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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Table 1 Clinical characteristics

Total number	142 (100)
Underlying disease	
ALL	33 (23.2)
AML	48 (33.8)
CML	43 (30.3)
MDS	10 (7.0)
NHL	6 (4.2)
SAA	2 (1.4)
Stage at transplant	
Standard	79 (55.6)
Advanced	52 (36.6)
Unknown	11 (7.8)
Conditioning regimen	
TBI containing	118 (83.0)
Non-TBI containing	24 (17.0)
GVHD prophylaxis	
C + M	128 (90.1)
C + M + F	1 (0.7)
C + M + P	1 (0.7)
F + M	7 (4.9)
F + M + P	2 (1.4)
F + P	1 (0.7)
F	2 (1.4)
Acute GVHD stage	
0	63 (44.4)
I	33 (23.2)
II	17 (12.0)
III	21 (14.8)
IV	8 (5.6)

ALL acute lymphocytic leukemia, *AML* acute myeloid leukemia, *CML* chronic myeloid leukemia, *MDS* myelodysplastic syndrome, *NHL* non-Hodgkin's lymphoma, *SAA* severe aplastic anemia, *TBI* total body irradiation, *C* cyclophosphamide, *M* methotrexate, *F* tacrolimus, *P* predonisolone, *GVHD* graft-versus-host disease

The characteristics of the 142 pairs are summarized in Table 1. Standard-risk disease was defined as acute myeloid leukemia or acute lymphoblastic leukemia in first remission, or chronic myeloid leukemia in first chronic phase. All other hematological malignancies, including Hodgkin's lymphoma and non-Hodgkin's lymphoma, were considered to be advanced disease. The pre-transplant conditioning regimen varied according to the disease or disease stage at transplantation. GVHD prophylaxis was achieved through treatment, including cyclosporine A or tacrolimus.

In addition, 479 Japanese CD patients were recruited from the Social Insurance Chuo General Hospital. Blood samples were collected, and DNA was extracted by standard protocols, as described previously [18]. Data for healthy controls were derived from a combination of the results from donor samples and the International HapMap Project data [19].

Informed consent was obtained from all patients, donors, and healthy controls. Approval of the study was obtained from the Institutional Review Board of the Institute of Medical Science, University of Tokyo (IMSUT), Advanced Industrial Science and Technology (AIST), JMDP, and the Institute of Physical and Chemical Research (RIKEN).

2.2 Evaluation of GVHD

The severity of acute GVHD in the skin, liver, and gastrointestinal tract was graded according to established clinical and histopathological criteria [20]: grade 0, none; grade I, mild; grade II, modest; grade III, severe; and grade IV, severe.

2.3 Detection of *NOD2* genetic variants in severe acute GVHD patients

Thirty-seven DNA samples, including sixteen samples from eight patients and eight donors in which the patient developed GVHD grade IV, and twenty-one from patients with GVDH grade III (Table 1), were screened for polymorphisms in the *NOD2* gene by direct sequencing of the entire *NOD2* coding region using the DNA primers listed in Table 2. Sequencing was performed using an ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA) with BigDye Terminator RR Mix (Applied Biosystems).

2.4 Genotyping

DNA samples from recipient–donor pairs of GVHD grade 0–II and donors of grade III; and CD patients were genotyped for the *NOD2* SNP (rs1078327: R471C) by direct sequencing of the relevant region using the following primers: forward, CAACCTCAAGGGCTTCTCTG; reverse, CTGGGCTGAGAACACGTAGC.

2.5 NOD2 functional assay

Construction of the pMX-471C-NOD2 plasmid has been described previously [2, 21]. NF-κB activation assays were performed as previously detailed. Briefly, HEK293T cells were co-transfected, in triplicate, with 12 ng of the reporter construct pBVI-Luc, 120 ng of pEF-BOS-β-gal, and the indicated amount of each NOD2-expression plasmid. Muramyl dipeptide (synthetic MDP; Peptide Institute, Inc., Osaka, Japan) was added to the cultures in the presence of calcium phosphate to allow its entry into the cells. At 24 h after transfection, cell extracts were prepared, and the relative luciferase activity was measured. The results were

Table 2 Primer sequences for amplifying the coding region of NOD2 gene

Exon	Forward primer	Reverse primer
2	TTCTGCTGGGGCTGACTTGC	AATCCCACGGACCAAGTTAC
3	AGTTGCAGTAATCAGTAAGC	CAAGGAAATTGAGTCATAGG
4 (upstream 1)	ACATTTCTCCCACCTTACAG	AACCTGAACTTGAACTCGTC
4 (upstream 2)	CTCAATGACGATGCGGACAC	CATGAGAAGACAGGCAGGTG
4 (midstream)	CCCTGCTCTTCAACCTTCTG	AGCAAAGCTGGTGGCACATC
4 (downstream 1)	TCCTGCACCTGGGCAGACTG	GGCGGGATGGAGTGGAAGTG
4 (downstream 2)	GCCACCAGCTTTGCTCAGAC	CTCGGTGCTCCCACACTTAG
5	TTCCAGGGTTCTTTAGTAGG	GTGCACAGCCGTCAGTCAAT
6	TGGGAAGCTGTGAGTGATGG	TACGGGCTAAAGGTCAAAAG
7	GCCTGGAATTGTCACACTGC	TCCAGGTGGTTTTAATATGC
8	GGAAGCGTATCTGAACTAAG	TACTCCATTGCCTAACATTG
9	GAATTTTGCCCTCCATAGGT	TCAATCACTCAATCATCCAC
10	TCGACTTCCCCTTATGTATC	TTTCCCTCATTGATAACTGG
11	CTGATGGTACTGAGCCTTTG	CCCCATTCCTACACTATCTC
12	TTCCTGAGCTGCCCTGGTTG	ACTGATGCTCCCTCCTCTGC

normalized for transfection efficiency using the values obtained from pEF-BOS-β-gal.

NOD2 protein expression levels were determined in HEK293T cells transfected, using Lipofectamine LTX (according to the manufacturer's instructions) with 950 ng of either pMX-471R-NOD2 or pMX-471C-NOD2, together with 50 ng of a plasmid encoding a FLAG-tagged control protein. Twenty-four hours after transfection, cells were lysed in 1% Triton X-100 lysis buffer, and the extracted proteins were resolved by 10% SDS-PAGE. Target proteins were detected by immunoblotting with anti-FLAG (Sigma-Aldrich, Tokyo, Japan) and anti-NOD2 (Roche, Tokyo, Japan) monoclonal antibodies.

2.6 Statistical analysis

The genotype frequencies in the cases and the controls were compared. Odds ratios were generated from 2×2 contingency tables, and statistical significance was determined using Fisher's exact test or χ^2 test with Yates correction. A p value of <0.05 was considered statistically significant.

3 Results

3.1 *NOD2* polymorphisms in severe acute GVHD patients

To investigate a possible association between polymorphisms in the *NOD2* gene and susceptibility to GVHD in a Japanese patient population, we conducted a systematic search for the presence of polymorphic variants of *NOD2* by directly sequencing the entire coding region in a total of 37 DNA samples obtained from eight patients and eight donors in which the patients developed grade IV severe acute GVHD, and 21 patients with grade III severe acute GVHD (Table 1). The analysis revealed one SNP (rs1078327) at nucleotide 1411 of the *NOD2* gene in two patients with grade IV severe acute GVHD. This SNP, a C-to-T substitution, would result in the replacement of an arginine residue with a cysteine at amino acid 471 (R471C) in the nucleotide-binding domain of NOD2. None of the three major NOD2 mutants (R702W, G908R, and 1007insC) reported previously in Western patient populations was found in our patients.

3.2 Functional analysis of *NOD2* SNP

To examine whether the R471C change affects the expression and/or function of NOD2, we constructed a NOD2 cDNA containing the R471C substitution. Immunoblot analysis of the expressed protein levels of the two variants revealed almost equal expression of both 471C- and 471R-NOD2 (Fig. 1a). We then evaluated the functional difference between 471C- and 471R-NOD2 by analyzing the NF-κB activity in response to MDP in HEK293T cells transfected with the vectors encoding the two variants. The activation of NF-κB was comparable between the 471C- and 471R-NOD2 proteins (Fig. 1b).

3.3 Association analysis of the R471C SNP of the *NOD2* gene

Because the R471C SNP was the only polymorphism in the *NOD2* gene detected in patients with severe acute GVHD, we analyzed the prevalence of the SNP by directly sequencing the relevant region of the gene in the remaining

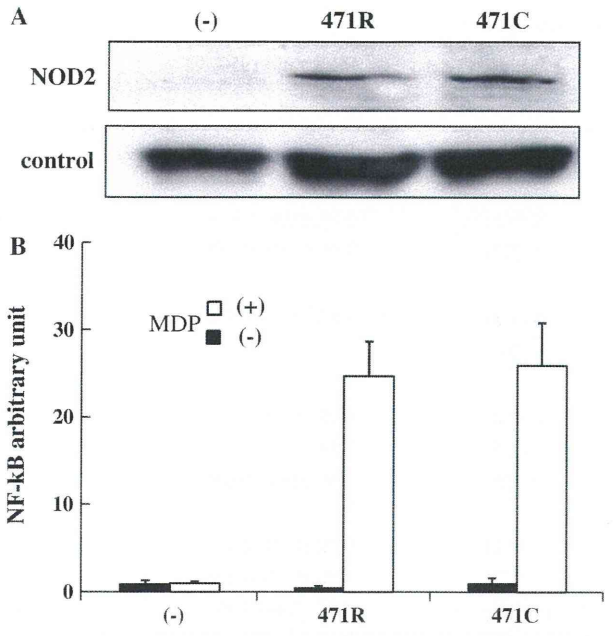

Fig. 1 Expression of NOD2 (R471C) and its response to MDP. **a** HEK293T cells were cotransfected with 950 ng 471R-NOD2 or 471C-NOD2 expression plasmid and 50 ng control plasmid encoding FLAG-tagged protein. Cell lysates were separated by 10% SDS-PAGE, and the resolved proteins were immunoblotted with anti-NOD2 and anti-FLAG antibodies. Representative results are shown. **b** HEK293T cells were transiently transfected with the reporter construct, alone (control) or in combination with 100 ng 471R-NOD2 or 471C-NOD2 expression plasmid. The transfected cells were incubated with (+) or without (−) MDP. Values represent the mean ± SD of normalized data from triplicate cultures

samples. The prevalence of this SNP was low in both healthy controls and patients with different grades of GVHD (2.7, 6.2, 6.9, 3.5, and 0% in healthy controls, grade 0–II patient, grade III–IV patients, grade 0–II donor, and grade III–IV donor, respectively (Table 3). The association between the allelic frequencies of the SNP and severity of GVHD was examined statistically (Table 4). The allelic frequencies of *NOD2* SNP in healthy control were not significantly different from those of both grade III–IV patients and grade III–IV donor. Thus, we detected no genetic alterations in the *NOD2* gene that influence the susceptibility to severe GVHD in our Japanese population. No conclusion could be reached regarding the possible influence of the R471C SNP on the prognosis (e.g., risk of relapse, overall survival rate) because of a lack of statistical significance, owing to the limited number of SNP samples available for analysis in this study.

We further examined the association between the *NOD2* R471C SNP and underlying diseases in the transplant patients in this study. The *NOD2* R471C SNP was present in 8.3, 3.0, and 7.0% of patients with acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and chronic myeloid leukemia (CML) patients, respectively (Table 3). The prevalence of the 471C allele was 5.2, 1.5, and 3.5% in AML, ALL, and CML, respectively (Table 4). Statistical analysis revealed a weak association between the 471C allele and AML ($p = 0.029$, odds ratio 4.08, 95% CI 1.22–13.67). These results suggest the possibility of weak association between the *NOD2* R471C SNP and susceptibility of AML; however, further analysis using more AML patients would be necessary for exactly deciding the association.

Table 3 Prevalence of NOD2 SNP in GVHD, leukemia, and Crohn's disease

	The number (%) of cases		
	Total	471R	471C
Healthy control	226 (100)	220 (97.3)	6 (2.7)
Patients			
0 + I + II	113 (100)	106 (93.8)	7 (6.2)
III + IV	29 (100)	27 (93.1)	2 (6.9)
Donor			
0 + I + II	113 (100)	109 (96.5)	4 (3.5)
III + IV	29 (100)	29 (100)	0 (0)
Underlying disease			
AML	48 (100)	44 (91.7)	4 (8.3)
ALL	33 (100)	32 (97.0)	1 (3.0)
CML	43 (100)	40 (93.0)	3 (7.0)
Crohn's disease			
Ileitis type	191 (100)	187 (97.9)	4 (2.1)
Other type	288 (100)	276 (95.8)	12 (4.2)
Total	479 (100)	463 (96.7)	16 (3.3)

AML acute myeloid leukemia, *ALL* acute lymphocytic leukemia, *CML* chronic myeloid leukemia

We also speculated that the R471C SNP may also be associated with susceptibility to CD, given that three major NOD2 mutants (R702W, G908R, and 1007insC) are significantly associated with both ileitis type CD and malignant diseases in Caucasians [6–8]. The prevalence of the 471C SNP was 2.1% in ileitis type CD patients (Table 3). Statistical analysis showed lack of significant association between the prevalence of SNP allele and susceptibility of ileitis type CD ($p = 0.761$, odds ratio 0.79, 95% CI 0.22–2.81) (Table 4).

4 Discussion

To investigate the possible role of NOD2 in the pathogenesis of GVHD in a Japanese patient population, we examined the *NOD2* gene in a large clinical cohort. We found no dysfunctional NOD2 variants in severe acute GVHD patients, implying that NOD2 is not a major

Table 4 Association study of NOD2 genotype with GVHD, leukemia, and Crohn's disease

	The number (%) of alleles			Odds ratio (95% Cl)	*P*
	Total	471R	471C		
Healthy control	452 (100)	446 (98.3)	6 (1.3)	1	
Patients					
0 + I + II	226 (100)	218 (96.5)	8 (3.5)	2.73 (0.94–7.96)	0.082
III + IV	58 (100)	56 (96.6)	2 (3.4)	2.66 (0.52–13.47)	0.228
Donor					
0 + I + II	226 (100)	222 (98.2)	4 (1.8)	1.34 (0.37–4.80)	0.738
III + IV	58 (100)	58 (100)	0 (0)	–	–
Underlying disease					
AML	96 (100)	91 (94.8)	5 (5.2)	4.08 (1.22–13.67)	0.029
ALL	66 (100)	65 (98.5)	1 (1.5)	1.14 (0.14–9.65)	1.000
CML	86 (100)	83 (96.5)	3 (3.5)	2.69 (0.66–10.96)	0.161
Crohn's disease					
Ileitis type	382 (100)	378 (99.0)	4 (1.0)	0.79 (0.22–2.81)	0.761
Other type	576 (100)	564 (97.9)	12 (2.1)	1.58 (0.59–4.25)	0.474
Total	958 (100)	942 (98.3)	16 (1.7)	1.26 (0.49–3.25)	0.819

AML acute myeloid leukemia, *ALL* acute lymphocytic leukemia, *CML* chronic myeloid leukemia

contributor to susceptibility to severe acute GVHD in our Japanese population.

Several possible mechanisms of NOD2 to affect the severity of acute GVHD have been described. Beelen et al. [22] reported that the intestinal bacterial microflora affects the pathogenesis of acute GVHD because antimicrobial therapy against intestinal bacteria in bone marrow transplant patients significantly reduces the severity of acute GVHD. NOD2 was hypothesized to influence the severity of acute GVHD by affecting the microflora because of the association of the type of gastrointestinal decontamination and the prognostic significance of NOD2 status [10]. Recently, Penack et al. [23] demonstrated the underlying mechanism of NOD2 to influence the severity of acute GVHD in experimental allogeneic bone marrow transplantation model. They hypothesized that NOD2 can negatively regulate the activity and function of host dendritic cells, resulting in increased alloactivation and proliferation of donor T cells in NOD2-impaired bone marrow transplant recipients.

Ethnic differences in the genetic susceptibility to CD have been reported for Caucasian and Japanese populations. CD is a chronic inflammatory disorder in the gastrointestinal tract. The association between genetic variants of *NOD2* and susceptibility to CD was first reported in Caucasians [5]. The three major variants relevant to CD were SNP8 (R702W, rs2066844), SNP12 (G908R, rs2066845), and a truncated mutant of SNP13 (1007fs, rs2066847) by a cytosine insertion. We previously characterized the function of the variants and found that SNP8 and SNP12, of the amino acid substitution type, showed a partially decreased response to ligand MDP, whereas the truncated mutant SNP13 showed a complete loss of responsiveness [2]. In a previous study comparing the NOD2 status in 906 CD patients with 206 healthy controls, the percentages of SNP8, SNP12, and SNP13 in the controls were 4.4, 1.0, and 1.9%, respectively, and 10.8, 6.1, and 10.6% in CD patients [24]. Some studies have reported that none of the common NOD2 variants that have been associated with CD in Caucasians are present in Japanese CD patients [25, 26]. Also, a mutational analysis of the entire coding region of *NOD2* in a Japanese CD population showed no common genetic variants [27]. HapMap data also provide evidence that SNP8 and SNP12 are absent in Japanese and Chinese populations. These results imply that the *NOD2* gene is not a major contributor to CD susceptibility in the Japanese population. The failure of the present study to detect the three major SNPs in the samples relating in severe acute GVHD is plausibly explained by no previous findings that these three SNPs are present in the Japanese population.

The initial report by Holler et al. was derived from data from 169 recipient–donor pairs from 2 independent cohorts in Europe. This report indicated that the presence of any of the three polymorphisms of *NOD2* (SNP8, SNP12, and SNP13) in donor or patient was associated with increased susceptibility to severe acute GVHD [9, 10]. Holler et al. suggested the need for future risk assessment or even donor selection through *NOD2* typing. These findings prompted studies by several groups to validate the link between the three major *NOD2* SNPs and susceptibility to acute GVHD. In particular, in their study of 85 Dutch recipients

of T-cell-depleted HSCT, Velden et al. [11] found that *NOD2* polymorphisms affected the severity of acute GVHD and transplantation-related mortality, which, in turn, had a significant impact on treatment outcome. Further, Elmaagacli et al. [12] showed higher incidence of severe acute GVHD in patients with *NOD2* gene variants, in a cohort of 304 patients in Germany. Although these findings support at least some of the earlier conclusions that the three major *NOD2* SNPs are risk factors for severe acute GVHD, other work has reported contrary findings. In a study of a cohort of 231 children in Germany, Gruhn et al. [13] reported no association between any of the *NOD2* polymorphisms and GVHD in either donor or recipient. Similarly in a study of a cohort of 304 patients in Germany, Wermke et al. [14] reported that the *NOD2* genotype of patient and donor was not associated with the occurrence of acute GVHD. Nguyen [15] showed no association of acute GVHD incidence with three intestinal bowel disease-associated alleles including NOD2, IL23R, and immunity-related GTPase family, M (IRGM) in a cohort of 390 patients in United States. In these reports, the authors have suggested that the contradictory results might be explained by ethnic diversity in the patient population [13], use of reduced-intensity conditioning therapy which causes less severe acute organ damage [14], and a lower incidence of NOD2 variants [15]. A comprehensive understanding of the role NOD2 in the pathogenesis of GVHD would require a prospective investigation.

The discrepancy in the susceptibility of acute GVHD among different ethnic populations has been discussed [28, 29]. Studies in a Japanese population reported the lower incidence of acute GVHD in Japanese bone marrow transplant patients and demonstrated that a genetic difference derived from HLA haplotype itself is associated with acute GVHD. The study from the Center for International Blood and Marrow Transplant Research (CIBMTR) compared the results of bone marrow transplantations for leukemia among different ethnic populations including Japanese, white Americans, African Americans, Scandinavians, and Irish [30]. Results of that study showed that Japanese or Scandinavian cohorts were at significantly lower risk for acute GVHD than other cohorts. They speculated that the degree of HLA diversity influences the risk of acute GVHD because Japanese population had the most restricted HLA representation among those cohorts. The results of our study which showed lack of impaired function of NOD2 may explain the relatively low incidence of severe acute GVHD in the Japanese population. However, further genetic variants of several factors such as IL10 promoter [31], Toll-like receptor 9 [32], IL23 receptor [33], and bactericidal permeability-increasing protein (BPI) [14] are recently reported to influence the degree of acute GVHD. Genetic analysis of these factors, in addition to NOD2 analysis, would be necessary to identify the reason for the relatively low incidence of GVHD in the Japanese population.

Our results suggest that the R471C substitution is weakly associated with AML, but not with acute lymphoblastic leukemia. This difference may be explained by the results of recent studies that revealed the important role of NOD2 in the differentiation of bone marrow $CD34^+$ hematopoietic cells: NOD2 mediates the induction of cytokines indispensable for cell differentiation towards the myeloid lineage [34]. It is possible that the R471C substitution causes abnormal cell differentiation by altering cytokine production, thereby increasing the incidence of AML.

The retrospective sample and the small study group might limit the present study. However, all available (142 pairs) DNA samples, from 2547 patients who underwent unrelated BMT between January 1993 and March 2000 and were registered to JMDP, were analyzed. In particular, we sequenced the entire coding region of 37 samples including eight patients and eight donors of GVHD grade IV and 21 patients of GVHD grade III. We infer that the status of genetic variants of *NOD2* gene might not be relevant to susceptibility to severe acute GVHD in the Japanese population, because our results found no genetic variant linked with the severity of GVHD.

In conclusion, the results of the present study indicate that NOD2 is not a major contributor to susceptibility to GVHD in a Japanese cohort.

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References

1. Inohara N, Chamaillard M, McDonald C, Nuñez G. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. Annu Rev Biochem. 2004;74:355–83.
2. Bonen DK, Ogura Y, Nicolae DL, Inohara N, Saab L, Tanabe T, et al. Crohn's disease-associated variants share a signaling defect in response to lipopolysaccharide and peptidoglycan. Gastroenterology. 2003;124(1):140–6.
3. Tanabe T, Chamaillard M, Ogura Y, Zhu L, Qiu S, Masumoto J, et al. Regulatory regions and critical residues of NOD2 involved in muramyl dipeptide recognition. EMBO J. 2004;23(7):1587–97.
4. Tanabe T, Ishige I, Suzuki Y, Aita Y, Furukawa A, Ishige Y, et al. Sarcoidosis and NOD1 variation with impaired recognition of intracellular Propionibacterium acnes. Biochim Biophys Acta. 2006;1762(9):794–801.
5. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature. 2001;411(6837):603–6.
6. Kurzawski G, Suchy J, Kladny J, Grabowska E, Mierzejewski M, Jakubowska A, et al. The NOD2 3020insC mutation and the risk of colorectal cancer. Cancer Res. 2004;64(5):1604–6.
7. Papaconstantinou I, Theodoropoulos G, Gazouli M, Panoussopoulos D, Mantzaris GJ, Felekouras E, et al. Association between

mutations in the CARD15/NOD2 gene and colorectal cancer in a Greek population. Int J Cancer. 2005;114(3):433–5.
8. Huzarski T, Lener M, Domagala W, Gronwald J, Byrski T, Kurzawski G, et al. The 3020insC allele of NOD2 predisposes to early-onset breast cancer. Breast Cancer Res Treat. 2005;89(1):91–3.
9. Holler E, Rogler G, Herfarth H, Brenmoehl J, Wild PJ, Hahn J, et al. Both donor and patient NOD2/CARD15 mutations associate with transplant-related mortality and GvHD following allogeneic stem cell transplantation. Blood. 2004;104(3):889–94.
10. Holler E, Rogler G, Brenmoehl J, Hahn J, Herfarth H, Greinix H, et al. Prognostic significance of NOD2/CARD15 variants in HLA-identical sibling hematopoietic stem cell transplantation: Effect on long-term outcome is confirmed in 2 independent cohorts and may be modulated by the type of gastrointestinal decontamination. Blood. 2006;107(10):4189–93.
11. van der Velden WJ, Blijlevens NM, Maas FM, Schaap NP, Jansen JH, van der Reijden BA, et al. NOD2 polymorphisms predict severe acute graft-versus-host and treatment-related mortality in T-cell-depleted haematopoietic stem cell transplantation. Bone Marrow Transplant. 2009;44(4):243–8.
12. Elmaagacli AH, Steckel N, Ditschkowski M, Hegerfeldt Y, Ottinger H, Trenschel R, et al. Toll-like receptor 9, NOD2 and IL23R gene polymorphisms influenced outcome in AML patients transplanted from HLA-identical sibling donors. Bone Marrow Transplant. 2010. doi:10.1038/bmt.2010.166.
13. Gruhn B, Intek J, Pfaffendorf N, Zell R, Corbacioglu S, Zintl F, et al. Polymorphism of interleukin-23 receptor gene but not of NOD2/CARD15 is associated with graft-versus-host disease after hematopoietic stem cell transplantation in children. Biol Blood Marrow Transplant. 2009;15(12):1571–7.
14. Wermke M, Maiwald S, Schmelz R, Thiede C, Schetelig J, Ehninger G, et al. Genetic variations of interleukin-23R (1143A>G) and BPI (A645G), but not of NOD2, are associated with acute graft-versus-host disease after allogeneic transplantation. Biol Blood Marrow Transplant. 2010;16(12):1718–27.
15. Nguyen Y, Al-Lehibi A, Gorbe E, Li E, Haagenson M, Wang T, et al. Insufficient evidence for association of NOD2/CARD15 or other inflammatory bowel disease-associated markers on GVHD incidence or other adverse outcomes in T-replete, unrelated donor transplantation. Blood. 2010;115(17):3625–31.
16. Sasazuki T, Juji T, Morishima Y, Kinukawa N, Kashiwabara H, Inoko H, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor Japan marrow donor program. N Engl J Med. 1998;339(17):1177–85.
17. Morishima Y, Sasazuki T, Inoko H, Juji T, Akaza T, Yamamoto K, et al. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. Blood. 2002;99(11):4200–6.
18. Yamazaki K, Onouchi Y, Takazoe M, Kubo M, Nakamura Y, Hata A. Association analysis of genetic variants in IL23R, ATG16L1 and 5p13.1 loci with Crohn's disease in Japanese patients. J Hum Genet. 2007;52(7):575–83.
19. International HapMap Consortium. The International HapMap Project. Nature. 2003;426(6968):789–96.
20. Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J, et al. Consensus conference on acute GVHD grading. Bone Marrow Transplant. 1994;15:825–8.
21. Onishi M, Kinoshita S, Morikawa Y, Shibuya A, Phillips J, Lanier LL, et al. Applications of retrovirus-mediated expression cloning. Exp Hematol. 1996;24:324–9.
22. Beelen DW, Elmaagacli A, Muller KD, Hirche H, Schaefer UW. Influence of intestinal bacterial decontamination using metronidazole and ciprofloxacin or ciprofloxacin alone on the development of acute graft-versus-host disease after marrow transplantation in patients with hematologic malignancies: final results and long-term follow-up of an open-label prospective randomized trial. Blood. 1999;93:3267–75.
23. Penack O, Smith OM, Cunningham-Bussel A, Liu X, Rao U, Yim N, et al. NOD2 regulates hematopoietic cell function during graft-versus-host disease. J Exp Med. 2010;115:2101–10.
24. Lesage S, Zouali H, Cézard JP, Colombel JF, Belaiche J, Almer S, et al. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. Am J Hum Genet. 2002;70(4):845–57.
25. Yamazaki K, Takazoe M, Tanaka T, Kazumori T, Nakamura Y. Absence of mutation in the NOD2/CARD15 gene among 483 Japanese patients with Crohn's disease. J Human Genet. 2002;47(9):469–72.
26. Inoue N, Tamura K, Kinouchi Y, Fukuda Y, Takahashi S, Ogura Y, et al. Lack of common NOD2 variants in Japanese patients with Crohn's disease. Gastroenterology. 2002;123:86–91.
27. Sugimura M, Kinouchi Y, Takahashi S, Aihara H, Takagi S, Negoro K, et al. CARD15/NOD2 mutational analysis in Japanese patients with Crohn's disease. Clin Genet. 2003;63(2):160–2.
28. Morishima Y, Morishita Y, Tanimoto M, Ohno R, Saito H, Horibe K, et al. Low incidence of acute graft-versus-host disease by the administration of methotrexate and cyclosporine in Japanese leukemia patients after bone marrow transplantation from human leukocyte antigen compatible siblings; possible role of genetic homogeneity. The Nagoya Bone Marrow Transplantation Group. Blood. 1989;74:2252–6.
29. Morishima S, Ogawa S, Matsubara A, Kawase T, Nannya Y, Kashiwase K, et al. Impact of highly conserved HLA haplotype on acute graft-versus-host disease. Blood. 2010;115:4664–70.
30. Oh H, Loberiza FR Jr, Zhang MJ, Ringdén O, Akiyama H, Asai T, et al. Comparison of graft-vs-host disease and survival after HLA-identical sibling bone marrow transplantation in ethnic populations. Blood. 2005;105(4):1408–16.
31. Lin MT, Storer B, Martin PJ, Tseng LH, Gooley T, Chen PJ, et al. Relation of an interleukin-10 promoter polymorphism to graft-versus-host disease and survival after hematopoietic-cell transplantation. N Engl J Med. 2003;349(23):2201–10.
32. Elmaagacli AH, Koldehoff M, Beelen DW. Improved outcome of hematopoietic SCT in patients with homozygous gene variant of Toll-like receptor 9 Bone. Marrow Transplant. 2009;44(5): 295–302.
33. Elmaagacli AH, Koldehoff M, Landt O, Beelen DW. Relation of an interleukin-23 receptor gene polymorphism to graft-versus-host disease after hematopoietic-cell transplantation. Bone Marrow Transplant. 2008;41(9):821–6.
34. Sioud M, Fløisand Y. NOD2/CARD15 on bone marrow CD34+ hematopoietic cells mediates induction of cytokines and cell differentiation. J Leukoc Biol. 2009;85(6):939–46.

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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References

Baccarani, M., Cortes, J., Pane, F., Niederwieser, D., Saglio, G., Apperley, J., Cervantes, F., Deininger, M., Gratwohl, A., Guilhot, F., Hochhaus, A., Horowitz, M., Hughes, T., Kantarjian, H., Larson, R., Radich, J., Simonsson, B., Silver, R.T., Goldman, J. & Hehlmann, R. (2009) Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. *Journal of Clinical Oncology*, **27**, 6041–6051.

Breccia, M., Orlandi, S.M., Latagliata, R., Grammatico, S., Diverio, D., Mancini, M., Loglisci, G., Salaroli, A., Federico, V., Santopietro, M. & Alimena, G. (2011) Suboptimal response to imatinib according to 2006–2009 European LeukaemiaNet criteria: a 'grey zone' at 3, 6 and 12 months identifies chronic myeloid leukaemia patients who need early intervention. *British Journal of Haematology*, **152**, 119–121.

Druker, B.J., Guilhot, F., O'Brien, S.G., Gathmann, I., Kantarjian, H., Gattermann, N., Deininger, M.W., Silver, R.T., Goldman, J.M., Stone, R.M., Cervantes, F., Hochhaus, A., Powell, B.L., Gabrilove, J.L., Rousselot, P., Reiffers, J., Cornelissen, J.J., Hughes, T., Agis, H., Fischer, T., Verhoef, G., Shepherd, J., Saglio, G., Gratwohl, A., Nielsen, J.L., Radich, J.P., Simonsson, B., Taylor, K., Baccarani, M., So, C., Letvak, L. & Larson, R.A. (2006) Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *New England Journal of Medicine*, **355**, 2408–2417.

Eliasson, L., Clifford, S., Barber, N. & Marin, D. (2010) Exploring chronic myeloid leukemia patients' reasons for not adhering to the oral anticancer drug imatinib as prescribed. *Leukemia Research*, Epub ahead of print; doi:10.1016/j.leukres.2010.10.017.

Kantarjian, H., Pasquini, R., Levy, V., Jootar, S., Holowiecki, J., Hamerschlak, N., Hughes, T., Bleickardt, E., Dejardin, D., Cortes, J. & Shah, N.P. (2009) dasatinib or high-dose imatinib for chronic-phase chronic myeloid leukemia resistant to imatinib at a dose of 400 to 600 milligrams daily two-year follow-up of a randomized phase 2 study (START-R). *Cancer*, **115**, 4136–4147.

de Lavallade, H., Apperley, J.F., Khorashad, J.S., Milojkovic, D., Reid, A.G., Bua, M., Szydlo, R., Olavarria, E., Kaeda, J., Goldman, J.M. & Marin, D. (2008) Imatinib for newly diagnosed patients with chronic myeloid leukemia: incidence of sustained responses in an intention-to-treat analysis. *Journal of Clinical Oncology*, **26**, 3358–3363.

Lucas, C.M., Wang, L., Austin, G.M., Knight, K., Watmough, S.J., Shwe, K.H., Dasgupta, R., Butt, N.M., Galvani, D., Hoyle, C.F., Seale, J.R. & Clark, R.E. (2008) A population study of imatinib in chronic myeloid leukaemia demonstrates lower efficacy than in clinical trials. *Leukemia*, **22**, 1963–1966.

Marin, D., Milojkovic, D., Olavarria, E., Khorashad, J.S., de Lavallade, H., Reid, A.G., Foroni, L., Rezvani, K., Bua, M., Dazzi, F., Pavlu, J., Klammer, M., Kaeda, J.S., Goldman, J.M. & Apperley, J.F. (2008) European LeukemiaNet criteria for failure or suboptimal response reliably identify patients with CML in early chronic phase treated with imatinib whose eventual outcome is poor. *Blood*, **112**, 4437–4444.

Marin, D., Bazeos, A., Mahon, F.X., Eliasson, L., Milojkovic, D., Bua, M., Apperley, J.F., Szydlo, R., Desai, R., Kozlowski, K., Paliompeis, C., Latham, V., Foroni, L., Molimard, M., Reid, A., Rezvani, K., de Lavallade, H., Guallar, C., Goldman, J. & Khorashad, J.S. (2010) Adherence is the critical factor for achieving molecular responses in patients with chronic myeloid leukemia who achieve complete cytogenetic responses on imatinib. *Journal of Clinical Oncology*, **28**, 2381–2388.

Saglio, G., Kim, D.W., Issaragrisil, S., le Coutre, P., Etienne, G., Lobo, C., Pasquini, R., Clark, R.E., Hochhaus, A., Hughes, T.P., Gallagher, N., Hoenekopp, A., Dong, M., Haque, A., Larson, R.A., Kantarjian, H.M. & Investigators, E. (2010) Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. *New England Journal of Medicine*, **362**, 2251–2259.

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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References

Creutzig, U., Reinhardt, D., Diekamp, S., Dworzak, M., Stary, J. & Zimmermann, M. (2005) AML patients with Down syndrome have a high cure rate with AML-BFM therapy with reduced dose intensity. *Leukemia*, **19**, 1355–1360.

Gamis, A.S., Woods, W.G., Alonzo, T.A., Buxton, A., Lange, B., Barnard, D.R., Gold, S. & Smith, F.O. (2003) Increased age at diagnosis has a significantly negative effect on outcome in children with Down syndrome and acute myeloid leukemia: a report from the Children's Cancer Group Study 2891. *Journal of Clinical Oncology*, **21**, 3415–3422.

Ghosh, L., Kumar, L., Seth, R. & Thavraj, V. (2009) Sustained remission achieved with ATRA and chemotherapy in second relapse of acute promyelocytic leukemia in Down syndrome. *Journal of Pediatric Hematology/oncology*, **31**, 539–540.

Hasle, H., Abrahamsson, J., Arola, M., Karow, A., O'Marcaigh, A., Reinhardt, D., Webb, D.K., van Wering, E., Zeller, B., Zwaan, C.M. & Vyas, P. (2008) Myeloid leukemia in children 4 years or older with Down syndrome often lacks GATA1 mutation and cytogenetics and risk of relapse are more akin to sporadic AML. *Leukemia*, **22**, 1428–1430.

Jain, D., Singh, T. & Arora, P. (2007) Down syndrome with microgranular variant of acute promyelocytic leukemia in a child: a case report. *Journal of Medical Case Reports*, **1**, 147.

Kudo, K., Kojima, S., Tabuchi, K., Yabe, H., Tawa, A., Imaizumi, M., Hanada, R., Hamamoto, K., Kobayashi, R., Morimoto, A., Nakayama, H., Tsuchida, M., Horibe, K., Kigasawa, H. & Tsukimoto, I. (2007) Prospective study of a pirarubicin, intermediate-dose cytarabine, and etoposide regimen in children with Down syndrome and acute myeloid leukemia: the Japanese Childhood AML Cooperative Study Group. *Journal of Clinical Oncology*, **25**, 5442–5447.

Kurkjian, C., Patel, S., Kamble, R., Dunn, S.T., Kern, W. & Kharfan-Dabaja, M.A. (2006) Acute promyelocytic leukemia and constitutional trisomy 21. *Cancer Genetics and Cytogenetics*, **165**, 176–179.

Lange, B.J., Kobrinsky, N., Barnard, D.R., Arthur, D.C., Buckley, J.D., Howells, W.B., Gold, S., Sanders, J., Neudorf, S., Smith, F.O. & Woods, W.G. (1998) Distinctive demography, biology, and outcome of acute myeloid leukemia and myelodysplastic syndrome in children with Down syndrome: Children's Cancer Group Studies 2861 and 2891. *Blood*, **91**, 608–615.

Sanz, M.A., Martin, G., Gonzalez, M., Leon, A., Rayon, C., Rivas, C., Colomer, D., Amutio, E., Capote, F.J., Milone, G.A., De La Serna, J., Roman, J., Barragan, E., Bergua, J., Escoda, L., Parody, R., Negri, S., Calasanz, M.J. & Bolufer, P. (2004) Risk-adapted treatment of acute promyelocytic leukemia with all-trans-retinoic acid and anthracycline monochemotherapy: a multicenter study by the PETHEMA group. *Blood*, **103**, 1237–1243.

Zwaan, C.M., Kaspers, G.J., Pieters, R., Hahlen, K., Janka-Schaub, G.E., van Zantwijk, C.H., Huismans, D.R., de Vries, E., Rots, M.G., Peters, G.J., Jansen, G., Creutzig, U. & Veerman, A.J. (2002) Different drug sensitivity profiles of acute myeloid and lymphoblastic leukemia and normal peripheral blood mononuclear cells in children with and without Down syndrome. *Blood*, **99**, 245–251.

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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

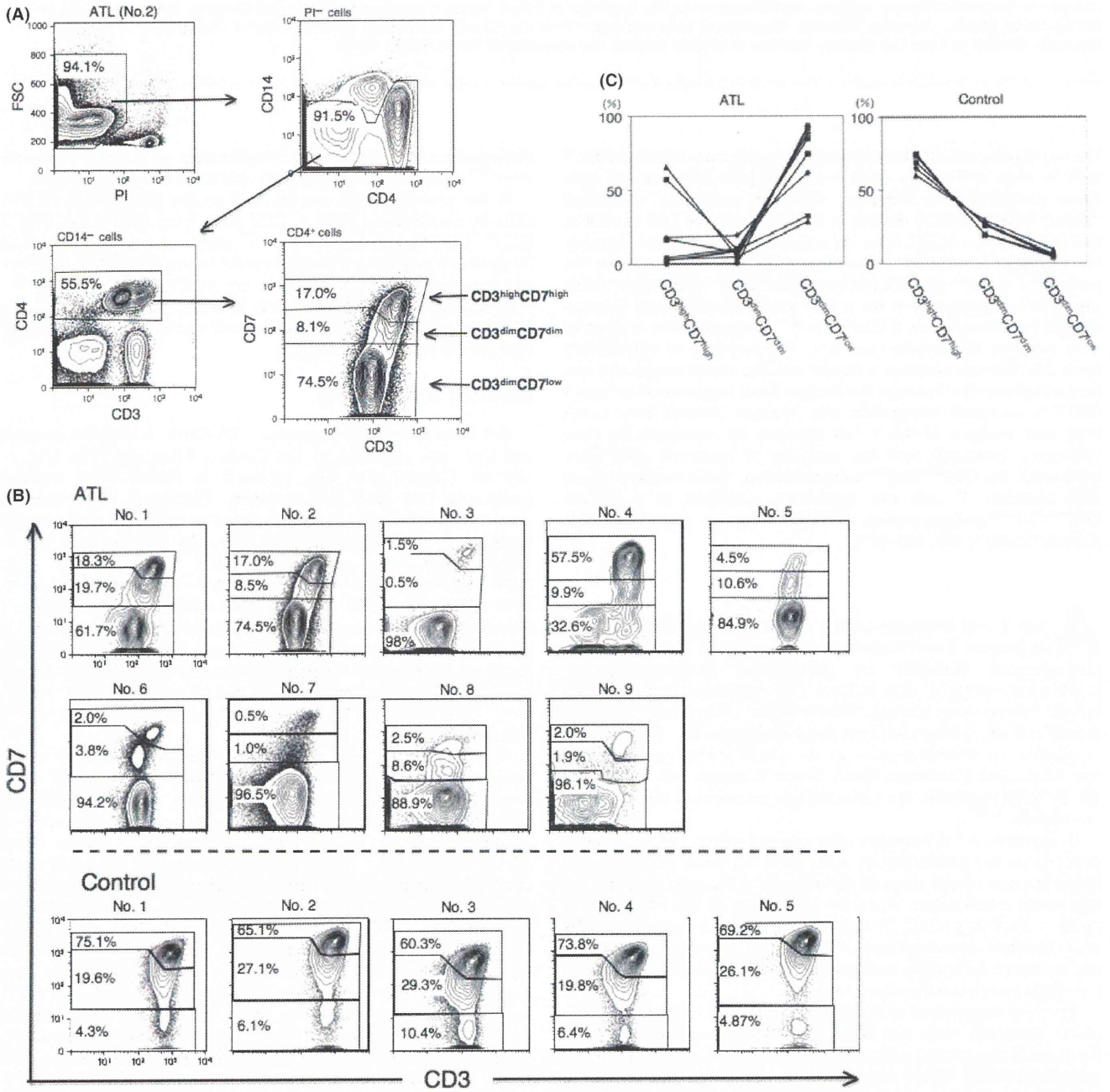

Fig. 1. CD3 *vs* CD7 plots from FACS analysis of patients with acute-type adult T-cell leukemia (ATL) and normal controls. (A) Representative flow cytometric analysis of a patient with acute-type ATL (patient no. 2). The CD3 *vs* CD7 plot in $CD4^+$ cells was constructed according to the gating procedure 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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Table 1. Clinical profile of nine acute-type ATL patients in the present study

No.	Age	Sex	WBC (/μL)	Lymph (%)	ATL cells† (%)	Organ involvement
1	60	M	5200	15.0	11.0	Skin
2	69	F	1600	43.5	9.0	Liver, LN, pleural effusion
3	61	M	18 620	24.7	43.7	Liver, uvea
4	59	F	6420	8.5	0.0	Liver, LN, skin
5	70	F	290	56.0	2.0	Liver, spleen, LN
6	60	F	4570	19.0	73.0	Skin
7	53	F	12 210	11.0	52.0	LN
8	74	F	6480	16.5	25.5	Liver, spleen, LN
9	63	F	34 810	21.5	33.5	Liver, spleen, LN, lung

†Proportion of ATL cells in the peripheral blood WBC evaluated by morphological examination. ATL, adult T-cell leukemia; LN, lymph nodes; Lymph, lymphocytes; WBC, white blood cells (normal range, 3500–9100/μL).

(Applied Biosystems) were subjected to 50 cycles of denaturation (95°C, 15 s) and annealing to extension (60°C, 1 min), following an initial Taq polymerase activation step (95°C, 10 min). The RNase P control reagent (Applied Biosystems) was used as an internal control for calculation of the input cell number (using VIC reporter dye). DNA extracted from TL-Om1 and normal human PBMC were used as positive and negative controls, respectively. The HTLV-I proviral load (%) was calculated as the copy number of the pX region per input cell number. To correct the deviation of acquired data in each experiment, data from TL-Om1 (positive control) were adjusted to 100% and the sample data was corrected by proportional calculation accordingly.

Inverse long PCR. For clonality analysis, inverse long PCR was performed. First, 1 μg of genomic DNA extracted from the FACS-sorted cells was digested with *Eco*RI, *Hin*dIII and *Pst*I at 37°C overnight. Purification of DNA fragments was performed using a QIAEX2 gel extraction kit (Qiagen). The purified DNA was self-ligated with T4 DNA ligase (Takara Bio, Otsu, Japan) at 16°C overnight. The circular DNA obtained from the *Eco*RI digestion fragment was then digested with *Mlu*I, which cuts the pX region of the HTLV-I genome and prevents amplification with the viral genome. Inverse long PCR was performed using Takara LA *Taq* polymerase (Takara Bio). The primer pairs for the *Eco*RI-treated template were: forward primer 5′-TGCCTGACCCTGCTTGCTCAACTCTACGTCTTTG-3′ and reverse primer 5′-AGTCTGGGCCCTGACCTTTTCAGACTTCTGTTTC-3′. For the *Hin*dIII-treated group, forward primer 5′-TAGCAGGAGTCTATAAAAGCGTGGAGACAG-3′ and reverse primer 5′-TGGGCAGGATTGCAGGGTTTAGAGTGG-3′ were used. For the *Pst*I-treated group, forward primer 5′-CAGCCCATTCTATAGCACTCTCCAGGAGAG-3′ and reverse primer 5′-CAGTCTCCAAACACGTAGACTGGGTATCCG-3 were used. Each 50-μL reaction mixture contained 0.4 mM of each dNTP, 25 mM $MgCl_2$, 10× LA PCR buffer II containing 20 mM Tris–HCl and 100 mM KCl, 0.5 mM primer, 2.5 U LA *Taq* polymerase and 50 ng of the processed genomic DNA. The reaction mixture of the *Eco*RI- or *Pst*I-treated group was subjected to 35 cycles of denaturation (94°C, 30 s) and annealing to extension (68°C, 8 min). For the *Hin*dIII group, the PCR conditions were denaturation (98°C, 30 s), annealing to extension (64°C, 10 min) for 5 cycles, followed by 30 cycles of denaturation (94°C, 30 s), annealing (64°C, 3 min) and extension (72°C, 15 min). Following PCR, the products were subjected to electrophoresis in 0.8% agarose gels. In the $CD3^{dim}CD7^{low}$ subpopulation from which a sufficient amount of DNA was extracted, PCR were performed in duplicate.

Cytospin and May–Giemsa staining. Cells enriched by cell sorting were washed twice with PBS. Aliquots of 100 μL of the cell suspension were mixed with 20 μL of 10% bovine serum albumin. The mixtures were centrifuged at 20*g* for 5 min onto glass slides. The fixed cells were air-dried and then subjected to May–Giemsa staining.

Statistical analyses. Data are expressed as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for statistical analyses, and $P < 0.05$ was taken to indicate statistical significance.

Results

Multi-color FACS, including CD3 *vs* CD7 plots, in patients with acute-type ATL. We constructed a gating procedure for flow cytometric analysis of acute-type ATL cells using a combination of CD3 and CD7. Figure 1A shows the representative flow cytometric data of an ATL sample (from patient no. 2 in Table 1). Dead cells (PI positive) were initially excluded on the forward scatter (FSC) *vs* PI plot. Next, monocytes ($CD4^{dim}$ $CD14^{+}$) were excluded on the CD4 *vs* CD14 plot. After $CD4^{+}$ T lymphocytes were gated on the CD3 *vs* CD4 plot, a CD3 *vs* CD7 plot was constructed. Based on the cell density and fluorescence intensity of CD3 and CD7, we designated three subpopulations on this plot: $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ (Fig. 1A). Using the same gating procedure, we analyzed nine patients with acute-type ATL and five normal controls (Fig. 1B). The patient characteristics analyzed in the present study are shown in Table 1. In normal controls, the expression pattern of CD3 *vs* CD7 was similar. The highest cell density was observed in the $CD3^{high}CD7^{high}$ subpopulation, and the $CD3^{dim}CD7^{dim}$ subpopulation was observed adjacent to it. The $CD3^{dim}CD7^{low}$

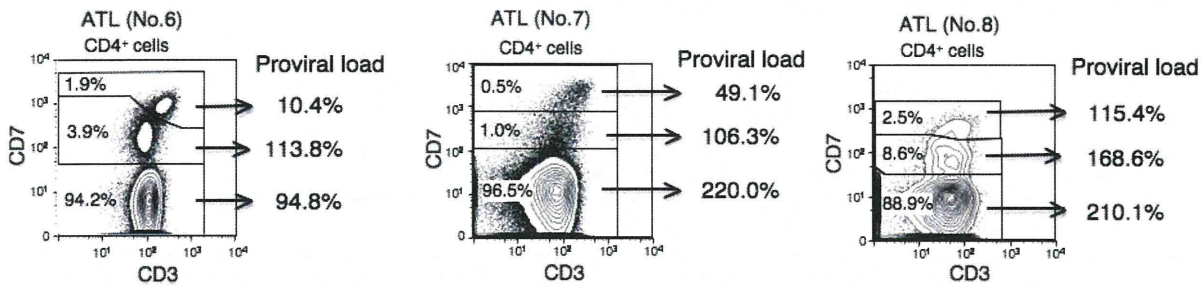

Fig. 2. Quantification of the human T-cell leukemia virus type 1 (HTLV-I) proviral load in $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations. Genomic DNA was extracted from the FACS-sorted cells of each subpopulation and subjected to real-time quantitative PCR Representative data of three cases (patients no. 6, 7 and 8) are shown.

subpopulation was a minor but distinct subpopulation. In contrast, the highest cell density was observed in the $CD3^{dim}CD7^{low}$ subpopulation in all acute-type ATL samples except for patient no. 4, from whom the sample was obtained under conditions of well-controlled ATL during chemotherapy. These subpopulations were distinct but the expression pattern of the CD3 *vs* CD7 plot, such as the degree of downregulation of CD3 and CD7, was variable among patients. The proportion of the $CD3^{dim}CD7^{low}$ subpopulation was significantly higher in acute-type ATL $CD4^+$ lymphocytes than in normal controls (Fig. 1C).

Analysis of the HTLV-I proviral load in $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations. We next estimated the HTLV-I proviral load by quantitative real-time PCR in each FACS-sorted subpopulation. Representative results from three patients with acute-type ATL (patients no. 6, 7 and 8) are shown in Figure 2. In all patient samples, HTLV-I proviral integration, analyzed by real-time PCR, was detected in all subpopulations. However, the proviral load (%) was significantly higher in $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations compared with the $CD3^{high}CD7^{high}$ subpopulation. The proviral load of the $CD3^{dim}CD7^{low}$ subpopulation in patients no. 7 and 8 was nearly 200%, indicating integration of two copies of the HTLV-I viral genome and that almost all of the cells were infected with HTLV-I. Similarly, in patient no. 6, the majority of the $CD3^{dim}CD7^{low}$ subpopulation was infected with HTLV-I. A substantial proportion of the $CD3^{dim}CD7^{dim}$ subpopulation was infected with HTLV-I in patients no. 7 and 8, and nearly all the cells in the same subpopulation in patient no. 6 were infected with HTLV-I.

Differences in the immunophenotype of $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations in patients with acute-type ATL. To further characterize these three subpopulations, we next examined CCR4 and CD25 expression in each subpopulation. Representative results of a normal control and a patient with acute-type ATL are shown in Figure 3A. The mean fluorescence intensities (MFI) of CD25 and CCR4 of each sub-

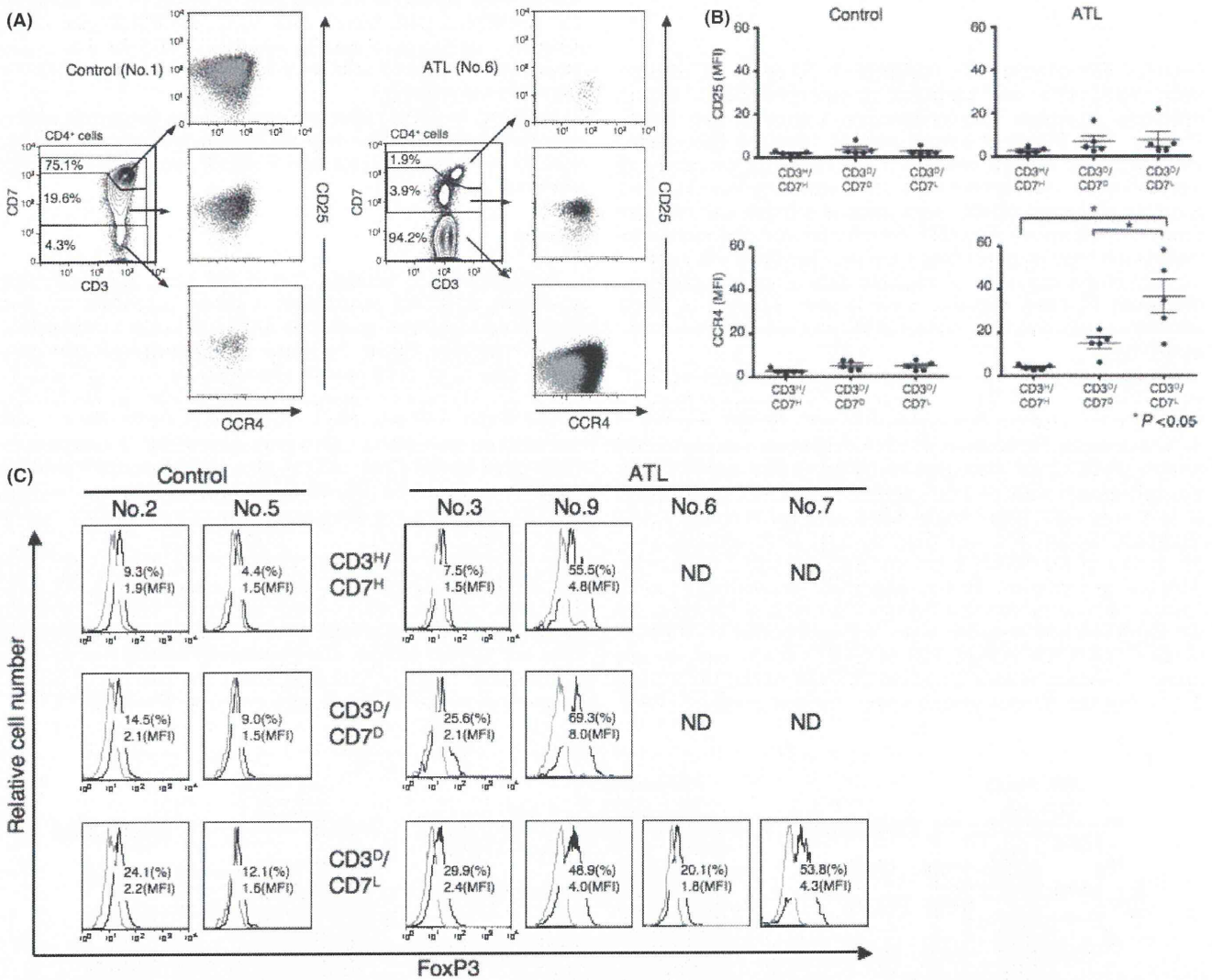

Fig. 3. Immunophenotypic analysis in $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations. (A) Expression of CCR4 and CD25 in each subpopulation. Representative FACS data of a normal control (no. 1) and a patient with adult T-cell leukemia (ATL) (no. 6) are shown Gray dots, isotype antibody-stained cells; black dots, specific antibody-stained cells. (B) Mean fluorescence intensity (MFI) of CD25 and CCR4 in each subpopulation from all normal controls and patients with ATL. The MFI is shown in arbitrary units defined as follows: MFI of specific antibody/MFI of isotype antibody. Each dot represents a sample $*P < 005$ by ANOVA. (C) Expression of FoxP3 in each subpopulation. ND, analysis could not be performed in the $CD3^{high}CD7^{high}$ and $CD3^{dim}CD7^{dim}$ subpopulations in patients no. 6 and 7 due to an insufficient number of cells.

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population in all patients with ATL and normal controls are shown in Figure 3B. Both CCR4 and CD25 expression levels were very low and maintained at similar levels throughout all subpopulations in normal control cells and in the $CD3^{high}CD7^{high}$ subpopulation of patients with ATL. In contrast, CCR4 expression was significantly upregulated in the $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations of patients with ATL compared with the $CD3^{high}CD7^{high}$ subpopulation. The expression of CD25 was also upregulated in these subpopulations but this difference was not significant ($P = 0.36$). The expression of Forkhead box P3 (FoxP3), a master regulator in the development and function of regulatory T (Treg) cells,[14] was also analyzed in some patients. As shown in Figure 3C, FoxP3 expression in the $CD3^{dim}CD7^{low}$ subpopulations was variably upregulated among patients. In addition, in patient no. 9, FoxP3 was upregulated in the $CD3^{high}CD7^{high}$ and $CD3^{dim}CD7^{dim}$ subpopulations.

Analysis of clonality in the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations by inverse long PCR. To further analyze the enrichment of ATL cells in the $CD3^{dim}CD7^{low}$ subpopulation, we estimated clonality in each FACS-sorted subpopulation by inverse long PCR in four patients with acute-type ATL (Fig. 4). An intense band, suggesting a major clone, was detected in the $CD3^{dim}CD7^{low}$ subpopulations in all patients. In the same subpopulation, multiple bands with weak intensity were also observed. As the levels of DNA extracted from the $CD3^{dim}CD7^{low}$ subpopulation were sufficient, we performed duplicate PCR in three patient samples (Fig. 4B–D). Detection of the major bands was consistent, but the presence of the minor bands was variable. In the $CD3^{dim}CD7^{dim}$ subpopulations, bands of the same size as those of the $CD3^{dim}CD7^{low}$ subpopulations were observed, indicating that a distinct population in the $CD3^{dim}CD7^{dim}$ subpopulations belonged to identical clones.

Clonality in the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations by flow cytometry-based TCR-Vβ repertoire analysis. To further confirm clonality and to evaluate the degree of enrichment in each subpopulation, we performed TCR-Vβ repertoire analysis by flow cytometry[15] in three ATL cases. The representative results are shown in Figure 5. In patient no. 3, over 95% of the $CD3^{dim}CD7^{low}$ subpopulation used specific TCR-Vβ (Vβ9) and their proportion was quite low in the $CD3^{high}CD7^{high}$ and $CD3^{dim}CD7^{dim}$ subpopulations. In addition, in the two other cases, over 90% of cells in the $CD3^{dim}CD7^{low}$ subpopulation used the same TCR-Vβ (data not shown). These results indicate that ATL cells are highly purified in the $CD3^{dim}CD7^{low}$ subpopulation.

Differences in morphology of the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$, and $CD3^{dim}CD7^{low}$ subpopulations in patients with acute-type ATL. We reviewed the glass-slide specimens of FACS-sorted samples to evaluate the morphology of each subpopulation on the CD3 *vs* CD7 plots. Representative results for two patients (no. 6 and 7) are shown in Figure 6A. In both patients, atypical lymphocytes with notched nuclei and/or basophilic cytoplasm were observed in all three subpopulations. In contrast, abnormal lymphocytes, including cells with multilobulated nuclei (flower cells) were mainly observed in the $CD3^{dim}CD7^{low}$ subpopulation in patient no. 6 (Fig. 6, left) and in the $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations in patient no. 7 (Fig. 6, right panel).

Discussion

To investigate the characteristics of ATL cells, the purification of tumor cells is essential. In the present study, we successfully discriminated the $CD3^{dim}CD7^{low}$ subpopulation in $CD4^+$ T cells in the peripheral blood of patients with acute-type ATL by constructing a CD3 *vs* CD7 plot of $CD4^+$ T cells from multi-color FACS (Fig. 1). Previously, Yokote *et al.*[10] reported that $CD3^{low}$ gating facilitated the discrimination of ATL cells by flow cytometry. If we constructed a CD4 *vs* either CD3 or CD7 plot, in which the downregulated cell subpopulation was not clearly separated, then we could not define distinct cell subpopu-

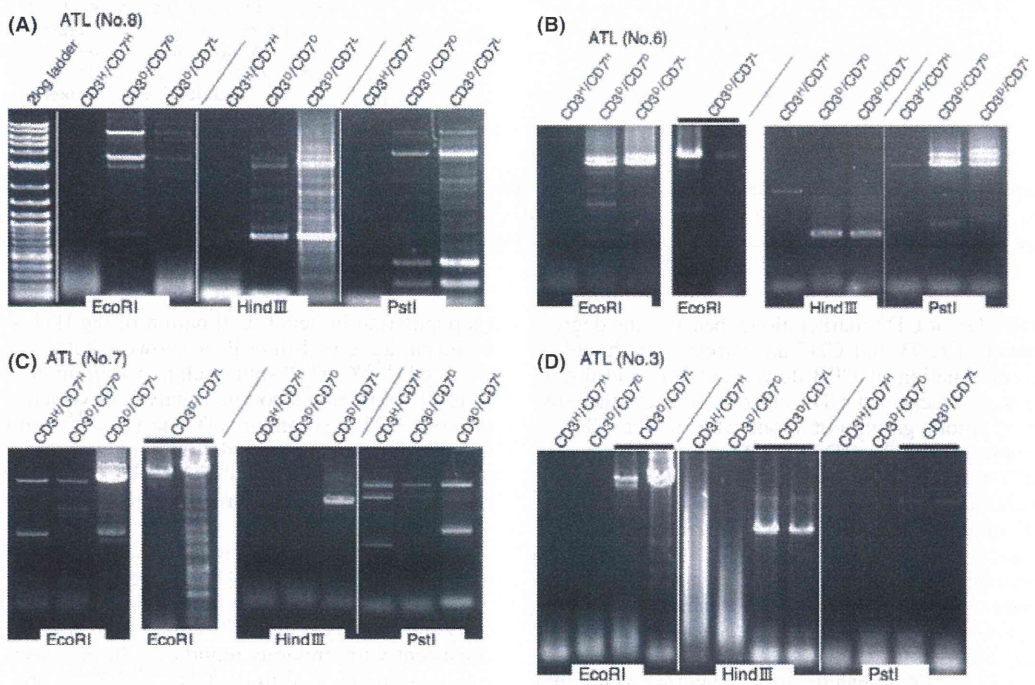

Fig. 4. Analysis of clonality in the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations using inverse long PCR. (A–D) Genomic DNA was extracted from FACS-sorted cells of each subpopulation and subjected to inverse long PCR. Representative data of four cases (patients no. 3, 6, 7 and 8) are shown For the $CD3^{dim}CD7^{low}$ subpopulations of patients no. 3, 6 and 7, PCR was performed in duplicate (black bars). ATL, adult T-cell leukemia.

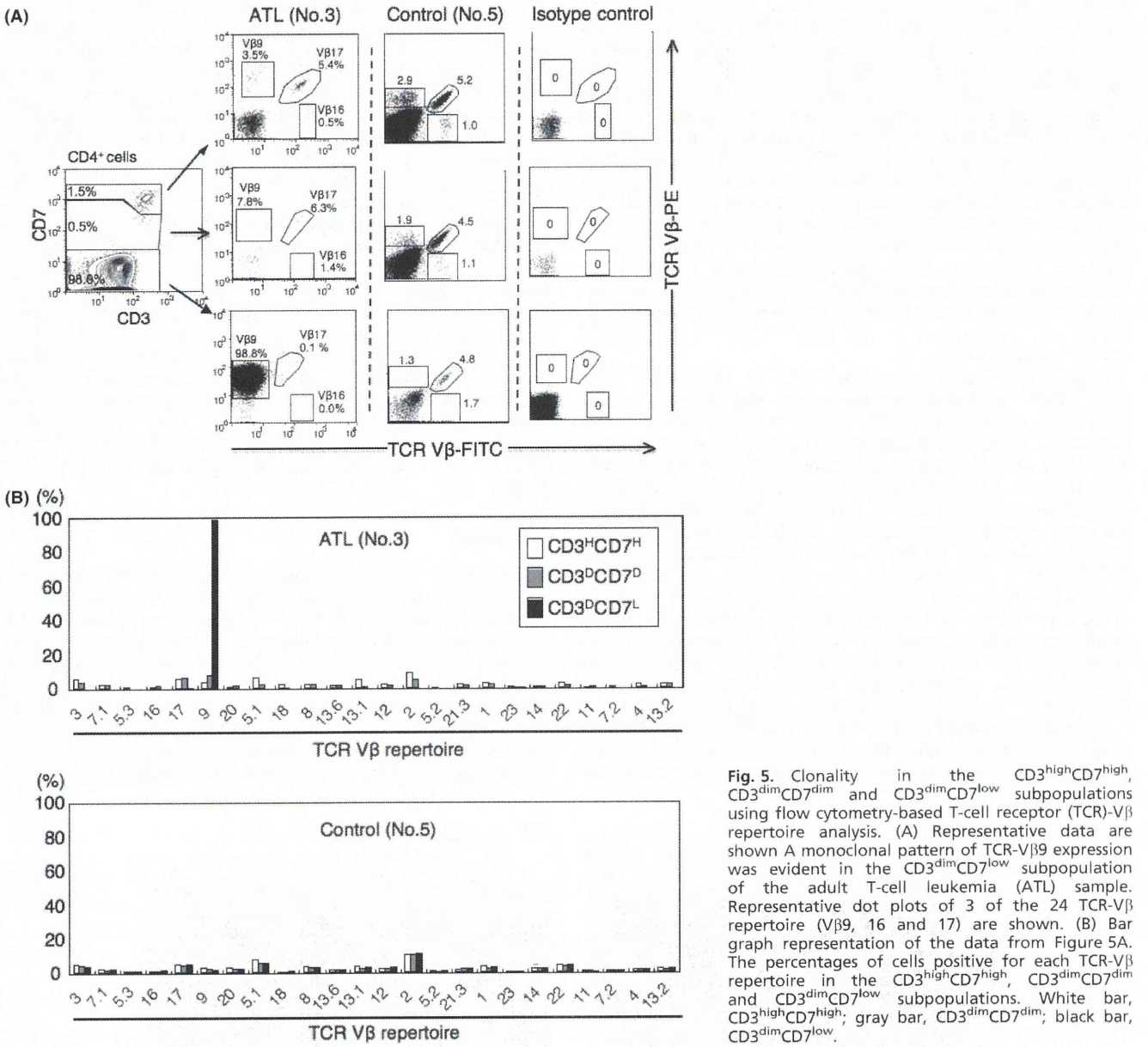

Fig. 5. Clonality in the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations using flow cytometry-based T-cell receptor (TCR)-Vβ repertoire analysis. (A) Representative data are shown A monoclonal pattern of TCR-Vβ9 expression was evident in the $CD3^{dim}CD7^{low}$ subpopulation of the adult T-cell leukemia (ATL) sample. Representative dot plots of 3 of the 24 TCR-Vβ repertoire (Vβ9, 16 and 17) are shown. (B) Bar graph representation of the data from Figure 5A. The percentages of cells positive for each TCR-Vβ repertoire in the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations. White bar, $CD3^{high}CD7^{high}$; gray bar, $CD3^{dim}CD7^{dim}$; black bar, $CD3^{dim}CD7^{low}$.

lations using the CD3 or CD7 marker alone, because the degrees of downregulation of CD3 and CD7 are variable. It should be noted that the combination of CD3 downregulation and diminished expression or absence of CD7 clearly indicates this subpopulation. In addition, gating-out monocytes in the CD4 *vs* CD14 plot is important for the CD3 *vs* CD7 plot because monocytes were CD3/CD7 dull-positive based on the nonspecific binding of the antibody.

A substantial subpopulation of T cells has been reported to be CD7-deficient under physiological[16,17] and certain pathological conditions, including autoimmune disorders and viral infection.[18–22] Consistent with these reports, the present study indicated that the proportion of $CD4^+CD7^-$ T cells in the peripheral blood of healthy adults is up to 10% (Fig. 1B,C). In ATL samples, the CD3 *vs* CD7 plot revealed various patterns, which may reflect the differences in clinical characteristics of each patient; however, the $CD3^{dim}CD7^{low}$ subpopulation, which was a minor population in the normal controls, was prominent in all ATL samples (Fig. 1B,C). These results prompted us to study this subpopulation in detail. Estimation of the HTLV-I proviral load by quantitative real-time PCR showed that the majority of cells in the $CD3^{dim}CD7^{low}$ subpopulation were infected with HTLV-I (Fig. 2). Immunophenotypic analysis revealed that the expression of CD25, a common ATL marker,[9,23] and CCR4, reported to be expressed in around 90% of cases of ATL,[24,25] were upregulated in the $CD3^{dim}CD7^{low}$ subpopulations of ATL samples in contrast to normal controls in which both markers were weakly expressed in the equivalent subpopulation (Fig. 3A,B). As several studies indicated that ATL cells originate from $CD4^+CD25^+FoxP3^+$ Treg cells,[26] we next analyzed FoxP3 expression in each subpopulation. In the $CD3^{dim}CD7^{low}$ subpopulation, the FoxP3 expression levels were variable, consistent with previous reports.[27] In one case, FoxP3 expression was upregulated in the $CD3^{high}CD7^{high}$ and $CD3^{dim}CD7^{dim}$ subpopulations suggesting that they were normal Treg cells.

The analysis of clonality is extremely important for determining whether cells are transformed and Southern blot analysis is usually used to confirm clonality. However, in the present study,

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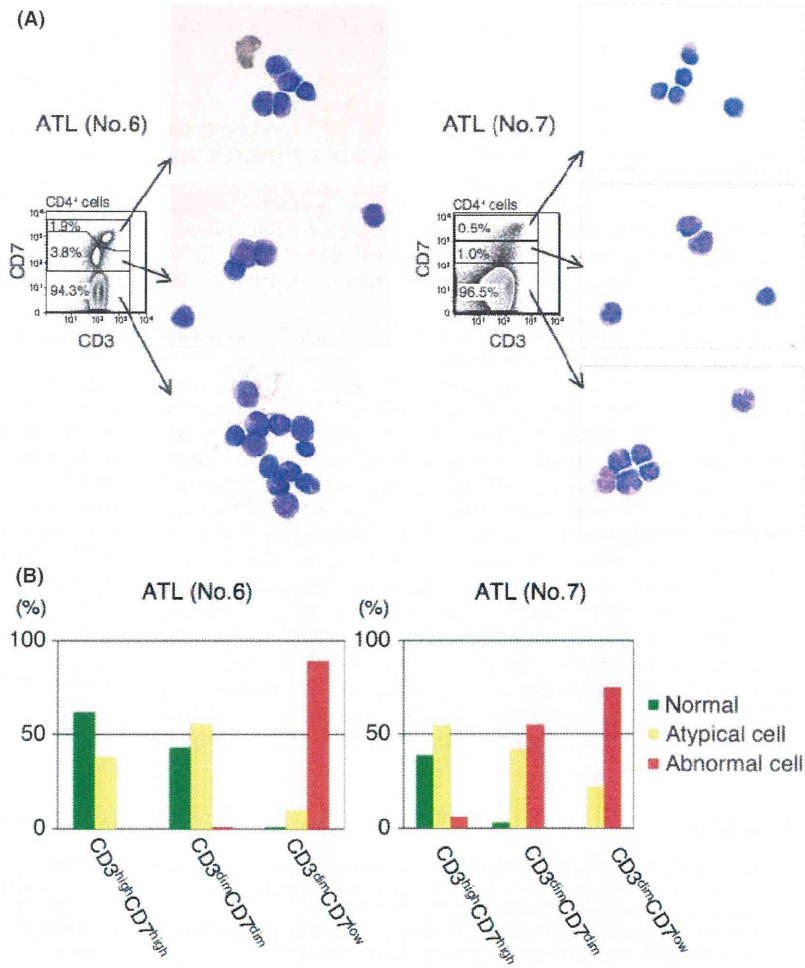

Fig. 6. Morphology of the $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations in two representative adult T-cell leukemia (ATL) samples (patients no. 6 and 7). (A) May–Giemsa staining of FACS-sorted cells from each subpopulation from two patients with acute-type ATL. Top, $CD3^{high}CD7^{high}$ subpopulation; middle, $CD3^{dim}CD7^{dim}$ subpopulation; bottom, $CD3^{dim}CD7^{low}$ subpopulation. (B) Percentages of cells with different morphology in each subpopulation. Normal, lymphocytes with normal morphology; atypical, lymphocytes with notched nuclei and basophilic cytoplasm; abnormal, lymphocytes with convoluted, deeply indented or multilobulated flower cells.

the cell number following cell sorting was not sufficient for Southern blotting, and thus inverse long PCR for clonality analysis of HTLV-I-infected cells was used.[25] Studies of four ATL samples revealed clonal expansion of ATL cells in the $CD3^{dim}CD7^{low}$ subpopulations, although minor clones may exist in the population (Fig. 4). When PCR was performed in duplicate, we found that the major bands were consistently detected in all cases. However, the detection of multiple minor bands was not consistent. As reported previously, the inverse long PCR method stochastically amplifies the template originating from small clones.[28,29] The minor bands observed in the present study will contain small clones. However, the presence of non-specific bands cannot be eliminated.

The inverse long PCR method is commonly used for clonality analysis; however, it cannot quantify the size of major/minor clones and the degree of enrichment in each subpopulation. Therefore, we tested the FACS-based TCR-Vβ repertoire analysis combined with our multi-color FACS system (Fig. 5). In ATL patient no. 3, almost all cells in the $CD3^{dim}CD7^{low}$ subpopulations were clonal cells with TCR-Vβ9. Inverse long PCR analysis in the same patient showed multiple minor bands in the $CD3^{dim}CD7^{low}$ subpopulations (Fig. 4D). These results did not conflict with those of the TCR-Vβ repertoire analysis, as the inverse long PCR method is a more sensitive method for detecting small clones compared with flow cytometry. Taken together, the series of analyses in the present study indicated that the $CD3^{dim}CD7^{low}$ subpopulations consist of highly purified ATL cells in patients with acute-type ATL.

A substantial proportion of cells in the $CD3^{dim}CD7^{dim}$ subpopulation consisted of morphologically abnormal lymphocytes (Fig. 6) that exhibited upregulation of CD25 and CCR4 expression (Fig. 3A,B). Using the inverse long PCR method, a similar band pattern between $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations was observed in patients no. 6 and 8, suggesting that these cells belonged to the same clone (Fig. 4A,B). However, not all of the cells in this subpopulation were infected with HTLV-I because the HTLV-I proviral load was less than that of the $CD3^{dim}CD7^{low}$ subpopulation (Fig. 2). Thus, at least a small number of the $CD3^{dim}CD7^{dim}$ cells were expected to be ATL cells. Those cells observed in the $CD3^{dim}CD7^{dim}$ subpopulation that were phenotypically different from the $CD3^{dim}CD7^{low}$ subpopulations were of particular interest. We detected a band of the same size on inverse long PCR in the $CD3^{dim}CD7^{dim}$ subpopulations as in the $CD3^{dim}CD7^{low}$ subpopulation. This may have been because the two subpopulations originated from the same clone that evolved from a $CD3^{dim}CD7^{dim}$ to a $CD3^{dim}CD7^{low}$ phenotype. Further studies are required to determine the characteristics of the $CD3^{dim}CD7^{dim}$ subpopulation in greater detail.

The results of the present study indicated that HTLV-I-infected cells distribute from a $CD7^{high}$ to a $CD7^{low}$ subpopulation, although the proportion of HTLV-I-infected cells was remarkably low in the $CD3^{high}CD7^{high}$ subpopulation (Fig. 2). A considerable proportion of cells in the $CD3^{high}CD7^{high}$ subpopulation consisted of morphologically atypical lymphocytes (Fig. 6), but the CD25 and CCR4 levels were not upregulated

(Fig. 3A,B). When analyzing the pattern of the inverse long PCR of the $CD3^{high}CD7^{high}$ subpopulations, we observed a difference from those of the $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations (Fig. 4). In patients no. 6 (Fig. 4B) and 7 (Fig. 4C), the band detected in the $CD3^{high}CD7^{high}$ subpopulation may represent an expanded clone that was not transformed. Most likely, these cells do not represent ATL cells, but oligoclonal HTLV-I-infected lymphocytes. Previous studies indicated that HTLV-I-infected cells undergo transformation through multi-step oncogenesis.[30] A detailed analysis of these three subpopulations may therefore provide some insight into the oncogenesis of HTLV-I-infected cells.

Accurate determination of ATL cells in peripheral blood is critical for estimating the response to chemotherapy. However, as discussed above, morphological studies (Fig. 6) have limitations in their ability to discriminate ATL from non-ATL cells.[31,32]. Recently, hematopoietic stem cell transplantation has been explored as a promising treatment to overcome the poor prognosis of this most incurable lymphoid malignancy,[33,34] and monitoring minimal residual disease following hematopoietic stem cell transplantation is more important. Our method of analyzing ATL cells may be particularly useful for monitoring minimal residual disease. Although the $CD3^{dim}CD7^{dim}$ subpopulation in our analysis may have included some ATL cells, this is a minor population in the peripheral blood of patients with acute-type ATL, and it is sufficient for practical use to monitor the $CD3^{dim}CD7^{low}$ subpopulation. Another possible use of our procedure is for the definitive classification of ATL subtypes according to Shimoyama's criteria.[8] A proportion of abnormal lymphocytes in peripheral blood comprise part of the criteria for ATL-subtype classification but it is sometimes confusing. Our multi-color FACS system may clearly quantify this proportion.

In conclusion, we have constructed a multi-color FACS system to purify ATL cells in the peripheral blood of patients with acute-type ATL. This system may be useful for precisely monitoring the disease during chemotherapy, detecting minimal residual disease and analyzing ATL cells. This system may be of great benefit in investigating oncogenesis in HTLV-I-infected cells.

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Disclosure Statement

The authors declare no financial conflicts of interest.

References

1 Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci U S A* 1982; **79** (6): 2031–5.
2 Matsuoka M. Human T-cell leukemia virus type I (HTLV-I) infection and the onset of adult T-cell leukemia (ATL). *Retrovirology* 2005; **2**: 27.
3 Nicot C. Current views in HTLV-I-associated adult T-cell leukemia/lymphoma. *Am J Hematol* 2005; **78** (3): 232–9.
4 Ginaldi L, Matutes E, Farahat N, De Martinis M, Morilla R, Catovsky D. Differential expression of CD3 and CD7 in T-cell malignancies: a quantitative study by flow cytometry. *Br J Haematol* 1996; **93** (4): 921–7.
5 Campana D, Coustan-Smith E. Minimal residual disease studies by flow cytometry in acute leukemia. *Acta Haematol* 2004; **112** (1–2): 8–15.
6 Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood* 2008; **111** (8): 3941–67.
7 Akl H, Badran B, Dobirta G *et al.* Progressive loss of CD3 expression after HTLV-I infection results from chromatin remodeling affecting all the CD3 genes and persists despite early viral genes silencing. *Virol J* 2007; **4**: 85.
8 Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984–87). *Br J Haematol* 1991; **79** (3): 428–37.
9 Tsukasaki K, Hermine O, Bazarbachi A *et al.* Definition, prognostic factors, treatment, and response criteria of adult T-cell leukemia-lymphoma: a proposal from an international consensus meeting. *J Clin Oncol* 2009; **27** (3): 453–9.
10 Yokote T, Akioka T, Oka S *et al.* Flow cytometric immunophenotyping of adult T-cell leukemia/lymphoma using CD3 gating. *Am J Clin Pathol* 2005; **124** (2): 199–204.
11 Yamada Y, Tomonaga M, Fukuda H *et al.* A new G-CSF-supported combination chemotherapy, LSG15, for adult T-cell leukaemia-lymphoma: Japan Clinical Oncology Group Study 9303. *Br J Haematol* 2001; **113** (2): 375–82.
12 Roncador G, Brown PJ, Maestre L *et al.* Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol* 2005; **35** (6): 1681–91.
13 Iwanaga M, Watanabe T, Utsunomiya A *et al.* Human T-cell leukemia virus type I (HTLV-I) proviral load and disease progression in asymptomatic HTLV-I carriers: a nationwide prospective study in Japan. *Blood* 2010; **116** (8): 1211–19.
14 Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; **6** (4): 345–52.
15 Morice WG, Kimlinger T, Katzmann JA *et al.* Flow cytometric assessment of TCR-Vbeta expression in the evaluation of peripheral blood involvement by T-cell lymphoproliferative disorders: a comparison with conventional T-cell immunophenotyping and molecular genetic techniques. *Am J Clin Pathol* 2004; **121** (3): 373–83.
16 Reinhold U, Abken H. CD4+ CD7- T cells: a separate subpopulation of memory T cells? *J Clin Immunol* 1997; **17** (4): 265–71.
17 Reinhold U, Abken H, Kukel S *et al.* CD7- T cells represent a subset of normal human blood lymphocytes. *J Immunol* 1993; **150** (5): 2081–9.
18 Aandahl EM, Quigley MF, Moretto WJ *et al.* Expansion of CD7(low) and CD7(negative) CD8 T-cell effector subsets in HIV-1 infection: correlation with antigenic load and reversion by antiretroviral treatment. *Blood* 2004; **104** (12): 3672–8.
19 Autran B, Legac E, Blanc C, Debre P. A Th0/Th2-like function of CD4 + CD7- T helper cells from normal donors and HIV-infected patients. *J Immunol* 1995; **154** (3): 1408–17.
20 Legac E, Autran B, Merle-Beral H, Katlama C, Debre P. CD4+CD7-CD57+ T cells: a new T-lymphocyte subset expanded during human immunodeficiency virus infection. *Blood* 1992; **79** (7): 1746–53.
21 Schmidt D, Goronzy JJ, Weyand CM. CD4+ CD7- CD28- T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J Clin Invest* 1996; **97** (9): 2027–37.
22 Willard-Gallo KE, Van de Keere F, Kettmann R. A specific defect in CD3 gamma-chain gene transcription results in loss of T-cell receptor/CD3 expression late after human immunodeficiency virus infection of a CD4+ T-cell line. *Proc Natl Acad Sci U S A* 1990; **87** (17): 6713–17.
23 Matsuoka M, Jeang KT. Human T-cell leukaemia virus type 1 (HTLV-I) infectivity and cellular transformation. *Nat Rev Cancer* 2007; **7** (4): 270–80.
24 Ishida T, Utsunomiya A, Iida S *et al.* Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma: its close association with skin involvement and unfavorable outcome. *Clin Cancer Res* 2003; **9** (10 Pt 1): 3625–34.
25 Yoshie O, Fujisawa R, Nakayama T *et al.* Frequent expression of CCR4 in adult T-cell leukemia and human T-cell leukemia virus type 1-transformed T cells. *Blood* 2002; **99** (5): 1505–11.
26 Yano H, Ishida T, Inagaki A *et al.* Regulatory T-cell function of adult T-cell leukemia/lymphoma cells. *Int J Cancer* 2007; **120** (9): 2052–7.
27 Abe M, Uchihashi K, Kazuto T *et al.* Foxp3 expression on normal and leukemic CD4+CD25+ T cells implicated in human T-cell leukemia virus type-1 is inconsistent with Treg cells. *Eur J Haematol* 2008; **81** (3): 209–17.

doi: 10.1111/j.1349-7006.2010.01833.x

28 Tanaka G, Okayama A, Watanabe T *et al.* The clonal expansion of human T lymphotropic virus type 1-infected T cells: a comparison between seroconverters and long-term carriers. *J Infect Dis* 2005; **191** (7): 1140–7.
29 Cavrois M, Wain-Hobson S, Wattel E. Stochastic events in the amplification of HTLV-I integration sites by linker-mediated PCR. *Res Virol* 1995; **146** (3): 179–84.
30 Verdonck K, Gonzalez E, Van Dooren S, Vandamme AM, Vanham G, Gotuzzo E. Human T-lymphotropic virus 1: recent knowledge about an ancient infection. *Lancet Infect Dis* 2007; **7** (4): 266–81.
31 Sakamoto Y, Kawachi Y, Uchida T *et al.* Adult T-cell leukaemia/lymphoma featuring a large granular lymphocyte leukaemia morphologically. *Br J Haematol* 1994; **86** (2): 383–5.
32 Tsukasaki K, Imaizumi Y, Tawara M *et al.* Diversity of leukaemic cell morphology in ATL correlates with prognostic factors, aberrant immunophenotype and defective HTLV-I genotype. *Br J Haematol* 1999; **105** (2): 369–75.
33 Okamura J, Utsunomiya A, Tanosaki R *et al.* Allogeneic stem-cell transplantation with reduced conditioning intensity as a novel immunotherapy and antiviral therapy for adult T-cell leukemia/lymphoma. *Blood* 2005; **105** (10): 4143–5.
34 Utsunomiya A, Miyazaki Y, Takatsuka Y *et al.* Improved outcome of adult T-cell leukemia/lymphoma with allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant.* 2001; **27** (1): 15–20.

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ORIGINAL ARTICLE

Unrelated cord blood transplantation after myeloablative conditioning in adults with advanced myelodysplastic syndromes

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We analyzed the disease-specific outcomes of adult patients with advanced myelodysplastic syndrome (MDS) treated with cord blood transplantation (CBT) after myeloablative conditioning. Between August 1998 and June 2009, 33 adult patients with advanced MDS were treated with unrelated CBT. The diagnoses at transplantation included refractory anemia with excess blasts ($n=7$) and MDS-related secondary AML (sAML) ($n=26$). All patients received four fractionated 12 Gy TBI and chemotherapy as myeloablative conditioning. The median age was 42 years, the median weight was 55 kg and the median number of cryopreserved nucleated cells was 2.51×10^7 cells per kg. The cumulative incidence of neutrophil recovery at day 50 was 91%. Neutrophil recovery was significantly faster in sAML patients ($P=0.04$). The cumulative incidence of plt recovery at day 200 was 88%. Plt recovery was significantly faster in CMV seronegative patients ($P<0.001$). The cumulative incidence of grade II–IV acute GVHD (aGVHD) and extensive-type chronic GVHD was 67 and 34%, respectively. Degree of HLA mismatch had a significant impact on the incidence of grade II–IV aGVHD ($P=0.021$). TRM and relapse at 5-years was 14 and 16%, respectively. The probability of EFS at 5 years was 70%. No factor was associated with TRM, relapse and EFS. These results suggest that adult advanced MDS patients without suitable related or unrelated BM donors should be considered as candidates for CBT.
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Introduction

The prognosis of advanced myelodysplastic syndrome (MDS) is poor. Although some patients with advanced MDS achieve remission with standard intensive chemotherapy, the duration is usually limited.[1] Therefore, Allo-SCT is considered as the only curative therapy for MDS patients. Alternative donor sources other than HLA-identical siblings have been used as allogeneic stem cell sources.[2–6] Recently, umbilical cord blood from unrelated donors has been used as an alternative stem cell source for adult patients.[7–15] However, reports of disease-specific outcomes for adult patients with advanced MDS after cord blood transplantation (CBT) are still limited. We have previously reported the results of a group of 19 adult patients with advanced MDS who received unrelated CBT.[16,17] Here, we have updated the results of unrelated CBT after myeloablative conditioning for 33 adult patients with advanced MDS. The main purpose of this retrospective single-center study was to confirm the safety and efficacy of unrelated CBT for adult advanced MDS patients after a myeloablative conditioning regimen, as well as to identify pretransplant factors related to the transplant outcomes on long-term follow-up.

Patients and methods

Patients

This was a retrospective single-center analysis. Between August 1998 and June 2009, 33 adult patients with advanced MDS were treated with unrelated CBT at The Institute of Medical Science, University of Tokyo. The diagnosis of MDS was made for all patients according to the World Health Organization classification. The diagnosis at transplantation included refractory anemia with excess blasts (1/2) ($n=7$) and MDS-related secondary AML (sAML) ($n=26$). MDS-related sAML was defined as AML that developed during the follow-up period of MDS. The cytogenetic subgroups according to a transplantation-specific cytogenetics grouping for MDS[18] were adverse risk (abnormalities of chromosome 7 and complex karyotype) in 10 patients and standard risk (all others) in 23 patients. Written informed consent for treatment was obtained from all patients.