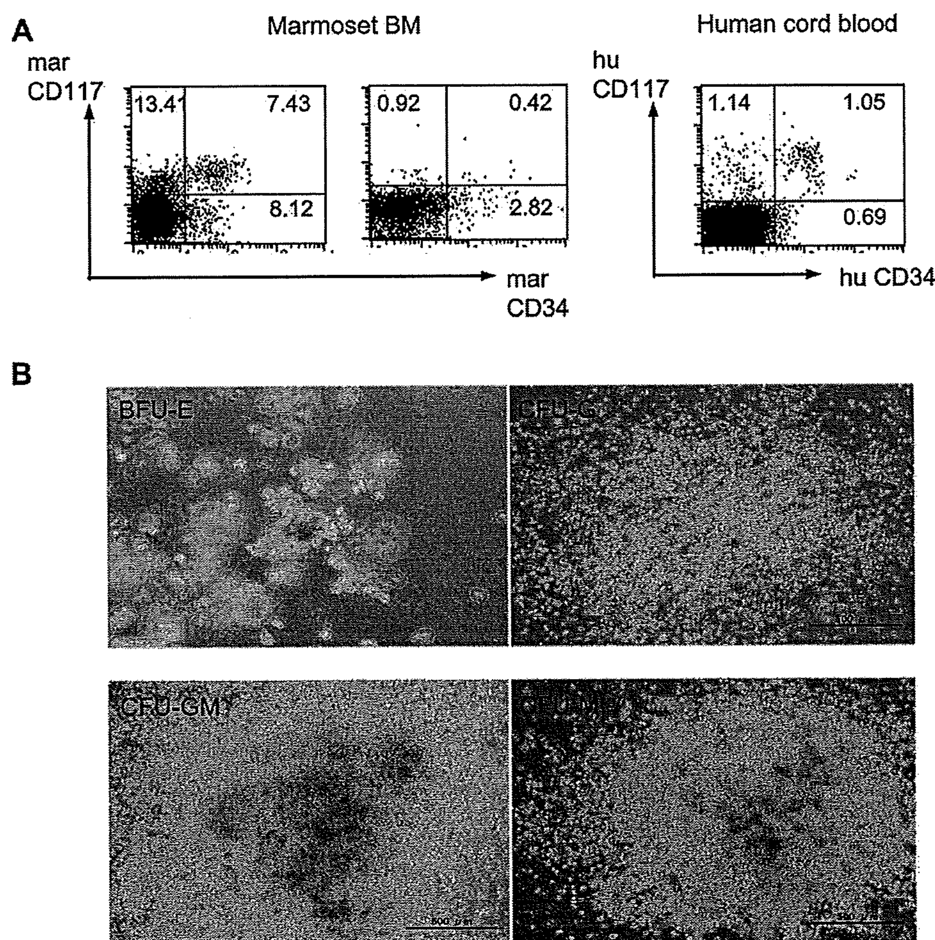

Figure 5. Hematopoietic progenitors in marmoset bone marrow as assayed in vitro. (A) Marmoset bone marrow (BM) cells and human cord blood cells were stained with the indicated monoclonal antibodies (mAbs), and their flow cytometrical patterns are displayed. The mAbs used were anti-marmoset CD117, anti-marmoset CD34, anti-human CD117, and anti-human CD34. (B) Morphological appearance of marmoset colonies formed in vitro. A $CD117^{+}CD34^{+}$ fraction was isolated from marmoset bone marrow, and assayed for colony-forming activity in the cytokine-supplemented culture. Phase contrast microscopic pictures are presented for burst-forming unit erythroid (BFU-E), colony-forming unit granulocyte (CFU-G), colony-forming unit macrophage (CFU-M), colony-forming unit granulocyte-macrophage (CFU-GM), respectively.

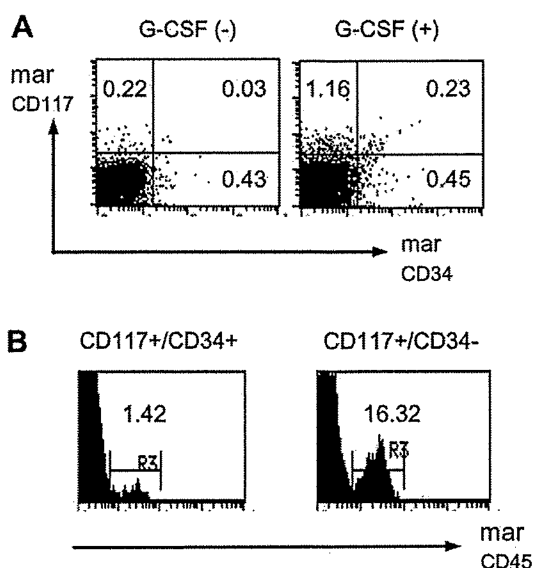

Figure 6. Hematopoietic progenitors in marmoset bone marrow as assayed in vivo. **(A)** Detection of peripherally mobilized progenitors in response to granulocyte colony-stimulating factor (G-CSF) injection. A marmoset individual was intravenously inoculated with human recombinant G-CSF, and peripheral blood was collected prior to (left panel) and 48 hours after the injection (right panel). Blood cells were stained using anti-marmoset CD117 and CD34 monoclonal antibodies (mAbs). **(B)** Lymphopoietic activity of marmoset progenitors as assayed in NOG mice. The $CD117^+CD34^+$ (left panel) and $CD117^+CD34^-$ (right panel) fractions isolated from marmoset bone marrow were inoculated into NOG mice, and 7 weeks after transplantation, peripheral blood was collected and stained using anti-marmoset CD45 mAb.

numbers of each colony were counted in each fraction and their average numbers are shown in Table 2. Quite similar numbers of colonies were formed from $CD117^+CD34^+$ and $CD117^+CD34^-$ fractions. However, only very weak colony-forming activity was detected for $CD117^-CD34^+$, and almost no activity for $CD117^-CD34^-$. This result suggests that hematopoietic activity as assayed in vitro resides mainly in the $CD117^+$ cells irrespective of CD34 expression.

Activity of marmoset hematopoietic progenitors as assayed in vivo

As described here, we could detect hematopoietic progenitors in marmoset bone marrow using marmoset-specific mAbs. Next, we examined the in vivo activity of marmoset progenitors. Human G-CSF was injected intravenously into marmosets. After 48 hours, peripheral blood was collected and stained with anti-marmoset mAbs (Fig. 6A). A $CD117^+$ fraction was not apparent in peripheral blood prior to G-CSF injection (left panel), but became evident after injection (right panel), corresponding to a sevenfold increase from 0.03% (left) to 0.23% (right). This indicates a mobilization of hematopoietic progenitors in response to G-CSF.

We also assayed the lymphopoietic activity of marmoset progenitors in vivo. For that, we used NOD/shi-SCID/IL-$2Rg^{null}$ (NOG) mice, a severe immunodeficient mouse cell line, because the mice have higher multipotential engraftment than any other mouse lines. The $CD117^+CD34^+$ and $CD117^+CD34^-$ fractions were isolated as mentioned here, and individually inoculated into NOG mice. After 7 weeks, peripheral blood was collected and stained for marmoset CD45 (Fig. 6B). In an individual inoculated with the $CD117^+CD34^-$ fraction, a substantial marmoset $CD45^+$ fraction emerged. In addition, the $CD20^+$ fraction was also observed in the $CD117^+CD34^-$-inoculated NOG mouse (data not shown). These observations indicate that the $CD117^+$ fraction developed into marmoset leukocytes, including a B-cell lineage, in vivo.

Discussion

In this study, to gain insight into how close or distant the immune system of the common marmoset is to that of humans and/or mice, we developed several mAbs directed toward marmoset antigens on T lymphocytes or on putative hematopoietic progenitors. CHO cells transfected with cDNAs and expressing marmoset proteins were initially used for screening the mAbs we produced against marmoset antigens. These cells were also used to examine the cross-reactivity of commercially available mAbs with human or mouse proteins. None of the examined anti-mouse mAbs showed any reactivity with marmoset antigens expressed on CHO cells, highlighting the great distance between marmoset and mouse epitopes. On the other hand, although mAbs against human CD34, CD45, and CD117 did not cross-react with respective marmoset antigens, other anti-human mAbs, including anti-CD4, anti-CD8, and anti-CD25 exhibited significant cross-reaction with the respective marmoset antigens. The reverse was not the case. The anti-marmoset CD4 or CD8 that we generated did not react with human CD4 or CD8. These observations suggest that use of anti-marmoset mAbs as was done in the present study would be ideal for examining marmoset immunity, although some anti-human mAbs could also be used as an alternative choice.

Marmoset $CD4^+$ T cells isolated from spleen cells and peripheral blood by using the established anti-marmoset mAbs showed various cytokine transcripts with TCR-mediated signaling. The induced cytokine transcripts included *IFN-γ*, *IL-4*, *IL-5*, *IL-10*, *IL-6*, and *IL-17*. These cytokines are produced from Th1, Th2, and Th17 T-cell subsets and play an important role in the immune response of humans and mice, suggesting that marmosets may have a similar immune function to humans. *TNF-α* and *TGF-β* transcripts were constitutively detected in marmoset $CD4^+$ cells even

prior to stimulation, which is not different from human and mouse. It must be emphasized that the present study established the authenticity of marmoset-derived cytokine transcripts because we designed the primers for PCR based on the marmoset sequences. On the other hand, the previous studies determined the marmoset cytokine transcripts by utilizing primers that were deduced by comparing the human and mouse orthologous genes [21,34]. Such an ambiguous method might allow the detection of only a few examples of cytokines.

TCR-stimulation caused an increment of *granzyme B* transcripts but not of *perforin 1* in these $CD8^+$ cells. In human, γδ T cells lose naïveness during childhood and start to express *perforin 1* constitutively [5,35]. Thus, one may speculate that the constitutive expression of *perforin 1* observed in the unstimulated marmoset $CD8^+$ T cells may not be detectable in resting cells but in already activated cells in some way. This is possible because the marmosets used were maintained under conventional, not pathogen-free, conditions. Alternatively, γδ T cells might be dominating in peripheral tissues of marmosets, which we could not yet determine as we have not cloned cDNA of any TCR chain genes, particularly γδ chains. Elucidation of this point requires development of anti-marmoset TCR γδ mAbs.

TSST-1 treatment of marmoset $CD4^+$ T cells enhanced their CD25 expression, reflecting the fact that CD25 expression is induced through the TCR-coupled activation process. To be noted is the existence of a $CD25^+$ subfraction in the freshly isolated splenic $CD4^-$ cells as well as $CD4^+$ T cells. The latter cells could be activated ones because they are maintained in non-specific pathogen free (SPF) environment as described here. Of a $CD4^-CD25^+$ fraction, they belong to non-T cells such as B cells and DCs, as shown in the past reports that these cells in human and mouse are induced to express CD25 if they are activated. In mouse and human, a $CD4^-CD25^+$ $CD20^+$ phenotype has been reported to represent B cells [36,37]. Further analyses of CD25 expression in marmoset resting B cells are required, particularly for developmental genetics/biology.

We generated and utilized anti-marmoset CD117 and CD34 mAbs for hematopoietic analysis. The percentage of $CD117^+CD34^+$ fraction was significantly increased in peripheral blood of marmosets receiving human G-CSF injection. These $CD117^+CD34^+$ cells exhibited multidifferentiating capabilities into myeloid and erythroid lineages by in vitro colony-forming assays. Furthermore, these double-positive cells, when transplanted into NOG mice, generated $CD45^+$ cells. Therefore, the $CD117^+CD34^+$ fraction most likely contains hematopoietic progenitor activity. The marmoset $CD34^+$ fraction reported to possess colony-forming activity [29] might have contained $CD117^+$ cells. In fact, as seen in Table 2, $CD117^+$ cells with and without CD34 expression showed higher hematopoietic activity than $CD117^-$ cells, while human $CD34^-CD117^+$ cells are reported to differentiate mainly to erythroid lineage cells [38]. A human $CD34^+$ fraction has been reported to possess progenitor activity into various cell lineages, including lymphocytes and DCs [39–41]. However, $CD34^+CD117^-$ cells are reported to possess less multipotentiality [41]. In mice, hematopoietic progenitor activity is present in $CD34^-CD117^+SCA\text{-}1^+$ cells [42,43]. Therefore, the previously known markers for hematopoietic progenitors may vary from species to species. The details of whether marmoset progenitors are more alike to mouse or human have to be investigated in greater detail.

In summary, the cell and cytokine profiles seen in marmoset helper and killer T cells were essentially reminiscent of those in human and mouse. Several marmoset antigens showed cross-reactivity with anti-human mAbs, suggesting a closer relationship of marmoset to human. The development of sophisticated analytical tools including mAbs should eventually contribute to the establishment of marmoset hematopoietic and immune systems as a human model.

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Conflict of Interest Disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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