3'). Sequencing analysis showed that the amplified products were compatible with the p190 BCR-ABL fusion gene (data not shown). Expression of p190 BCR-ABL protein was confirmed by western blot analysis in our previous report [15]. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was simultaneously amplified as an internal control. In some experiments, real-time quantitative PCR analysis of p190 BCR-ABL was performed as previously described [16].

2.5. Enrichment of $CD34^+$ Bone Marrow Cells

Marmoset bone marrow $CD34^+$ cells were isolated with the immunobeads system using streptavidin microbeads (MACS, Miltenyi Biotec, Sunnyvale, CA, USA). Marmoset bone marrow MNCs were prepared from fresh bone marrow samples and stained with a biotin-labeled anti-marmoset CD34 monoclonal antibody (clone MA24) for 30 min at 4°C [17]. The cells were washed and incubated with streptavidin-conjugated microbeads for 15 min at 4°C. The $CD34^+$ cells were washed and separated using immunomagnetic columns (MACS) according to the manufacturer's instructions.

2.6. *Ex Vivo* p190 BCR-ABL Transduction and Autologous Peripheral Blood Stem Cell Transplantation (PBSCT)

The time course and treatment protocol are described in **Table 1**. Briefly, 10 μg/kg/day of recombinant human granulocyte colony-stimulating factor (G-CSF, Roche, Basel, Switzerland) was subcutaneously administered to individual marmosets for five days. Peripheral blood (1 mL) was collected daily from the femoral vein of each marmoset for seven consecutive days. MNCs were isolated by Ficoll-Hypaque centrifugation. Red cell lysis was performed when necessary. The cells were frozen using a programmed freezer and stored in liquid nitrogen until use. Ninety milligrams of busulfan (Sigma Aldrich, St Louis, MO, USA) was dissolved in 15 mL of DMA/PEG (1:2) solution, and a 6 mg/mL busulfan solution was made. All marmosets received the busulfan solution (10 mg/kg) from day −3 to day −2 before PBSCT as previously described [18,19].

Three days before transplantation, the MNCs were thawed and incubated overnight in IMDM supplemented with 10% FBS and human cytokines (10 ng/mL hSCF, hIL-3 and hTPO) as previously described with some modifications [12]. After pre-stimulation, the cells were collected and centrifuged. The cell pellet was incubated with the concentrated viral supernatant at 37°C in 5% CO_2 for 2 hrs. The cells of two marmoset (No. 591 and 2338) were infected with the CMV-p190 BCR-ABL lentiviral vector. Then, the cells were cultured in IMDM supplemented with 10% FBS and human cytokines (hSCF, hIL-3, hTPO) in 24-well plates for 48 hrs. The transduced cells were collected, filtered with 40 μm nylon mesh, washed with serum-free IMDM and suspended in 1.5 mL normal saline. The cells (2×10^6) were transplanted via the femoral vein using a syringe and 27 G needle.

2.7. Direct Injection of Lentiviral Vectors into the Bone Marrow

The time course and treatment protocol are described in **Table 2**. Briefly, marmosets received 25 mg/body of 5-fluorouracil (Kyowa Hakko Kirin, Tokyo, Japan) on day −5 and prednisolone from day −3 to day 1. After anesthesia, *in vivo* transduction of bone marrow cells was performed by directly injecting the viral supernatant into the bone marrow cavity using an aspiration needle on day 0. One marmoset (No. 2129) received the CMV-p190 BCR-ABL lentiviral vector, whereas the other (No. 2223) received the PGK-p190 BCR-ABL lentiviral vector. All marmosets received both oral fluconazol (2 mg/body/day) and intramuscular injections of ampicillin (10 mg/body/day) from day −5 to day 7 and pentamidine (1.5 mg/body/day) once on day −5.

2.8. Colony Formation Assay

A colony formation assay (progenitor cell assay) was performed as previously described with minor modifications [17]. Bone marrow cells were washed twice, plated in methylcellulose containing human hematopoietic

Table 1. Time coure and treatment of *ex vivo* BCR-ABL transduction method.

Treatment	−14	−13	−12	−11	−10	−9	−8	−7	−6	−5	−4	−3	−2	−1	0	(days)
G-CSF (10 μg/body)	●	●	●	●	●											
PBMC collection					●	●	●	●	●	●	●					
Busulfan (10 mg/body)												●	●			
ex vivo gene transduction (p190 BCR-ABL)													●	●		
PBSCT															▼	

G-CSF: granulocyte colony stimulationg factor; PBMC: peripheral blood mononuclear cell; PBSCT: peripheral blood stem cell transplantation.

Table 2. Time coure and treatment of BCR-ABL direct *in vivo* injection method.

Treatment	–5	–4	–3	–2	–1	0	1	2	3	4	5	6	7	(days)
5FU (25 mg/body)	•													
PSL (8 mg/body)			•	•	•									
PSL (4 mg/body)						•								
PSL (2 mg/body)							•							
BM injection (p190 BCR-ABL)						▼								
Pentamidine (1.5 mg/body)	•													
ABPC (10 mg/body)	•	•	•	•	•	•	•	•	•	•	•	•	•	
FCZ (2 mg/body)	•	•	•	•	•	•	•	•	•	•	•	•	•	

5FU: 5-fluorouracil, PSL: predonisolone, BM: bone marrow, ABPC: ampicillin, FCZ: fluconazole

cytokines (Methocult GF+, StemCell Technologies, Vancouver, BC, Canada) and incubated at 37°C in 5% CO_2. Then, 1.5×10^3 MACS-sorted $CD34^+$ cells or 3×10^4 non-sorted cells were plated in one dish. On day 14, individual colonies were picked to identify BCR-ABL gene transduction by RT-PCR and examined by May-Giemsa staining.

3. Results

3.1. Lentiviral Vector Expressing p190 BCR-ABL Functionally and Efficiently Transduced Hematopoietic Stem/Progenitor Cells

First, we produced third generation VSV.G pseudotyped lentiviral vectors expressing the p190 BCR-ABL fusion gene (HIV-CMV/PGK-BCR-ABL). Plasmids including the lentiviral transfer vector were transduced into 293T cells by the calcium phosphate method. After 48 and 72 hrs, the viral supernatants were collected and ultra-centrifuged. The DNA titers of HIV-CMV-BCR-ABL and HIV-PGK-BCR-ABL were 5.59×10^7/ml and 2.53×10^8/ml, respectively. To determine that the vector functions properly, Ba/F3 cells, a mIL-3-dependent murine hematopoietic cell line, were transduced with this vector and cultured without mIL-3. These cells rapidly expanded after 12 days, indicating that p190 BCR-ABL gene expression allowed the Ba/F3 cells to grow autonomously regardless of the promoter used (**Figure 1(a)**). RT-PCR confirmed that p190 BCR-ABL was expressed in the transduced Ba/F3 cells (**Figure 1(b)**). We then performed quantitative RT-PCR (**Figure 1(c)**). Although the MOI of the lentiviral vector was not high, p190 BCR-ABL expression was detected in the Ba/F3 cell line. Next, to check whether marmoset hematopoietic stem/progenitor cells were efficiently transduced with the lentiviral vector, MACS-sorted bone marrow $CD34^+$ cells were transduced with the lentiviral vector (HIV-CMV/PGK-BCR-ABL) and subjected to the colony formation assay (**Figure 1(d)**). In more than 80% of colonies examined, p190 BCR-ABL transduction was detected regardless of the promoter. Taken together, the above findings indicate that this oncogene was efficiently transduced into marmoset stem/progenitor cells.

3.2. *Ex Vivo* BCR-ABL Transduction Method

To establish a marmoset leukemia model, we first tried the *ex vivo* BCR-ABL transduction method (**Table 1**). After mobilizing the hematopoietic stem/progenitor cells by administering G-CSF, peripheral blood MNCs were collected according to the schedule in **Table 1**. The collected cells were pre-cultured with cytokines (10 ng/ml hSCF, hIL-3 and hTPO) to improve the transduction efficiency. After the cells were transduced with the lentiviral vector containing the p190 BCR-ABL fusion gene *ex vivo*, they were transplanted into common marmosets that were previously treated with pre-conditioning busulfan (**Table 1**). On days 28 and 56 post transplantation, BCR-ABL expression was detected by RT-PCR in the peripheral blood MNCs of two marmosets (**Figure 2(a)**, upper). The time course for BCR-ABL expression after transplantation is shown in **Figure 2(a)** (lower). Although we expected stable BCR-ABL expression, BCR-ABL expression disappeared after days 56 and 100 in two marmosets. Because this result was thought to partially result from low-level transgene expression due to ineffective gene transduction in hematopoietic stem/progenitor cells, we changed the gene transduction method.

3.3. *In Vivo* BCR-ABL Direct Transduction Method

Previous reports showed that directly injecting lentiviral vectors *in vivo* resulted in stable gene expression [20].

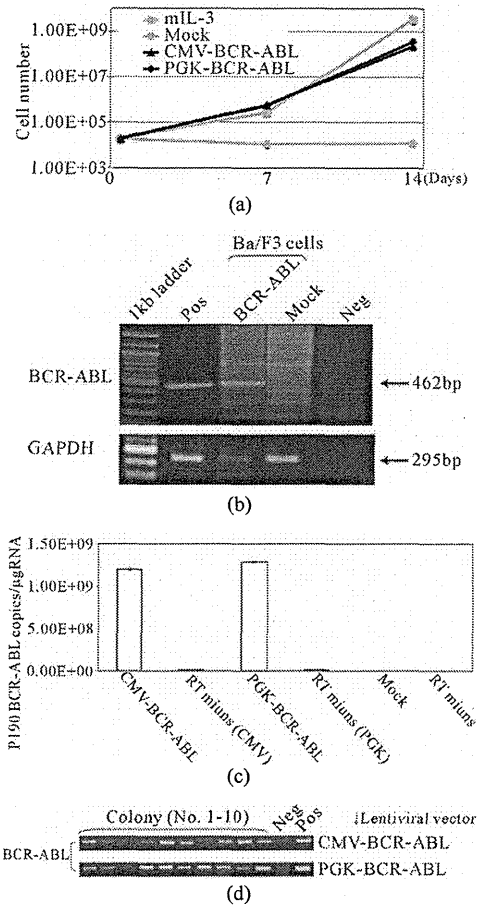

Figure 1. (a) Ba/F3, a murine hematopoietic cell line that is dependent on mouse interleukin-3 (mIL-3), was transduced with the p190 BCR-ABL lentiviral vector (HIV-CMV/ PGK-BCR-ABL) and cultured without mIL-3. As positive and negative controls, mock transfected cells were cultured with or without mIL-3. The number of live cells was determined by the trypan blue exclusion method. All experiments were done in triplicate and also repeated three independent times, and data were plotted as mean ± SD; (b) Detection of the BCR-ABL fusion gene in transduced Ba/F3 cells. Three days after lentiviral transduction, RT-PCR for the BCR-ABL gene was performed in BCR-ABL- or mock-transduced cell lines. Pos: positive control (KOPN30 cell line harbouring p190 BCR-ABL), Neg: negative control (ddw: deionized distilled water); (c) Quantitative analysis of transduced p190 BCR-ABL fusion gene expression. Three days after lentiviral transduction, quantitative RT-PCR for p190 BCR-ABL was performed. RT minus indicates the control PCR reaction without reverse transcriptase; (d) Lentiviral transduction of the BCR-ABL gene into colony-forming hematopoietic stem/progenitor cells. MACS-sorted marmoset CD34$^+$ cells were transduced with the lentiviral vector expressing p190 BCR-ABL under a CMV or PGK promoter, and then 1.5 × 10^3 cells were plated in methylcellulose containing human hematopoietic growth factors. After 14 days of culture, colonies were randomly picked and examined for BCR-ABL gene expression by RT-PCR.

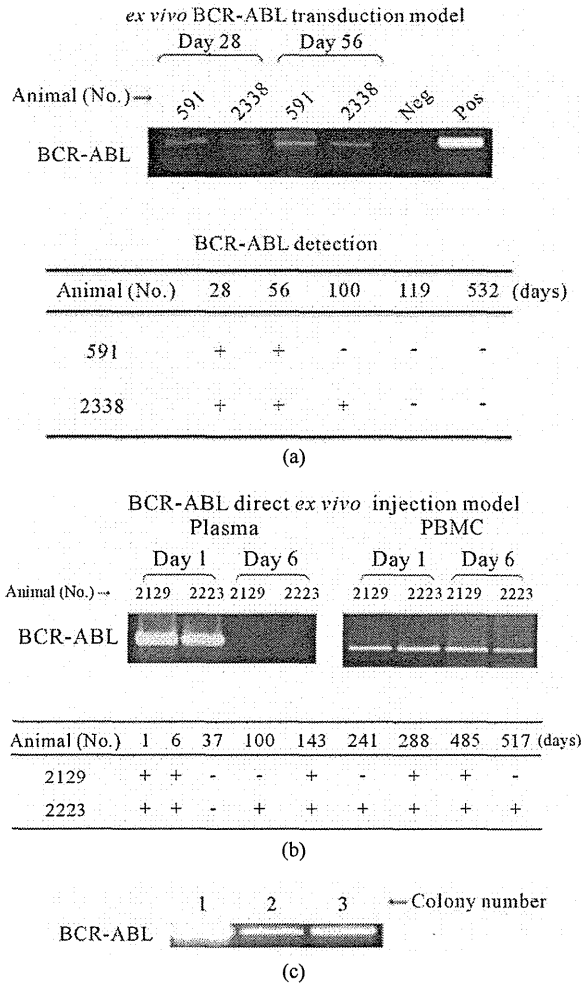

Figure 2. (a) Detection of the BCR-ABL fusion gene with the *ex vivo* BCR-ABL transduction method. Upper: RT-PCR of the BCR-ABL fusion gene in samples on day 28 and 56 after the BCR-ABL-transduced CD34$^+$ cells were transplanted. Lower: detection of BCR-ABL in samples from day 28 to 532 in two marmosets. BCR-ABL expression disappeared in two marmosets after day 56 and 100. GAPDH expression was confirmed for all samples (data not shown). No. 591 and No. 2338: CMV-p190 BCR-ABL; (b) Detection of BCR-ABL fusion gene with the *in vivo* BCR-ABL direct transduction method. Upper: RT-PCR of the BCR-ABL fusion gene in plasma and PBMC samples on days 1 and 6. Lower: detection of BCR-ABL in samples from day 1 to day 517 in two marmosets. Compared to the *ex vivo* BCR-ABL transduction method, two marmosets maintained long-term BCR-ABL expression. No. 2129: CMV-p190 BCR-ABL. No. 2223: PGK-p190 BCR-ABL; (c) Detection of the BCR-ABL fusion gene in colony-forming hematopoietic stem/progenitor cells. Bone marrow MNCs were isolated from marmoset No. 2223 on day 517, and 3 × 10^4 cells were plated in methylcellulose containing several human hematopoietic growth factors. After 14 days of culture, colonies were randomly picked and examined for the BCR-ABL gene. Three out of nine colonies were positive for BCR-ABL.

Therefore, we administered an immunosuppressive pretreatment therapy consisting of 5-fluouracil and prednisolone and then injected the lentiviral vector into the bone marrow cavity (**Table 2**). BCR-ABL was detected in the plasma on day 1 but not day 6. In contrast, BCR-ABL was detected in peripheral blood MNCs on day 1 and 6 in two marmosets. **Figure 2(b)** (lower) shows the time course of BCR-ABL expression after injection. With this *in vivo* BCR-ABL direct transduction method, BCR-ABL expression was sustained in two marmosets (No. 2129: positive until day 485, No. 2223: positive until day 517). To determine if the p190 BCR-ABL fusion gene was successfully transduced into hematopoietic stem/progenitor cells, we performed a colony formation assay. Thirty thousand bone marrow MNCs were isolated from marmoset No. 2223 on day 517 and plated in methylcellulose. After 14 days of culture, random colonies were examined for BCR-ABL gene expression. Three of nine colonies were positive for BCR-ABL expression (**Figure 2(c)**). In the same experiment, all colonies for the control marmoset were negative for BCR-ABL expression (data not shown).

3.4. Myelofibrosis in a Marmoset That Was Directly Transduced with BCR-ABL *in Vivo*

Marmoset No. 2223, which received a direct *in vivo* injection and maintained BCR-ABL expression, became lethargic and lost weight. A blood cell count indicated anemia and thrombocytopenia (**Figure 3(a)**). This marmoset was sacrificed on day 686 post injection and examined pathologically. BCR-ABL expression was detected in the spleen, liver, kidney, heart and peripheral blood MNCs by RT-PCR. However, the bone marrow sample was negative for BCR-ABL. Hematoxylin-Eosin staining of the bone marrow showed that the bone marrow cavity was replaced by a marked noncellular component (**Figure 3(b)**). Bone marrow fibrosis was confirmed by Masson staining. An examination of the liver revealed extramedullary hematopoiesis. Naphthol AS-D chloroacetate (ASD) and Myeloperoxidase (MPO) staining confirmed that the cells were of myeloid lineage (**Figure 3(c)**).

4. Discussion

Previously, we reperted the usefulness of the common marmoset as a hematopoietic stem/progenitor cell transplantation model, analytic results of major histocompatibility antigens, and the production of anti-marmoset CD34 monoclonal antibodies [4,17,21,22]. These studies helped to establish and analyze a primate disease model. In this study, we lentivirally transduced common marmoset hematopoietic cells with the p190 BCR-ABL fu-

Animal (No.)	RBC ($\times 10^4$/μL)	WBC ($\times 10^3$/μL)	Plt ($\times 10^4$/μL)
BCR-ABL (2223)	175	13.9	14.1
Normal	623±74	9.7±3.2	65.0±17.0
(range)	(373-717)	(3.3-17.4)	(40.5-111.2)

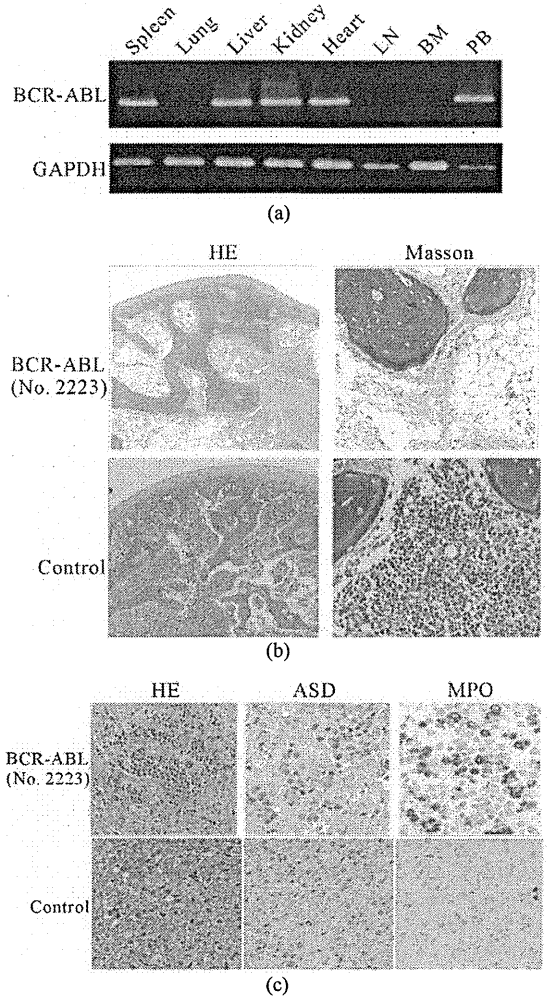

Figure 3. Pathological examination of marmoset No. 2223 (transduced using the *in vivo* BCR-ABL direct transduction method) with myelofibrosis-like disease. (a) Upper: Peripheral blood count of No. 2223 on day −7 before sacrifice. Data on the blood cell count of healthy marmosets were obtained from a textbook. Lower: The marmoset was sacrificed and pathologically examined on day 686 after p190 BCR-ABL was directly injected into the bone marrow. BCR-ABL gene expression was detected by a RT-PCR method in several organs. No. 2223: PGK-p190 BCR-ABL; (b) Left: Hematoxylin-Eosin (HE) staining of the bone marrow. Right: Masson staining. Bone marrow fibrosis is stained in blue. Upper: Experimental marmoset (No. 2223). Lower: Control marmoset; (c) Extramedullary hematopoiesis in the liver in the experimental marmoset (upper). ASD: Naphthol AS-D chloroacetate stain, MPO: Myeloperoxidase stain.

sion gene using either an *ex vivo* BCR-ABL transduction method or *in vivo* BCR-ABL direct transduction method. Then, we examined the expression of the fusion oncogene in transduced progenitor cells *in vivo* and monitored the marmosets for leukemogenic events.

With the *ex vivo* BCR-ABL transduction method, BCR-ABL expression was not detected by RT-PCR after day 100 (**Figure 2(a)**). This lack of sustained expression may result from a low gene transduction efficiency of hematopoietic stem/progenitor cells even though the high transduction efficiency at progenitor levels. Additionally, the transduced cells may have been immunologically rejected. Anti-human G-CSF neutralizing antibodies appeared in the transplanted marmosets even with an immunosuppressive pre-conditioning regimen (data not shown). The presence of these antibodies supports the latter possibility. Majority of the marmosets used in this study were four to six years old, which is an immunologically competent and equivalent to an adult human. Treating the marmosets with immune suppressants might solve this problem.

Previously, our group successfully transduced G-CSF-mobilized hematopoietic stem/progenitor cells in a marmoset model with the MDR1 gene using a retroviral vector [21]. In this study, a consistent increase in progenitor cells in the peripheral blood was observed using the same G-CSF mobilization protocol. Retroviral vectors mainly transduce dividing cells. In contrast, lentiviral vectors transduce both dividing and non-dividing cells [12,20]. And recent reports showed that the retroviral vector, but not lentiviral vector, is integrated near transcriptional genes and induce leukemia [23]. Moreover, in a previous study, we successfully transduced cord blood hematopoietic stem/progenitor cells using a lentiviral vector [12]. For these reasons, we chose a lentiviral vector to transduce p190 BCR-ABL in order to obtain a high transduction efficiency. However, the p190 BCR-ABL gene is approximately 6 kilobase pairs in length, and its large size hinders the production of a high-titer lentiviral vector. This limitation may be one reason why BCR-ABL was expressed for a limited time with the *ex vivo* BCR-ABL transduction method (**Figure 2(a)**).

This was our first attempt to directly inject a lentiviral vector *in vivo* into the bone marrow of common marmosets, although a previous study reported efficient gene transduction by direct injection into the central nervous system of rats [24]. BCR-ABL was detected in the plasmaon day 1 but not day 6. It is likely that vectors injected into the bone marrow, which is rich in blood vessels, transiently leaked into the circulation. Of note, we observed long-term BCR-ABL expression in hematopoietic cells (**Figure 2(b)**). Further optimization of the experimental procedures, such as altering the pre-conditioning regimen, using a higher titer vector, and treating with immunosuppressive drugs after injecting the lentiviral vector, might be effective to obtain stable gene expression in hematopoietic stem/progenitor cells *in vivo*.

To date, mouse models have been predominantly used to study human cancers [25]. However, primates are more genetically related to humans than rodents. For this reason, much effort has focused on establishing a primate model that can be used to more precisely evaluate new cancer therapies in pre-clinical studies. However, thus far, there have been no successful reports of a primate model that mimics human cancers, and this study also faced challenges. The primary reason for these difficulties may be because primate and rodent hematopoietic stem/progenitor cells have different susceptibilities to oncogene transduction [26,27]. In gene therapy clinical trials in France for X chromosome-linked severe combined immune deficiency, leukemia developed two to three years after the common γ chain receptor gene was transduced into hematopoietic stem/progenitor cells [26-28]. In contrast, in a mouse model of oncogene transduction, the duration of developing leukemia is generally less than one year [9-11]. Therefore, these findings support the hypothesis mentioned above.

Moreover, the susceptibility to malignant transformation by each oncogene reportedly differs based on age in humans. For example, for the chromosomal translocations in ALL, MLL-AF4 is dominant in infants, TEL-AML1 in children, and BCR-ABL in adults [29]. Furthermore, cord blood cells were used in all previous reports of BCR-ABL transduction into human hematopoietic stem/progenitor cells in a SCID mouse model [30]. There have been no studies that used adult hematopoietic progenitor cells. In this study, all of the marmosets were adults. Considering these findings, it will be important to consider using younger marmosets in future studies.

Furthermore, it is well known that multiple genetic mutations are required for leukemogenesis [31]. The number of required gene mutations may not be the same between primates and rodents. In the marmoset model, subsequent gene mutations in addition to p190 BCR-ABL may be required for malignant transformation [32]. Thus, it may be necessary to test the cotransduction of p190 BCR-ABL and another oncogene, mutated tumor suppressor gene, or anti-apoptosis gene in order to achieve leukemogenesis in the marmoset model.

Unexpectedly, one marmoset that was transduced using the *in vivo* BCR-ABL direct transduction method developed myelofibrosis-like disease (**Figures 3(a)-(c)**). BCR-ABL gene expression was detected in various organs. However, BCR-ABL was not expressed in the lung, lymph nodes and bone marrow. BCR-ABL expression could be detected where extramedullar hematopoiesis

was observed (liver and spleen) and in organs rich in blood perfusion (heart, kidney and peripheral blood). These differences might contribute to the observed tissue specificities, although this is speculative because the data are only from one marmoset. Bone marrow was negative for BCR-ABL. We believe that our inability to detect BCR-ABL expression in this sample was because we could obtain insufficient hematopoietic cells from the fibrotic bone marrow. Recently, a mutation in JAK2 gene was reported to cause myelofibrosis [33]. We hypothesized that the lentiviral vector inserted into the host genome and unregulated the expression of genes such as JAK2. We performed LAM-PCR (linear amplification-mediated PCR), which identifies the sequence flanking the integrated vector genome, to identify the unregulated gene that may be responsible for myelofibrosis [34]. However, due to the insufficient bone marrow samples, this attempt was unsuccessful. If the phenomenon is reproduced in other marmosets, further analyses will be required to understand this pathology.

In conclusion, we stably expressed an oncogene *in vivo* in a marmoset model, although several steps may be required to develop hematological malignancy in this model. The results provide information that can be used to establish a marmoset disease model in which hematopoietic stem/progenitor cells are targeted for oncogene delivery.

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ORIGINAL ARTICLE

CD30 Ligand/CD30 Interaction Is Involved in Pathogenesis of Inflammatory Bowel Disease

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Abstract

Background and Aims Although CD30 has long been recognized as an important marker in many lymphomas of diverse origin, and as an activation molecule on B and T cells, its primary function has remained obscure. Soluble CD30 (sCD30) is released from CD30 on the cell membrane by enzymatic cleavage. This study investigated the role of CD30 ligand (CD30L)/CD30 signals in intestinal mucosal damage.

Methods Serum sCD30 in patients with ulcerative colitis (UC) and Crohn's disease (CD) and healthy individuals was assessed. A model of enteritis induced by anti-CD3 monoclonal antibody injection was studied in wild-type mice and in CD30L knockout mice.

Results Increased sCD30 was observed in UC and CD patients, and the level was correlated with disease activity in both conditions. In a murine model of enteritis, histological intestinal damage was significantly reduced in CD30L knockout mice with decreased Th1 and Th17 cytokine levels. Moreover, blocking of CD30L/CD30 signals by CD30-immunoglobulin (CD30-Ig) resulted in reduced inflammation.

Conclusions Increased sCD30 expression correlating with disease activity suggested that CD30L/CD30 signals play an important role in pathogenesis of UC and CD. CD30L/CD30 pathway acts as an accelerator of enteritis in a murine disease model. Successful blockade of enteritis by

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CD30-Ig suggests a potential tool for future therapy of inflammatory bowel diseases.

Keywords CD30 · CD30 ligand · Inflammatory bowel disease · Ulcerative colitis · Crohn's disease

Introduction

CD30 and CD30 ligand (CD30L) are glycoproteins that belong to the tumor necrosis factor receptor (TNFR) and tumor necrosis factor (TNF) superfamily, respectively [1, 2]. CD30 is expressed by activated lymphoid and lymph node cells in the parafollicular area, decidua, and in endometrial cells with deciduoid changes. The extracellular part of membrane-bound CD30 can be proteolytically cleaved by the action of a zinc metalloprotease [3], which produces a soluble form of CD30 (sCD30) with a molecular mass of 85/90 kDa. Elevated levels of sCD30 have been observed in patients with various diseases such as systemic lupus erythematosus, rheumatoid arthritis and human immunodeficiency virus-1 infection [4]. The source of sCD30 is assumed to be CD30-expressing cells. Although its function remains to be clarified, sCD30 can bind to CD30L and block the interaction between CD30 and its ligand. CD30L/CD30 signaling is thought to augment T and B cell differentiation and proliferation, and negative selection of T cells in the thymus [2, 5–9]. We have previously reported that triggering of CD30 signals in a lymphoma cell line can down-modulate lymphocyte effector function and proliferation, while directing the cells to lymph nodes and increasing their susceptibility to certain apoptotic signals [10]. We have constructed CD30L knockout mice (CD30LKO), and have found that CD30L/CD30 signals play potent roles in regulation of $CD4^+$ T-cell-mediated graft-versus-host disease [11], and in the differentiation of long-lived central memory $CD8^+$ T cells following infection [12].

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is of unknown etiology. Recent reports have suggested that chronic inflammation occurs as a consequence of aberrant intestinal immune responses to the bacterial microflora in genetically susceptible individuals [13]. It is believed that T cells, especially T helper (Th) cells, play a crucial role in disease development. Some reports have suggested that Th cells in the intestinal mucosa in CD are polarized to Th1 and to Th2 in UC [13]. In addition, recent evidence has suggested that a new Th subset, Th17, is implicated in the pathogenesis of CD and UC [14, 15]. As regards the involvement of CD30L/CD30 signaling in IBD, serum sCD30 concentration is elevated in UC but not in CD [16]. We have reported previously [17] that an experimental colitis model induced by oxazolone enema was worsened in CD30LKO mice with increased expression of Th2 cytokines including interleukin (IL)-4 and IL-13. On the contrary, CD30LKO mice were resistant to another experimental colitis by trinitrobenzene sulfonic acid (TNBS) enema, with significant reduction in Th1 cytokine, interferon (IFN)-γ expression. The latter showed the involvement of CD30L/CD30 signaling in Th1-mediated colitis and is inconsistent with the former, which suggests that CD30L/CD30 signals play a role in Th2 responses in UC [16]. We thus considered that many issues remained to be clarified about the role of CD30L/CD30 pathways in the pathogenesis of IBD. To address such issues, we investigated the expression of sCD30 in IBD patients and the effect of inhibiting CD30L/CD30 signals in a murine experimental model of enteritis.

Methods

Patients

All patients in this study were admitted to Kyushu University Hospital, Saiseikai Fukuoka General Hospital, National Fukuoka-Higashi Medical Center, or Harasanshin Hospital between 2003 and 2004. The protocol was approved by the institutional ethical committee at all institutions. Written informed consent was obtained from all patients. We obtained peripheral blood samples from 25 UC patients, 16 CD patients, and 12 hospital employees as control subjects. Nine UC and five CD patients were subjected to examination before and after medical treatment. For diagnosis of UC, all patients underwent colonoscopy and a pathological examination of a colonic biopsy sample, to rule out CD, ischemic colitis, or infectious colitis. The disease activity was scored according to the Rachmilewitz Clinical Activity Index [18]. CD patients underwent colonoscopy and/or X-ray examination of the small intestine, and a diagnosis was made based on the presence of typical longitudinal ulcers and/or a cobblestone appearance, and in some cases, the detection of granuloma by histological examination. UC, ischemic colitis, Behçet's disease, and infectious enterocolitis were all ruled out.

Mice

BALB/cN Sea mice were purchased from Kyudo (Saga, Japan). CD30LKO mice on a BALB/c background were obtained by backcrossing CD30LKO mice with wild-type (WT) BALB/c mice for more than nine generations [11].

Genotypes of CD30LKO mice were screened by PCR as described previously [11]. All mice were 8–12 weeks of age, housed in a pathogen-free facility, and handled according to the recommended guidelines of the Animal Centre of Kyushu University.

Reagents

Anti-mouse CD3 monoclonal antibody (mAb) (145-2C11) was purchased from R&D Systems (Minneapolis, MN, USA), or purified from culture supernatant of a hybridoma purchased from American Type Culture Collection (Manassas, VA, USA). To produce recombinant protein CD30-immunoglobulin (CD30-Ig), murine CD30 cDNA was truncated at the extracellular domain next to the transmembrane segment (nucleotide 906) and fused to the hinge region of murine IgG1, as described previously [6].

sCD30

sCD30 concentrations in human serum were determined by a specific ELISA kit purchased from Bender Med Systems (Burlingame, CA, USA). sCD30 concentration in mouse serum was determined by a specific ELISA kit (GT, Minneapolis, MN, USA).

Experimental Enteritis Model of Anti-CD3 mAb Injection

Anti-CD3 induced enteritis is a well-established experimental model of T-cell-dependent enteritis in mice [19]. The toxic effects of anti-CD3 antibody are caused by activated $CD4^+$ and $CD8^+$ effector cells in the gut mucosa. WT and CD30LKO mice were injected i.p. with 100 μg anti-CD3 mAb in 500 μl PBS. After 2, 4, 24, and 72 h, peripheral blood was obtained and analyzed. Body weight was measured every day after injection of anti-CD3 mAb. Mice injected with anti-CD3 mAb were killed 24 h after injection, and the terminal ileum was removed. The tissue sections were stained with hematoxylin and eosin (H&E) and examined by optical microscopy. The occurrence of apoptosis in the ileal section was assessed by TUNEL staining using the in situ Apoptosis Detection Kit (Takara Bio, Shiga, Japan).

Cytokine Measurement

Peripheral blood was collected from mice at 2 or 4 h after the injection of anti-CD3 antibodies. Sera were separated by centrifugation (4 °C, 200 × *g*, 20 min). TNF-α, IFN-γ, IL-4, and IL-17 concentrations in mouse serum were determined with specific ELISA kits (GT).

Statistical Analysis

Student's *t* test was used for statistical comparisons between two groups. $p < 0.05$ was considered statistically significant.

Results

Expression of CD30 in IBD

We analyzed sera obtained from 25 UC and 16 CD patients, and 12 healthy volunteers, whose demographics are summarized in Table 1. The levels of sCD30 in UC and CD patients were significantly higher than those in healthy subjects (Fig. 1a). To investigate the relationship between disease activity and sCD30 expression, levels of sCD30 measured before and after treatment were compared in nine UC and five CD patients (Fig. 1b). Disease activity was reduced in all patients after treatment; similarly, serum levels of sCD30 decreased after treatment in all but one patient with UC. sCD30 levels and clinical scores in UC and CD were assessed and were found to be significantly correlated (Fig. 1c). sCD30 is thought to be generated as a consequence of shedding of CD30 from the membrane of CD30-expressing cells; therefore, these results suggested increased CD30 expression in IBD in association with disease activity.

CD30LKO Mice Are Resistant to Anti-CD3 Antibody Enteritis

To validate a role for CD30 signals in mucosal immunity, we studied anti-CD3-induced enteritis in WT and

Table 1 Patient demographics

	Healthy controls	UC	CD
Number	12	25	16
Male:female	6:6	15:10	10:6
Age (years; mean)	33.6	36.5	36.8
Affected area		Pancolitis: 19	Without colonic lesion: 5
		Left-sided: 5	With colonic lesion: 11
		Proctitis: 1	
Treatment		5-ASA: 22	5-ASA: 10
		Steroid: 12	Steroid: 2
		Apheresis: 7	Infliximab: 1

ASA 5-aminosalicylic acid

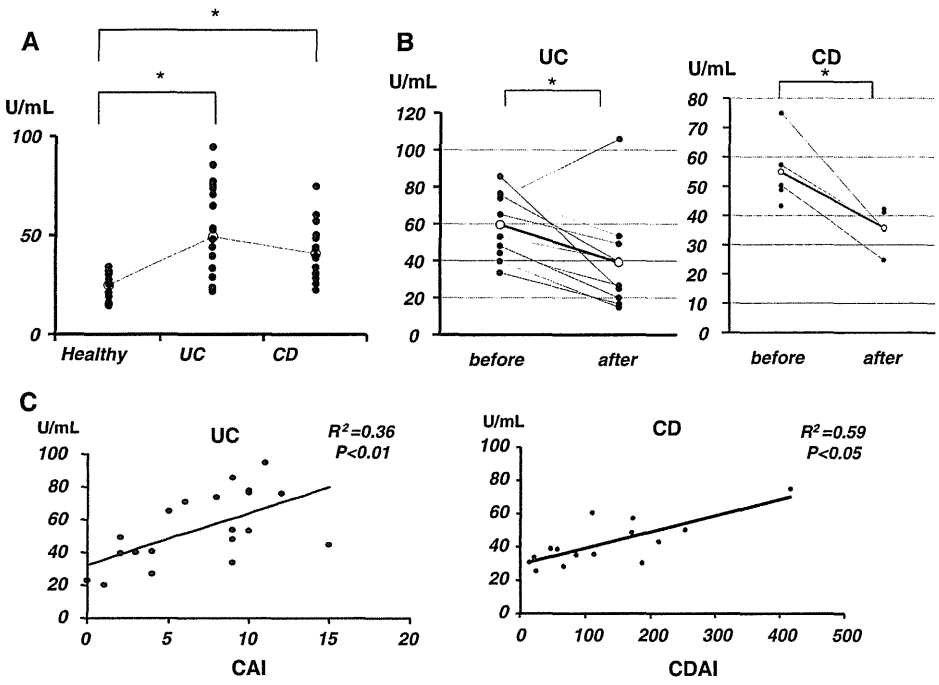

Fig. 1 Expression of sCD30 in serum of IBD patients. **a** Concentration of sCD30 in serum obtained from 12 healthy controls and 25 UC and 16 CD patients was analyzed by sCD30-specific ELISA. *Open circles* represent medians of each group. **b** Concentrations of serum sCD30 before and after treatment were compared in nine UC and five CD patients. *Open circles* represent medians of each group. **c** Correlations of sCD30 and clinical scores in UC and CD are shown. $*p < 0.05$, *CAI* clinical activity index, *CDAI* Crohn's disease activity index

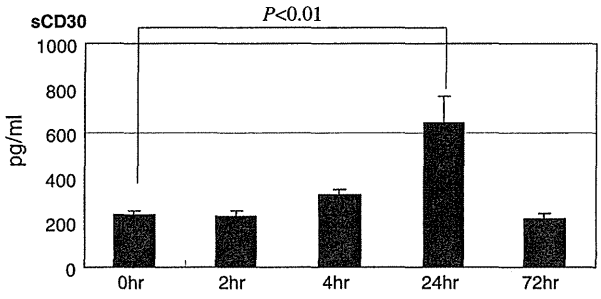

Fig. 2 sCD30 expression in an anti-CD3-induced enteritis model. Time-course of sCD30 levels in mouse serum detected by ELISA at 0, 2, 4, 24, and 72 h after injection of anti-CD3. Serum sCD30 at 24 h after injection was significantly higher than that at 0 h

CD30LKO mice. In this model, the systemic administration of anti-CD3 induces small bowel inflammation and causes diarrhea, piloerection, and changes in overall mobility [19]. We analyzed levels of serum sCD30 in WT mice by ELISA after injection of anti-CD3 antibody. Serum sCD30 levels increased after anti-CD3 antibody treatment and reached a peak at 24 h (Fig. 2), which suggested upregulation of CD30 expression.

To examine the impact of the absence of CD30 signals on anti-CD3-induced enteritis, changes in body weight of WT and CD30LKO mice were measured daily after administration of anti-CD3 (Fig. 3a). Body weight loss in CD30LKO mice was significantly less than that in WT mice at days 2, 4, 5, 6, and 7, which indicated that the absence of CD30 signals diminished anti-CD3-induced enteritis. Differences in the histopathology of enteritis induced by anti-CD3 in WT and CD30LKO mice were assessed at 24 h after injection. The absence of CD30L was associated with diminished anti-CD3-induced inflammation in the terminal ileum (Fig. 3b), with inhibition of reduction in the villous/crypt length ratio (Fig. 3c). Apoptotic bodies detected by TUNEL staining were counted in five villous crypt units of each group of WT and CD30LKO mice (Fig. 3d). The average number of apoptotic bodies in CD30LKO was significantly lower than that in WT mice (Fig. 3e). In addition, serum levels of IFN-γ (Fig. 4a), TNF-α (Fig. 4b), and IL-17 (Fig. 4c) in CD30LKO were significantly lower than those in WT mice. The level of IL-4 was not significantly altered (Fig. 4d). These results indicated that inflammation of the intestine induced by anti-CD3 antibody was reduced in the absence of CD30L/CD30 signals.

Recombinant Fusion Protein CD30-Ig Suppresses Anti-CD3-induced Enteritis

Recombinant fusion protein (CD30-Ig) contained the extracellular domain of CD30 fused to the hinge, CH2, and CH3 domains of murine IgG1, as described previously [6]. CD30-Ig has appropriate binding activity for the CD30L and blocks CD30L/CD30 signals. One hundred micrograms of CD30-Ig was administered to WT mice i.p. 1 h before and after injection of anti-CD3 mAb. The terminal ileum was removed from mice 24 h later, and tissue sections were stained with H&E and examined by optical

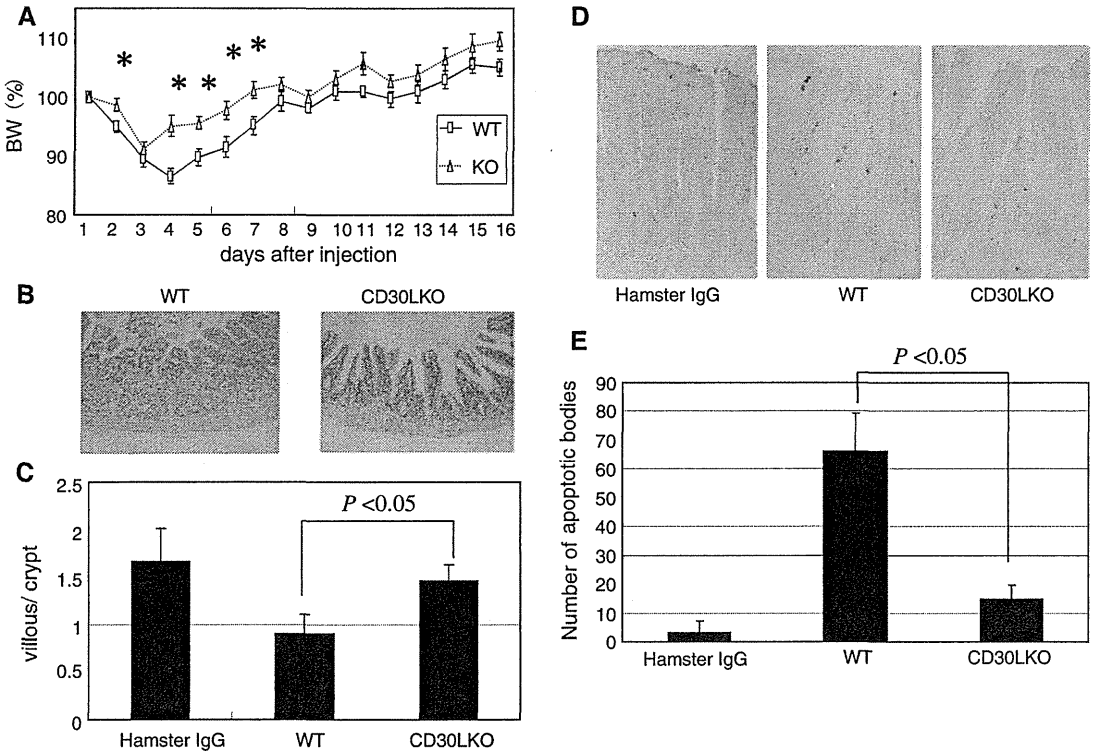

Fig. 3 CD30LKO mice were resistant to anti-CD3-induced enteritis. **a** Body weight changes in WT and CD30LKO mice were measured every day after injection of anti-CD3 mAb. The body weight losses in CD30LKO mice were less than those in WT mice at days 2, 4, 5, 6, and 7 (average of five mice), $*p < 0.05$. **b** Histological findings of anti-CD3-induced enteritis in WT and CD30LKO mice. Terminal ileum was removed from mice injected with anti-CD3 mAb at 24 h after injection. Tissue sections were stained with H&E and examined by optical microscopy. **c** Five villous crypt units were measured in each mouse. The average villous/crypt length ratio was calculated from three mice in each group. **d** Terminal ileum was removed 24 h after injection, and apoptotic bodies were detected by TUNEL staining. **e** The number of apoptotic bodies was counted in five villous crypt units of each group of mice

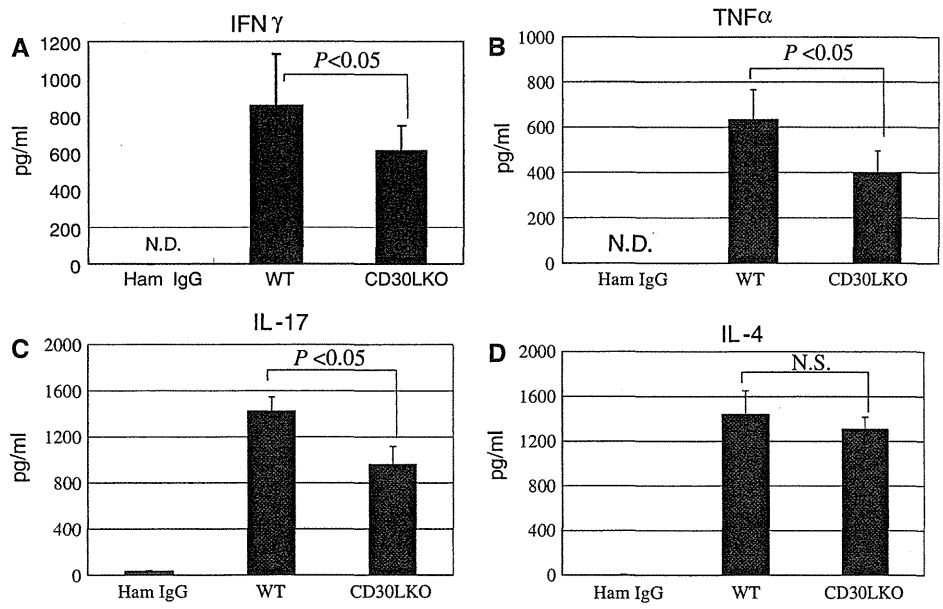

Fig. 4 Serum IFN-γ was measured in WT and CD30LKO mice at 4 h after anti-CD3 injection (**a**). Serum IFN-γ was higher in WT than CD30LKO mice. Serum TNF-α (**b**), IL-17 (**c**), and IL-4 (**d**) were measured in WT and CD30LKO mice at 2 h after anti-CD3 injection. *N.S.* not significant

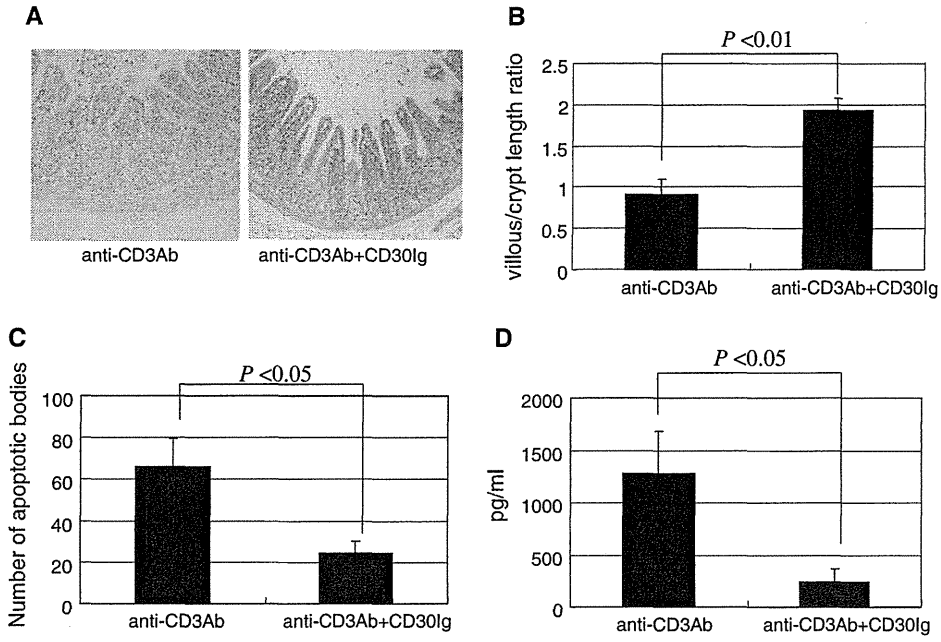

Fig. 5 Recombinant fusion protein CD30-Ig suppressed anti-CD3-induced enteritis. **a** CD30-Ig (100 μg) was injected i.p. 1 h before and after injection of anti-CD3 mAb. Terminal ileum was removed from mice at 24 h after injection of anti-CD3 mAb. Tissue sections were stained with H&E and examined by optical microscopy. **b** Five villous crypt units were measured in each mouse. The average villous/crypt length ratio was calculated from three mice in each group. **c** The number of apoptotic bodies was counted in five villous crypt units of each group of mice. **d** Serum IFN-γ was measured at 4 h after anti-CD3 antibody injection and was lower in mice with CD30-Ig injection

microscopy (Fig. 5a). Enteritis was significantly reduced in mice that received CD30-Ig. Similar to the experiment with CD30LKO, villous crypt units were measured and villous/crypt length ratio was significantly greater in CD30-Ig-treated mice than in controls (Fig. 5b). Furthermore, the number of apoptotic bodies and the level of serum IFN-γ were also diminished in mice that received CD30-Ig (Fig. 5c, d).

Discussion

The role of CD30L/CD30 signals in mucosal immunity has not received much attention. Giacomelli et al. [16] have reported elevated levels of sCD30 in UC patients. On the contrary, in CD patients, the levels of sCD30 were not significantly higher than those of normal controls. They concluded that, as CD30-expressing cells produce Th2 cytokines, these differences come from the Th2 immune response in UC. Likewise, Elewaut et al. [20] have reported that lymphocytes obtained from UC patients overexpress CD30 when compared to those from CD patients. These studies have indicated that CD30L/CD30 signals play a more important role in UC than in CD. However, after the discovery of Th17 cells, Th reactions in human disorders need to be considered in a new axis of Th1/Th2/Th17 rather than a classical Th1/Th2 paradigm. Therefore, the involvement of Th2 reactions in the pathogenesis of UC needs to be re-evaluated. We thus considered that many issues remained to be investigated on the role of CD30L/CD30 signaling in IBD and conducted this current study. Contrary to a previous report, we found sCD30 levels were elevated in both CD and UC patients, and were correlated with disease activity. To clarify the reason for these contradictions, analysis of larger numbers of patients is needed. Nevertheless, our results suggest that CD30L/CD30 signals play some role in the development of intestinal inflammation in IBD.

Anti-CD3 antibody enteritis is induced purely by activated T cells. It is a useful murine enteritis model for discerning the role of T cells in enteritis. Merger et al. [19] have reported that apoptosis and mucosal injury are significantly reduced in perforin knockout mice, and a further significant reduction is observed in mice with perforin and Fas ligand (FasL) knockout. Both FasL and CD30L are members of the TNF superfamily. We have reported previously that the CD30 signal induces perforin expression [10]. Thus, we used the anti-CD3-induced enteritis model to analyze CD30L/CD30 signals in CD30LKO mice. As expected, serum sCD30 increased at a peak of 24 h after anti-CD3 antibody injection. sCD30 recovered to normal levels at 72 h after injection of anti-CD3, with recovery of intestinal mucosa indicating that sCD30 level reflected severity of inflammation. These results suggest that reduced sCD30 after treatment in IBD patients is also caused by convergence of disease activity, and is not simply caused by immunosuppressants.

Recently, we have reported that oxazolone and TNBS induce colitis in CD30LKO mice [17]. We have found that CD30LKO mice are susceptible to oxazolone-induced colitis but are resistant to TNBS-induced acute colitis, and have concluded that CD30L plays a crucial role in

deviating $CD30^+$ Th cells to Th1 cells in the colon [17]. In anti-CD3-induced enteritis, body weight loss, histological appearance, and serum levels of IFN-γ and IL-17 were significantly suppressed in CD30LKO mice, which suggests that CD30L/CD30 signals deviate Th cells not only to Th1 but also to Th17 phenotype. In addition, CD30-Ig fusion protein, which blocks CD30L, significantly decreased disease activity of anti-CD3-induced enteritis in WT mice, which ruled out other genetic effects due to CD30L deletion in CD30LKO mice. Compared to the agonistic anti-CD30 mAb used in the former study [17], CD30-Ig fusion protein blocked CD30L/CD30 signals and was shown to be suitable for reduction of enteritis in vivo. These results suggest that blocking agent of CD30L/CD30 signaling, such as CD30-Ig or antagonistic anti-CD30 antibodies could be new therapeutic tools for IBD.

In this study, the decrease in weight changes in CD30LKO mice in comparison with WT mice was relatively small and did not correlate with the marked differences in histology and the number of apoptotic cells. However, it correlated well with relatively small differences in cytokine productions between CD30LKO and WT mice. It was therefore suggested that weight loss in this model may be due to the augmented expression of inflammatory cytokines as well as intestinal tissue damage itself. Improvement of inflammation seemed less efficient by the blocking of CD30-signal in CD30LKO mice than in the CD30-Ig-treated mice, especially in inflammatory cytokine production. This might be because of the redundancy of CD30L/CD30-signaling. It is well known that TNF/TNFR-signaling is frequently redundant. It is, therefore, reasonable to consider that another signal was augmented to overcome the absence of CD30-signaling in CD30LKO mice. Such mechanism does not work in CD30-Ig-treated mice.

In summary, we showed that expression of sCD30 was elevated in patients with UC and CD, suggesting the involvement of CD30L/CD30 signaling in the pathogenesis of IBD. Using a murine enteritis model and recombinant protein CD30-Ig, we clarified that CD30L/CD30 signals play an important role in induction of enteritis in mice. We also introduced a new strategy for treatment of IBD and other diseases in which CD30L/CD30 signals are involved.

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Conflict of interest The authors have no conflicts of interest to declare.

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

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Abstract

Mast cells (MCs) are developed from hematopoietic progenitor cells and play an important role in inflammation. Study of the kinetics of development and accumulation of primate MC *in vivo* is crucial for the control of human inflammatory diseases, as evolution of the immune system is quite rapid and inflammation including MC response is considered to be different between mouse and human. In the present study, we examined the development of MC from hematopoietic progenitors of *Callithrix jacchus* (common marmoset), an experimental animal of nonhuman primates. Bone marrow cells were fractionated for the expression of CD34 and CD117 by cell sorting. MCs were developed *in vitro* or by transplanting the cells to NOD/SCID/IL-2γc knockout (NOG) mice. *In vitro* culture of $CD34^+CD117^+$ (double positive, DP) cells with stem cell factor could generate high-affinity Fc epsilon receptor (FcεR)-expressing $CD117^+$ cells with typical granules. The developed MC released β-hexosaminidase and produced leukotriene C_4 after the stimulation of FcεRI. Transplantation of DP cells gave rise to a marked expansion of $CD34^-CD45^+CD117^+Fc\varepsilon R^+$ cells in NOG mice. They expressed transcripts encoding chymase 1 and tryptase β. Differentiation of $CD34^-CD117^+$ cells to MCs was relatively limited compared with the DP cells, similarly to human MCs. These results suggest that this marmoset system provides a good model for human MC development.

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

Introduction

Allergic inflammation is associated with various human diseases including pollinosis and asthma. Molecular mechanisms involved in the onset and progression of allergic inflammation have been extensively studied at both cellular and tissue levels (1,2). In parallel, intense efforts have been made to develop animal models using mammals such as rodents, expecting to mimic or reflect the human pathology of allergic inflammation (3–6). However, rodents (mouse) and

primates (human) are evolutionarily distant from each other, and they exhibit many differing features in their immune and allergic reactions (7). Especially, the engagement of cytokines and their receptors differs between humans and mice. For example, T_h17, a subset of T helper cells, is closely involved in allergy. IL-6, IL-21 and TGF-β are necessary for mouse T_h17 cell differentiation, whereas IL-1, IL-6 and IL-23 are important in the case of human T_h17 cells (8). Furthermore, gene configurations that encode allergy-suppressive signaling molecules such as Siglec and leukocyte Ig-like receptors/Ig-like transcripts are substantially different between mice and humans (9).

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

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

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

One trial to examine the development of cell lineage is the xenotransplantation of cells in immunodeficient mice. Hematopoietic progenitors prepared from bone marrow, granulocyte-colony stimulating factor-mobilized peripheral blood or cord blood of humans have been transferred into immune-deficient mice such as NOD/SCID/IL-2γc knockout (NOG) mice (19–21). Then, in these mice, T and B lymphocytes of human origin have been successfully regenerated to certain extents, but immune reactions to antigen exposure have been very limited. In reconstituted mice, formation of a germinal center is not evident, and production of antigen-specific antibodies is very poor (20,22,23). Under these circumstances, it was striking to witness the successful development of human MCs in NOG mice transplanted with hematopoietic progenitors (24).

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

Methods

Animals

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

Preparation of marmoset cells

Spleens were taken from marmosets and cells were released from tissues. After red blood cells were lysed with low osmotic buffer (20 mM Tris–HCl, pH 7.4, 0.15 M NH_4Cl), they were suspended in RPMI1640 medium (Nissui, Tokyo, Japan) containing 10% (v/v) heat-inactivated fetal calf serum (FCS; SAFC Biosciences, Tokyo, Japan). Bone marrow cells of marmosets were collected into a heparinized tube and centrifuged on Lymphocepar (IBL Co., Takasaki, Japan) at 2000 r.p.m. for 30 min. Mononuclear cells were collected and remaining erythrocytes were lysed.

MC-inducing culture

In the first experimental series, marmoset bone marrow cells were used directly without fractionation. To induce MC differentiation, cells were cultured in RPMI1640 medium under

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

Flow cytometry

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

Immunoblotting

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

Assay of degranulation and leukotriene C_4 production

IgE-sensitized and unsensitized cells were washed twice with PBS and then stimulated with rabbit anti-human IgE polyclonal antibody at the indicated concentrations for 30 min. Degranulation was determined by β-hexosaminidase release, as described previously (6). The percentage of β-hexosaminidase release was calculated as follows: (supernatant optical density of cells)/(total cell lysate optical density of cells) × 100. The leukotriene C_4 (LTC_4) production was analyzed with an LTC_4 EIA kit (Cayman Chemical, Ann Arbor, MI).

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

The IgE-sensitized and unsensitized cells were washed twice with PBS and then loaded with 4 μM Fluo3-AM (Dojindo, Kumamoto, Japan) for 30 min. The cells were resuspended in 1 × Tyrode's buffer (126 mM NaCl, 4 mM KCl, 641 μM KH_2PO_4, 10 mM HEPES, 5.6 mM D-glucose, 1 mM $CaCl_2$, 600 μM $MgCl_2$ and 0.1% BSA) and stimulated with rabbit anti-human IgE polyclonal antibody. Changes in dye fluorescence upon addition of stimuli were monitored at 200-ms intervals by a flow cytometer (FACSCalibur, BD Biosciences).

Histological analyses

Cells in suspension were placed on glass slides pretreated with 3-aminopropylethoxysilane (Digene, Beltsville, MD), incubated in 5% (v/v) CO_2 in air at 37°C in a humidified chamber for 30–40 min and centrifuged at 100 *g* for 15 min using Cytospin 3 (Shandon, Pittsburgh, PA). Cells settled onto glass surfaces were fixed with 99.8% pure methanol. In case of tissues such as spleen, they were fixed with 20%-buffered formalin and embedded in paraffin. A paraffin block was microsectioned, deparaffinized and postfixed. Cells or tissue sections on glass slides were conventionally stained with hematoxylin–eosin. In order to stain metachromatic granules specifically present in MCs, acidic toluidine blue (pH 1.0), alcian blue (pH 2.5) or 0.1% safranin O solution was used (Merck, Darmstadt, Germany). Immunohistochemistry was performed using Bond-max automatic slide stainer (Leica Microcystems, Tokyo, Japan) and associated Bond Refine Polymer Detection Kit. Primary antibody used was anti-human-HLA clone EMR8-5 (Hokudo, Sapporo, Japan). Sections were examined by microscope DP71 (Olympus, Tokyo, Japan).

Confocal immunoscopy

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

Reverse transcription–polymerase chain reaction

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

Table 1. Primers for RT–PCR

Animal	Genes	Forward strand primer	Reverse strand primer
Marmoset	chymase 1	5′-TCTATAACAGT CACCCTTGG-3′	5′-AGCTTCACCTCTT GCAGAGT-3′
	tryptase β	5′-AAGCAGGTGA AGGTCCCCAT-3′	5′-TGTAGATGCCAG GCCGGTT-3′
	HPRT	5′-TGACCAGTCA ACAGGGGAC-3′	5′-GCTCTACTAAGC AGATGGC-3′
Mouse	β-actin	5′-TCTCCCCAAG TTAGGTTTTGTC-3′	5′-ATCATGTTTGAGA CCTTCAACAC-3′

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

Transplantation of marmoset hematopoietic cells into NOG mice

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

Miscellaneous

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

Results

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

In humans, MC progenitors are present in bone marrow. We first examined whether MC progenitors also existed in bone marrow of the common marmoset by culturing bone marrow cells *in vitro*. Mouse stem cell factor (SCF), IL-3 and/or IL-6 were added in combination to the medium as indicated in Fig. 1A. Mouse SCF was used because it can induce differentiation of human MCs more efficiently than human SCF, when hematopoietic progenitors from human cord blood are cultured *in vitro* (24). After culture was continued for 4 weeks, the cell surface expression of high-affinity IgE receptors (FcεRI) was monitored as a MC marker. This was done by incubating cells with human IgE and subsequently with FITC-labeled anti-human IgE. As seen by flow cytometry (Fig. 1A), approximately 15% of cells in the culture showed phenotypes of SSC^{lo} $Fc\varepsilon RI^+$, and similar percentages were observed irrespective of the cytokine combinations added. Thus, it became clear that the addition of SCF alone was sufficient for *in vitro* differentiation of MCs from marmoset bone marrow cells. As mouse IL-3 did not affect the development of marmoset MCs, we cut IL-3 in the following culture.

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

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

Function of in vitro*-developed MCs*

Next we examined whether the developed $Fc\varepsilon R^+$ cells were functional MCs. We used the cells cultured for 10 weeks in the presence of mouse SCF, as most of the cells became $Fc\varepsilon R^+$ cells (Fig. 2A). At first we examined whether FcεRI stimulation induces activation of signaling molecules. Cultured cells were sensitized with human IgE and stimulated by cross-linking the FcεR-bound IgE molecule with anti-human IgE antibody. We observed the upregulation of tyrosine phosphorylation level of spleen tyrosine kinase (Syk) (Fig. 2B). Calcium mobilization was also observed, as shown in Fig. 2C. The release of β-hexosaminidase by MCs was enhanced significantly by the cross-linking (Fig. 2D, upper panel). LTC_4 was significantly produced by MCs in a dose-dependent manner (Fig. 2D, lower panel). These results suggest that the developed MCs were functional MCs.

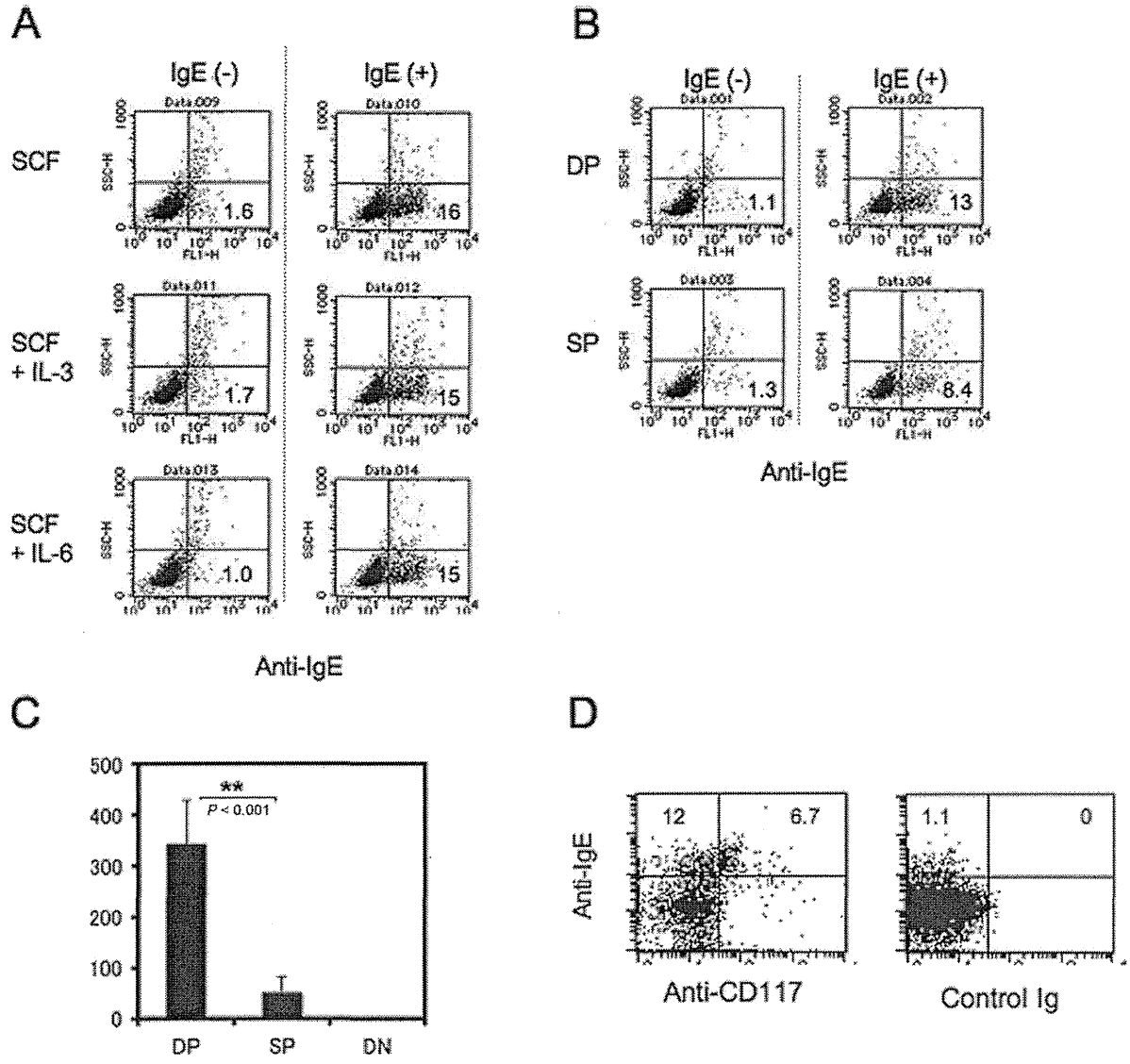

Fig. 1. *In vitro* differentiation of MCs from marmoset bone marrow cells. (A) Effects of cytokines on IgE receptor expression. Bone marrow cells of common marmoset were subjected to *in vitro* culture in the presence of mouse SCF, IL-3 and IL-6 as indicated. After 4 weeks of culture, human IgE was added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

Development of marmoset MCs in transplanted NOG mice

Next, we transplanted the marmoset bone marrow cell fractions into immunodeficient NOG mice, and the possible existence of a MC population in the leukocyte fraction *in vivo* was examined. The monocyte-gated fraction was examined for CD34 and CD117 expressions (Fig. 3A). A substantial population of $CD34^{-}CD117^{+}$ cells was detected in splenocytes (15% in DP-NOG and 13% in SP-NOG). Because the transplanted cells were originally $CD34^{+}CD117^{+}$ in the case of DP, the emergence of $CD34^{-}CD117^{+}$ cells suggests their differentiation toward MC lineage in NOG mice. The expression of FcεR was further examined by staining the CB-NOG spleen