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Bioimaging analysis of nuclear factor-κB activity in Philadelphia chromosome-positive acute lymphoblastic leukemia cells reveals its synergistic upregulation by tumor necrosis factor-α-stimulated changes to the microenvironment

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To gain an insight into the microenvironmental regulation of nuclear factor (NF)-κB activity in the progression of leukemia, we established a bioluminescent imaging model of Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) cells transduced with a NF-κB/luciferase (Luc) reporter and cocultured with murine stromal cells and cytokines. Stromal cells alone did not augment Luc activity, taken as an index of NF-κB, but Luc activity was synergistically upregulated by the combination of stromal cells and tumor necrosis factor (TNF)-α. Dehydroxymethylepoxyquinomicin (DHMEQ), a specific inhibitor of NF-κB DNA binding, rapidly induced the apoptosis of Ph+ALL cells, indicating that NF-κB is necessary for the growth and survival of these cells. However, the DHMEQ-induced suppression of NF-κB activity and the apoptosis of leukemia cells were attenuated by the presence of stromal cells and TNF-α. In NOD-SCID mice transplanted with NF-κB/Luc reporter-containing Ph+ALL cell lines and monitored periodically during the progression of the leukemia, murine TNF-α was significantly expressed in lesions in which the leukemia cells emitted a significant NF-κB signal. These results support the notion that TNF-α also triggers microenvironmental upregulation of NF-κB activity *in vivo*. Collectively, the results indicated that TNF-α-stimulated microenvironment may contribute to the survival and progression of Ph+ALL cells through the synergistic upregulation of NF-κB activity. (*Cancer Sci* 2011; 102: 2014–2021)

There are five members in the nuclear factor (NF)-κB family, namely RelA (p65), c-Rel, RelB, p50 (and its precursor p105) and p52 (and its precursor p100). These proteins are assembled as homo- or heterodimers bound to IκB family proteins and retained in the cytoplasm in unstimulated cells.[1,2] Responding to extracellular stimuli, NF-κB signaling is activated and plays a crucial role in the regulation of innate and adaptive immune responses.[3,4] More recently, a significant role for NF-κB signaling in cancer development and progression has been demonstrated.[5,6] Activation of NF-κB has been noted in a variety of hematopoietic malignancies.[7–9] In addition, NF-κB is absolutely required for the growth and survival of certain hematologic malignancies.[10–12]

Development of Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) is essentially attributed to genomic recombination between the *ABL* gene and *BCR* gene, which results in the formation of p190 or p210 Bcr-Abl tyrosine kinase.[13,14] Approximately 20–30% of adult ALL is Ph+ALL, which is the most refractory subtype.[15–17] Despite progress in targeted molecular therapy,[18–21] clinical outcomes are not as promising as expected, mainly due to acquired drug resistance.[22,23] Although a series of Abl kinase domain mutations play a crucial role in acquired drug resistance, it is well documented that a close interaction with stromal cells, the so-called microenvironment, can protect leukemia cells from spontaneous and/or drug-induced apoptosis through diverse mechanisms,[24–26] one of which may be due to the activation of the NF-κB signal in leukemia cells. Constitutively active NF-κB has been detected in primary blast cells and cell lines derived from Ph+ALL,[27] and is required for Bcr–Abl-mediated transformation of bone marrow cells and interleukin (IL)-3-dependent cell lines, as well as tumorigenicity in nude mice.[28,29] However, the role of NF-κB in the progression of Ph+ALL is not yet well understood. In addition, microenvironmental regulation of NF-κB activity in Ph+ALL has not been clarified. Therefore, in the present study, we transduced Ph+ALL cells with an NF-κB/luciferase (κB/Luc) reporter construct and established a bioluminescence imaging model for *in vitro* and *in vivo* analysis. The aim of the present study was to elucidate the role of cytokines and cellular interactions in the regulation of NF-κB activity in Ph+ALL cells during the progression of leukemia.

Materials and Methods

Cell lines. The p190-expressing Ph+ALL cell lines Sup-B15, OM9:22, KOPN-30, and KOPN-72 (gift from Dr Oyashiki, Tokyo Medical College, Tokyo, Japan) were maintained in our laboratory as described previously.[30] The p210-expressing IMS-PhL1 cell line was established in our laboratory (Division of Molecular Therapy, Institute of Medical Science, The University of Tokyo, Tokyo, Japan) from a Ph+ALL patient, as reported previously.[31] The culture of the murine bone marrow stromal cell line HESS-5 was as described elsewhere.[32] The leukemia cell lines were cultured in RPMI-1640 containing 10% FBS. When IMS-PhL1 and Sup-B15 cells were seeded onto an

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HESS5 monolayer, the culture medium was changed to α-minimum essential medium (α-MEM) containing 10% FBS.

Reagents. All cytokines, namely tumor necrosis factor (TNF)-α, IL-3, Flt3-ligand (Flt3L), stem cell factor (SCF), granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-1α, and interferon (IFN)-γ, were purchased from PeproTech (Rocky Hill, NJ, USA). Fibronectin was purchased from Invitrogen (Carlsbad, CA, USA) and imatinib was obtained from Novartis Pharmaceuticals (East Hanover, NJ, USA). Dehydroxymethylepoxyquinomicin (DHMEQ), a potent inhibitor of the DNA binding of the NF-κB complex,[33] was dissolved in 100% of DMSO prior to use in the experiments. The final maximal concentration of DMSO that the cells were exposed to was 500 fold dilution of pure DMSO (0.2%).

Construction and production of lentiviral vectors. A self-inactivating lentiviral vector encoding humanized Renilla reniformis (*R. reniformis*) green fluorescent protein (GFP) driven by a CMV promoter (HIV-CMV/hrGFP) was produced as described previously.[30] To construct NF-κB reporter vectors, this expression cassette was replaced by a firefly luciferase gene downstream of three copies of NF-κB-responsive elements linked to a basal TATA promoter (HIV-NF-κB/Luc). The NF-κB specificity of these constructs was confirmed by comparing reporter activity with a mutant enhancer-containing reporter (HIV-mTA/Luc) in various cell lines and with various stimuli. In addition, HIV-EF1α/Luc and Venus were prepared as constitutively expressing vectors. Viral supernatant and high titer stocks were prepared as described previously.[30] The functional titers of these vectors was determined by flow cytometry of infected HeLa cells and exceeded 1×10^9 units/mL.

Transduction of leukemia cells and analysis of NF-κB activity. For viral transduction of leukemia cells, 2×10^5 cells were pelleted and incubated with viral supernatant at a multiplicity of infection (MOI) of 10 in 1.5-mL Eppendorf tubes. After incubation for 2 h at 37°C in 5% CO_2, infected cells were seeded onto an HESS-5 monolayer and cultured for 1 week. Single cell sorting was performed using a BD FACSAria (Becton Dickinson, Franklin Lakes, NJ, USA) and the resulting cell clones were cocultured with HESS5 cells in 96-multiwell dishes for 3 weeks. A number of reporter vector-transduced clones were selected by screening TNF-α-induced Luc activity on an ARVO MX multilabel counter (PerkinElmer, Waltham, MA, USA) and used in further experiments. Selected clone-derived cells were cultured in 6-cm dishes and subjected to *in vitro* bioluminescence imaging (BLI) analysis with a cooled charge-coupled device (CCD) camera system (IVIS Imaging System 100; Xenogen, Alameda, CA, USA).[34]

Apoptosis assay. The IMS-PhL1 cells were cultured in 24-multiwell plates and treated with 10 μg/mL of DHMEQ for 0, 24 and 48 h. Treated cells were collected, labeled with annexin V–phycoerythrin (PE) and 7-aminoactinomycin D (7-AAD), and then analyzed on a BD FACSCalibur (Becton Dickinson).

Cell proliferation assay (WST1 assay). Samples (in triplicate) of 5×10^4 leukemia cells were incubated with serial twofold dilutions of TNF-α (from 100 ng/mL to 0) and DHMEQ (from 10 μg/mL to 0) in 96-multiwell plates. Cultures were maintained at 37°C in 5% CO_2 for 48 h. Then, 10 μL of Tetracolor1 solution (water-soluble tetrazolium salt; Seikagaku, Tokyo, Japan) was added to each well and cells were incubated for a 1 h. Absorbance was measured at 450 nm using an ARVO MX multilabel counter (PerkinElmer).

Other methods including electrophoretic mobility shift analysis, animal study, and polymerase chain reaction analysis are provided in Data S1.

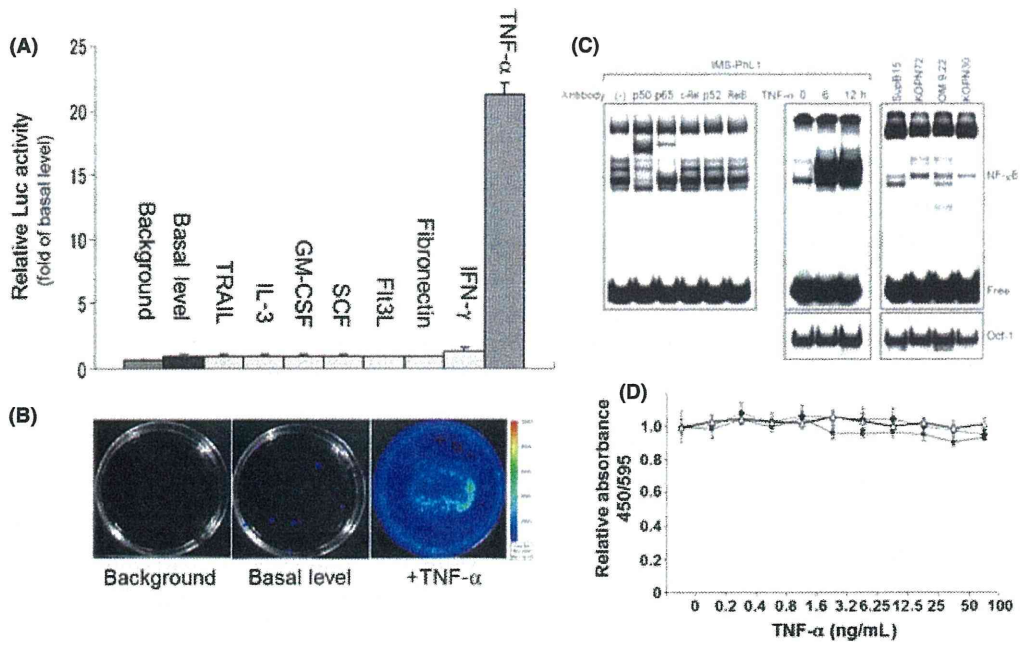

Fig. 1. Nuclear factor (NF)-κB is constitutive and inducible in Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) cells. (A) Relative NF-κB/luciferase (Luc) activity compared with basal levels. The IMS-PhL1 cells were stimulated with the different cytokines (TRAIL 200 ng/mL; IL-3 10 ng/mL; GM-CSF 10 ng/mL; SCF 50 ng/mL; Flt3L 50 ng/mL; Fibronectin 100 μg/mL; IFN-γ 1000 IU/mL) as indicated for 24 h and luc activity was measured using a multilabel counter. Data show the mean ± SD. TRAIL, TNF-related apoptosis inducing ligand; IL-3, interleukin-3; GM-CSF, granulocyte–macrophage colony-stimulating factor; SCF, stem cell factor; Flt3L, Flt3-ligand; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α. (B) Bioluminescent images on the IVIS imaging system (Xenogen) showing background, basal and TNF-α-triggered NF-κB/Luc activity. (C) Nuclear extracts prepared from Ph+ALL cell lines, treated with or without TNF-α, were subjected to electrophoretic mobility shift assay (EMSA) or supershift assay using the antibodies indicated. Oct-1 served as a loading control for the EMSA. Free indicates nuclear acid not bound with NF-κB. (D) Results of the WST1 assay for the Ph+ALL cell lines (◆, IMS-PhL1; △, Sup-B15; ■, OM9;22) treated with TNF-α for 48 h. Data show the mean ± SEM.

RESEARCH ARTICLE

Gaussia Luciferase for Bioluminescence Tumor Monitoring in Comparison with Firefly Luciferase

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Abstract

Gaussia luciferase (Gluc) is a secreted reporter, and its expression in living animals can be assessed by in vivo bioluminescence imaging (BLI) or blood assays. We characterized Gluc as an in vivo reporter in comparison with firefly luciferase (Fluc). Mice were inoculated subcutaneously with tumor cells expressing both Fluc and Gluc and underwent Fluc BLI, Gluc BLI, blood assays of Gluc activity, and caliper measurement. In Gluc BLI, the signal from the tumor peaked immediately and then decreased rapidly. In the longitudinal monitoring, all measures indicated an increase in tumor burden early after cell inoculation. However, the increase reached plateaus in Gluc BLI and Fluc BLI despite a continuous increase in the caliper measurement and Gluc blood assay. Significant correlations were found between the measures, and the correlation between the blood signal and caliper volume was especially high. Gluc allows tumor monitoring in mice and should be applicable to dual-reporter assessment in combination with Fluc. The Gluc blood assay appears to provide a reliable indicator of viable tumor burden, and the combination of a blood assay and in vivo BLI using Gluc should be promising for quantifying and localizing the tumors.

BIOLUMINESCENCE IMAGING (BLI) using firefly luciferase (Fluc) as a reporter is applied to small-animal experiments for various purposes, and tumor monitoring is the most popular application.[1,2] In vivo BLI is a highly sensitive, high-throughput technology, and its noninvasiveness allows the visualization of disease progression and the longitudinal assessment of therapeutic effects in given animals. Detected light signals serve as quantitative indicators of tumor burden, which has been validated in many tumor models.[3–13]

Dual-reporter imaging enables the evaluation of given disease model animals from two different aspects simultaneously. For example, the spatial relationship between tumor cells and effector cells and the relationship between the tumor cell number and signal transduction activity can be evaluated. Renilla luciferase (Rluc) is commonly used in combination with Fluc in dual-reporter imaging.[14] The substrates for Fluc and Rluc are D-luciferin and coelenterazine, respectively, and cross-reactivity is not present between the two substrates. The injection of D-luciferin and coelenterazine into mice with both Fluc and Rluc causes light emission reflecting Fluc activity and Rluc activity, respectively.

Gaussia luciferase (Gluc), another reporter applicable to in vivo BLI,[15–18] causes a stronger light emission than Rluc after the addition of coelenterazine. Because it is naturally secreted from cells, blood or urine assays and in vivo BLI allow us to assess Gluc expression in living mice. Gluc appears to be superior to Rluc as an in vivo reporter, and the combination of Fluc and Gluc may be useful for dual-reporter assessment.

In this study, we compared BLI tumor monitoring using Fluc and Gluc. Mice were inoculated subcutaneously with tumor cells expressing Fluc and Gluc under the control of the same promoter and underwent Fluc BLI, Gluc BLI, and Gluc blood assays. Dual-reporter imaging requires comparable results for two reporters expressed in parallel. We aimed to characterize Gluc as an in vivo reporter and to evaluate the validity of dual-reporter imaging using Fluc and Gluc.

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Materials and Methods

Cell Lines

The human colon cancer cell line HCT116 was cotransfected with both the Fluc and Gluc genes using a retroviral method described previously.[19] The Fluc and Gluc genes were excised from the pGL3-basic vector (Promega, Madison, WI) and pcDNA3/GL Vector (LUX Biotechnology, Edinburgh, UK), respectively. A clone was selected from cells expressing both genes stably and named HCT116-Fluc/Gluc. The cells were maintained in McCoy's 5A medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) and 1% penicillin-streptomycin (Invitrogen). Both Fluc and Gluc were stably expressed under the control of the long terminal repeat of Moloney murine leukemia virus in these cells. Cell cultures were incubated at 37°C under 5% CO_2.

Animals

Eight-week-old, female, severe combined immunodeficiency mice were inoculated subcutaneously in the shoulder with 1×10^5 HCT116-Fluc/Gluc cells mixed with the same volume of Matrigel (BD Biosciences, San Jose, CA). The injection volume was 40 μL. Fur near the shoulder was removed with depilatory mousse before cell inoculation, and if necessary, fur regrowth was cut with scissors. The mice were obtained from CLEA Japan (Tokyo, Japan) and handled in accordance with the guidelines of the host institution. The experiments were approved by the committee for animal research at the institution.

Time Course of Bioluminescence after Substrate Injection

The time course of bioluminescence was evaluated for Gluc BLI using the intravenous injection of coelenterazine and Fluc BLI using the intravenous or subcutaneous injection of D-luciferin. In a single day, the three in vivo BLI studies were performed successively 10 and 14 days after subcutaneous inoculation of HCT116-Fluc/Gluc cells. Three mice were studied on each day, and six sets of imaging data were acquired. First, mice were intravenously injected into the tail vein with 2 mg/kg of coelenterazine (LUX Biotechnology) under isoflurane anesthesia and, immediately after injection, imaged with a cooled charge-coupled device (CCD) camera system (IVIS Imaging System 100, Xenogen/Caliper Life Sciences, Alameda, CA) to assess Gluc activity. Dorsal luminescent images of 1-second exposure time were sequentially acquired at a rate of 1 image per 10 seconds for 4 minutes and then at a rate of 1 image per 30 seconds for 8 minutes. Approximately 1 hour after coelenterazine injection, the mice were intravenously injected into the tail vein with 25 mg/kg of D-luciferin (beetle luciferin, potassium salt; Promega) and imaged using the aforementioned imaging protocol to assess Fluc activity. Approximately 3 hours later, 75 mg/kg of D-luciferin was subcutaneously injected in the dorsal flank, and 1-second luminescent images were sequentially acquired at a rate of one image per minute from 5 to 40 minutes after substrate injection.

For quantitative analysis, an elliptical region of interest (ROI) was placed over the tumor, and the total signal (photons/s) in the ROI was determined using *Living Image* software version 2.50 (Xenogen/Caliper Life Sciences). The same ROI was applied to all images acquired sequentially, and a time-intensity curve was generated. Peak time, the interval from substrate injection to peak of light emission, was determined from the curve. Half-time was also determined from the curves following the intravenous injection of coelenterazine or D-luciferin. An exponential curve was fitted to the declining phase of the time-intensity curve using the following formula: $y = a \times \exp(kt)$, where a and k are constants and t is time after injection. The end of the declining phase used for data analysis was determined as the time point when the signal decreased to less than half of the peak signal. The half-time of bioluminescence was calculated as $\ln(0.5)/k$. Peak time and half-time were compared statistically between intravenous injections of coelenterazine and D-luciferin using the Wilcoxon signed rank test, and a p value less than .05 was considered statistically significant.

Long-Term Tumor Monitoring

Mice bearing subcutaneous tumors underwent Fluc BLI, Gluc BLI, blood assays of Gluc activity, and caliper measurement of the tumor size to monitor tumor growth. The tumor burden was assessed in six mice 4, 7, 10, 14, 21, and 28 days after subcutaneous inoculation of HCT116-Fluc/Gluc cells. First, mice were intravenously injected with 2 mg/kg of coelenterazine, and immediately 1-second dorsal luminescent images were sequentially acquired at a rate of one image per 10 seconds for 4 minutes. Approximately 1 hour later, the mice were subcutaneously injected with 75 mg/kg of D-luciferin, and beginning 5 minutes after injection, 10-second luminescent images were sequentially acquired at a rate of one image per

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ORIGINAL ARTICLE

Association analysis of the *NOD2* gene with susceptibility to graft-versus-host disease in a Japanese population

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Abstract Members of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family participate in the innate immune system, exerting widespread effects on cytokine secretion, autophagy, and apoptosis. Recent studies in Caucasians revealed the association between mutants of NOD2, a member of the NLR family, and severity of acute graft-versus-host disease (GVHD). *NOD2* polymorphism screening has been recommended for donor selection and risk assessment at bone marrow transplantation. To investigate whether NOD2 plays a role in the pathogenesis of GVHD in a Japanese population, we examined DNA from 142 bone marrow transplant patient/donor pairs to detect genetic variation in the *NOD2* gene. No genetic variants of *NOD2* were associated with the severity of acute GVHD in our patients. However, a weak association between a single nucleotide polymorphism in

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the *NOD2* gene (R471C) and acute myeloid leukemia in the bone marrow patients ($p = 0.029$, odds ratio 4.08, 95% CI 1.22–13.67) was detected. This polymorphism was not prevalent in 479 Crohn's disease (CD) patients in Japan. These results suggest that, in the Japanese population, unlike the Caucasian, NOD2 is not a major contributor to susceptibility to severe acute GVHD.

Keywords NLR · Crohn's disease · Innate immunity · GVHD · Leukemia · NOD2

1 Introduction

In the response to microorganisms, hosts use two types of immune mechanisms, adaptive system and innate system, to effectively eliminate the invading pathogen. Fast innate immune responses are mediated by a set of non-clonal, germline-encoded pattern-recognition receptors (PRRs) that sense conserved structures in pathogens, called pathogen-associated molecular patterns (PAMPs) [1]. PAMPS include lipopolysaccharides, unmethylated CpG DN, and endogenous danger signals, such as heat shock proteins (HSP) and uric acid. The nucleotide binding oligomerization domain (NOD)-like receptor (NLR) family consists of cytoplasmic PRRs that play a pivotal role in sensing PAMPs in the cytosol. As a member of the NLR family, NOD2 recognizes muramyl dipeptide (MDP), a component of peptidoglycan that is found in both Gram-positive and Gram-negative bacteria. NOD2, a critical mediator of inflammation, participates in the formation of a protein complex known as the inflammasome, which has important roles in innate immunity, cytokine secretion, cell survival, autophagy, and apoptosis.

We have previously shown that multiple genetic variants of NLR are associated with susceptibility to several granulomatous diseases. Notably, NOD2 loss-of-function and gain-of-function mutations are involved in the pathogenesis of Crohn's disease (CD) [2] and Blau syndrome [3], respectively. Furthermore, we have shown that impaired recognition of intracellular *Propionibacterium acnes* resulting from a mutation in the *NOD1* gene affects susceptibility to Sarcoidosis in a Japanese patient population [4]. Accumulating evidence suggests that three major *NOD2* mutants (R702W, G908R, and 1007insC) identified in CD patients [5] increase the risk for colorectal cancer and bowel cancer in Caucasian populations [6–8].

Hematopoietic stem cell transplantation is currently the only curative treatment for patients with severe hematopoietic disease. Despite recent advances, transplantation-related mortality remains high, and acute graft-versus-host disease (GVHD) remains the major and most severe complication of transplantation. Therefore, defining the variables that predispose patients to GVHD is vital. Holler et al. [9, 10] initially reported an association between single nucleotide polymorphisms (SNPs) in the *NOD2* gene and the incidence and severity of acute GVHD in two separate patient cohorts in Caucasians, and proposed to use *NOD2* polymorphism screening to optimize donor selection. However, subsequent results from other groups were somewhat contradictory; some studies demonstrated that *NOD2*-SNPs are a risk factor for severe acute GVHD [11, 12] but other reports did not support this conclusion [13–15]. The effect of *NOD2* SNPs on susceptibility to acute GVHD in Japanese patients has not been reported.

To investigate whether NOD2 plays a role in the pathogenesis of GVHD in Japanese populations, we examined this gene in a large clinical population.

2 Materials and methods

2.1 Patients

The study population was selected from patients who received a bone marrow transplant from an unrelated donor, matched through the Japanese Marrow Donor Program (JMDP), between January 1993 and March 2000. The selection criteria for the patients and donors in the study were: (a) patient/donor pairs matched for all genotypes of HLA-A, HLA-B, and HLA-C and DRB1; (b) intensive myeloablative pre-transplant conditioning regimen; (c) unmanipulated marrow graft; (d) use of cyclosporine A or tacrolimus as GVHD prophylaxis; (e) available DNA samples for genotyping; and (f) available clinical outcome data. The genotypes of each allele at the HLA-A, HLA-B, and HLA-C and DRB1 loci were determined by high-resolution DNA typing, as described previously [16, 17]. The number of patients who underwent unrelated BMT between January 1993 and March 2000 was 2547, and registered with the Japanese Marrow Donor Program; samples of DNA from 142 patients and their respective donors were available at the time of the design of the present study. We analyzed all available DNA samples.

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Adequate assessment of the efficacy of first line treatment options in CML CP is necessary to fully appreciate the role of second line treatment options. While in the rest of Europe the use of second generation TKI is possibly going to be extended to newly diagnosed CP CML patients, in the UK there is a risk that a higher proportion of patients than originally thought could be denied such an effective treatment as a second line option.

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Reduced dose chemotherapy for acute promyelocytic leukaemia with adult Down syndrome

Down syndrome (DS) is associated with an increased risk of acute myeloid leukaemia (AML). The most common subtype of AML described in patients with DS is acute megakaryoblastic leukaemia (AMKL), which occurs before 4 years of age (Lange *et al*, 1998). The occurrence of acute promyelocytic leukaemia (APL), an AML subtype showing distinct molec-

ular, biological and clinical features, is extremely rare in DS patients, especially adults. Indeed, only one adult case and five childhood cases have been reported. Among them, one adult and two childhood cases were treated with variety of standard dose chemotherapies for non-DS AML and achieved complete remission (CR) (Kurkjian *et al*, 2006; Hasle *et al*, 2008; Ghosh *et al*, 2009), although the reported details of three childhood cases were unclear (Lange *et al*, 1998; Jain *et al*, 2007). Thus, there have been no established protocols for adult and paediatric cases of APL patients with DS. We here report a 23-year-old male with DS diagnosed as having APL, who was successfully treated with reduced dose chemotherapy in combination with all-*trans* retinoic acid (ATRA) and maintained molecular complete remission (CR) for over 1 year.

A 23-year-old male with DS admitted to the local hospital because of pancytopenia, pneumonia and disseminated intravascular coagulation (DIC) after influenza A (H1N1) infection. Upon examination he was suspected to have acute leukaemia, and was referred to our hospital. On admission, he had anaemia and petechiae. He had no history of transient abnormal myelopoiesis during the newborn period or cardiac problems. Haemogram findings were as follows: haemoglobin 92 g/l; white blood cell (WBC) count $5{\cdot}37 \times 10^9$/l; platelet count 32×10^9/l. A peripheral blood (PB) smear showed 86·0% blasts. A bone marrow (BM) aspirate was performed, which revealed increased cellularity comprising 91·3% of abnormal promyelocytes with prominent cytoplasmic granules and multiple Auer rods. Flowcytometric analysis showed that the immature myeloid cells were $CD13^+$, $CD33^+$, $CD34^-$, and $HLA\text{-}DR^-$, consistent with APL. Karyotype analysis of BM cells showed the presence of *t*(15;17) with +21, and reverse transcription polymerase chain reaction confirmed the presence of *PML/RARA* chimeric mRNA, but no *FLT3* internal tandem duplication. Thus a diagnosis of APL was made, and the patient was commenced on ATRA (45 mg/m^2). Immediately after the ATRA treatment, DIC began to improve and WBC and APL cells in PB began to decrease. However, as the WBC count increased again, together with APL cells after 10 days of ATRA treatment, reduced dose chemotherapy was added, which consisted of pirarubicin (25 mg/m^2) for 2 days (days 1–2), etoposide (150 mg/m^2) for 3 days (days 3–5), and cytarabine (100 mg/m^2) for 7 days (days 1–7). This was a modification of the DS protocol proposed by the Japanese Childhood AML Cooperative study Group (Kudo *et al*, 2007). ATRA had been administrated throughout the induction therapy of 5 weeks. The patient tolerated the induction therapy well and entered haematological CR after the induction therapy. He subsequently received four courses of intensification therapy composed of etoposide (150 mg/m^2) for 3 days (days 3–5), and cytarabine (100 mg/m^2) for 7 days (days 1–7) with or without pirarubicin (25 mg/m^2) for 2 days (days 1–2) in combination with ATRA (45 mg/m^2) for 7 days (day 8–15). He achieved molecular CR after the first intensification therapy, and under close follow-up for 13 months has been disease-free whilst receiving maintenance therapy with ATRA (45 mg/m^2) for 15 days every 3 months at the time of writing.

The outcome of adult and paediatric patients with APL has improved considerably because of the introduction of ATRA to the treatment. The PETHEMA (Programa de Estudio y Tratamiento de las Hemopatias Malignas) group showed that using ATRA for induction and consolidation therapy resulted in improved antileukaemic efficacy (Sanz *et al*, 2004). Furthermore, the BFM (Berlin/Frankfurt/Muenster) group showed that the reduction of cumulative anthracycline dose in the treatment using ATRA did not affect the cure rate but promoted the decrease of long-term adverse effects in paediatric APL patients (Creutzig *et al*, 2005). On the other hand, several cooprerative clinical trials showed that reduced dose chemotherapy produced lower treatment-related mortality and higher cure rate in AML patients with DS (Creutzig *et al*, 2005; Kudo *et al*, 2007), most of whom were younger than 4 years old, based on *in vitro* studies demonstrating that DS AML cells were significantly more sensitive to cytarabine, anthracyclines, and etoposide than non-DS AML cells (Zwaan *et al*, 2002). However, the reports regarding outcome in older AML patients with DS have been controversial. The Children's Cancer Group Study 2891 showed that increased age at diagnosis had a negative effect on outcome in AML patients with DS (Gamis *et al*, 2003). By contrast, the BFM 98 study found no difference in outcome between those aged 2 years or younger and those older than 2 years (Creutzig *et al*, 2005). The Japanese Childhood AML Cooperative Study Group also did not identify age older than 2 years as a risk factor in the multivariate analysis in a less intensive regimen using a combination of cytarabine, etoposide, and pirarubicin (Kudo *et al*, 2007), which was modified in the treatment for the patient reported here.

This is the first report of a DS patient with APL treated with reduced dose chemotherapy and ATRA. The patient successfully achieved molecular CR without major toxicity and has remained disease-free for over 1 year after ATRA and reduced intensity chemotherapy consisting of cumulative doses of cytarabine 2500 mg, etoposide 1350 mg, and pirarubicin 250 mg, which were lower than previous reports (Creutzig *et al*, 2005; Kudo *et al*, 2007). Our experience showed that less intensive chemotherapy in combination with ATRA seemed to be effective for older DS patients with APL, even in adults.

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Leukemic T cells are specifically enriched in a unique $CD3^{dim}CD7^{low}$ subpopulation of $CD4^+$ T cells in acute-type adult T-cell leukemia

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The morphological discrimination of leukemic from non-leukemic T cells is often difficult in adult T-cell leukemia (ATL) as ATL cells show morphological diversity, with the exception of typical "flower cells." Because defects in the expression of CD3 as well as CD7 are common in ATL cells, we applied multi-color flow cytometry to detect a putative leukemia-specific cell population in the peripheral blood from ATL patients. $CD4^+CD14^-$ cells subjected to two-color analysis based on a CD3 *vs* CD7 plot clearly demonstrated the presence of a $CD3^{dim}CD7^{low}$ subpopulation in each of nine patients with acute-type ATL. The majority of sorted cells from this fraction showed a flower cell-like morphology and carried a high proviral load for the human T-cell leukemia virus type 1 (HTLV-I). Genomic integration site analysis (inverse long-range PCR) and analysis of the T cell receptor Vβ repertoire by flow cytometry indicated that the majority of leukemia cells were included in the $CD3^{dim}CD7^{low}$ subpopulation. These results suggest that leukemic T cells are specifically enriched in a unique $CD3^{dim}CD7^{low}$ subpopulation of $CD4^+$ T cells in acute-type ATL. (*Cancer Sci* 2011; 102: 569–577)

Adult T-cell leukemia (ATL) is a malignant disorder caused by human T-cell leukemia virus type 1 (HTLV-I)[1] and is characterized clinically by generalized lymphadenopathy, hepatosplenomegaly, skin lesions, hypercalcemia and a characteristic morphology termed "flower cells." Importantly, ATL is one of the most incurable lymphoid malignancies. This disease is endemic to several regions in the world, including sub-Saharan Africa, the Caribbean basin, South America and Japan, and 10–20 million people are estimated to be infected by this virus worldwide.[2,3]

Evaluation of the response after chemotherapy for ATL partly depends on the proportion of ATL cells in the peripheral blood. However, the morphological diversity of ATL cells may lead to inaccurate estimations. Accurate estimation of the chemotherapeutic effect is pivotal in clinical practice because ATL cells often become chemoresistant, even during chemotherapy. Methods to detect ATL cells with greater precision than morphological examination are therefore required.

Aberrant expression of cell-surface antigens in myeloid/lymphoid leukemia cells has been studied extensively.[4–6] Using fluorescence-activated cell sorting (FACS) analysis, gating cells with diminished CD45 expression in acute myeloid/lymphoid leukemia is widely used for purifying leukemia cells. However, in ATL there are only limited data regarding the identification of transformed leukemia cells by similar methods. Previous studies indicated that most ATL cells lack CD7 and exhibit diminished CD3 expression.[7–10] Although a study using CD3 gating by FACS analysis has indicated that ATL cells were distinguishable from normal lymphocytes as a $CD3^{low}$ population,[10] these cells were not well characterized as ATL cells.

In the present study, we focused on the enrichment of ATL cells by constructing CD3 *vs* CD7 plots from multi-color FACS. $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ cells were extensively studied and compared with normal control samples. Taken together, our data suggest that ATL cells are purified in $CD3^{dim}CD7^{low}$ subpopulations. The purification of ATL cells by FACS may therefore allow monitoring of disease activity and yield insight into the biology of this disease.

Materials and Methods

Cell lines and patient samples. TL-Om1, a HTLV-I-infected cell line, was provided by Dr. Toshiki Watanabe (The University of Tokyo), and was cultured in RPMI 1640 medium containing 10% fetal bovine serum. Peripheral blood samples were collected from patients admitted to our hospital (Research Hospital, Institute of Medical Science, The University of Tokyo, Tokyo, Japan) during the period from August 2009 to April 2010 with written informed consent. All patients were diagnosed with acute-type ATL according to Shimoyama's criteria.[8] Blood samples were collected before treatment using the LSG15 protocol[11] or during the recovery phase between chemotherapy sessions. Samples collected from five healthy volunteers (median age, 45 years) were used as normal controls. The present study was approved by the institutional review board of our hospital.

Flow cytometry and cell sorting. Peripheral blood mononuclear cells (PBMC) were isolated from heparin-treated whole blood by density gradient centrifugation using Lymphoprep (Axis-Shield, Dundee, UK) and subsequently suspended in phosphate-buffered saline (PBS) containing 5% mouse serum (DAKO, Glostrup, Denmark) for prevention of nonspecific antibody binding. Cells were stained using a combination of phycoerythrin (PE)-CD7, PE-Cy7-CCR4, allophycocyanin (APC)-CD25, APC-Cy7-CD3, Pacific Blue-CD4 and Pacific Orange-CD14. Pacific Orange-CD14 was purchased from Caltag-Invitrogen (Carlsbad, CA, USA). All other antibodies were obtained from BD BioSciences (San Jose, CA, USA). Propidium iodide (PI; Sigma, St Louis, MO, USA) was added to the samples to stain dead cells immediately prior to FACS analysis. Cells were also stained with APC-FoxP3 (eBioscience, San Diego, CA, USA) using intracellular staining methods as previously described.[12] A TCR-Vβ repertoire kit (Beckman Coulter, Miami, FL, USA) was used for T-cell receptor (TCR) Vβ repertoire analysis according to the manufacturer's instructions.

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A BD FACS Aria (BD Immunocytometry Systems, San Jose, CA, USA) was used for all multi-color FACS analysis and cell sorting. Data were analyzed using FlowJo software (Treestar, San Carlos, CA, USA).

Quantification of HTLV-I proviral load by real-time quantitative polymerase chain reaction (PCR). The HTLV-I proviral load in PBMC was quantified by real-time quantitative polymerase chain reaction (PCR; TaqMan method) using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) as previously described.[13] Briefly, a total of 50 ng of genomic DNA was extracted from human PBMC using a QIAamp DNA blood Micro kit (Qiagen, Hilden, Germany). Triplicate samples of the DNA were amplified. Each PCR mixture containing a HTLV-I pX region-specific primer pair at 0.1 μM (forward primer 5′-CGGATACCCAGTCTACGTGTT-3′ and reverse primer 5′-CAGTAGGGCGTGACGATGTA-3′), FAM-labeled probe at 0.1 μM (5′- CTGTGTACAAGGC-GACTGGTGCC-3′) and 1× TaqMan Universal PCR master mix

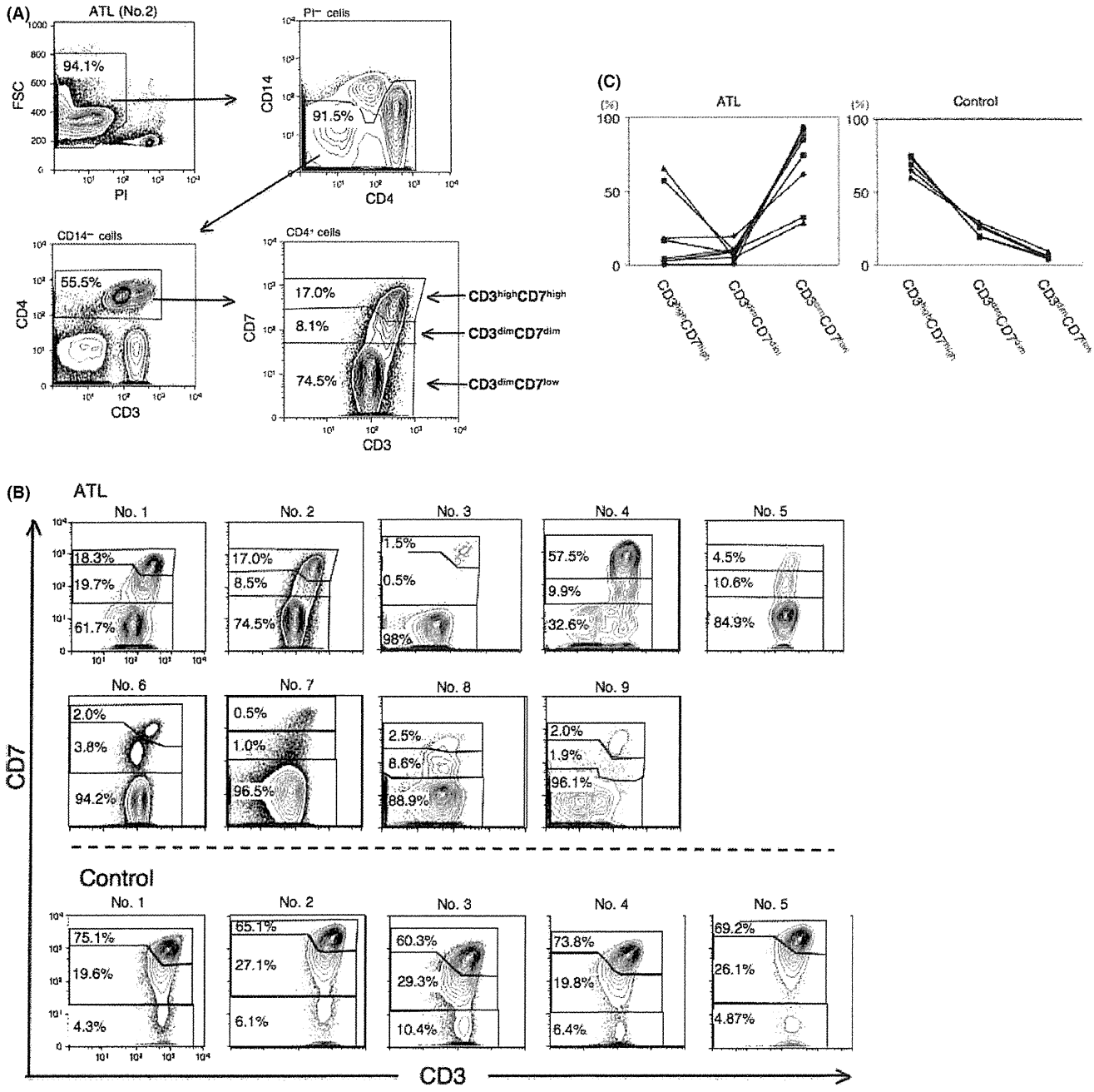

Fig. 1. CD3 *vs* CD7 plots from FACS analysis of patients with acute-type adult T-cell leukemia (ATL) and normal controls. (A) Representative flow cytometric analysis of a patient with acute-type ATL (patient no. 2). The CD3 *vs* CD7 plot in $CD4^+$ cells was constructed according to the gating procedure shown in this figure. In the plot, we designated three subpopulations: $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$. (B) Flow cytometric profile of the CD3 *vs* CD7 plot in patients with acute-type ATL and normal controls. (C) Percentages of $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations in $CD4^+$ T cells in patients with acute-type ATL and normal controls. Each line represents an individual sample. ATL group, $n = 9$; control group, $n = 5$; FSC, forward scatter; PI, Propidium iodide.

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