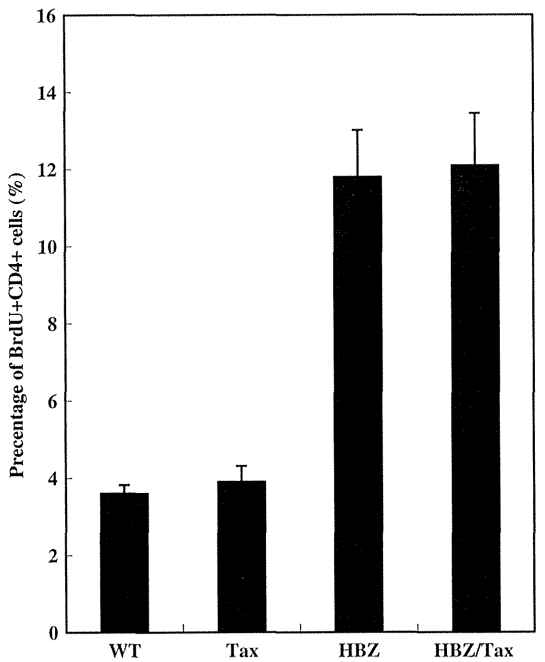

Fig. 4 CD4+ T cells proliferated in HBZ/Tax transgenic mice. BrdU was injected into mice twice a day for three days, and splenocytes were stained with antibodies to BrdU and CD4

HTLV-1 infected individuals. However, Tax single transgenic mice did not develop major health problems. This study highlights the importance of HBZ in HTLV-1-associated disease.

Acknowledgments This work was supported by a grant from National Natural Science Foundation of China to TZ (No.31200128); a Grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan to MM. The authors thank Aaron Coutts for proofreading the manuscript.

Conflict of interest The authors declare that they have no competing interests.

References

1. Arnulf B, Villemain A, Nicot C, Mordelet E, Charneau P, Kersual J, Zermati Y, Mauviel A, Bazarbachi A, Hermine O (2002) Human T-cell lymphotropic virus oncoprotein Tax represses TGF-beta 1 signaling in human T cells via c-Jun activation: a potential mechanism of HTLV-I leukemogenesis. Blood 100:4129–4138
2. Basbous J, Arpin C, Gaudray G, Piechaczyk M, Devaux C, Mesnard JM (2003) The HBZ factor of human T-cell leukemia virus type I dimerizes with transcription factors JunB and c-Jun and modulates their transcriptional activity. J Biol Chem 278:43620–43627
3. Fan J, Ma G, Nosaka K, Tanabe J, Satou Y, Koito A, Wain-Hobson S, Vartanian JP, Matsuoka M (2010) APOBEC3G generates nonsense mutations in human T-cell leukemia virus type 1 proviral genomes in vivo. J Virol 84:7278–7287
4. Gaudray G, Gachon F, Basbous J, Biard-Piechaczyk M, Devaux C, Mesnard JM (2002) The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription. J Virol 76:12813–12822
5. Grassmann R, Aboud M, Jeang KT (2005) Molecular mechanisms of cellular transformation by HTLV-1 Tax. Oncogene 24:5976–5985
6. Grossman WJ, Kimata JT, Wong FH, Zutter M, Ley TJ, Ratner L (1995) Development of leukemia in mice transgenic for the tax gene of human T-cell leukemia virus type I. Proc Natl Acad Sci USA 92:1057–1061
7. Hagiya K, Yasunaga J, Satou Y, Ohshima K, Matsuoka M (2011) ATF3, an HTLV-1 bZip factor binding protein, promotes proliferation of adult T-cell leukemia cells. Retrovirology 8:19
8. Hasegawa H, Sawa H, Lewis MJ, Orba Y, Sheehy N, Yamamoto Y, Ichinohe T, Tsunetsugu-Yokota Y, Katano H, Takahashi H, Matsuda J, Sata T, Kurata T, Nagashima K, Hall WW (2006) Thymus-derived leukemia-lymphoma in mice transgenic for the Tax gene of human T-lymphotropic virus type I. Nat Med 12:466–472
9. Kannagi M, Harada S, Maruyama I, Inoko H, Igarashi H, Kuwashima G, Sato S, Morita M, Kidokoro M, Sugimoto M et al (1991) Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX gene products by human CD8+ cytotoxic T cells directed against HTLV-I-infected cells. Int Immunol 3:761–767
10. Karube K, Ohshima K, Tsuchiya T, Yamaguchi T, Kawano R, Suzumiya J, Utsunomiya A, Harada M, Kikuchi M (2004) Expression of FoxP3, a key molecule in CD4CD25 regulatory T cells, in adult T-cell leukaemia/lymphoma cells. Br J Haematol 126:81–84
11. Lee DK, Kim BC, Brady JN, Jeang KT, Kim SJ (2002) Human T-cell lymphotropic virus type 1 tax inhibits transforming growth factor-beta signaling by blocking the association of Smad proteins with Smad-binding element. J Biol Chem 277:33766–33775
12. Lemasson I, Lewis MR, Polakowski N, Hivin P, Cavanagh MH, Thebault S, Barbeau B, Nyborg JK, Mesnard JM (2007) Human T-cell leukemia virus type 1 (HTLV-1) bZIP protein interacts with the cellular transcription factor CREB to inhibit HTLV-1 transcription. J Virol 81:1543–1553
13. Ma G, Yasunaga J, Fan J, Yanagawa S, Matsuoka M (2012) HTLV-1 bZIP factor dysregulates the Wnt pathways to support proliferation and migration of adult T-cell leukemia cells. Oncogene 32:4222–4230
14. Matsumoto J, Ohshima T, Isono O, Shimotohno K (2005) HTLV-1 HBZ suppresses AP-1 activity by impairing both the DNA-binding ability and the stability of c-Jun protein. Oncogene 24:1001–1010
15. Matsuoka M, Jeang KT (2007) Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. Nat Rev Cancer 7:270–280
16. Matsuoka M, Green PL (2009) The HBZ gene, a key player in HTLV-1 pathogenesis. Retrovirology 6:71
17. Mori N, Morishita M, Tsukazaki T, Giam CZ, Kumatori A, Tanaka Y, Yamamoto N (2001) Human T-cell leukemia virus type I oncoprotein Tax represses Smad-dependent transforming growth factor beta signaling through interaction with CREB-binding protein/p300. Blood 97:2137–2144
18. Nerenberg M, Hinrichs SH, Reynolds RK, Khoury G, Jay G (1987) The tat gene of human T-lymphotropic virus type 1 induces mesenchymal tumors in transgenic mice. Science 237:1324–1329
19. Nicot C, Harrod RL, Ciminale V, Franchini G (2005) Human T-cell leukemia/lymphoma virus type 1 nonstructural genes and their functions. Oncogene 24:6026–6034

20. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC (1980) Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc Natl Acad Sci USA 77:7415–7419
21. Satou Y, Yasunaga J, Yoshida M, Matsuoka M (2006) HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. Proc Natl Acad Sci USA 103:720–725
22. Satou Y, Yasunaga J, Zhao T, Yoshida M, Miyazato P, Takai K, Shimizu K, Ohshima K, Green PL, Ohkura N, Yamaguchi T, Ono M, Sakaguchi S, Matsuoka M (2011) HTLV-1 bZIP factor induces T-cell lymphoma and systemic inflammation in vivo. Plos Pathogens 7:e1001274
23. Toulza F, Heaps A, Tanaka Y, Taylor GP, Bangham CR (2008) High frequency of CD4+ FoxP3+ cells in HTLV-1 infection: inverse correlation with HTLV-1-specific CTL response. Blood 111:5047–5053
24. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H (1977) Adult T-cell leukemia: clinical and hematologic features of 16 cases. Blood 50:481–492
25. Yamamoto-Taguchi N, Satou Y, Miyazato P, Ohshima K, Nakagawa M, Katagiri K, Kinashi T, Matsuoka M (2013) HTLV-1 bZIP Factor Induces Inflammation through Labile Foxp3 Expression. Plos Pathogens 9:e1003630
26. Yamano Y, Takenouchi N, Li HC, Tomaru U, Yao K, Grant CW, Maric DA, Jacobson S (2005) Virus-induced dysfunction of CD4 + CD25 + T cells in patients with HTLV-I-associated neuroimmunological disease. J Clin Invest 115:1361–1368
27. Zhao T, Yasunaga J, Satou Y, Nakao M, Takahashi M, Fujii M, Matsuoka M (2009) Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-kappaB. Blood 113:2755–2764
28. Zhao T, Satou Y, Sugata K, Miyazato P, Green PL, Imamura T, Matsuoka M (2011) HTLV-1 bZIP factor enhances TGF-beta signaling through p300 coactivator. Blood 118:1865–1876
29. Zhao T, Matsuoka M (2012) HBZ and its roles in HTLV-1 oncogenesis. Front Microbiol 3:247

Biol Blood Marrow Transplant 20 (2014) 1968–1974

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

Treatment of Patients with Adult T Cell Leukemia/Lymphoma with Cord Blood Transplantation: A Japanese Nationwide Retrospective Survey

Koji Kato [1,*], Ilseung Choi [2], Atsushi Wake [3], Naokuni Uike [2], Shuichi Taniguchi [3], Yukiyoshi Moriuchi [4], Yasushi Miyazaki [5], Hirohisa Nakamae [6], Eijirou Oku [7], Makoto Murata [8], Tetsuya Eto [9], Koichi Akashi [1], Hisashi Sakamaki [10], Koji Kato [11], Ritsuro Suzuki [12], Takeharu Yamanaka [13], Atae Utsunomiya [14]

[1] *Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan*
[2] *Department of Hematology, National Kyushu Cancer Center, Fukuoka, Japan*
[3] *Department of Hematology, Toranomon Hospital, Tokyo, Japan*
[4] *Department of Hematology, Sasebo City General Hospital, Sasebo, Japan*
[5] *Department of Hematology and Molecular Medicine Unit, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Science, Nagasaki, Japan*
[6] *Department of Hematology, Osaka City University Graduate School of Medicine, Osaka, Japan*
[7] *Department of Hematology, Kurume University Graduate School of Medicine, Kurume, Japan*
[8] *Department of Hematology, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[9] *Department of Hematology, Hamanomachi Hospital, Fukuoka, Japan*
[10] *Department of Hematology, Tokyo Metropolitan Cancer and Infectious Disease Center, Komagome Hospital, Tokyo, Japan*
[11] *Department of Hematology and Oncology, Children's Medical Center, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan*
[12] *Department of HSCT Data Management/Biostatistics, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[13] *Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Japan*
[14] *Department of Hematology, Imamura Bun-in Hospital, Kagoshima, Japan*

Article history:
Received 9 June 2014
Accepted 12 August 2014

Key Words:
Adult T cell leukemia/lymphoma (ATTL)
Cord blood transplantation
Graft-versus-adult T cell leukemia/lymphoma

A B S T R A C T

Allogeneic bone marrow and peripheral blood stem cell transplantations are curative treatment modalities for adult T cell leukemia/lymphoma (ATLL) because of the intrinsic graft-versus-ATLL effect. However, limited information is available regarding whether cord blood transplantation (CBT) induces a curative graft-versus-ATLL effect against aggressive ATLL. To evaluate the effect of CBT against ATLL, we retrospectively analyzed data from 175 patients with ATLL who initially underwent single-unit CBT. The 2-year overall survival (OS) rate was 20.6% (95% confidence interval [CI], 13.8% to 27.4%). A multivariate analysis revealed that the development of graft-versus-host disease (GVHD) was a favorable prognostic factor for OS (hazard ratio, .10; 95% CI, .01 to .94; $P = .044$). Furthermore, the 2-year OS (42.7%; 95% CI, 28.1% to 56.6%) of patients with grade 1 to 2 acute GVHD was higher than that of patients without acute GVHD (24.2%; 95% CI, 11.2% to 39.8%; $P = .048$). However, the cumulative incidence of treatment-related mortality (TRM) was high (46.1%; 95% CI, 38.2% to 53.7%), and early death was particularly problematic. In conclusion, CBT cures patients with ATLL partly through a graft-versus-ATLL effect. However, novel interventions will be required, particularly in the early phase, to reduce TRM and optimize GVHD.

INTRODUCTION

Adult T cell leukemia/lymphoma (ATLL), an aggressive peripheral T cell neoplasm caused by the human T cell lymphotropic/leukemia virus type-1, has an extremely poor prognosis [1]. Intensive chemotherapy and autologous stem cell transplantation have not been shown to improve this prognosis [2,3]. As a curative treatment, allogeneic hematopoietic stem cell transplantation (allo-HSCT) can confer long-term remission via a graft-versus-ATLL effect in a proportion of patients with ATLL [4-7]. Recent reports have demonstrated that allo-HSCT using bone marrow (BM) or peripheral blood stem cells (PBSC) from a related or unrelated donor can effectively treat ATLL, yielding a 3-year overall survival rate

Financial disclosure: See Acknowledgments on page 1973.

* Correspondence and reprint requests: Koji Kato, MD, PhD, Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Science, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail address: kojikato@intmed1.med.kyushu-u.ac.jp (K. Kato).

http://dx.doi.org/10.1016/j.bbmt.2014.08.012
1083-8791/

(OS) of approximately 30% [8-16]. However, patients with ATLL typically lack a suitable HLA-identical sibling donor because both the recipients and donors are typically elderly and because the aggressive ATLL tumor burden reduces the available time to find a suitable unrelated donor within the Japan Marrow Donor Program. Umbilical cord blood, which can serve as an alternative to BM or PBSC as a source of stem cells, has been used primarily to treat children; however, the number of unrelated-donor cord blood transplantation (CBT) procedures used to treat adult patients with ATLL is increasing in Japan. The rapid availability of CBT may provide a great advantage for patients who require urgent allo-HSCT to treat aggressive ATLL [17].

Currently, the outcome of CBT in patients with acute leukemia is comparable to that of other graft sources [18,19]; however, there are few reports on the outcomes of CBT in patients with ATL [20,21]. Moreover, it is difficult to draw firm conclusions regarding the efficacy of this procedure because of the small number of cases. Therefore, to evaluate the role of CBT for ATLL in a larger and more recent cohort, we performed a nationwide retrospective study of patients with ATLL who underwent CBT as the initial allo-HSCT.

PATIENTS AND METHODS

Data Collection

We analyzed nationwide survey data from the Japan Society for Hematopoietic Cell Transplantation regarding patients with ATLL who had undergone an initial CBT between March 2001 and December 2009 (n = 175). This analysis included the patients' clinical characteristics, such as the age at transplantation, gender, disease status at transplantation, date of transplantation, time from diagnosis to transplantation, conditioning regimens, and number of infused cells. The number of mismatches was counted with respect to HLA-A, HLA-B (low-resolution typing), and DRB1 (high-resolution typing). The present study was approved by the data management committees of the Japan Society for Hematopoietic Cell Transplantation as well as the institutional ethics committee of the Kyushu University Graduate School of Medical Sciences.

Definitions

OS was defined as the time from transplantation until death, and patients who remained alive at the time of the last follow-up were censored. The causes of death were reviewed and categorized as either ATLL-related or transplantation-related mortality (TRM). *ATLL-related mortality* was defined as death caused by a relapse or progression of ATLL, whereas *TRM* was defined as any death related to transplantation other than ATLL-related mortality, according to the judgment of each institution. The patients were divided into 2 groups according to the conditioning regimen: full-intensity conditioning (FIC) and reduced-intensity conditioning (RIC). FIC and RIC were defined according to the proposals of Giralt et al. [22] and Bacigalupo et al. [23], respectively, with slight modifications. In the present study, conditioning regimens that included ≥5 Gy of total body irradiation (TBI) in a single fraction or ≥8 Gy of TBI in multiple fractions, oral busulfan (BU) at >8 mg/kg, intravenous BU at >6.4 mg/kg, or melphalan (Mel) at >140 mg/m^2 were considered FIC; all others were classified as RIC.

Statistical Analysis

Descriptive statistics were used to summarize the variables related to patient demographics and transplantation characteristics. The probability of the OS time was estimated according to the Kaplan-Meier method. To evaluate the influences of confounding factors on acute graft-versus-host disease (GVHD) and survival, the log-rank test and proportional hazards modeling were used for the univariate and multivariate analyses, respectively. The Cox proportional hazard model was used for the multivariate analyses of OS in which all independent variables were incorporated in the model, followed by the use of a stepwise selection method [24]. Fine and Gray proportional hazard modeling was used to estimate the effects of the same variables used in the multivariate analysis for OS on the cumulative incidence rates of TRM and ATLL-related mortality [25,26]. In these regression models, the occurrence of GVHD was treated as a time-dependent covariate [27]. In the analysis of acute GVHD, patients were assigned to the "no acute GVHD group" at the time of transplantation and transferred to the "acute GVHD group" at the onset of the maximum grade of acute GVHD. The landmark method was used to evaluate the effects of GVHD on OS and the cumulative incidence of disease-associated and treatment-related deaths among patients who remained alive at 60 days for acute GVHD and at 100 days for chronic GVHD after transplantation. Factors associated with at least borderline significance ($P \leq .10$) in the univariate analysis were subjected to a multivariate analysis using a backward stepwise covariate selection. All P values were 2-tailed, and P values ≤.05 were considered statistically significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University), a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0) [28].

RESULTS

Patient Characteristics

The characteristics of 175 ATLL patients who received a single CBT are shown in Table 1. The median age at CBT was 55 years (range, 27 to 79 years). The cohort comprised 70 women and 105 men with the following ATLL statuses at CBT: complete remission (CR; n = 50), not in CR (n = 116), and unknown (n = 9). The conditioning regimen intensity was classified as FIC in 63 (36%) patients and RIC in 128 (62%) patients. FIC was further subdivided into 2 groups as follows: TBI (n = 47) or non-TBI (n = 15). RIC was also subdivided into 3 groups as follows: fludarabine (Flu) + Mel (n = 75), Flu + BU (n = 15), and other types (n = 15). Cyclosporine and tacrolimus were administered for prophylaxis to 90 (51%) and 77 patients (44%), respectively. Cyclosporine-based prophylaxis was subdivided into 3 groups as follows: (1) cyclosporine

Table 1
Patient Characteristics at Cord Blood Transplantation

Variables	No. of Patients (n = 175)
Age at transplantation, median (range), yr	55 (27-79)
Gender	
Male	105
Female	70
Disease status at transplantation	
CR	50
Not in CR	116
Unknown	9
Conditioning regimen	
FIC	63
RIC	108
Unknown	4
GVHD prophylaxis	
Cyclosporine-based	90
Tacrolimus-based	77
Unknown	8
Time from diagnosis to transplantation, d	
<200	94
≥200	75
Unknown	6
Year of transplantation	
<2005	71
≥2005	104
HLA matching*	
0 mismatched loci	5
1 mismatched locus	36
2 mismatched loci	73
≥3 mismatched loci	42
Unknown	19
ABO matching	
Matched	56
Minor mismatched	49
Major mismatched	69
Unknown	1
Nucleated cells infused per 10^7/kg, median (range)	2.58 (.36-5.34)
CD34-positive cells infused per 10^5/kg, median (range)	.85 (.07-5.39)

* Number of mismatches was counted among HLA-A, -B (low-resolution typing), and DRB1 (high-resolution typing).

alone (n = 33), (2) cyclosporine + short-term methotrexate (MTX) (n = 45), and (3) cyclosporine + mycophenolate mofetil (MMF; n = 12). Tacrolimus-based prophylaxis was subdivided into 4 groups as follows: (1) tacrolimus alone (n = 37), (2) tacrolimus + short-term MTX (n = 32), (3) tacrolimus + MMF (n = 5), (4) and tacrolimus + prednisolone (n = 3). Ninety-four patients (54%) received CBT < 200 days after diagnosis. One hundred twenty-four (71%) patients underwent CBT with 2 HLA-mismatched loci. The numbers of infused nucleated and CD34-positive cells were 2.58×10^7/kg (range, .36 to 5.34×10^7/kg) and $.85 \times 10^5$/kg (range, .07 to 5.39×10^5/kg), respectively. Engraftment evaluation was possible in 125 patients (71%) within a median interval of 19 days after CBT (range, 7 to 46 days). Among the survivors, the median follow-up duration was 22.5 months (range, 0 to 74.5 months).

Prognostic Factors for Survival

The OS rates of 175 patients with ATLL who received CBT were 30.2% (95% confidence interval [CI], 23.0% to 37.4%) at 1 year and 20.6% (95% CI, 13.8% to 27.4%) at 2 years (Figure 1A). The cumulative incidence rates of ATLL-related mortality and TRM at 2 years were 31.9% (95% CI, 24.8% to 39.3%) and 46.4% (95% CI, 38.5% to 54.0%), respectively (Figure 1B). The following confounding factors affected survival: age, gender, disease status at transplantation, days from diagnosis to transplantation, date of transplantation, age at transplantation, conditioning regimen, number of infused nucleated and CD34-positive cells, ABO compatibility, HLA compatibility, GVHD prophylaxis, and the development of acute GVHD. A univariate analysis revealed that higher OS ($P < .05$) correlated with CR at transplantation, minor ABO incompatibility, the addition of other agents to calcineurin inhibitors (MTX or MMF), and the development of acute GVHD (Table 2). A multivariate analysis was performed to further examine the effects of an age <55 years, the development of acute GVHD as a time-dependent covariate coincident with CR at transplantation, minor ABO incompatibility, and the addition of other agents to calcineurin inhibitors (Table 3). Compared with the absence of GVHD, the development of acute GVHD was associated independently with higher OS (hazard ratio [HR], .10; 95% CI, .01 to 0.94; $P = .044$).

Effects of Acute GVHD on Survival

To further validate the effect of acute GVHD on OS, we examined survival according to the acute GVHD grade in a landmark analysis. The median time to onset of acute GVHD of any grade after transplantation was 21 days (range, 5 to 100 days). Acute GVHD occurred in 80 patients (46%) as

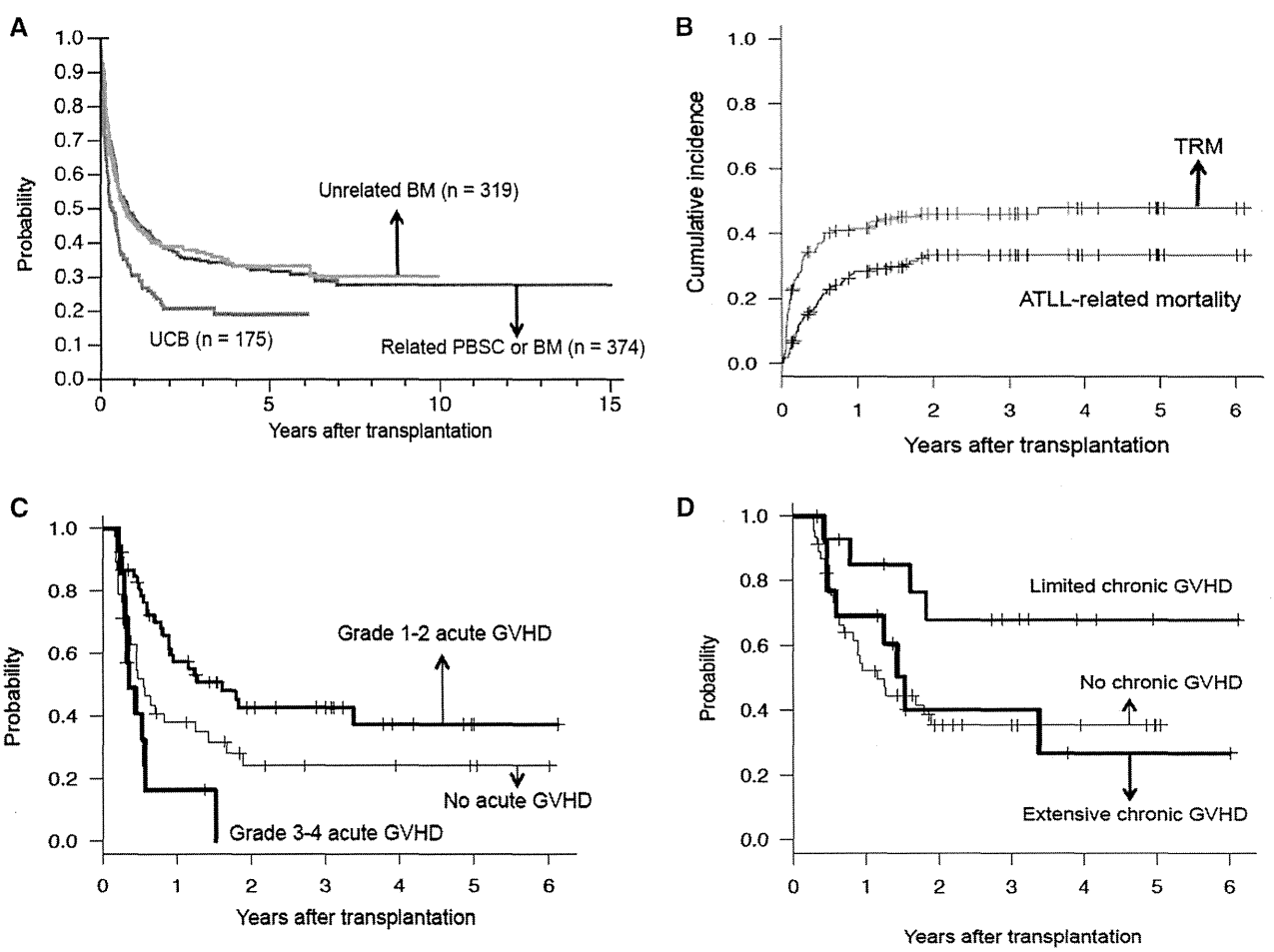

Figure 1. Survival, adult T cell leukemia/lymphoma (ATLL)-related mortality rates, and transplantation-related mortality (TRM) rates of patients receiving cord blood transplantation (CBT). (A) Kaplan-Meier curves of the estimated overall survival rates (OS) of ATLL patients treated with CBT. UCB, umbilical cord blood; PBSC, peripheral blood stem cells; BM, bone marrow, GVHD, graft-versus-host disease. (B) Cumulative incidence curves of ATLL-related mortality and TRM in patients treated with CBT. (C) Landmark plots of OS to determine the effects of acute GVHD. (D) Landmark plots of OS to determine the effects of chronic GVHD.

Table 2
Univariate Analysis of Risk Factors for Overall Survival

Variables		No.	OS		
			Two-Year OS (%)	95% CI	*P* Value
Age 1	<60 yr	134	23.0	15.0-31.0	.080
	≥60 yr	41	12.0	6.0-22.4	
Age 2	<55 yr	85	25.4	15.0-35.8	.100
	≥55 yr	90	15.6	7.0-24.2	
Sex	Female	70	22.3	11.5-33.1	.453
	Male	105	19.4	10.8-28.0	
Disease status at transplantation	CR	50	40.3	25.5-55.1	.003
	Not in CR	116	14.3	7.1-21.7	
Time from diagnosis to transplantation	<200 d	94	22.4	12.8-32.0	.752
	≥200 d	75	19.9	9.7-30.1	
Yr of transplantation	<2005	71	17.6	8.2-27.0	.160
	≥2005	104	23.1	13.5-31.5	
Conditioning regimen	FIC	63	20.2	9.8-30.6	.740
	RIC	108	20.2	11.8-28.6	
Infused nucleated cell dose ($\times 10^7$/kg)	<2	19	10.8	0-29.3	.290
	≥2	145	22.6	14.9-30.3	
Infused CD34 cell dose ($\times 10^5$/kg)	<1	97	23.3	13.9-32.7	.396
	≥1	66	19.1	8.0-30.2	
ABO matching	Matched	56	12.8	3.4-22.2	.024
	Minor mismatched	49	30.5	15.5-45.5	
	Major mismatched	69	20.5	9.9-31.1	
HLA matching	0 mismatched	5	30.0	0-77.4	.525
	1 mismatched	36	21.6	5.6-37.6	
	2 mismatched	73	24.6	14.3-35.9	
	≥3 mismatched	42	18.1	3.9-32.3	
GVHD prophylaxis 1	Cyclosporine-based	90	21.9	12.5-31.4	.710
	Tacrolimus-based	77	20.3	10.0-30.4	
GVHD prophylaxis 2 (cyclosporine/tacrolimus + other drug)	No	70	12.4	4.8-20.0	.003
	Yes	97	32.7	21.1-44.3	
Acute GVHD	No	59	16.8	5.7-27.9	<.0001
	Yes	80	29.4	18.2-40.6	

follows: grade 1, n = 23 patients; grade 2, n = 37 patients; grade 3, n = 14 patients; and grade 4, n = 6 patients. There was no significant difference in OS between patients with grades 1 and 2 GVHD ($P = 1.00$), in contrast to the difference between patients with grades 1 and 3 GVHD ($P = .013$). Moreover, based on the previous national survey analysis of the effect of acute GVHD on survival in patients with ATLL [5,15], the effect of acute GVHD on OS in the present study was evaluated using landmark plots (landmark day 60) according to the following 3 categories: (1) no acute GVHD (n = 38), (2) grade 1 to 2 acute GVHD (n = 53), and (3) grade 3 to 4 acute GVHD (n = 14). The 2-year OS rates for patients according to the acute GVHD grade were as follows: 24.2% (95% CI, 11.2% to 39.8%) without acute GVHD; 42.7% (95% CI, 28.1% to 56.6%) with grade 1 to 2 GVHD; and 0% with grade 3 to 4 GVHD (Figure 1C). These analyses demonstrated that the development of grade 1 to 2 acute GVHD was associated with higher OS compared with the absence of acute GVHD ($P = .048$), whereas the development of grade 3 to 4 acute GVHD was associated with lower OS compared with that in patients with grade 1 to 2 acute GVHD ($P = .0003$). The cumulative 2-year ATLL-related mortality rates according to the GVHD grades were as follows: 32.6% (95% CI, 19.7% to 46.1%) for grade 1 to 2 acute GVHD; 29.8% (95% CI, 8.2% to 55.6%) for grade 3 to 4 acute GVHD; and 45.9% (95% CI, 29.0% to 61.3%) for no acute GVHD. There was a trend toward a lower risk of relapse or progression in those who developed grade 1 to 2 acute GVHD relative to those without GVHD. Among patients with non-CR at transplantation, there was also a trend toward higher 2-year OS (36.7%; 95% CI, 18.7% to 54.9%) in those who developed grade 1 to 2 acute GVHD than in those without GVHD (15.6%; 95% CI, 3.4% to 35.9%). These data suggested a graft-versus-ATLL effect induced by CBT.

Table 3
Multivariate Analysis of Risk Factors for OS

Variables	OS		
	HR	95% CI	*P* Value
Age, yr			
<55	1		
≥55	1.15	.63-2.09	.652
Disease status at transplantation			
CR	1		
Not in CR	1.38	.73-2.63	.190
ABO matching			
Matched	1		
Minor mismatched	.56	.25-1.24	.152
Major mismatched	.77	.39-1.48	.337
GVHD prophylaxis (cyclosporine/ tacrolimus + other drug)			
No	1		
Yes	.76	.42-1.38	.365
Acute GVHD (time-dependent covariate)			
No	1		
Yes	.10	.01-.94	.044

Effects of Chronic GVHD on Survival

Chronic GVHD was evaluated in 74 patients who survived for at least 100 days after transplantation. Chronic GVHD occurred in 28 patients (37%) with a median time to onset of 115 days (range, 73 to 1287 days) after CBT. The effect of chronic GVHD on OS was evaluated using landmark plots (landmark day 100), and the 2-year OS results were as follows: no chronic GVHD (n = 46), 35.6% (95% CI, 21.0% to 50.0%); limited chronic GVHD (n = 15), 68.1% (95% CI, 35.4%

to 86.8%); and extensive chronic GVHD (n = 13), 40.4% (95% CI, 13.4% to 66.4%) (Figure 1D). There was a trend toward a higher OS among patients with limited chronic GVHD, but there were no significant differences relative to patients without chronic GVHD ($P = .10$) and those with extensive chronic GVHD ($P = .12$).

Cause of Death

At the last follow-up, 46 patients remained alive and 129 were deceased. The median follow-up time among the survivors was 22.5 months (range, 0 to 74.5 months). Disease progression (n = 52) was the leading cause of death. Infection was the cause of death in 40 patients (31%; bacterial, n = 27 patients; fungal, n = 3; viral, n = 8; and others, n = 2). Viral infection-related deaths were caused by the following pathogens: cytomegalovirus, n = 3; adenovirus, n = 2; human herpesvirus-6, n = 2; and varicella-zoster virus, n = 1. Among the 27 patients who succumbed to bacterial infection, 16 died before engraftment at a median of 17 days after CBT (range, 7 to 38 days). Among the 20 patients who developed severe acute grade 3 to 4 GVHD, 2 remain alive without disease progression. However, 9 of the 20 patients died of GVHD, 5 of disease progression, and 4 of infection.

The Fine and Gray proportional hazards model was applied to identify the variables affecting ATLL-related mortality and TRM. The pretransplantation variables included age, gender, disease status at CBT, days from diagnosis to transplantation, age at transplantation, conditioning regimen, number of infused nucleated cells, ABO compatibility, HLA compatibility, and GVHD prophylaxis. The following pretransplantation factors associated with a higher risk of ATLL-related mortality were identified in a multivariate analysis: not in CR at CBT (HR, 3.37; 95% CI, 1.12 to 10.2; $P = .032$) and an age > 55 years at CBT (HR, 2.32; 95% CI, .98 to 5.48; $P = .054$). The following pretransplantation factors were associated with a marginally higher risk of TRM: lower number of infused nucleated cells ($\geq 2 \times 10^7$/kg versus $< 2 \times 10^7$/kg; HR, .56; 95% CI, .30 to 1.02; $P = .059$) and GVHD prophylaxis with a calcineurin inhibitor alone (additional agents plus calcineurin inhibitors versus calcineurin inhibitors alone; HR, .60; 95% CI, .34 to 1.07; $P = .064$).

DISCUSSION

We present here the results of the largest retrospective study of ATLL patients receiving CBT; these results have extended our knowledge relative to that gained from other studies, which were limited by the numbers of cases [15,20,21]. Because graft source selection is strongly influenced by the donor availability, it is difficult to directly compare the outcomes of CBT with those of other allo-HSCT modalities. Nevertheless, the outcome of CBT for ATLL in the previous nationwide survey, with a 3-year OS rate of 17%, was clearly unsatisfactory because the study period corresponded with the developmental phase of CBT in adult patients [15]. Recent improvements in the outcome of CBT have been expected after optimization of the number of cells used for CBT and the improved HLA-compatibility of cord blood units [29-31]. Consequently, a recent nationwide survey data of adults with acute non-ATLL leukemia revealed no differences in the outcome of CBT in comparison with those of other allo-HSCT modalities [18,19]. However, the updated data (through December 2009) indicated that CBT for ATLL remained associated with a poorer 3-year OS of 20.6%, compared with OS of 34.4% among the 374 patients who received related BM or PBSC and 37.1% among the 319 patients who received unrelated BM ($P < .0001$) (Figure 1A). Therefore, the aim of the present study focused on the feasibility of CBT in the context of a larger cohort of patients with ATLL.

In the present study, 2 important findings were identified regarding CBT for ATLL. First, CBT cured patients with ATLL partly through a graft-versus-ATLL effect. Second, the high rate of TRM (approximately 50%) remains a significant problem. The OS curve for ATLL patients who received CBT reached a plateau by 3 years, suggesting long-term survival of selected patients, although the outcome of CBT for ATLL (3-year OS, 20%) did not compare favorably with those of other allo-HSCT modalities. Regarding the prognostic factors affecting survival, our present univariate analysis identified the 5 following significant variables associated with higher OS: (1) age, (2) disease status at transplantation, (3) ABO compatibility, (4) addition of agents such as MTX or MMF to calcineurin inhibitors for GVHD prophylaxis, and (5) development of acute GVHD. Further, the multivariate analysis revealed that the development of acute GVHD was independently associated with better OS relative to the absence of acute GVHD. A landmark analysis showed that the development of grade 1 to 2, or so called mild-to-moderate acute GVHD, was associated with better OS when compared with the absence of acute GVHD. There was also a trend toward a lower risk of relapse or progression with the development of acute GVHD when compared with the absence of GVHD and better OS in patients with limited chronic GVHD. Taken together, these data suggest the presence of a curative graft-versus-ATLL effect conferred by CBT.

However, it is typically difficult for physicians to optimize the effects of acute GVHD to prevent disease progression via graft-versus-ATLL. Therefore, a more realistic attempt would be the control of pretransplantation factors that might affect the CBT outcome and, thus, enhance the benefit of allo-HSCT. The multivariate analysis performed herein with respect to ATLL-related deaths identified disease status at CBT as the most important factor. ATLL usually resists conventional chemotherapy and must be treated soon after diagnosis because of the rapid proliferation of tumor cells, which generates a high tumor burden [2,3]. In the future, novel agents, such as mogamulizumab, a humanized anti-CCR4 monoclonal antibody, might improve CBT-associated survival by decreasing the tumor burden before transplantation [32-35]. Another possibility for improving survival might be reducing the time from diagnosis to transplantation while patients with ATLL remain chemosensitive. Moreover, CBT provides a considerable advantage for patients who require urgent allo-HSCT to combat aggressive ATLL.

In the present study, we have shown that CBT is feasible and curative. However, the high rate of TRM remained a significant problem. Bacterial infection caused the highest incidence of death (21%) during the neutropenic period. The infusion of lower numbers of nucleated cells ($< 2 \times 10^7$/kg), which is usually associated with delayed engraftment, was marginally associated with TRM. Neutrophil recovery is slower in patients treated via CBT, and immunosuppressed patients with ATLL might be at an increased risk of developing more frequent opportunistic infections [36]. Improved supportive care to prevent bacterial infection is required after CBT for patients experiencing a prolonged neutropenic period. The ongoing development of better graft engineering [37] or double-CBT [38] might facilitate rapid neutrophil recovery and, thus, help to reduce the TRM rate in CB recipients.

The present study has several limitations. First, our results concerning the effect of chronic GVHD on survival should be interpreted with caution because the relatively small number of patients who developed chronic GVHD did not allow us to evaluate the effect of this condition on survival in a multivariate analysis. Instead, we were limited to performing a landmark analysis of OS according to the severity of chronic GVHD. Certainly, we detected a trend toward higher OS in patients with limited chronic GVHD when compared with patients without chronic GVHD, suggesting the possible presence of a graft-versus-ATLL effect. However, these results might be biased because of insufficient statistical power. Our future studies will assess the effect of chronic GVHD on the outcome of CBT for the treatment of ATLL after a long-term follow-up. Although the present study employed, to our knowledge, the largest cohort of CBT-treated patients to date and our results demonstrated that CBT is a feasible and effective treatment, this was a retrospective analysis. Therefore, this finding requires confirmation in prospective studies. To establish reliable criteria for CBT administration, a prospective multicenter clinical trial is underway in Japan to evaluate the safety and efficacy of CBT combined with Flu, Mel, and low-dose TBI (4 Gy) along with GVHD prophylaxis (tacrolimus and MMF [39]).

In conclusion, CBT is feasible and effective for patients with ATLL and acts via a graft-versus-ATLL effect. However, the outcome of CBT is unsatisfactory when compared with those of other allo-HSCT modalities. The high rate of TRM must be reduced, and the development of novel strategies is required to further improve the outcome of CBT.

ACKNOWLEDGMENTS

The authors thank the physicians and data managers at the institutes who contributed valuable data regarding ATLL-related transplantation to the Japan Society for Hematopoietic Cell Transplantation. We also thank all members of the data management committees of the JSHCT. This work was supported in part by MEXT KAKENHI Grant Number 25461453 (K.K.) and H22-Ganrinsho-Ippan-028 (N.U.).

Conflict of interest disclosure: The authors declare no competing financial interests.

Authorship contributions: K.K., I.C., A.W., and A.U. designed the study; K.K., I.C., A.W., N.U., S.T., Y. Moriuchi, Y. Miyazaki, H.N., E.O., M.M., T.E., K.A., H.S., K. Kato, R.S., T.Y., and A.U. collected and analyzed the data; K.K. and T.Y. performed the statistical analysis. K.K. wrote the manuscript and created the figures and tables; all authors critically reviewed the manuscript and read and approved the final version of the manuscript.

Financial disclosure: The authors have nothing to disclose.

REFERENCES

1. Uchiyama T, Yodoi J, Sagawa K, et al. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood*. 1977;50:481-492.
2. Tsukasaki K, Maeda T, Arimura K, et al. Poor outcome of autologous stem cell transplantation for adult T cell leukemia/lymphoma: a case report and review of the literature. *Bone Marrow Transplant*. 1999;23:87-89.
3. Tsukasaki K, Utsunomiya A, Fukuda H, et al. VCAP-AMP-VECP compared with biweekly CHOP for adult T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study JCOG9801. *J Clin Oncol*. 2007;25:5458-5464.
4. Harashima N, Kurihara K, Utsunomiya A, et al. Graft-versus-Tax response in adult T-cell leukemia patients after hematopoietic stem cell transplantation. *Cancer Res*. 2004;64:391-399.
5. Kanda J, Hishizawa M, Utsunomiya A, et al. Impact of graft-versus-host disease on outcomes after allogeneic hematopoietic cell transplantation for adult T-cell leukemia: a retrospective cohort study. *Blood*. 2012;119:2141-2148.
6. Ishida T, Hishizawa M, Kato K, et al. Allogeneic hematopoietic stem cell transplantation for adult T-cell leukemia-lymphoma with special emphasis on preconditioning regimen: a nationwide retrospective study. *Blood*. 2012;120:1734-1741.
7. Itonaga H, Tsushima H, Taguchi J, et al. Treatment of relapsed adult T-cell leukemia/lymphoma after allogeneic hematopoietic stem cell transplantation: the Nagasaki Transplant Group experience. *Blood*. 2013;121:219-225.
8. 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;27:15-20.
9. Kami M, Hamaki T, Miyakoshi S, et al. Allogeneic haematopoietic stem cell transplantation for the treatment of adult T-cell leukemia/lymphoma. *Br J Haematol*. 2003;120:304-309.
10. Fukushima T, Miyazaki Y, Honda S, et al. Allogeneic hematopoietic stem cell transplantation provides sustained long-term survival for patients with adult T-cell leukemia/lymphoma. *Leukemia*. 2005;19:829-834.
11. Nakase K, Hara M, Kozuka T, et al. Bone marrow transplantation from unrelated donors for patients with adult T-cell leukemia/lymphoma. *Bone Marrow Transplant*. 2006;37:41-44.
12. Kato K, Kanda Y, Eto T, et al. Allogeneic bone marrow transplantation from unrelated human T-cell leukemia virus-I-negative donors for adult T-cell leukemia/lymphoma: retrospective analysis of data from the Japan Marrow Donor Program. *Biol Blood Marrow Transplant*. 2007;13:90-99.
13. Shiratori S, Yasumoto A, Tanaka J, et al. A retrospective analysis of allogeneic hematopoietic stem cell transplantation for adult T cell leukemia/lymphoma (ATL): clinical impact of graft-versus-leukemia/lymphoma effect. *Biol Blood Marrow Transplant*. 2008;14:817-823.
14. Yonekura K, Utsunomiya A, Takatsuka Y, et al. Graft-versus-adult T-cell leukemia/lymphoma effect following allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2008;41:1029-1035.
15. Hishizawa M, Kanda J, Utsunomiya A, et al. Transplantation of allogeneic hematopoietic stem cells for adult T-cell leukemia: a nationwide retrospective study. *Blood*. 2010;116:1369-1376.
16. Choi I, Tanosaki R, Uike N, et al. Long-term outcomes after hematopoietic SCT for adult T-cell leukemia/lymphoma: results of prospective trials. *Bone Marrow Transplant*. 2011;46:116-118.
17. Takizawa J, Aoki S, Kurasaki T, et al. Successful treatment of adult T-cell leukemia with unrelated cord blood transplantation. *Am J Hematol*. 2007;82:1113-1115.
18. Atsuta Y, Morishima Y, Suzuki R, et al. Comparison of unrelated cord blood transplantation and HLA-mismatched unrelated bone marrow transplantation for adults with leukemia. *Biol Blood Marrow Transplant*. 2012;18:780-787.
19. Atsuta Y, Suzuki R, Nagamura-Inoue T, et al. Disease-specific analyses of unrelated cord blood transplantation compared with unrelated bone marrow transplantation in adult patients with acute leukemia. *Blood*. 2009;113:1631-1638.
20. Nakamura T, Oku E, Nomura K, et al. Unrelated cord blood transplantation for patients with adult T-cell leukemia/lymphoma: experience at a single institute. *Int J Hematol*. 2012;96:657-663.
21. Fukushima T, Itonaga H, Moriuchi Y, et al. Feasibility of cord blood transplantation in chemosensitive adult T-cell leukemia/lymphoma: a retrospective analysis of the Nagasaki Transplantation Network. *Int J Hematol*. 2013;97:485-490.
22. Giralt S, Ballen K, Rizzo D, et al. Reduced-intensity conditioning regimen workshop: defining the dose spectrum. Report of a workshop convened by the center for international blood and marrow transplant research. *Biol Blood Marrow Transplant*. 2009;15:367-369.
23. Bacigalupo A, Ballen K, Rizzo D, et al. Defining the intensity of conditioning regimens: working definitions. *Biol Blood Marrow Transplant*. 2009;15:1628-1633.
24. Scrucca L, Santucci A, Aversa F. Competing risk analysis using R: an easy guide for clinicians. *Bone Marrow Transplant*. 2007;40:381-387.
25. Scrucca L, Santucci A, Aversa F. Regression modeling of competing risk using R: an in depth guide for clinicians. *Bone Marrow Transplant*. 2010;45:1388-1395.
26. Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. *Stat Med*. 1999;18:695-706.
27. Cortese G, Andersen PK. Competing risks and time-dependent covariates. *Biom J*. 2010;52:138-158.
28. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant*. 2013;48:452-458.
29. Laughlin MJ, Eapen M, Rubinstein P, et al. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *N Engl J Med*. 2004;351:2265-2275.
30. Rocha V, Labopin M, Sanz G, et al. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *N Engl J Med*. 2004;351:2276-2285.

31. Sanz MA. Cord-blood transplantation in patients with leukemia—a real alternative for adults. *N Engl J Med.* 2004;351:2328-2330.
32. Yamamoto K, Utsunomiya A, Tobinai K, et al. Phase I study of KW-0761, a defucosylated humanized anti-CCR4 antibody, in relapsed patients with adult T-cell leukemia-lymphoma and peripheral T-cell lymphoma. *J Clin Oncol.* 2010;28:1591-1598.
33. Ishida T, Joh T, Uike N, et al. Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: a multicenter phase II study. *J Clin Oncol.* 2012;30:837-842.
34. Ito Y, Miyamoto T, Chong Y, et al. Successful treatment with anti-CC chemokine receptor 4 MoAb of relapsed adult T-cell leukemia/lymphoma after umbilical cord blood transplantation. *Bone Marrow Transplant.* 2013;48:998-999.
35. Kato K, Miyamoto T, Numata A, et al. Diffuse panbronchiolitis after humanized anti-CCR4 monoclonal antibody therapy for relapsed adult T-cell leukemia/lymphoma. *Int J Hematol.* 2013;97:430-432.
36. Itonaga H, Taguchi J, Fukushima T, et al. Distinct clinical features of infectious complications in adult T cell leukemia/lymphoma patients after allogeneic hematopoietic stem cell transplantation: a retrospective analysis in the Nagasaki transplant group. *Biol Blood Marrow Transplant.* 2013;19:607-615.
37. de Lima M, McNiece I, Robinson SN, et al. Cord-blood engraftment with ex vivo mesenchymal-cell coculture. *N Engl J Med.* 2012;367:2305-2315.
38. Rocha V, Crotta A, Ruggeri A, et al. Double cord blood transplantation: extending the use of unrelated umbilical cord blood cells for patients with hematological diseases. *Best Pract Res Clin Haematol.* 2010;23:223-229.
39. Uchida N, Wake A, Nakano N, et al. Mycophenolate and tacrolimus for graft-versus-host disease prophylaxis for elderly after cord blood transplantation: a matched pair comparison with tacrolimus alone. *Transplantation.* 2011;92:366-371.

Tomohiro Ishigaki*, Yuji Zaike, Masanori Nojima, Seiichiro Kobayashi, Nobuhiro Ohno, Kaoru Uchimaru, Arinobu Tojo, Hiromitsu Nakauchi and Nobukazu Watanabe

Quantification of adult T-cell leukemia/lymphoma cells using simple four-color flow cytometry

Abstract

Background: The absolute number of adult T-cell leukemia/lymphoma (ATL) cells in peripheral blood is an essential indicator to evaluate disease status. However, microscopically counting ATL cells based on morphology requires experience and tends to be inaccurate due to the rarity of ATL.

Methods: Based on our research showing that acute-type ATL cells are specifically enriched in the CD4+/CD7- (CD7N) fraction, a new analytical method to accurately quantify ATL cells was established using an internal bead standard and simple four-color flow cytometry. This method was verified by comparison with microscopic examination of 49 peripheral blood samples and used to follow up patients.

Results: A strong correlation was observed between the number of CD7N cells measured by flow cytometry and the number of abnormal lymphocytes measured microscopically by experienced technicians [Pearson's R, 0.963; Spearman's rho, 0.921; intercorrelation coefficient, 0.962]. The linear regression coefficient was close to 1 (β=1.013). Our method could detect 1 cell/μL, and the limit of quantitation was between 2.9 and 9.8 cells/μL. The frequency of CD7N cells among CD4+ cells changed during chemotherapy, which reflected differences between chemosensitive and chemoresistant cases. Kaplan-Meier analysis with a log-rank test showed that patients with decreased CD7N proportion after chemotherapy had significantly longer disease-specific survival (p=0.003).

Conclusions: Our newly established method quantified tumor cells in patients with acute-type ATL. Furthermore, this method was useful for assessing the efficacy of chemotherapy, and the change of the CD7N proportion could be more important to predict prognosis.

Keywords: adult T-cell leukemia/lymphoma (ATL); flow cytometry; HAS-Flow; human T-cell leukemia virus type 1 (HTLV-1).

DOI 10.1515/cclm-2014-0183
Received February 19, 2014; accepted June 18, 2014

***Corresponding author: Tomohiro Ishigaki,** Clinical FACS Core Laboratory, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan, Phone: +81 3 5449 5765, Fax: +81 3 5449 5750, E-mail: ishigaki@ims.u-tokyo.ac.jp; and Institute of Medical Science, Division of Stem Cell Therapy, University of Tokyo, Tokyo, Japan
Yuji Zaike: Institute of Medical Science, Department of Laboratory Medicine, Research Hospital, University of Tokyo, Tokyo, Japan
Masanori Nojima: Institute of Medical Science, Division of Advanced Medicine Promotion, University of Tokyo, Tokyo, Japan
Seiichiro Kobayashi: Institute of Medical Science, Division of Molecular Therapy, University of Tokyo, Tokyo, Japan
Nobuhiro Ohno and Kaoru Uchimaru: Institute of Medical Science, Department of Hematology and Oncology, Research Hospital, University of Tokyo, Tokyo, Japan
Arinobu Tojo: Institute of Medical Science, Division of Molecular Therapy, University of Tokyo, Tokyo, Japan; and Institute of Medical Science, Department of Hematology and Oncology, Research Hospital, University of Tokyo, Tokyo, Japan
Hiromitsu Nakauchi: Institute of Medical Science, Division of Stem Cell Therapy, University of Tokyo, Tokyo, Japan
Nobukazu Watanabe: Clinical FACS Core Laboratory, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Introduction

Adult T-cell leukemia/lymphoma (ATL) is a mature T-cell neoplasm caused by human T-cell leukemia virus type 1 (HTLV-1). According to the classification of the Japanese Lymphoma Study Group (Shimoyama classification) [1], ATL is classified into four subtypes: smoldering, chronic, lymphoma, and acute-type. Chemotherapy should be offered to patients with acute, lymphoma, and chronic-type ATL with unfavorable prognostic factors.

A quantitative analysis of ATL cells is essential to evaluate the therapeutic effect. The number of ATL cells is currently estimated based on morphological abnormalities. Cells with abnormally hyperlobulated nuclei, which are termed 'flower cells', are characteristic ATL cells, but ATL cells are not always typical flower cells. ATL cells are morphologically diverse among cases, and the histological feature of ATL is diffuse proliferation of abnormal cells that vary in size and shape [2]. As discriminating ATL cells

morphologically from other lymphocytes, particularly from reactive atypical lymphocytes, is difficult, experience is required. Consequently, these difficulties cause differences between examiners and errors; therefore, morphological quantification of ATL cells tends to be inaccurate.

Many researchers have attempted to develop other counting methods or to identify markers that reflect the number of ATL cells [3–9]. The most prevalent method is HTLV-1 proviral load (PVL), measured by quantitative real-time polymerase chain reaction [5–9]. Although PVL in peripheral blood mononuclear cells (PBMCs) is a surrogate marker of the number of HTLV-1-infected cells, it has several problems. First, PVL can be affected by the total number of PBMCs, as it is only expressed as a percentage in PBMCs and not an absolute value. Second, PVL reflects only the burden of infection and is not specific for ATL cells. Third, PVL may overestimate the frequency of ATL cells when ATL cells harbor multiple copies within a single cell [5]. Furthermore, PVL measurements vary widely among laboratories, and should be standardized [10]. Finally, the method is time-consuming and is not sufficiently easy to use frequently for clinical testing. Therefore, establishing a new method to quantify ATL cells more accurately and easily is required.

We assessed a number of samples from patients with ATL using 12-color flow cytometry, and have established the flow cytometric method named 'HAS (HTLV-1 Analyzing System)-Flow,' to analyze ATL cells. Downregulation of CD3 and CD7 is observed in ATL cells, and we reported that CD4-positive cells in patients with ATL can be classified into three groups of: $CD3^{positive}/CD7^{positive}$ (CD7P), $CD3^{dimly\ positive}/CD7^{dimly\ positive}$ (CD7D), and $CD3^{dimly\ positive}/CD7^{negative}$ (CD7N) [11]. Examining the PVL showed that HTLV1-infected cells were concentrated mainly in the CD7N fraction in patients with acute-type ATL. Moreover, the Vβ repertoire revealed that tumor cells are specifically enriched in the CD7N fraction, usually to almost 100% after assessing clonality in the context of the T-cell receptor [11]. Therefore, the number of CD7N cells reflects the number of ATL cells. We applied these findings to a new clinical test, and established a new analytical method using simple four-color flow cytometry.

Materials and methods

Patient samples

Peripheral blood samples were collected from patients with acute-type ATL who were admitted to the Research Hospital at the Institute of Medical Science, University of Tokyo (IMSUT) between June 2011 and December 2012. Some of the patients were transferred to our hospital after a few courses of chemotherapy. This study was approved by the Research Ethics Committee of IMSUT, and written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. All patients were diagnosed with acute-type ATL according to Shimoyama's criteria [1] and had not received hematopoietic stem cell transplantation (HSCT). In total, 49 samples from 14 patients were collected before treatment or just before a course of chemotherapy (Table 1). The effectiveness of chemotherapy was evaluated using the ATL response criteria [12].

Sample preparation and immunofluorescence staining

Peripheral blood was obtained in Vacutainer Hemogard Plus tubes (BD Biosciences, San Jose, CA, USA) by conventional venipuncture. As the volume required for our method was only 100 μL, the rest of peripheral blood samples used for routine laboratory tests were applied for measurement. A ProCOUNT method using Trucount tubes (BD Biosciences), in which a known number of fluorescent reference beads are included, was adopted to measure the absolute number of cells. First, fluorescently labeled antibodies, consisting of fluorescein isothiocyanate (FITC)-CD4 (BioLegend, San Diego, CA, USA), phycoerythrin (PE)-CD7 (BD Pharmingen, San Jose, CA, USA), allophycocyanin (APC)-CD3 (BioLegend), and PerCP-Cy5.5-CD14 (BioLegend), were mixed in a Trucount tube. Then, 100 μL of whole peripheral blood were added to the tube and mixed well. The cells were stained for 15 min at room temperature. After staining, 1 mL of Cell Lysis Buffer (BD Biosciences) was added to lyse the red blood cells. After 15 min, the sample was vortexed gently for 10 s and analyzed with a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA) as soon as possible.

Sorting, cytospin, and Wright-Giemsa staining

A FACS Aria II SORP flow cytometer (BD Immunocytometry Systems) was used for cell sorting. Sorted cells were fixed on glass slides by cytospinning (20×*g*, 5 min) and subjected to Wright-Giemsa staining.

Flow cytometric analysis

Flow cytometry data were analyzed with FlowJo 9.6 (Treestar, San Carlos, CA, USA). We defined CD4-positive cells according to the gating procedure shown in Figure 1. The first gate on the FSC versus SSC plot was set relatively wide so as not to miss lymphocytes (Figure 1A), because adjacent monocytes and hemolytic debris could be excluded by subsequent CD14-negative selection (Figure 1B) and CD4-positive selection steps (Figure 1C). Purified CD4-positive cells were drawn in a pseudo-color plot (Figure 1E) and in a contour plot (Figure 1F). The borders among CD7P, CD7D, and CD7N in the CD7 versus CD3 plot were drawn according to contour lines (Figure 1F). Reference beads were gated in the PE versus FITC plot, according to the manufacturer's instructions (Figure 1D).

Table 1 Forty-nine samples from 14 patients with acute-type ATL were analyzed.

Patient ID	Age	Sex	Number of analysis	Average interval of analysis (range)	At the first flow cytometric evaluation						CD3 expression of ATL cells	HSCT before/during analysis
					CD7P, /μL	CD7D, /μL	CD7N, /μL	WBC, /μL	Normal lymphocytes, %	Abnormal lymphocytes, %		
1	62	M	2	14	12.9	36.4	538.0	2340	7.0	38.0	Dim	–
2	38	F	3	38.5 (14–63)	247.8	92.9	15,144.9	25,020	5.7	71.2	Dim	–
3	43	M	4	28.0 (13–43)	81.1	173.6	200.9	10,790	7.4	2.4	Dim	–
4	69	M	4	25.0 (14–40)	63.4	60.7	116.9	3450	7.5	3.0	Dim	–
5	62	M	4	17.7 (14–20)	68.0	172.7	838.5	11,310	8.5	8.5	Dim	–
6	62	M	2	27	907.7	5214.6	13,191.0	27,230	8.4	65.4	Dim	–
7	63	F	7	44.5 (30–57)	116.2	26.8	544.6	3620	12.4	21.4	Dim	–
8	67	M	6	36.2 (12–53)	189.5	266.4	290.3	10,280	15.3	3.8	Dim	–
9	50	M	6	30.0 (14–54)	86.3	112.9	6442.3	19,050	3.3	33.0	Dim	–
10	50	M	1	–	21.7	52.8	142.7	3800	10.3	3.0	Dim	–
11	44	M	1	–	683.8	184.4	6815.1	22,840	11.0	33.0	Negative	–
12	59	M	3	27.5 (27–28)	312.9	480.3	1951.4	7550	16.0	38.0	Dim	–
13	72	M	4	28.0 (25–32)	129.6	28.9	397.8	11,520	4.0	5.5	Dim	–
14	63	M	2	29	167.1	164.0	115.7	4590	23.5	1.0	Dim	–

HSCT, hematopoietic stem cell transplantation

The absolute number of cells was accurately calculated from the ratio of beads to cells in the region of the interest (Figure 1G). For example, when the total number of beads in a Trucount tube was 52187, the number of CD7N cells in the case in Figure 1 was calculated as follows: CD7N cells=(5482/100)×(52187/5201)=550.1/μL.

Validation of the flow cytometric quantification

Cryopreserved PBMCs of acute-type ATL patients were used for validation of this assay, and CD4+CD7N cells were quantified in the same way indicated above. As a blank control, phosphate buffered saline (PBS) was used. The intra-assay variation was assessed by calculating the coefficient of variation (CV) with 10 different density gradients ranging from 0 to 30000 cells/μL. Each sample was assayed six times. The limits of detection (LoD) and quantitation (LoQ) were also assessed. The LoQ was determined by the lowest concentration whose six replicates had a CV <20%. The inter-assay variation was assessed by calculating the CV of multiple determinations of a same sample measured on different days.

Conventional assessment of morphologically abnormal lymphocytes

When samples were examined by flow cytometry, total white blood cell (WBC) counts (normal range, 3500–9100/μL) were performed mechanically using an XE-2100 system (Sysmex, Kobe, Japan), and 300–400 WBCs per sample were classified by clinical technicians who were skilled in morphologically classifying ATL cells. Abnormal lymphocytes were classified according to the guideline of Japanese Association of Medical Technologists (JAMT). Briefly, lymphocytes with nuclear abnormalities, such as lobulated nuclei, multiple nuclei, evident nucleoli, or high nucleo-cytoplasmic ratio, were classified as abnormal lymphocytes. The absolute number of morphologically abnormal lymphocytes was calculated by multiplying their percentage by the total number of WBCs.

Measurement of LDH and sIL-2R

Disease status was also followed by lactate dehydrogenase (LDH) and soluble IL-2 receptor (sIL-2R). LDH activities were measured by the lactate-to-pyruvate assay according to the recommendations of the Japanese Society of Clinical Chemistry (normal range, 106–211 IU/L). Serum sIL-2R levels were measured by ELISA (normal range, 145–519 U/mL).

Statistical analysis

The correlation between the number of CD7N cells measured by flow cytometry and the number of abnormal lymphocytes measured by microscopic counting was assessed by Pearson's correlation coefficients (R), Spearman's correlation coefficients (rho), intra-class correlation coefficients (ICC), and linear regression coefficients.

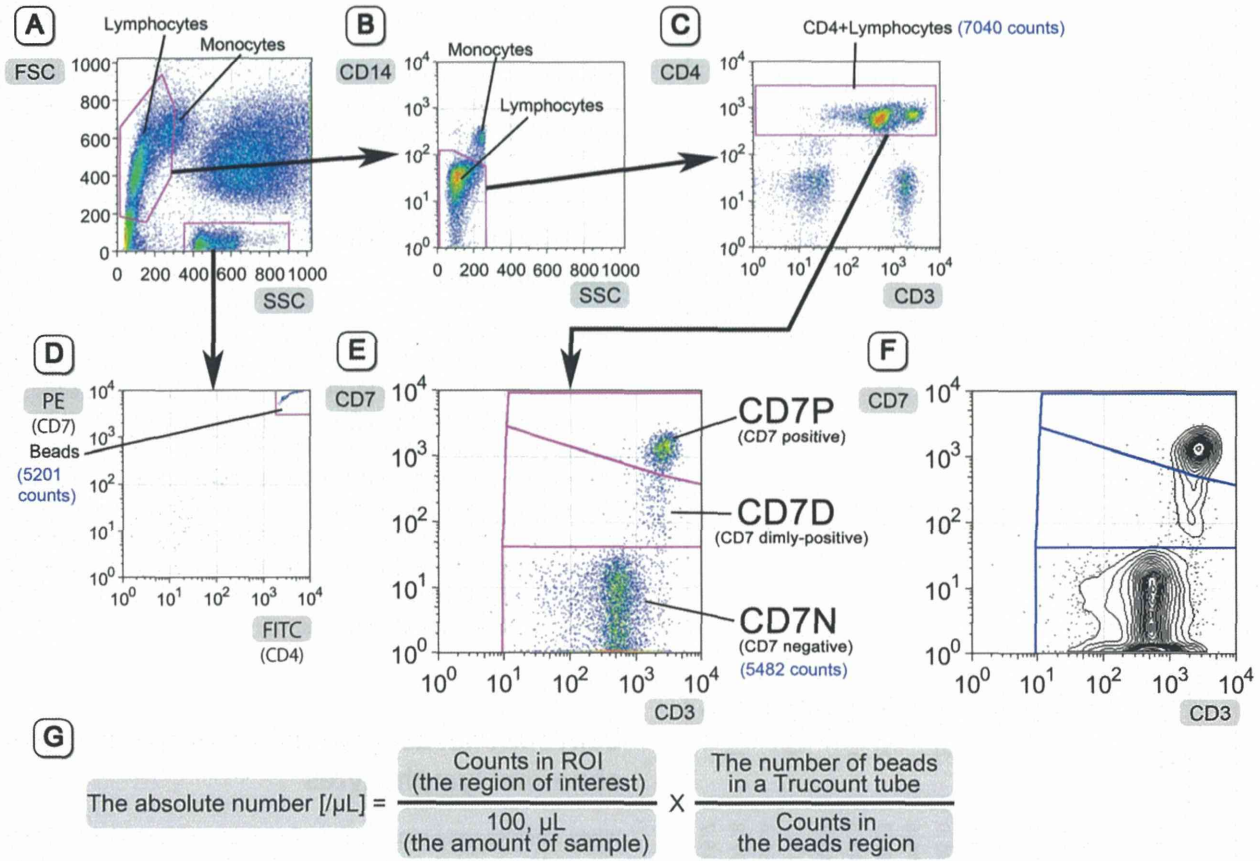

Figure 1 Flow cytometric gating procedure.
CD4-positive cells were properly defined according to the procedure shown here (A–C). CD4-positive cells were classified into CD7P, CD7D, and CD7N cells according to the contour lines of a CD7 versus CD3 plot (E, F). Reference beads were gated in the PE versus FITC plot (D). The absolute number of CD7N cells was calculated using this formula (G).

As data from both measurements followed a log-normal distribution, these analyses were performed after log-transformation. Bland-Altman analysis was used to calculate the agreement between flow cytometric and microscopic counting. In addition, the inverse probability weighting (IPW) method and mixed model (varying intercepts and slopes) were used due to the imbalance in sample number and individual differences between the patients. Kaplan-Meier analysis with a post hoc log-rank test was performed for disease-specific survival stratified by whether relative decrease of the CD7N proportion after the chemotherapy was over 5% or not. Statistical analyses were performed using the GraphPad Prism software, ver. 6.0c (GraphPad Software Inc., San Diego, CA, USA) and SPSS software ver. 20 (IBM Corp., Armonk, NY, USA).

Results

Abnormal lymphocytes are enriched in the CD7N fraction

Along with our previous research using 12-color flow cytometry, simple four-color flow cytometry could classify CD4-positive lymphocytes into three groups of: CD7P, CD7D, and CD7N (Figure 1). Almost all of CD7-negative cells were dimly-positive for CD3, except for a case in which CD7-negative cells were all negative for CD3. In the representative case of acute-type ATL where the proportions of CD7P, CD7D, and CD7N in CD4+ cells were 2.7%, 1.7%, and 95.6%, the proportions of abnormal lymphocytes in these three fractions were 0.0%, 6.0%, and 99.0%, respectively. Morphological evaluation showed that almost all CD7N cells were morphological abnormal, whereas none of the CD7P cells were abnormal.

Intra- and inter-assay variation of this flow cytometric quantification was low

To evaluate the precision of our assay, we assessed the intra-assay CVs of 10 different density gradients. The intra-assay CVs of samples with the average of 1.2, 2.9, 9.8, 20.5, 42.7, 442.7, 1073.7, 8081.2, and 30,729.8 cells/μL were 30.69, 31.17, 5.94, 4.89, 3.64, 4.02, 4.61, 2.45, and 4.98%, respectively. Six determinations of a blank control were all measured to be

0 cells/μL. The limit of detection was about 1 cell/μL, and the limit of quantitation was estimated to be between 2.9 and 9.8 cells/μL. The inter-assay CV was 5.81%.

Correlation between CD7N cells and morphologically abnormal lymphocytes

We found a significant correlation between the number of CD7N cells and the number of morphologically abnormal lymphocytes (Pearson's R=0.963, Spearman's ρ=0.921, and ICC=0.962; Figure 2A). Moreover, the linear regression coefficient was close to 1 [β=1.013, 95% confidence interval (CI), 0.991–1.034]. The calculated number of CD7N cells was often similar to the number of morphologically abnormal lymphocytes. In two cases, flow cytometric analysis detected ATL cells but microscopic counting did not.

In addition, we reassessed the correlation using the IPW method and the mixed model considering the imbalance in sample number and individual differences between patients. Using the IPW method, Pearson's R=0.973, Spearman's ρ=0.942, and the linear regression coefficient was 1.010 (95% CI 0.990–1.030). The regression coefficient was 0.979 (95% CI 0.893–1.064) in the mixed model. Almost all results were improved from the crude analysis, and the influences of sampling imbalance and individual differences were limited.

The Bland-Altman plot showed good agreement between both measurements (Figure 2B). There seemed to be little additive or proportional bias.

The change in the CD7-CD3 profile was useful to evaluate the effectiveness of chemotherapy

We then compared the change in a CD7 versus CD3 plot of CD4-positive cells during chemotherapy between chemoresistant and chemosensitive cases. The proportion of CD7N cells in chemoresistant cases increased or did not change, although the absolute number of CD7N cells decreased slightly. In the case of Figure 3A, i.e., both the number of ATL cells and lactate dehydrogenase (LDH) decreased after the first course of chemotherapy, and the response seemed good. Nevertheless, the CD7-CD3 profile was almost unchanged. After the second course, all parameters, including the number of ATL cells and the LDH and soluble interleukin (IL)-2 receptor levels, increased. The disease was not controlled by chemotherapy, and the patient died after 1 month.

In contrast, the CD7-CD3 profile changed dramatically in clinically good responders who achieved a complete response or partial response. Representative data are shown in Figure 3B. In addition to an abrupt decrease in the absolute number of CD7N cells, the frequency of CD7N cells among CD4-positive cells decreased significantly, whereas the frequency of CD7P cells increased. While a significant proportion of patients with acute-type ATL cannot undergo HSCT because of uncontrollable disease, the patient in Figure 3B received allogeneic HSCT after several courses of chemotherapy.

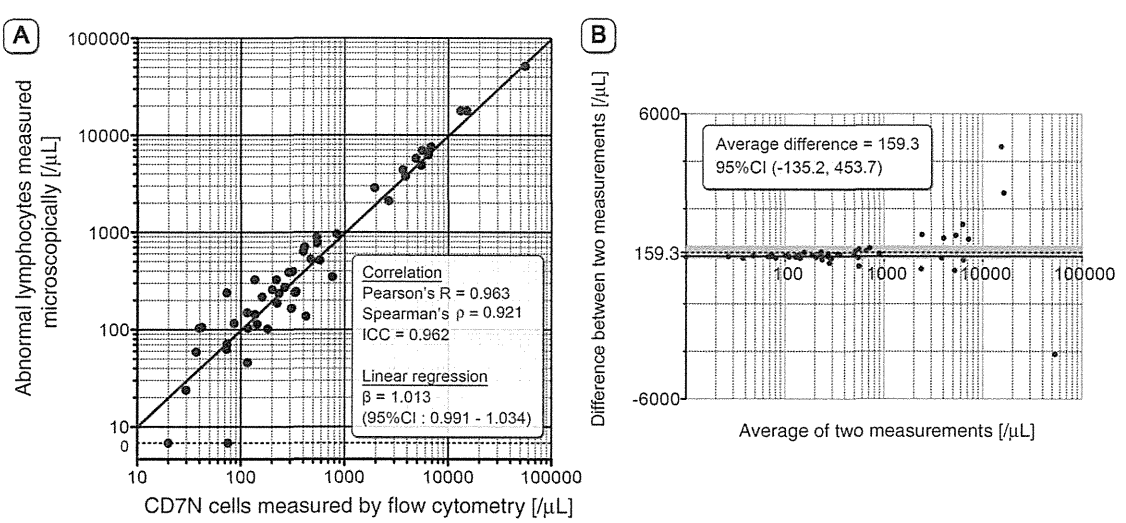

Figure 2 The correlation between CD7N cells and abnormal lymphocytes.
The correlation between CD7N cells measured by flow cytometry and abnormal lymphocytes measured microscopically was evaluated using three correlation tests and a linear regression analysis (A). The agreement between the two measurements was analyzed with a Bland-Altman plot (B).

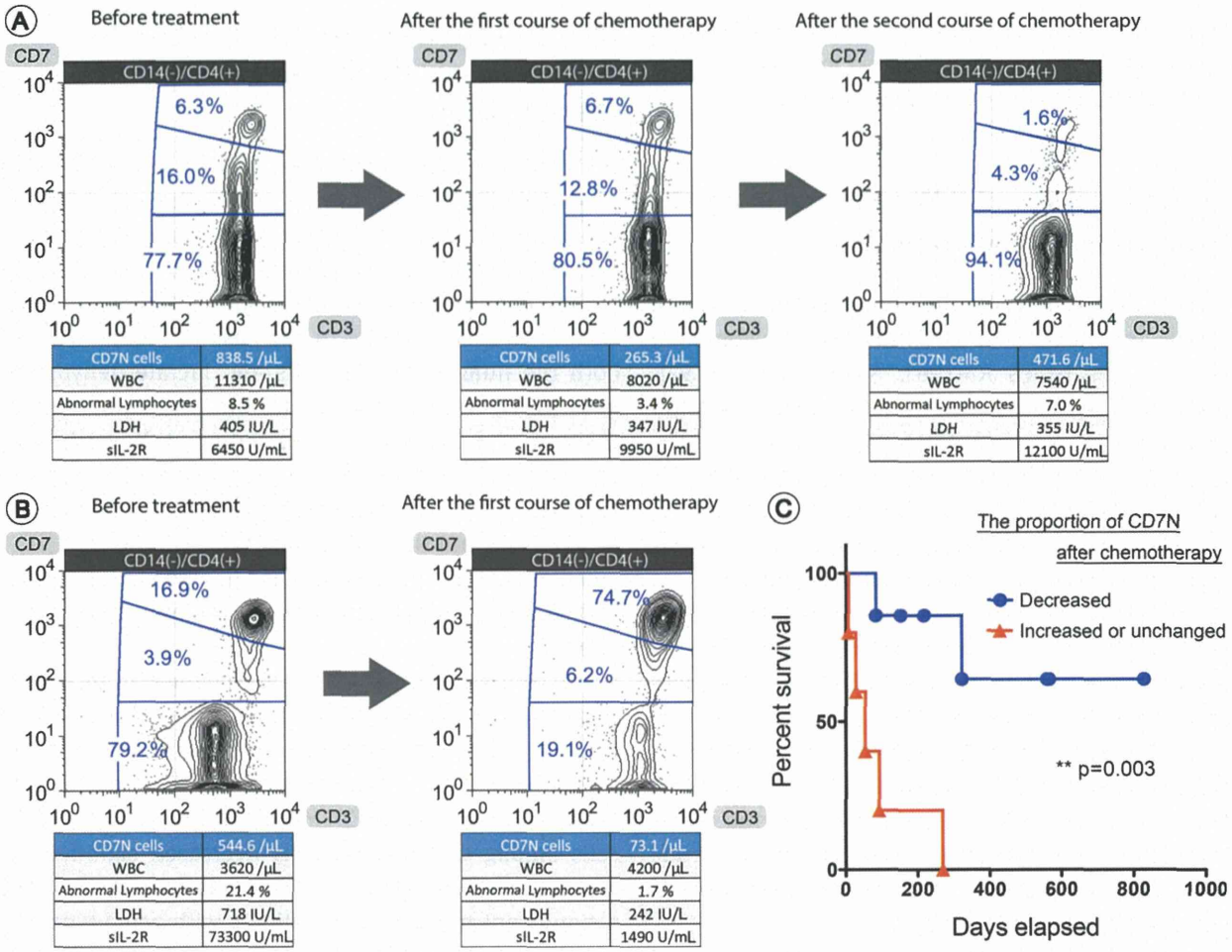

Figure 3 The CD7-CD3 profile allowed for an assessment of chemotherapy efficacy and could predict prognosis.
A patient with an almost unchanged CD7-CD3 profile after the first course of chemotherapy had an unfavorable prognosis after the second course (A). In contrast, a patient that showed a good clinical response to chemotherapy exhibited marked changes in the CD7-CD3 profile after only one course of chemotherapy (B). Kaplan-Meier suvival curves showed ATL patients with decreased CD7N proportion after chemotherapy showed significantly longer disease-specific survival (p=0.003) (C).

The pattern of change in the CD7-CD3 profile was observed repeatedly in many patients to reflect the effectiveness of chemotherapy, and these results suggested that the change in the CD7-CD3 profile is useful to assess the effect of chemotherapy.

ATL patients with decreased CD7N proportion after chemotherapy showed longer disease-specific survival

Next, we focused on the first chemotherapy during flow cytometric analyses periods, and examined the relation between the prognosis and the change of clinical parameters (Table 2). Consequently, the CD7N proportion was picked up, and acute-type ATL patients were classified into two groups by the relative change of the CD7N proportion after the chemotherapy. The CD7N proportion was considered decreased when a relative decrease of more than 5% was achieved after chemotherapy compared with the one before the chemotherapy. ATL patients with decreased CD7N proportion after chemotherapy showed longer survival than ones with unchanged or increased (Figure 3C). A log-rank test showed that there was a significant difference between the two groups (p=0.003). The difference suggested that the change in the CD7N proportion could be more important to predict the prognosis.

Discussion

Based on our previous studies [11, 13], we applied research findings to a clinical test. We made the procedure as simple as possible to maximize practicality after several

Table 2 The change of clinical parameters after the first chemotherapy.

Patient ID	Before chemotherapy				After chemotherapy				Change of CD7N proportion after chemotherapy	HSCT after flow cytometric analysis	Outcome (days after post-chemotherapy evaluation)
	Proportion of CD7N, %	Absolute number of CD7N, /μL	LDH, IU/L	sIL-2R, U/mL	Proportion of CD7N,%	Absolute number of CD7N, /μL	LDH, IU/L	sIL-2R, U/mL			
1	91.6	538.0	774	22,600	96.1	115.2	651	–	Increased	–	Died of ATL (7)
2	97.8	15,144.9	272	24,100	97.4	5479.9	108	27,700	Unchanged	–	Died of ATL (93)
3	44.1	200.9	376	4200	37.6	72.8	273	7420	Decreased	+	Died of ATL (322)
4	48.5	116.9	446	4040	60.7	181.2	717	22,200	Increased	–	Died of ATL (28)
5	77.7	838.5	405	6450	80.5	265.3	347	9950	Increased	–	Died of ATL (54)
6	68.3	13,191.0	633	11,700	57.9	2640.0	283	3500	Decreased	+	Died of ATL (83)
7	79.2	544.6	718	73,300	19.1	73.1	242	1490	Decreased	+	Alive in CR (828)
8	38.9	290.3	259	3500	30.9	796.5	200	2300	Decreased	–	Died of infection (152)[a]
9	97.0	6442.3	655	17,200	96.2	3859.2	392	21,400	Unchanged	+	Died of ATL (270)
12	71.1	1951.4	582	15,500	65.2	577.1	238	2840	Decreased	+	Alive in CR (565)
13	71.5	397.8	251	6340	59.6	335.6	169	1420	Decreased	–	Alive in CR (559)
14	25.9	115.7	293	5230	14.3	29.7	452	7820	Decreased	–	Transferred to another hospital (217)[a]

[a]ATL was under control when patients were censored.

trials, and finally established a practical flow cytometry method to quantify acute-type ATL cells.

Flow cytometry is highly sensitive in detecting minimal residual disease (MRD) of hematological malignancies. Similar flow cytometric approach for detection of MRD in ATL was reported previously, and a multi-parametric approach using CD2, CD3, CD4, CD5, CD7, CD25, CD26, and CD27 was useful for detection of ATL cells [14]. We established here a more practical and easier flow cytometric method using only CD3, CD4, CD7, and CD14. Moreover we limited the use to acute-type ATL cases because our previous studies showed the expression of cell surface antigens on ATL cells was slightly different among subtypes.

Combination of gates is also important and characteristic of our method. We showed here all the procedures including gating and quantification so that the method could be easily applied in other hospitals. This test provides accurate quantification of CD7N lymphocytes achieved by establishing an appropriate gating procedure (Figure 1). As a first gate, two methods are generally used to gate lymphocytes. One is FSC versus SSC gating, and the other is CD45 versus SSC gating [15]. The latter method is often used to analyze malignant hematologic diseases and eliminate red blood cell debris but is not convenient to analyze ATL cells because these occasionally lose [16] or express a high level of CD45. Therefore, we applied FSC versus SSC gating for lymphocyte gating. Moreover, eliminating monocytes is necessary to precisely enumerate CD7N lymphocytes because monocytes are weakly positive for CD4 and negative for CD7. Hence, we gated out monocytes carefully by CD14 staining before CD4-positive selection. Combination of the first wide lymphocyte gate and the following two strict gates enabled purification of CD4-positive lymphocytes without excess or deficiency. Then, CD7N cells were defined according to the contour lines in a plot of CD4-positive lymphocytes. Drawing the border between CD7D and CD7N cells was easy because contour lines clearly and horizontally separated these two populations. In contrast, the border between CD7P and CD7D cells was sometimes difficult to determine; thus, further improvement may be needed. However, the latter border is not necessary to estimate the number of ATL cells using this method.

Although the number of ATL cells is currently estimated based on morphologically abnormal cells, morphological evaluation of ATL cells often differs between examiners. Particularly, morphological enumeration by inexperienced examiners tends to be inaccurate. We therefore assessed here the correlation between the number of CD7N cells and the number of morphologically

abnormal lymphocytes evaluated only by experienced technicians (Figure 2A). As expected, a strong correlation was identified. It is noteworthy that the regression coefficient was very close to 1.0. This new flow cytometric method is useful for accurate evaluation of ATL cells. Furthermore, only flow cytometry detected ATL cells in two cases. As the validation study of the assay showed very low limits of detection and quantitation, flow cytometry could detect ATL cells more sensitively than microscopic counting.

The Bland-Altman plot also showed good agreement and the relatively small average difference between the two measurements (Figure 2B). However, the three samples with the highest WBCs had high differences between the two methods. While flow cytometry allows rapid and accurate analysis of a much larger number of cells, manual counting has limitations in accuracy and the number of counts. Since the absolute number of morphologically abnormal lymphocytes was calculated by multiplying their percentage by the total number of WBCs, the margin of error in microscopic counting tended to get larger as the number of WBCs got higher. Although the relative differences between the two methods were generally not so high, these findings suggested the number of abnormal lymphocytes in samples with high WBC counts should be carefully examined.

This flow cytometry-based method has many advantages compared to PVL. First, flow cytometry allows for calculation of the absolute number of ATL cells, which is not affected by the number of other cells. Second, intra- and inter-assay variations of our method were confirmed to be low. The inter- and intra-laboratory variabilities of the ProCOUNT method are also known to be low [17]. Therefore, this flow cytometric method is precise and can be easily standardized. Third, it is practical and takes no more than 1 h.

In addition to its usefulness for quantitation, we also found that the change in the CD7-CD3 profile discriminated cases sensitive to chemotherapy and may predict prognosis (Figure 3A and B). Serum sIL-2R and LDH levels are also clinically important [18, 19], but they are not as specific for disease status because they are influenced by other factors, such as infection, inflammation, and hemolysis. Patients with a better prognosis tended to have a markedly better change in the CD7-CD3 profile after only one course of chemotherapy. In contrast, cases with unchanged or worse CD7-CD3 profiles after one course of chemotherapy tended to have an unfavorable prognosis after the second course, even if the number of ATL cells decreased, or the levels of sIL-2R and LDH improved initially.

From various parameters of the CD7-CD3 profile, we had tried picking up prognostic indicators. We focused on the first chemotherapy, and examined the change of various clinical parameters (Table 2) and their relation with survival. Needless to say, the number of CD7N cells, as the number of ATL cells, was important to evaluate the disease status through the follow-up, and robust reduction of the number of CD7N cells was necessary for a better prognosis. However, the change of the CD7N proportion seemed more sensitive. We examined the relationship between the disease-specific survival and the relative change of the CD7N proportion after chemotherapy. Although the number of patients was limited, it is noteworthy that patients with decreased CD7N proportion had significantly longer survival (Figure 3C). Further accumulation of cases and longer follow-up are warranted to elucidate the ability of this method to predict prognosis.

Our experience suggests that this method can be applied to almost all patients with acute-type ATL. However, some limitations should be noted. First, this method did not accurately detect ATL cells in patients who had undergone HSCT, as downregulation of CD7 in CD4-positive non-ATL lymphocytes was observed in most cases [20]. Second, this method cannot identify ATL cells in rare cases in which ATL tumor cells lack CD4 expression or express CD7. Therefore, a brief confirmation of the ATL phenotype using other surface markers is recommended before flow cytometric quantification.

In summary, we established a clinical test to accurately quantify ATL cells in patients with acute-type ATL using simple four-color flow cytometry. This newly established clinical application of 'HAS-Flow' will provide more accurate enumeration of ATL cells and assessment of chemosensitivity.

Acknowledgments: We would like to thank Dr. Naoki Oyaizu and Dr. Naoyuki Isoo (Laboratory Medicine, Research Hospital, Institute of Medical science) for their kind permission to introduce this clinical test, as well as Mr. Yukihisa Tanaka, Ms. Etsuko Nagai, and Ms. Motoko Mizukami for their excellent morphological classification.
Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. 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;79:428–37.
2. Ohshima K. Pathological features of diseases associated with human T-cell leukemia virus type I. Cancer Sci 2007;98:772–8.
3. de Souza JG, Fonseca FG, Martins-Filho OA, Teixeira-Carvalho A, Martins CP, Carvalho LD, et al. Diagnostic tool based on an HTLV-1-Tax expression system in eukaryotic cells using a poxvirus vector. J Virol Methods 2010;166:65–71.
4. Sadamori N. Clinical and biological significance of serum tumor markers in adult T-cell leukemia. Leuk Lymphoma 1996;22:415–9.
5. Kamihira S, Dateki N, Sugahara K, Hayashi T, Harasawa H, Minami S, et al. Significance of HTLV-1 proviral load quantification by real-time PCR as a surrogate marker for HTLV-1-infected cell count. Clin Lab Haematol 2003;25:111–7.
6. Waters A, Oliveira AL, Coughlan S, de Venecia C, Schor D, Leite AC, et al. Multiplex real-time PCR for the detection and quantitation of HTLV-1 and HTLV-2 proviral load: addressing the issue of indeterminate HTLV results. J Clin Virol 2011;52:38–44.
7. Moens B, Lopez G, Adaui V, Gonzalez E, Kerremans L, Clark D, et al. Development and validation of a multiplex real-time PCR assay for simultaneous genotyping and human T-lymphotropic virus type 1, 2, and 3 proviral load determination. J Clin Microbiol 2009;47:3682–91.
8. Takenouchi H, Umeki K, Sasaki D, Yamamoto I, Nomura H, Takajo I, et al. Defective human T-lymphotropic virus type 1 provirus in asymptomatic carriers. Int J Cancer 2011;128:1335–43.
9. Altamirano NA, Rocco C, Aulicino P, Sen L, Mangano A. Quantitation of HTLV-I proviral load by a real-time PCR assay using SYBR Green: comparison of two methods for DNA isolation. J Virol Methods 2010;170:160–4.
10. Kamihira S, Yamano Y, Iwanaga M, Sasaki D, Satake M, Okayama A, et al. Intra- and inter-laboratory variability in human T-cell leukemia virus type-1 proviral load quantification using real-time polymerase chain reaction assays: a multi-center study. Cancer Sci 2010;101:2361–7.
11. Tian Y, Kobayashi S, Ohno N, Isobe M, Tsuda M, Zaike Y, et al. 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. Cancer Sci 2011;102:569–77.
12. Tsukasaki K, Hermine O, Bazarbachi A, Ratner L, Ramos JC, Harrington W Jr., 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;27:453–9.
13. Kobayashi S, Tian Y, Ohno N, Yuji K, Ishigaki T, Isobe M, et al. The CD3 versus CD7 plot in multicolor flow cytometry reflects progression of disease stage in patients infected with HTLV-I. PLoS One 2013;8:e53728.
14. Shao H, Yuan CM, Xi L, Raffeld M, Morris JC, Janik JE, et al. Minimal residual disease detection by flow cytometry in adult T-cell leukemia/lymphoma. Am J Clin Pathol 2010;133:592–601.
15. Borowitz MJ, Guenther KL, Shults KE, Stelzer GT. Immunophenotyping of acute leukemia by flow cytometric analysis. Use of CD45 and right-angle light scatter to gate on leukemic blasts in three-color analysis. Am J Clin Pathol 1993;100:534–40.
16. Weil R, Levraud JP, Dodon MD, Bessia C, Hazan U, Kourilsky P, et al. Altered expression of tyrosine kinases of the Src and Syk families in human T-cell leukemia virus type 1-infected T-cell lines. J Virol 1999;73:3709–17.
17. Schnizlein-Bick CT, Spritzler J, Wilkening CL, Nicholson JK, O'Gorman MR. Evaluation of TruCount absolute-count tubes for determining CD4 and CD8 cell numbers in human immunodeficiency virus-positive adults. Site Investigators and The NIAID DAIDS New Technologies Evaluation Group. Clin Diagn Lab Immunol 2000;7:336–43.
18. Motoi T, Uchiyama T, Uchino H, Ueda R, Araki K. Serum soluble interleukin-2 receptor levels in patients with adult T-cell leukemia and human T-cell leukemia/lymphoma virus type-I seropositive healthy carriers. Jpn J Cancer Res 1988;79:593–9.
19. Araki K, Harada K, Nakamoto K, Shiroma M, Miyakuni T. Clinical significance of serum soluble IL-2R levels in patients with adult T cell leukaemia (ATL) and HTLV-1 carriers. Clin Exp Immunol 2000;119:259–63.
20. Leblond V, Othman TB, Blanc C, Theodorou I, Choquet S, Sutton L, et al. Expansion of CD4+CD7- T cells, a memory subset with preferential interleukin-4 production, after bone marrow transplantation. Transplantation 1997;64:1453–9.

Supplemental Material: The online version of this article (DOI: 10.1515/cclm-2014-0183) offers supplementary material, available to authorized users.

Biol Blood Marrow Transplant 20 (2014) 183–191

Mammalian Target of Rapamycin Inhibitors Permit Regulatory T Cell Reconstitution and Inhibit Experimental Chronic Graft-versus-Host Disease

Haruko Sugiyama [1], Yoshinobu Maeda [1,*], Hisakazu Nishimori [1], Yoshiko Yamasuji [1], Ken-ichi Matsuoka [1], Nobuharu Fujii [1], Eisei Kondo [1], Katsuji Shinagawa [1], Takehiro Tanaka [2], Kengo Takeuchi [3], Takanori Teshima [4], Mitsune Tanimoto [1]

[1] *Department of Hematology and Oncology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan*
[2] *Department of Pathology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan*
[3] *Pathology Project for Molecular Target, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan*
[4] *Department of Hematology, Hokkaido University, Hokkaido, Japan*

Article history:
Received 4 June 2013
Accepted 1 November 2013

Key Words:
Chronic graft-versus-host disease (GVHD)
Cyclosporine
Mammalian target of rapamycin (mTOR) inhibitor
Regulatory T cell

A B S T R A C T

Chronic graft-versus-host disease (GVHD) remains a major late complication of allogeneic bone marrow transplantation (BMT). In a previous study, impaired thymic negative selection of the recipients permitted the emergence of pathogenic T cells that cause chronic GVHD using MHC class II-deficient (H2-Ab1 KO) B6 into C3H model and $CD4^+$ T cells isolated from chronic GVHD mice caused chronic GVHD when administered into the secondary recipients. In this study, we evaluated the kinetics of regulatory T cell (Treg) reconstitution in wild type B6 into C3H model. After myeloablative conditioning, host Tregs disappeared rapidly, followed by expansion of Tregs derived from the donor splenic T cell inoculum. However, the donor splenic T cell–derived Treg pool contracted gradually and was almost completely replaced by newly generated donor bone marrow (BM)-derived Tregs in the late post-transplantation period. Next, we compared the effects of cyclosporine (CSA) and mammalian target of rapamycin (mTOR) inhibitors on Treg reconstitution. Administration of CSA significantly impaired Treg reconstitution in the spleen and thymus. In contrast, BM-derived Treg reconstitution was not impaired in mTOR inhibitor-treated mice. Histopathological examination indicated that mice treated with CSA, but not mTOR inhibitors, showed pathogenic features of chronic GVHD on day 120. Mice treated with CSA until day 60, but not mTOR inhibitors, developed severe chronic GVHD followed by adoptive transfer of the pathogenic $CD4^+$ T cells isolated from H2-Ab1 KO into C3H model. These findings indicated that long-term use of CSA impairs reconstitution of BM-derived Tregs and increases the liability to chronic GVHD. The choice of immunosuppression, such as calcineurin inhibitor-free GVHD prophylaxis with mTOR inhibitor, may have important implications for the control of chronic GVHD after BMT.

INTRODUCTION

Chronic graft-versus-host disease (GVHD) is the most serious late complication after allogeneic hematopoietic stem cell transplantation, but the pathophysiology and treatment strategy of chronic GVHD remain poorly defined [1-3]. GVHD prophylaxis using calcineurin inhibitors, such as cyclosporine (CSA) and tacrolimus, reduces the expansion of effector T cells by blocking interleukin (IL)-2 and prevents acute GVHD, but fails to reduce chronic GVHD [4,5]. Administration of CSA for up to 24 months, longer than the standard 6 months of CSA, also did not decrease the risk of chronic GVHD [6]. Several studies have indicated that the efficacy and safety of mammalian target of rapamycin (mTOR) inhibitor, rapamycin (RAPA), in refractory chronic GVHD patients [7-10]. However, a recent randomized trial showed that the combination of RAPA and tacrolimus as GVHD prophylaxis failed to reduce chronic GVHD compared with tacrolimus and methotrexate [11].

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) have been shown to play an important role in the establishment of tolerance between recipient tissues and donor-derived immunity. A series of animal studies indicated that Tregs in the inoculum can prevent acute GVHD when injected together with donor T cells [12-14]. Based on the role of Tregs in the prevention of GVHD and on their dependence on IL-2, there is considerable concern regarding the impact of blocking IL-2 signaling or IL-2 production by the immunosuppressive agents used for prophylaxis of GVHD. Zeiser et al. reported that Tregs showed relative resistance to RAPA as a result of reduced usage of the mTOR pathway and functional phosphatase and tensin homolog, a negative regulator of the phosphatidyl-inositol 3-kinase/Akt/mTOR pathway in Tregs

Financial disclosure: See Acknowledgments on page 190.

* Correspondence and reprint requests: Yoshinobu Maeda, MD, PhD, Department of Hematology and Oncology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Shikata-cho 2-5-1, Kita-ku, Okayama city, Okayama 700-8558, Japan.
E-mail address: yosmaeda@md.okayama-u.ac.jp (Y. Maeda).

1083-8791/$ – see front matter © 2014 American Society for Blood and Marrow Transplantation.
http://dx.doi.org/10.1016/j.bbmt.2013.11.018

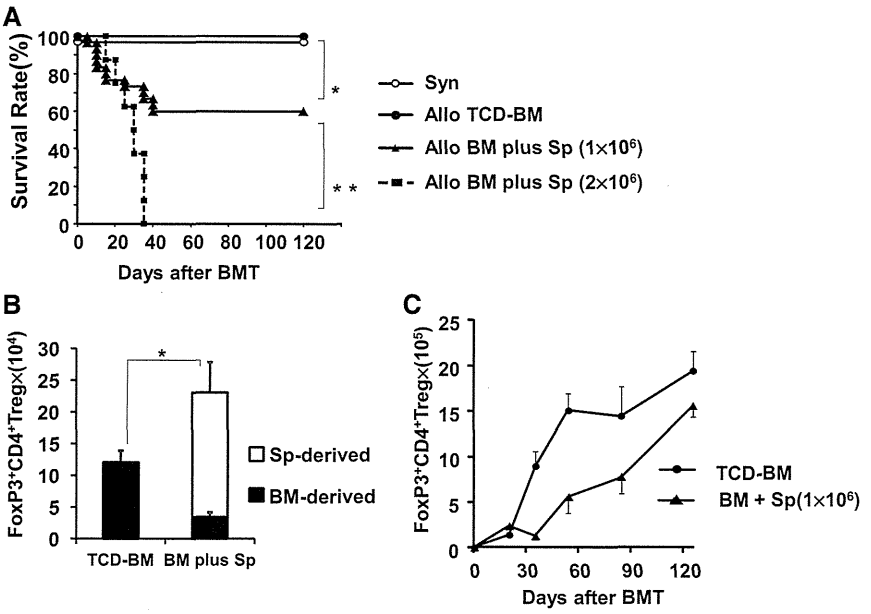

Figure 1. Regulatory T cell reconstitution after allogeneic BMT. Lethally irradiated C3H (H-2^k) recipient mice received 10×10^6 T cell–depleted bone marrow (TCD-BM) cells from B6.Ly-5a (H-2^b,CD45.1) mice with/without 1 to 2×10^6 spleen cells from B6 (H-2^b,CD45.2) mice. The syngeneic group received transplantation from C3H mice. (A) Survival: the recipients of allogeneic BM plus 1×10^6 spleen cells (BM plus Sp cells) showed a survival rate of 60% by day 120. Open circle, syngeneic; closed circle, TCD-BM cells only; triangle, -with 1×10^6 spleen cells; square, -with 2×10^6 spleen cells. (B) Origin of $CD4^+Foxp3^+$ Treg in the spleen on day 21 post transplantation: $CD45.2^+$ splenic T cell–derived (white bars) and $CD45.2^-$ BM–derived (black bars) are shown. (C) The absolute numbers of Treg in the recipients of BM plus Sp cells (triangles) and TCD-BM (closed circles) are shown. Each group consisted of 7 to 25 mice. The means (±SE) of each group are shown. Data are from a representative of at least 3 independent experiments. $^*P < .05$; $^{**}P < .01$.

compared with conventional T cells [15]. In contrast to CSA, RAPA allowed expansion of adoptively transferred Treg cells and led to reduction of alloreactive T cell expansion when animals received Treg treatment in combination with RAPA. They also showed that a combination of RAPA plus IL-2 increased both expansion of donor natural Tregs and conversion of induced Tregs from donor conventional T cells, and suppressed acute GVHD [16]. These animal data suggest that RAPA and CSA have differential effects on peripheral Tregs after bone marrow transplantation (BMT).

IL-2 signaling is pivotal for Treg homeostasis in the periphery and is also essential for naturally occurring Treg development in the thymus [17-19]. T cell repopulation after BMT is composed of 2 subsets: T cells derived from the donor splenic T cell inoculum and newly arising T cells from bone marrow (BM) inoculum. It has been shown that Tregs from the former pathway play an important role in acute GVHD, whereas, no previous study evaluated whether use of CSA for an extended period affects donor BM-derived Treg generation. We hypothesized that BM-derived Tregs comprise the long-term peripheral Treg pool and that CSA, but not mTOR inhibitors, causes impaired BM-derived Treg reconstitution, which has a negative effect on chronic GVHD. In the present study, we therefore evaluated effects of different immunosuppressants on 2 distinct Treg expansion reconstitution pathways and on the development of chronic GVHD.

MATERIALS AND METHODS

Mice

Female C57BL/6 (B6: H-2^b, $CD45.2^+$) and C3H/HeN (C3H: H-2^k) mice were purchased from Charles River Japan (Yokohama, Japan) or from the Okayama University mouse colony (Okayama, Japan). B6-Ly5a (H-2^b, $CD45.1^+$) and C3.SW (H-2^b, $CD45.2^+$) mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6-background MHC class II-deficient H2-$Ab1^{-/-}$ mice (B6.129-*H2-Ab1*^{tm1Gru} N12) were from Taconic Farms (Germantown, NY) [20]. Mice between 8 and 18 weeks of age were maintained under specific pathogen-free conditions and received normal chow and hyperchlorinated drinking water after transplantation. All experiments involving animals were approved by the Institutional Animal Care and Research Advisory Committee, Okayama University Advanced Science Research Center.

BMT

Mice underwent transplantation according to the standard protocol described previously [21,22]. Briefly, recipient mice received 2 split doses of either 500 cGy (allogeneic C3H and C3.SW recipients) or 650 cGy (syngeneic B6 recipients) total-body irradiation (TBI) 3 to 4 hours apart. Recipients were injected with 10×10^6 T cell–depleted bone marrow (TCD-BM) cells plus 1 or 2×10^6 whole spleen cells from B6 donors. [*H2-Ab1*$^{-/-}$→C3H] chimeras were produced by reconstituting lethally irradiated C3H mice with 5×10^6 TCD-BM cells from *H2-Ab1*$^{-/-}$ mice, as described previously [23]. T cell depletion was performed using anti-CD90–microbeads and an AutoMACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Donor cells were injected intravenously into the recipients on day 0.

Immunosuppressive Treatment

RAPA was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Everolimus (RAD) and CSA were synthesized and provided by Novartis Pharma AG (Basel, Switzerland). Everolimus emulsion was dissolved in distilled water at a concentration of 625 μg/mL and administered to recipients by oral gavage at a dose of 5 mg/kg. RAPA and CSA were given as suspensions in carboxymethylcellulose sodium salt: CMC (C5013; Sigma-Aldrich, St. Louis, MO) at a final concentration of .2% CMC. RAPA and CSA were administered to recipients by peritoneal injection at doses of .5 and 20 mg/kg, respectively [15,24]. Immunosuppressive treatments were performed once daily, starting on day 0 and continuing until death or end of the observation period (day 110 to 125).

Adoptive Transfer

Splenocytes were isolated from [*H2-Ab1*$^{-/-}$→C3H] chimeras 6 to 11 weeks after TCD-BMT. $CD4^+$ T cells were negatively selected from splenocytes by depletion of $CD8^+$, $DX5^+$, $CD11b^+$, Ter-119^+, and $B220^+$ cells using the AutoMACS system, as described previously [23]. A total of 2×10^7 $CD4^+$ T cells per mouse were injected intravenously into recipients after immunosuppressive therapy for 70 days after BMT.

Assessment of GVHD

After BMT, survival was monitored daily, and weight changes were assessed twice per week. The degree of clinically acute GVHD was assessed twice per week using a scoring system that sums changes in 5 clinical