13 patients who developed grade 2 to 3 skin disorders, five patients achieved CR/PR. Of the 24 patients who developed grade 1 or no skin disorders, eight patients achieved CR/PR. Hence, no clear correlation between skin disorders and response rate was observed in the present study.

As shown in Figure A1, mogamulizumab caused a significant and persistent reduction in the number of Treg cells. This may be responsible for the increased incidence of skin disorders seen in patients with ATL.[30,40] Skin disorders were observed in 19 patients (51%), with grade 3/4 in four cases (11%). This was lower than the proportion of patients who developed skin disorders (67%, 22% in grade 3/4) in a previous study.[30] One patient (4%) with ATL developed Stevens-Johnson syndrome (SJS)[30] and four patients with ATL developed SJS/toxic epidermal necrolysis in postmarketing surveillance of mogamulizumab[40]; however, no cases of SJS/toxic epidermal necrolysis were observed in the present study. Similarly, four of 21 patients with ATL (19%) developed symptoms consistent with SJS[41] after treatment with pralatrexate, whereas no SJS was observed in patients with PTCL[10] after pralatrexate treatment. The risk of severe skin disorders may therefore be lower in patients with PTCL, compared with patients with ATL.

In conclusion, this phase II study revealed that mogamulizumab had promising efficacy and tolerability in patients with relapsed CCR4-positive PTCL and CTCL. Given its novel mechanism of action and favorable toxicity profile compared with multiagent cytotoxic chemotherapy, we might expect the use of mogamulizumab in combination with other agents. Further preclinical and clinical studies of combination therapy will be needed.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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REFERENCES

1. WHO: WHO classification of tumours of haematopoietic and lymphoid tissues (ed 4). Lyon, France, International Agency for Research on Cancer (IARC), 2008
2. O'Leary HM, Savage KJ: Update on the World Health Organization classification of peripheral T-cell lymphomas. Curr Hematol Malig Rep 4:227-235, 2009
3. Vose J, Armitage J, Weisenburger D, et al: International peripheral T-cell and natural killer/T-cell lymphoma study: Pathology findings and clinical outcomes. J Clin Oncol 26:4124-4130, 2008
4. Lymphoma Study Group of Japanese Pathologists: The World Health Organization classification of malignant lymphomas in Japan: Incidence of recently recognized entities. Pathol Int 50:696-702, 2000
5. Aoki R, Karube K, Sugita Y, et al: Distribution of malignant lymphoma in Japan: Analysis of 2260 cases, 2001-2006. Pathol Int 58:174-182, 2008
6. Wollina U: Cutaneous T cell lymphoma: Update on treatment. Int J Dermatol 51:1019-1036, 2012
7. NCCN Clinical Practice Guidelines in Oncology. Non-Hodgkin's lymphomas. Version 1.2013. Fort Washington, PA, NCCN Clinical Practice Guidelines in Oncology, 2013
8. Savage KJ, Chhanabhai M, Gascoyne RD, et al: Characterization of peripheral T-cell lymphomas in a single North American institution by the WHO classification. Ann Oncol 15:1467-1475, 2004
9. Savage KJ: Therapies for peripheral T-cell lymphomas. Hematology Am Soc Hematol Educ Program 2011:515-524, 2011
10. O'Connor OA, Pro B, Pinter-Brown L, et al: Pralatrexate in patients with relapsed or refractory peripheral T-cell lymphoma: Results from the pivotal PROPEL study. J Clin Oncol 29:1182-1189, 2011
11. Coiffier B, Pro B, Prince HM, et al: Results from a pivotal, open-label, phase II study of romidepsin in relapsed or refractory peripheral T-cell lymphoma after prior systemic therapy. J Clin Oncol 30:631-636, 2012
12. Pro B, Advani R, Brice P, et al: Brentuximab vedotin (SGN-35) in patients with relapsed or refractory systemic anaplastic large-cell lymphoma: Results of a phase II study. J Clin Oncol 30:2190-2196, 2012
13. Sugaya M, Hamada T, Kawai K, et al: Guidelines for the management of cutaneous lymphomas (2011): A consensus statement by the Japanese Skin Cancer Society-Lymphoma Study Group. J Dermatol 40:2-14, 2013
14. Olsen E, Vonderheid E, Pimpinelli N, et al: Revisions to the staging and classification of mycosis fungoides and Sézary syndrome: A proposal of the International Society for Cutaneous Lymphomas (ISCL) and the cutaneous lymphoma task force of the European Organization of Research and Treatment of Cancer (EORTC). Blood 110:1713-1722, 2007
15. Kim YH, Liu HL, Mraz-Gernhard S, et al: Long-term outcome of 525 patients with mycosis fungoides and Sézary syndrome. Clinical prognostic factors and risk for disease progression. Arch Dermatol 139:857-866, 2003
16. Diamandidou E, Colome-Grimmer M, Fayad L, et al: Transformation of mycosis fungoides/Sezary syndrome: Clinical characteristics and prognosis. Blood 92:1150-1159, 1998
17. Olsen EA, Kim YH, Kuzel TM, et al: Phase IIB multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous T-cell lymphoma. J Clin Oncol 25:3109-3115, 2007

18. Olsen E, Duvic M, Frankel A, et al: Pivotal phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T-cell lymphoma. J Clin Oncol 19:376-388, 2001

19. Whittaker SJ, Demierre MF, Kim EJ, et al: Final results from a multicenter, international, pivotal study of romidepsin in refractory cutaneous T-cell lymphoma. J Clin Oncol 28:4485-4491, 2010

20. Jones D, O'Hara C, Kraus MD, et al: Expression pattern of T-cell-associated chemokine receptors and their chemokines correlates with specific subtypes of T-cell non-Hodgkin lymphoma. Blood 96:685-690, 2000

21. Ishida T, Inagaki H, Utsunomiya A, et al: CXC chemokine receptor 3 and CC chemokine receptor 4 expression in T-cell and NK-cell lymphomas with special reference to clinicopathological significance for peripheral T-cell lymphoma, unspecified. Clin Cancer Res 10:5494-5500, 2004

22. Ohshima K, Karube K, Kawano R, et al: Classification of distinct subtypes of peripheral T-cell lymphoma unspecified, identified by chemokine and chemokine receptor expression: Analysis of prognosis. Int J Oncol 25:605-613, 2004

23. Nakagawa M, Nakagawa-Oshiro A, Karnan S, et al: Array comparative genomic hybridization analysis of PTCL-U reveals a distinct subgroup with genetic alterations similar to lymphoma-type adult T-cell leukemia/lymphoma. Clin Cancer Res 15:30-38, 2009

24. Yagi H, Seo N, Ohshima A, et al: Chemokine receptor expression in cutaneous T cell and NK/T-cell lymphomas: Immunohistochemical staining and in vitro chemotactic assay. Am J Surg Pathol 30: 1111-1119, 2006

25. Shinkawa T, Nakamura K, Yamane N, et al: The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem 278:3466-3473, 2003

26. Niwa R, Sakurada M, Kobayashi Y, et al: Enhanced natural killer cell binding and activation by low-fucose IgG1 antibody results in potent antibody-dependent cellular cytotoxicity induction at lower antigen density. Clin Cancer Res 11:2327-2336, 2005

27. Niwa R, Shoji-Hosaka E, Sakurada M, et al: Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T-cell leukemia and lymphoma. Cancer Res 64:2127-2133, 2004

28. Yano H, Ishida T, Inagaki A, et al: Defucosylated anti CC chemokine receptor 4 monoclonal antibody combined with immunomodulatory cytokines: A novel immunotherapy for aggressive/refractory Mycosis fungoides and Sezary syndrome. Clin Cancer Res 13:6494-6500, 2007

29. 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 28:1591-1598, 2010

30. 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 10:837-842, 2012

31. Duvic M, Pinter-Brown L, Foss F, et al: Results of a phase 1/2 study for KW-0761, a monoclonal antibody directed against CC chemokine receptor type 4 (CCR4), in CTCL patients. Presented at the 53rd Annual Meeting of the American Society of Hematology, San Diego, CA, December 10-13, 2011 (abstr 962)

32. Cheson BD, Horning SJ, Coiffier B, et al: Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. J Clin Oncol 17:1244-1253, 1999

33. 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 27:453-459, 2009

34. Stevens SR, Ke MS, Parry EJ, et al: Quantifying skin disease burden in mycosis fungoides-type cutaneous T-cell lymphomas: The severity-weighted assessment tool (SWAT). Arch Dermatol 138:42-48, 2002

35. Olsen EA, Whittaker S, Kim YH, et al: Clinical end points and response criteria in mycosis fungoides and Sézary syndrome: A consensus statement of the International Society for Cutaneous Lymphomas, the United States Cutaneous Lymphoma Consortium, and the Cutaneous Lymphoma Task Force of the European Organisation for Research and Treatment of Cancer. J Clin Oncol 29:2598-2607, 2011

36. Ishida T, Ueda R: CCR4 as a novel molecular target for immunotherapy of cancer. Cancer Sci 97:1139-1146, 2006

37. d'Amore F, Radford J, Relander T, et al: Phase II trial of zanolimumab (HuMax-CD4) in relapsed or refractory non-cutaneous peripheral T cell lymphoma. Br J Haematol 150:565-573, 2010

38. Hodi FS, O'Day JS, McDermott FD, et al: Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363:711-723, 2010

39. Campbell JJ, Haraldsen G, Pan J, et al: The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. Nature 400:776-780, 1999

40. Ishida T, Ito A, Sato F, et al: Stevens-Johnson syndrome associated with mogamulizumab treatment of adult T-cell leukemia/lymphoma. Cancer Sci 104:647-650, 2013

41. Lunning MA, Gonsky J, Ruan J, et al: Pralatrexate in relapsed/refractory HTLV-1 associated adult T-cell lymphoma/leukemia: A New York City multi-institutional experience. Blood 120 (ASH Annual Meeting). 2012 (abstr 2735)

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Appendix

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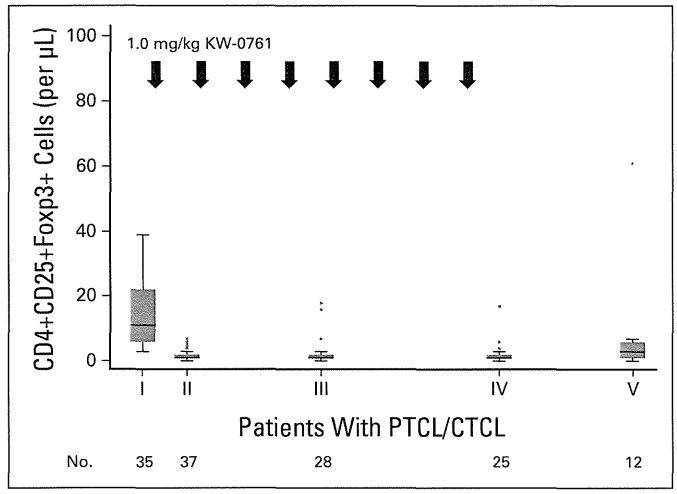

Fig A1. T-cell subset analysis. Numbers of CD4+CD25+Foxp3+ (regulatory T) cells are presented. Blood samples collected at times indicated in the protocol were analyzed. Blood samples were taken (I) just before the first mogamulizumab infusion, (II) just before the second infusion, (III) just before the fifth infusion, (IV) 1 week after the eighth infusion, and (V) 4 months after the eighth infusion. The number of samples used for analysis at each point is indicated below the graph. CTCL, cutaneous T-cell lymphoma; PTCL, peripheral T-cell lymphoma.

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Impact of Graft-versus-Host Disease on Allogeneic Hematopoietic Cell Transplantation for Adult T Cell Leukemia-Lymphoma Focusing on Preconditioning Regimens: Nationwide Retrospective Study

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A B S T R A C T

Allogeneic hematopoietic cell transplantation (HCT), but not autologous HCT, can provide long-term remission in some patients with adult T cell leukemia-lymphoma (ATL). We retrospectively analyzed the effects of acute graft-versus-host disease (GVHD) among the 616 patients with ATL who survived at least 30 days after allogeneic HCT with other than cord blood grafts. Multivariate analyses treating the occurrence of GVHD as a time-varying covariate demonstrated an association between grade I-II acute GVHD and favorable overall survival (OS) (hazard ratio [HR], 0.634; 95% confidence interval [CI], 0.477 to 0.843), whereas grade III-IV acute GVHD showed a trend toward unfavorable OS (HR, 1.380; 95% CI, 0.988 to 1.927) compared with nonacute GVHD. In subsequent multivariate analyses of patients who survived at least 100 days after HCT (n = 431), the presence of limited chronic GVHD showed a trend toward favorable OS (HR, 0.597; 95% CI, 0.354 to 1.007), and extensive chronic GVHD had a significant effect on OS (HR, 0.585; 95% CI, 0.389 to 0.880). There were no significant interactions between myeloablative conditioning or reduced-intensity conditioning with OS even when acute GVHD was absent or present at grade I-II or grade III-IV or when chronic GVHD was absent, limited, or extensive. This study demonstrates the actual existence of graft-versus-ATL effects in patients with ATL regardless of whether myeloablative conditioning or reduced-intensity conditioning is used.

INTRODUCTION

Adult T cell leukemia-lymphoma (ATL) is an aggressive peripheral T cell neoplasm caused by human T cell lymphotropic/leukemia virus type 1 (HTLV-1). It has a very poor prognosis, and it has been generally accepted that conventional chemotherapeutic agents alone, even including zidovudine/IFN-α, yield few or no long-term remissions or potential cures in patients with ATL [1-6]. Although early experience in myeloablative chemoradiotherapy together with autologous hematopoietic cell rescue for ATL has been associated with high incidences of relapse and fatal toxicities [7], allogeneic hematopoietic cell transplantation (HCT) has been explored as a promising alternative treatment that can provide long-term remission in a proportion of patients with ATL [8-10].

We previously performed a nationwide retrospective study of patients with ATL who underwent allogeneic HCT in Japan, with special emphasis on the effect of the graft source. We concluded that allogeneic HCT using currently available sources is an effective treatment in selected patients with ATL, but that the use of unrelated cord blood as a stem cell source is associated with lower survival [11]. Our results suggest that allogeneic bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT) could be considered the more standard transplantation forms compared with unrelated cord blood transplantation (CBT) for ATL.

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As the next step, we conducted a nationwide retrospective study of patients with ATL who underwent allogeneic HCT other than CBT, with special emphasis on the effects of the preconditioning regimen, whether conventional myeloablative conditioning (MAC) or reduced-intensity conditioning (RIC). No significant difference in overall survival (OS) was observed between patients receiving MAC and those receiving RIC, but a trend toward RIC contributing to better OS in older patients was noted. Thus, we conclude that allogeneic HCT not only with MAC, but also with RIC, is an effective treatment resulting in long-term survival in selected patients with ATL [12].

ATL has a long latency and occurs in older individuals at a median age of nearly 66 years. The median age at diagnosis of ATL has been increasing over the last few decades [13]. Accordingly, the proportion of patients with ATL undergoing HCT with RIC is currently increasing in relation to HCT with MAC. It is thought that compared with HCT with MAC, allogeneic HCT with RIC depends more on donor cellular immune effects after transplantation and less on the cytotoxic effects of the conditioning regimen to eradicate residual tumor cells. In this context, RIC might be suitable for ATL, given that several reports have indicated the high immunogenicity of ATL cells [14-18] and even the existence of graft-versus-HTLV-1 and/or graft-versus-ATL effects [19-21].

Although we previously reported the impact of post-transplantation immune reactions, graft-versus-host disease (GVHD), on outcomes in patients with ATL [21], our cohort included CBT recipients whose OS curve had a quite different trajectory from that of BMT and PBSCT recipients [12]. Thus, in the present study, we evaluated whether acute and chronic GVHD affect outcomes in patients with ATL undergoing allogeneic HCT other than unrelated CBT, with special emphasis on the effects of the preconditioning regimen. Our present analysis included the previous cohort (1996 to 2005) [21] with updated clinical information, as well as data on 1 patient who underwent allogeneic HCT in 1995 and patients who underwent allogeneic HCT between 2006 and 2010.

PATIENTS AND METHODS

Data Collection

Data on patients with ATL who had undergone a first allogeneic BMT, PBSCT, or BMT + PBSCT were collected from nationwide survey data of the Japan Society for Hematopoietic Cell Transplantation (JSHCT). Cases with missing preconditioning information, acute GVHD, or survival data were excluded, leaving 679 patients. Because the association between the occurrence of acute GVHD and disease-associated mortality was difficult to evaluate in the event of early toxic death, patients who died within 30 days or were censored within 29 days of transplantation (n = 63) were excluded; thus, 616 patients who underwent HCT between March 1995 and December 2010 were included in our analysis.

Data collected for analysis included clinical characteristics, such as age at HCT, sex, disease status at HCT, date of HCT, time from diagnosis of ATL to HCT, performance status (PS) according to the Eastern Cooperative Oncology Group criteria at transplantation, stem cell source, donor–recipient relationship, ATL clinical subtype [22], preconditioning regimen, type of GVHD prophylaxis, date alive at last follow-up, date and cause of death, date of occurrence of acute GVHD and disease-associated mortality was difficult to evaluate in the event of early toxic death, patients who died within 30 days or were censored within 29 days of transplantation (n = 63) were excluded; thus, 616 patients who underwent HCT between March 1995 and December 2010 were included in our analysis.

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Definitions

OS was defined as the time from HCT until death, and patients who remained alive at the time of the last follow-up were censored. Reported causes of death were reviewed and categorized into ATL-related mortality or treatment-related mortality (TRM). ATL-related mortality was defined as death caused by relapse or progression of ATL based on the judgment of each institution. TRM was defined as any death other than ATL-related mortality.

Table 1
Patient and Transplantation Characteristics by Type of Conditioning Regimen

Characteristic	MAC	RIC	*P* Value
Total patients, n (%)	284 (46.1)	332 (53.9)	
Age at HCT, y, n (%)			
<50	178 (62.7)	43 (13.0)	<.0001
51-55	79 (27.8)	91 (27.4)	
56-60	20 (7.0)	125 (37.7)	
61+	7 (2.5)	73 (22.0)	
Sex, n (%)			
Male	159 (56.0)	160 (48.2)	.0628
Female	125 (44.0)	172 (51.8)	
Disease status at HCT, n (%)			
CR	104 (36.6)	128 (38.6)	.1013
Not in CR	161 (56.7)	194 (58.4)	
Unknown	19 (6.7)	10 (3.0)	
GVHD prophylaxis, n (%)			
CyA + MTX	129 (45.4)	112 (33.7)	<.0001
FK506 + MTX	142 (50.0)	147 (44.3)	
CyA	6 (2.1)	58 (17.5)	
FK506	5 (1.8)	13 (3.9)	
Unknown	2 (0.7)	2 (0.6)	
Stem cell source, n (%)			
BM	216 (76.1)	213 (64.2)	.0015
PBSCs	68 (23.9)	117 (35.2)	
BM + PBSCs	0 (0.0)	2 (0.6)	
Donor–recipient relationship, n (%)			
HLA-matched related	98 (34.5)	120 (36.1)	.3649
HLA-mismatched related	24 (8.5)	40 (12.0)	
Unrelated	160 (56.3)	171 (51.5)	
Unknown	2 (0.7)	1 (0.3)	
PS at HCT, n (%)			
0	111 (39.1)	144 (43.4)	.0012
1	127 (44.7)	154 (46.4)	
2	26 (9.2)	27 (8.1)	
3	3 (1.1)	5 (1.5)	
4	1 (0.4)	1 (0.3)	
Unknown	16 (5.6)	1 (0.3)	
ATL clinical subtype, n (%)			
Chronic/smoldering	11 (3.9)	10 (3.0)	.5278
Acute	171 (60.2)	189 (56.9)	
Lymphoma	80 (28.2)	97 (29.2)	
Unknown	22 (7.7)	36 (10.8)	
Time from diagnosis to HCT, d, n (%)			
16-153	82 (28.9)	72 (21.7)	.0632
154-204	64 (22.5)	88 (26.5)	
205-307	75 (26.4)	78 (23.5)	
308-4355	63 (22.2)	91 (27.4)	
Unknown	0 (0.0)	3 (0.9)	
Time of HCT, n (%)			
March 1995 to March 2005	75 (26.4)	79 (23.8)	.3119
April 2005 to May 2007	75 (26.4)	79 (23.8)	
June 2007 to February 2009	73 (25.7)	81 (24.4)	
March 2009 to December 2010	61 (21.5)	93 (28.0)	
Grade of acute GVHD, n (%)			
No acute GVHD	80 (28.2)	128 (38.6)	.0111
Grade I-II	148 (52.1)	159 (47.9)	
Grade III-IV	56 (19.7)	45 (13.6)	

Acute GVHD was diagnosed and graded using traditional criteria [23] by the physicians who performed HCT at each institution, as was chronic GVHD [24]. Among the 487 patients who survived at least 100 days after HCT, 431 patients with complete information on the grade and the day of occurrence of chronic GVHD were included in the analysis for chronic GVHD.

Patients undergoing allogeneic BMT or PBSCT were divided into 2 groups, MAC and RIC, based on the preconditioning regimen. MAC and RIC were defined according to Giralt et al. [25] and Bacigalupo et al. [26] with slight modifications. In the present study, MAC was defined as any regimen that includes (1) ≥5 Gy of total body irradiation (TBI) as a single fraction or ≥8 Gy fractionated, (2) busulfan >8 mg/kg orally or the i.v. equivalent, or (3) melphalan >140 mg/m^2. All other regimens were classified as RIC.

Statistical Analysis

Comparisons among the groups were performed using Fisher's exact test as appropriate for categorical variables. The probability of survival was

estimated by the Kaplan-Meier method. TRM and ATL-related mortality were estimated using cumulative incidence curves to accommodate the competing events ATL-related mortality for TRM and TRM for ATL-related mortality [27]. Semilandmark plots were used to illustrate the effects of GVHD on survival and the cumulative incidence of ATL-related mortality and TRM. This landmark method was used to exclude bias that might have arisen from including patients who died too early to develop GVHD in the group without GVHD [28,29]. For patients with acute or chronic GVHD, the probability of survival and the cumulative incidences of ATL-related mortality and TRM were plotted as functions of time from the onset of acute or chronic GVHD. Day 25, the median day of onset for acute GVHD (range, 6 to 166 days), was designated the landmark day for acute GVHD. Day 126, the median day of onset for chronic GVHD (range, 52 to 1203 days), was designated the landmark day for chronic GVHD.

Multivariate Cox proportional hazards regression models were used to evaluate variables potentially affecting OS, and Fine and Gray proportional subdistribution hazards models [30] were used to evaluate variables potentially affecting ATL-related mortality and TRM. In these regression models, the occurrence of acute and chronic GVHD was treated as a time-varying covariate [31]. In the analysis of acute GVHD, patients were assigned to the no acute GVHD group at the time of HCT and then transferred to the grade I-II acute GVHD group or to the grade III-IV acute GVHD group at the onset of acute GVHD. In the analysis of chronic GVHD, patients were assigned to the no chronic GVHD group at the time of HCT and then transferred to the limited chronic GVHD group or to the extensive chronic GVHD group at the onset of chronic GVHD. We also assessed the interaction between acute and chronic GVHD and the preconditioning regimen in the multivariate models.

The heterogeneities of the effects of grade I-II or III-IV acute GVHD on OS according to background transplantation characteristics were evaluated by forest plots stratified by variables included in the regression analyses. Results are expressed as hazard ratio (HR) with 95% confidence interval (CI). All tests were 2-sided, and a P value <.05 was considered to indicate statistical significance. All statistical analyses were performed by Kureha Special Laboratory (Tokyo, Japan) using SAS 9.3 (SAS Institute, Cary, NC).

RESULTS

Patient Characteristics

Among the 616 patients in the study cohort, 284 received MAC and the remaining 332 received RIC. Characteristics of these patients are summarized in Table 1. Compared with MAC recipients, significantly fewer RIC recipients belonged to the youngest age group (<50 years), and significantly more were in the 2 oldest age groups (56 to 60 and 61+ years). In addition, significantly fewer RIC recipients received cyclosporin A (CyA) + methotrexate (MTX), but significantly more received CyA without MTX. PBSCT was significantly more frequent in RIC recipients compared with MAC recipients. There was no significant difference between MAC and RIC recipients regarding PS distribution from 0 to 4, but an unknown PS was observed significantly more frequently in the MAC recipients. A significantly greater number of RIC recipients did not have acute GVHD, and significantly fewer had grade III-IV acute GVHD.

Effects of Acute GVHD on Survival

In the 208 patients with ATL and no acute GVHD, the unadjusted 1-year and 3-year probabilities of survival from the landmark day for acute GVHD were 45.4% (95% CI, 38.3 to 52.2%) and 37.3% (95% CI, 30.3 to 44.4%), respectively. The unadjusted 1-year and 3-year probabilities of survival from the onset of acute GVHD were 60.1% (54.2 to 65.5%) and 49.1% (43.0 to 55.0%), respectively, in the 307 patients with grade I-II acute GVHD and 36.4% (26.9 to 46.0%) and 21.7% (13.9 to 30.6%), respectively, in the 101 patients with grade III-IV acute GVHD (Figure 1A).

Forest plots revealed that the development of grade I-II acute GVHD was associated with longer OS compared with the absence of acute GVHD in patients with the following characteristics: age <56 years, either male or female, not in complete remission (CR), receiving FK506 + MTX,

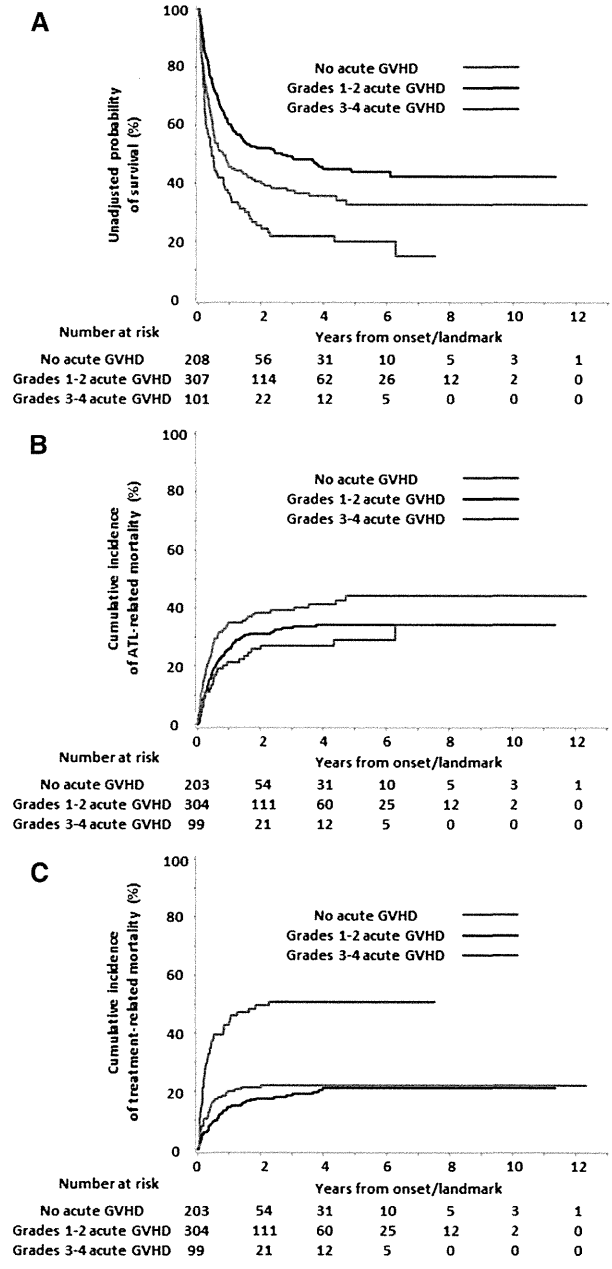

Figure 1. Semi-landmark plots illustrating the effects of acute GVHD on survival, ATL-related mortality, and TRM. (A) HR for survival in patients with grade I-II and grade III-IV acute GVHD compared with patients with no acute GVHD: 0.681 (95% CI, 0.537 to 0.863) versus 1.437 (95% CI, 1.082 to 1.910). (B) HR for ATL-related mortality in patients with grade I-II and grade III-IV acute GVHD compared with patients with no acute GVHD: 0.729 (95% CI, 0.540 to 0.984) versus 0.624 (95% CI, 0.403 to 0.967). (C) HR for TRM in patients with grade I-II and grade III-IV acute GVHD compared with patients with no acute GVHD: 0.824 (95% CI, 0.552 to 1.232) versus 2.793 (95% CI, 1.884 to 4.229).

undergoing either BMT or PBSCT, having an unrelated donor, PS 1 at transplantation, acute type of ATL, interval between ATL diagnosis and HCT of 308 to 4355 days, and date of HCT between June 2007 and February 2009. The development of grade I-II acute GVHD was also significantly associated with longer OS compared with the absence of acute GVHD regardless of whether the patient received MAC or RIC. On the other hand, this comparison revealed a shorter OS in the patients with lymphoma type ATL (Figure 2). These plots also revealed that the development of grade III-IV acute GVHD

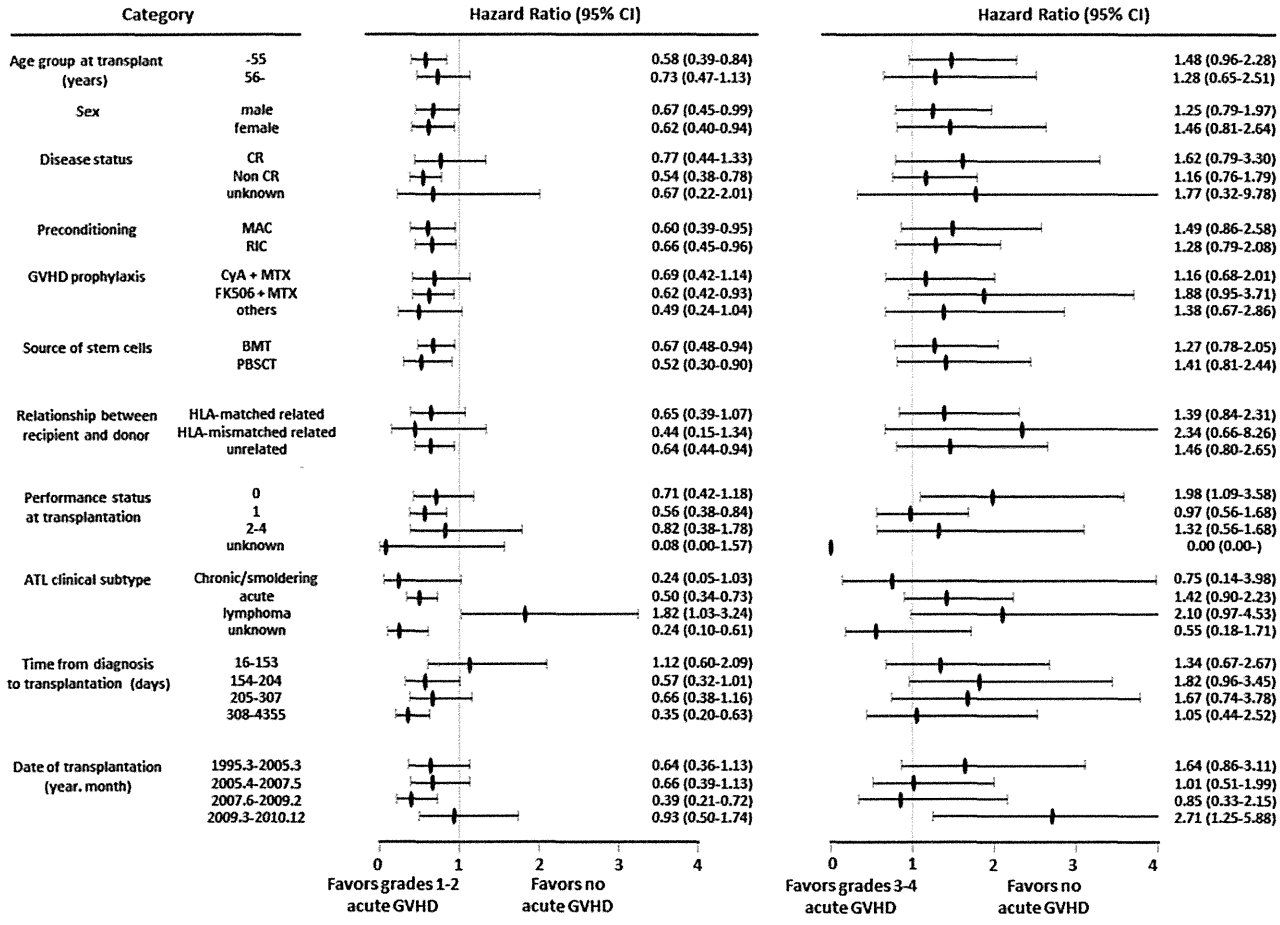

Figure 2. Impact of the grade of acute GVHD on OS in each stratified category. Effects of grade I-II (A) and grade III-IV acute GVHD (B) on OS are shown as forest plots. Closed ellipses on lines indicates HRs compared with the no acute GVHD group, and horizontal lines represent the corresponding 95% CI.

was significantly associated with shorter OS compared with the absence of acute GVHD in patients with PS 0 and who underwent HCT between March 2009 and December 2010. However, this comparison revealed no significant findings for OS according to whether the patient received MAC or RIC (Figure 2).

Multivariate analysis of the 616 study patients was performed to examine whether acute GVHD affects OS using the following variables: age (15 to 55 or 56 to 72 years), sex, disease status at HCT (CR, not CR, or unknown), preconditioning regimen (MAC or RIC), GVHD prophylaxis (CyA + MTX, FK506 + MTX, or other/unknown), relationship between recipient and donor (HLA-matched related, HLA-mismatched related, or unrelated), PS (0, 1, 2 to 4, or unknown), ATL clinical subtype (chronic/smoldering, acute, lymphoma, or unknown), time from diagnosis to HCT (16 to 153, 154 to 204, 205 to 307, or 308 to 4355 days or unknown), date of HCT (March 1995 to March 2005, April 2005 to May 2007, June 2007 to February 2009, or March 2009 to December 2010), and source of stem cells (bone marrow [BM], peripheral blood stem cells [PBSCs], or BM + PBSCs), as well as acute GVHD as a time-dependent covariate (no, grade I-II, or grade III-IV). There was a significant positive impact of grade I-II acute GVHD on OS (HR, 0.634; 95% CI, 0.477 to 0.843) compared with no acute GVHD (Table 2).

To further investigate the clinical significance of acute GVHD for OS, we divided acute GVHD into 5 categories (none or grade I, II, III, or IV) and then performed multivariate analysis in the same manner as described above. HRs for OS of patients with grade I, II, III, and IV acute GVHD compared with the absence of acute GVHD were 0.568 (95% CI, 0.402 to 0.801), 0.688 (95% CI, 0.501 to 0.946), 1.199 (95% CI, 0.831 to 1.730), and 2.245 (95% CI, 1.354 to 3.722), respectively.

Interactions of the Preconditioning Regimen with Acute GVHD for OS

We tested statistical interactions between the preconditioning regimens and acute GVHD with regard to OS by adding an interaction term to the multivariate analysis. This analysis included the same variables as the multivariate Cox proportional hazards regression models for OS. Among the 616 patients, when the HR for death of MAC recipients with no acute GVHD was set as 1.000, the HRs in MAC recipients with grade I-II acute GVHD and in RIC recipients with no GVHD and with grade I-II acute GVHD were 0.659, 0.971, and 0.592, respectively ($P_{\text{interaction}} = .7962$), and the HRs in MAC and RIC recipients with grade III-IV acute GVHD were 1.343 and 1.387, respectively ($P_{\text{interaction}} = .7603$) (Figure 3A).

Effects of Acute GVHD on ATL-Related Mortality and TRM

Among the 616 patients receiving allogeneic BMT or PBSCT, 10 patients could not be assigned to either the ATL-related mortality or TRM category because of missing detailed information on the cause of death. The cumulative incidences of ATL-related mortality at 1 year and 3 years from the landmark day for acute GVHD were 35.0% (95% CI,

Table 2
Effect of Acute GVHD on OS, ATL-related Mortality, and TRM after Allogeneic HCT

Outcome	HR (95% CI)	*P* Value
OS*		
No acute GVHD	1.000	Reference
Grade I-II acute GVHD	0.634 (0.477-0.843)	.0017
Grade III-IV acute GVHD	1.380 (0.988-1.927)	.0590
ATL-related mortality†		
No acute GVHD	1.000	Reference
Grade I-II acute GVHD	0.833 (0.566-1.224)	.3511
Grade III-IV acute GVHD	0.599 (0.373-0.964)	.0347
TRM‡		
No acute GVHD	1.000	Reference
Grade I-II acute GVHD	0.645 (0.407-1.023)	.0624
Grade III-IV acute GVHD	2.474 (1.495-4.095)	.0004

* Other than acute GVHD, the following 4 variables significantly affected OS: older age (56 to 72 yr compared with 15 to 55 yr: HR, 1.356; 95% CI, 1.033 to 1.781), male sex (HR, 1.404; 95% CI, 1.127 to 1.750), not in CR compared with CR (HR, 1.877; 95% CI, 1.459 to 2.416), and worse PS (1 compared with 0: HR, 1.486; 85% CI, 1.168 to 1.889; 2 to 4 compared with 0: HR, 2.691; 95% CI, 1.918 to 3.777).

† Other than acute GVHD the following 2 variables significantly affected ATL-related mortality: not in CR compared with CR (HR, 2.633; 95% CI, 1.818 to 3.814) and worse PS (2 to 4 compared with 0: HR, 3.272; 95% CI, 2.100 to 5.099).

‡ Other than acute GVHD, the following 3 variables significantly affected TRM: older age (56 to 72 yr compared with 15 to 55 yr: HR, 1.663; 95% CI, 1.025 to 2.697), male sex (HR, 1.545; 95% CI, 1.078 to 2.214), and transplantation from an unrelated donor compared with an HLA-matched related donor (HR, 2.098; 95% CI, 1.131 to 3.895).

27.6% to 42.5%) and 39.2% (95% CI, 31.1% to 47.2%), respectively, in the 203 patients with no acute GVHD. Those of TRM were 20.0% (95% CI, 13.5% to 27.5%) and 22.0% (95% CI, 14.8% to 30.1%), respectively (Figure 1B and C). The cumulative incidences of ATL-related mortality at 1 year and 3 years from the onset of acute GVHD were 25.8% (95% CI, 20.5% to 31.4%) and 33.0% (95% CI, 26.9% to 39.3%), respectively, in the 304 patients with grade I-II acute GVHD, whereas those of TRM were 14.5% (95% CI, 10.1% to 19.6%) and 18.5% (95% CI, 13.0% to 24.6%), respectively (Figure 1B and C). In the 99 patients with grade III-IV acute GVHD, the cumulative incidences of ATL-related mortality at 1 year and 3 years from the onset of acute GVHD were 21.2% (95% CI, 10.8% to 33.8%) and 27.0% (95% CI, 14.2% to 41.5%), respectively, and those of TRM were 42.7% (95% CI, 31.8% to 53.3%) and 50.7% (95% CI, 38.6% to 61.7%), respectively (Figure 1B and C).

We next applied the Fine and Gray proportional hazards model to the 606 patients. The analysis included the same variables as in the multivariate Cox proportional hazards regression models for OS. There were significant associations between grade III-IV acute GVHD and lower ATL-related mortality (HR, 0.599; 95% CI, 0.373 to 0.964) and higher TRM (HR, 2.474; 95% CI, 1.495 to 0.964) compared with no acute GVHD (Table 2).

In investigating the clinical significance of acute GVHD for ATL-related mortality or TRM, we divided acute GVHD into 5 categories (none and grade I, II, III, and IV) and conducted the analysis in the same manner as described above. HRs for ATL-related mortality in patients with grade I, II, III, and IV acute GVHD compared with the absence of acute GVHD were 0.809 (95% CI, 0.517 to 1.268), 0.857 (95% CI, 0.558 to 1.315), 0.585 (95% CI, 0.347 to 986), and 0.654 (95% CI, 0.298 to 1.435), respectively. HRs for TRM in patients with grade I, II, III, and IV acute GVHD compared with the absence of acute GVHD were 0.519 (95% CI, 0.282 to 0.955), 0.747 (95% CI, 0.455 to 1.227), 2.153 (95% CI, 1.267 to 3.659), and 4.114 (95% CI, 2.033 to 8.326), respectively.

Effects of Chronic GVHD on Survival

Among the 431 patients evaluable for chronic GVHD, 199 received MAC and 232 received RIC. In the MAC group, limited and extensive chronic GVHD occurred in 26 (13.1%) and 67 patients (33.7%), respectively, and in the RIC group, limited and extensive chronic GVHD occurred in 35 (15.1%) and 65 patients (28.0%), respectively. Regarding the incidence and grade of chronic GVHD, there were no significant differences between MAC and RIC recipients. In the 214 patients with no chronic GVHD, the unadjusted 1-year and 3-year probabilities of survival from the landmark day for chronic GVHD were 58.7% (95% CI, 51.6 to 65.1%) and 51.0% (95% CI, 43.6 to 57.9%), respectively,. Those probabilities from the onset of chronic GVHD were 77.4% (64.2 to 86.2%) and 61.7% (46.7 to 73.6%), respectively, in the 60 patients with limited chronic GVHD and were 70.4% (61.7 to 77.5%) and 55.1% (45.5 to 63.7%), respectively, in the 132 patients with extensive chronic GVHD. Twenty-five patients were excluded from this semilandmark plot because they were censored or died before the landmark day for chronic GVHD (Figure 4A).

We performed a multivariate analysis of data on 431 patients to examine whether chronic GVHD affects OS using the following variables: age, sex, disease status, preconditioning regimen, GVHD prophylaxis, donor–recipient relationship, PS, ATL clinical subtype, time from diagnosis to

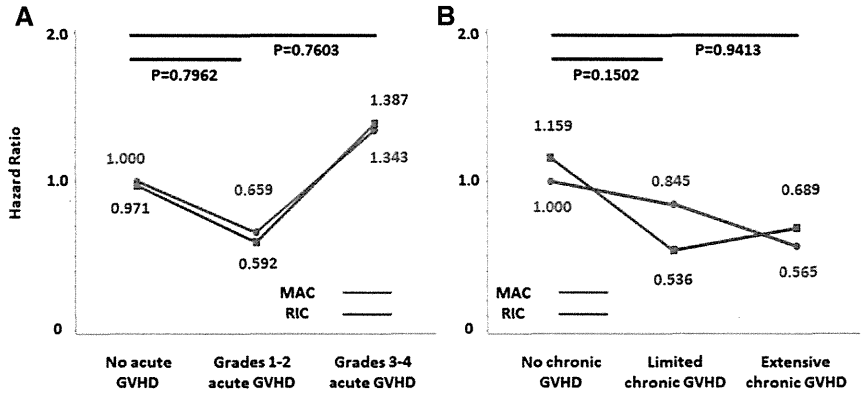

Figure 3. Interactions of the preconditioning regimen with acute GVHD for overall survival. Statistical interactions between the preconditioning regimens (MAC or RIC) and acute GVHD (absent versus grade I-II or grade II-IV; A) and chronic GVHD (absent versus limited or extensive type; B) for overall survival were analyzed.

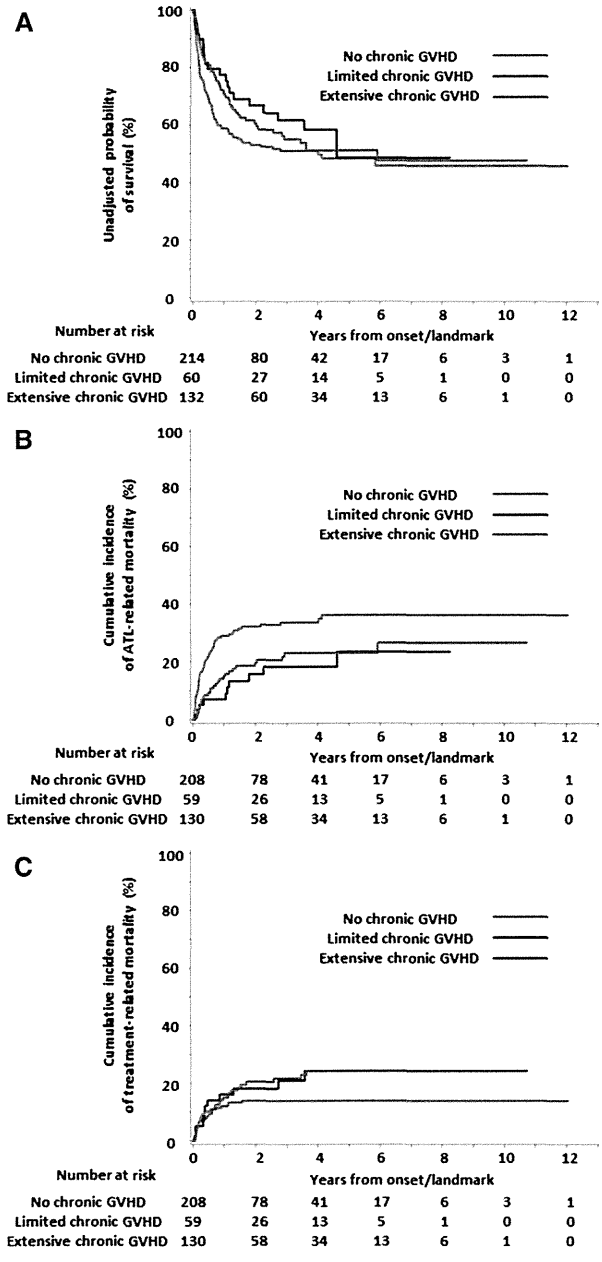

Figure 4. Semilandmark plots illustrating the effects of chronic GVHD on survival, ATL-related mortality, and TRM. (A) HR for survival in patients with limited and extensive chronic GVHD compared with patients with no chronic GVHD: 0.719 (95% CI, 0.457 to 1.131) versus 0.796 (95% CI, 0.576 to 1.100). (B) HR for ATL-related mortality in patients with limited and extensive chronic GVHD compared with patients with no chronic GVHD: 0.461 (95% CI, 0.237 to 0.897) versus 0.570 (95% CI, 0.366 to 0.886). (C) HR for TRM in patients with limited and extensive chronic GVHD compared with patients with no chronic GVHD: 1.609 (95% CI, 0.815 to 3.175) versus 1.620 (95% CI, 0.955 to 2.749).

HCT, date of HCT, and stem cell source, as well as chronic GVHD as a time-dependent covariate. We found a significant positive impact of extensive chronic GVHD on OS compared with no chronic GVHD (HR, 0.585; 95% CI, 0.389 to 0.880) (Table 3).

Interactions of the Preconditioning Regimen with Chronic GVHD for OS

We tested the statistical interactions between the preconditioning regimens and chronic GVHD for OS by adding an interaction term into the multivariate analysis. The analysis included the same variables as the multivariate Cox proportional hazards regression models for OS with chronic GVHD. Among the 431 patients, when the HR for death of MAC recipients with no chronic GVHD was set as 1.000, the HRs in MAC recipients with limited chronic GVHD and RIC recipients with no GVHD and limited chronic GVHD were 0.845, 1.159, and 0.536, respectively ($P_{interaction} = .1502$), and the HRs in MAC and RIC recipients with extensive chronic GVHD were 0.565 and 0.689, respectively ($P_{interaction} = .9413$) (Figure 3B).

Table 3
Effect of Chronic GVHD on OS, ATL-related Mortality, and TRM after Allogeneic HCT

Outcome	HR (95% CI)	*P* Value
OS*		
No chronic GVHD	1.000	Reference
Limited chronic GVHD	0.597 (0.354-1.007)	.0533
Extensive chronic GVHD	0.585 (0.389-0.880)	.0100
ATL-related mortality†		
No chronic GVHD	1.000	Reference
Limited chronic GVHD	0.395 (0.184-0.847)	.0170
Extensive chronic GVHD	0.421 (0.240-0.740)	.0026
TRM‡		
No chronic GVHD	1.000	Reference
Limited chronic GVHD	1.549 (0.704-3.409)	.2767
Extensive chronic GVHD	1.204 (0.659-2.201)	.5462

* Other than chronic GVHD, the following 3 variables significantly affected OS: male sex (HR, 1.480; 95% CI, 1.103 to 1.986), not in CR compared with CR (HR, 1.629; 95% CI, 1.171 to 2.266), worse PS (1 compared with 0: HR, 1.446; 95% CI, 1.057 to 1.980, 2 to 4 compared with 0: HR, 2.828; 95% CI, 1.751 to 4.568).

† Other than chronic GVHD, the following 2 variables significantly affected ATL-related mortality: not in CR compared with CR (HR, 2.499; 95% CI, 1.563 to 3.994), worse performance score (1 compared with 0: HR, 1.524; 95% CI, 1.013 to 2.294, 2 to 4 compared with 0: HR, 2.383; 95% CI, 1.216 to 4.669).

‡ The following 4 variables significantly affected TRM: older age (56 to 72 yr compared with 15 to 55 yr: HR, 2.022; 95% CI, 1.045 to 3.913), male sex (HR, 2.254; 95% CI1.322 to 3.844), worse performance score (2 to 4 compared with 0: HR, 3.127; 95% CI, 1.260 to 7.762), and ATL clinical subtype (acute compared with chronic/smoldering type: HR, 0.288; 95% CI, 0.093 to 0.897; lymphoma compared with chronic/smoldering type: HR, 0.249; 95% CI, 0.078 to 0.794).

Effects of Chronic GVHD on ATL-Related Mortality and TRM

Among the 406 patients analyzed by a semilandmark plot for survival, 9 could not be assigned to either the ATL-related mortality or TRM category. The cumulative incidences of ATL-related mortality at 1 year and 3 years from the landmark day for chronic GVHD were 29.0% (95% CI, 22.4% to 35.8%) and 33.7% (95% CI, 26.4% to 41.1%), respectively, in the 208 patients with no chronic GVHD, whereas those of TRM were 12.0% (95% CI, 7.3% to 18.1%) and 13.9% (95% CI, 8.5% to 20.7%), respectively (Figure 4B and C). In the 59 patients with limited chronic GVHD, the cumulative incidences of ATL-related mortality at 1 year and 3 years from the onset of chronic GVHD were 7.0% (95% CI, 2.1% to 16.1%) and 18.3% (95% CI, 7.7% to 32.4%), respectively, and those of TRM were 16.0% (95% CI, 7.5% to 27.4%) and 20.8% (95% CI, 10.1% to 34.1%), respectively (Figure 4B and C). In the 130 patients with extensive chronic GVHD, the cumulative incidences of ATL-related mortalities at 1 year and 3 years from the onset of chronic GVHD were 15.1% (95% CI, 9.0% to 22.6%) and 23.1% (95% CI, 14.7% to 32.6%), respectively, and those of TRM were 15.0% (95% CI, 9.1% to 22.3%) and 21.6% (95% CI, 13.9% to 30.5%), respectively (Figure 4B and C).

We next applied the Fine and Gray proportional hazards model to the 422 patients evaluable for chronic GVHD who could be assigned to either the ATL-related mortality or the TRM category. The analysis included the same variables as the multivariate Cox proportional hazards regression models for OS. Chronic GVHD was significantly associated with reduced ATL-related mortality. HRs for recipients with limited and extensive chronic GVHD compared with the absence of chronic GVHD were 0.395 (95% CI, 0.184 to 0.847) and 0.421 (95% CI, 0.240 to 0.740), respectively (Table 3). On the other hand, chronic GVHD was not significantly associated with TRM.

DISCUSSION

To the best of our knowledge, this is the largest retrospective study reported to date analyzing the impact of acute and chronic GVHD on clinical outcomes in ATL. As shown in Table 1, the associations with no acute GVHD and without grade III-IV acute GVHD were significant in RIC recipients compared with MAC recipients. Those findings are consistent with reports of an association between dose-intensified conditioning, especially regimens including TBI, and acute GVHD [32,33]. Our results also show no significant difference in the occurrence of chronic GVHD between MAC and RIC recipients. This may be because the effects of older age and more frequent PBSCT, which increase the occurrence of chronic GVHD, were counterbalanced by the lower frequency of history of previous acute GVHD, which reduces the incidence of chronic GVHD, in the RIC recipients [32,34].

Forest plots revealed that the development of grade I-II acute GVHD was associated with favorable OS compared with the absence of acute GVHD in most categories, with the exception of lymphoma in the ATL clinical subtype category. The reason for this exception is unclear, however. Our forest plots also show that the occurrence of grade III-IV acute GVHD was associated with unfavorable OS in most categories.

The significant positive impact of grade I-II acute GVHD on OS identified by multivariate analysis confirmed the results presented in our previous report [21]. However, in the present study, we found that grade I-II acute GVHD had no significant association with ATL-related mortality, in disagreement with our previous report showing a significant association between grade I-II acute GVHD and decreased ATL-related mortality in ATL patients undergoing allogeneic HCT [21]. We surmise that the incompatibility might stem from 2 factors, the influence of unrelated CBT, which was included in the previous study [21], and the progress in transplantation-related medicine from 2006 onward. The clear trend of decreased TRM in patients with grade I-II acute GVHD observed here seems a bit puzzling, but we have no suitable explanation. With respect to preconditioning, there were no significant interactions between MAC and RIC for OS even when post-transplantation acute GVHD was absent or present at grade I-II or III-IV.

Our multivariate analysis revealed a clear trend toward a favorable OS with limited chronic GVHD and a significant association with lower ATL-related mortality. These findings are consistent with previous reports by our group [20] and others [35]. The latter report included a variety of hematologic diseases. Even though our univariate analyses revealed a trend toward better survival (but without significance) in patients with extensive chronic GVHD in the semilandmark plots (Figure 4A), our multivariate analysis demonstrated that a significant association between extensive chronic GVHD and a favorable OS. This finding is in disagreement with our previous report [21] and another study demonstrating a negative impact of extensive chronic GVHD on OS [35]. Extensive chronic GVHD had a significant association with lower ATL-related mortality, but not with TRM. The former finding was reasonable and expected, but the latter was not consistent with our previous report demonstrating significant associations between extensive chronic GVHD and greater TRM [21]. Although the present study found a significant association between extensive chronic GVHD and favorable OS in the patients with ATL, we also must pay special attention to the fact that quality of life after HCT is highly compromised by chronic GVHD [36]. With respect to preconditioning, there were also no significant interactions between MAC and RIC with OS even when chronic GVHD was absent, limited, or extensive.

Several promising new agents for treating ATL are currently under development [37-40]. These novel treatments should increase the number of patients with a sufficient disease control status and who have maintained a good PS who could become suitable candidates for HCT [12]. These agents will also contribute to the establishment of better rescue strategies for patients relapsing after HCT [41]. Among the novel agents, we should pay special attention to mogamulizumab (humanized anti-CCR4 monoclonal antibody) [42], which was approved for the treatment of ATL in Japan in 2012, because of its potent activity that depletes regulatory T (Treg) cells, leading to enhanced antitumor activity [38,43,44]. The occurrence and severity of GVHD are closely associated with low Treg frequency [45]; thus, a decrease in Treg cells caused by mogamulizumab not only may lead to enhanced GVHD, but also may provoke an anti-HTLV-1/ATL immune effect.

Although this study reports significant novel findings on GVHD in patients with ATL, it also has inherent limitations common to observational retrospective studies. First, eligibility for HCT as well as choice of transplantation protocol, including the selection of MAC or RIC, were determined by physicians at each institution. Second, regarding analysis of mortality, it was not always easy to determine whether death after allogeneic HCT was an ATL-related mortality or TRM, in part because patients with relapsed ATL sometimes achieve partial or complete remission after decreasing or discontinuing immunosuppressive agents, donor lymphocyte infusions, or chemotherapy, which can result in long-term remission and survival [20]. Third, acute GVHD is occasionally induced in some patients considered at high risk for relapse by treating clinicians. Finally, the evaluation of chronic GVHD according to the 2005 National Institutes of Health consensus criteria [46] is not possible in this study, which was based on nationwide survey data of the JSHCT.

In conclusion, we found that the development of mild to moderate (grade I-II) acute GVHD was significantly associated with favorable OS, as was the development of both limited and extensive chronic GVHD. Regarding preconditioning, we found no difference in the clinical impact of acute GVHD and chronic GVHD on OS between patients receiving MAC and those receiving RIC. These findings confirm the actual existence of graft-versus-HTLV-1 and/or graft-versus-ATL effects in recipients of HCT for ATL regardless of whether MAC or RIC was used. New strategies that enhance the post- transplantation allogeneic anti–HTLV-1 effect targeting HTLV-1–associated antigens, such as Tax and/or HBZ [14-17], and/or the anti-ATL effect targeting tumor-specific antigens, such as cancer testis antigens [18],

which do not provoke GVHD, lead to improved outcomes in patients undergoing allogeneic HCT for ATL.

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Authorship statement: T.I., M.H., K.K., R.T., and A.U. designed the research, organized the project, and wrote the manuscript. T.I. helped with statistical analysis. H.S. and R.S. collected data from the JSHCT, and Y.M. collected data from the JMDP. All authors interpreted data and reviewed and approved the final manuscript.

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. Vose J, Armitage J, Weisenburger D. International T-Cell Lymphoma Project. International peripheral T-cell and natural killer/T-cell lymphoma study: pathology findings and clinical outcomes. *J Clin Oncol.* 2008;26:4124-4130.
3. 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;27:453-459.
4. 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.
5. Katsuya H, Yamanaka T, Ishitsuka K, et al. Prognostic index for acute- and lymphoma-type adult T-cell leukemia/lymphoma. *J Clin Oncol.* 2012;30:1635-1640.
6. Bazarbachi A, Plumelle Y, Carlos Ramos J, et al. Meta-analysis on the use of zidovudine and interferon-alfa in adult T-cell leukemia/lymphoma showing improved survival in the leukemic subtypes. *J Clin Oncol.* 2010;28:4177-4183.
7. 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.
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. 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;105:4143-4145.
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. 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.
12. 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.
13. Iwanaga M, Watanabe T, Yamaguchi K. Adult T-cell leukemia: a review of epidemiological evidence. *Front Microbiol.* 2012;3:322.
14. 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.
15. Suzuki S, Masaki A, Ishida T, et al. Tax is a potential molecular target for immunotherapy of adult T-cell leukemia/lymphoma. *Cancer Sci.* 2012; 103:1764-1773.
16. Arnulf B, Thorel M, Poirot Y, et al. Loss of the ex vivo but not the reinducible $CD8^-$ T-cell response to Tax in human T-cell leukemia virus type 1–infected patients with adult T-cell leukemia/lymphoma. *Leukemia.* 2004;18:126-132.
17. Masaki A, Ishida T, Suzuki S, et al. Autologous Tax-specific CTL therapy in a primary ATL cell-bearing NOD/Shi-scid, IL-$2R\gamma^{null}$ mouse model. *J Immunol.* 2013;191:135-144.
18. Nishikawa H, Maeda Y, Ishida T, et al. Cancer/testis antigens are novel targets of immunotherapy for adult T-cell leukemia/lymphoma. *Blood.* 2012;119:3097-3104.
19. 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.
20. 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.
21. 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.
22. 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;79:428-437.
23. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant.* 1995;15: 825-828.
24. Sullivan KM, Agura E, Anasetti C, et al. Chronic graft-versus-host disease and other late complications of bone marrow transplantation. *Semin Hematol.* 1991;28:250-259.
25. 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.
26. Bacigalupo A, Ballen K, Rizzo D, et al. Defining the intensity of conditioning regimens: working definitions. *Biol Blood Marrow Transplant.* 2009;15:1628-1633.
27. 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.
28. Anderson JR, Cain KC, Gelber RD. Analysis of survival by tumor response. *J Clin Oncol.* 1983;1:710-719.
29. Dafni U. Landmark analysis at the 25-year landmark point. *Circ Cardiovasc Qual Outcomes.* 2011;4:363-371.
30. Fine JP, Gray RJ. A proportional hazards model for subdistribution of a competing risk. *J Am Stat Assoc.* 1999;94:496-509.
31. Cortese G, Andersen P. Competing risks and time-dependent covariates. *Biomed J.* 2010;52:138-158.
32. Flowers ME, Inamoto Y, Carpenter PA, et al. Comparative analysis of risk factors for acute graft-versus-host disease and for chronic graft-versus-host disease according to National Institutes of Health consensus criteria. *Blood.* 2011;117:3214-3219.
33. Jagasia M, Arora M, Flowers ME, et al. Risk factors for acute GVHD and survival after hematopoietic cell transplantation. *Blood.* 2012;119: 296-307.
34. Arora M, Pidala J, Cutler CS, et al. Impact of prior acute GVHD on chronic GVHD outcomes: a chronic graft versus host disease consortium study. *Leukemia.* 2013;27:1196-1201.
35. Ozawa S, Nakaseko C, Nishimura M, et al. Chronic graft-versus-host disease after allogeneic bone marrow transplantation from an unrelated donor: incidence, risk factors and association with relapse. A report from the Japan Marrow Donor Program. *Br J Haematol.* 2007; 137:142-151.
36. Pidala J, Kurland B, Chai X, et al. Patient-reported quality of life is associated with severity of chronic graft-versus-host disease as measured by NIH criteria: report on baseline data from the Chronic GVHD Consortium. *Blood.* 2011;117:4651-4657.
37. 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.
38. 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.

39. Ishida T, Ueda R. Antibody therapy for adult T-cell leukemia-lymphoma. *Int J Hematol.* 2011;94:443-452.
40. Tanosaki R, Tobinai K. Adult T-cell leukemia-lymphoma: current treatment strategies and novel immunological approaches. *Expert Rev Hematol.* 2010;3:743-753.
41. 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.
42. Ishii T, Ishida T, Utsunomiya A, et al. Defucosylated humanized anti-CCR4 monoclonal antibody KW-0761 as a novel immunotherapeutic agent for adult T-cell leukemia/lymphoma. *Clin Cancer Res.* 2010;16:1520-1531.
43. Ishida T, Ueda R. Immunopathogenesis of lymphoma: focus on CCR4. *Cancer Sci.* 2011;102:44-50.
44. Ishida T, Ito A, Sato F, et al. Stevens-Johnson syndrome associated with mogamulizumab treatment of adult T-cell leukemia/lymphoma. *Cancer Sci.* 2013;104:647-650.
45. Mielke S, Rezvani K, Savani BN, et al. Reconstitution of FOXP3^{+} regulatory T cells (Tregs) after CD25-depleted allotransplantation in elderly patients and association with acute graft-versus-host disease. *Blood.* 2007;110:1689-1697.
46. Filipovich AH, Weisdorf D, Pavletic S, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease, I: Diagnosis and Staging Working Group Report. *Biol Blood Marrow Transplant.* 2005;11:945-956.

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Development of a novel redirected T-cell–based adoptive immunotherapy targeting human telomerase reverse transcriptase for adult T-cell leukemia

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Regular Article

LYMPHOID NEOPLASIA

Development of a novel redirected T-cell–based adoptive immunotherapy targeting human telomerase reverse transcriptase for adult T-cell leukemia

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Key Points

- The efficacy and safety of a novel redirected T-cell–based adoptive immunotherapy targeting hTERT for patients with adult T-cell leukemia.
- hTERT-specific T-cell receptor gene-transduced $CD8^+$ T cells lyse ATL cells, but not normal cells, both in vitro and in vivo.

Although adult T-cell leukemia (ATL) has a poor prognosis, successful allogeneic hematopoietic stem cell transplantation (allo-HSCT) in some cases suggests that a cellular immune-mediated strategy can be effective. So far, however, no effective target for anti-ATL immunotherapy has been defined. Here we demonstrated for the first time that human telomerase reverse transcriptase (hTERT) is a promising therapeutic target for ATL, and we developed a novel redirected T-cell–based immunotherapy targeting hTERT. *hTERT* messenger RNA was produced abundantly in ATL tumor cells but not in steady-state normal cells. Rearranged human leukocyte antigen-A*24:02 (HLA-A*24:02) –restricted and $hTERT_{461-469}$ nonameric peptide-specific T-cell receptor (TCR) α/β genes were cloned from our previously established cytotoxic T lymphocyte clone (K3-1) and inserted into a novel retroviral TCR expression vector encoding small interfering RNAs for endogenous *TCR* genes in redirected T cells (hTERT-*siTCR* vector). Consequently, allogeneic or autologous gene-modified $CD8^+$ T cells prepared using the hTERT-*siTCR* vector successfully killed ATL tumor cells, but not normal cells including steady-state hematopoietic progenitors, in an HLA-A*24:02-restricted manner both in vitro and in vivo. Our experimental observations support the development of a novel hTERT-targeting redirected T-cell–based adoptive immunotherapy for ATL patients, especially those for whom suitable allo-HSCT donors are lacking. (*Blood*. 2013;121(24):4894-4901)

Introduction

Adult T-cell leukemia (ATL) is an aggressive peripheral T-cell neoplasm caused by human T-cell lymphotropic virus I (HTLV-I).[1] It is estimated that there are more than 1 million HTLV-I carriers in Japan, about 5% of whom develop ATL at around 60 years of age or older.[2] Because ATL tumor cells soon acquire chemotherapy resistance and compromise host immunity against infectious pathogens, ATL has a poor prognosis.[3] Although most ATL patients are ineligible for allogeneic hematopoietic stem cell transplantation (allo-HSCT) because of advanced age, age-related comorbidity, or lack of suitable donors,[4] the number of ATL patients who are treated successfully with allo-HSCT and achieve prolonged survival has been increasing.[5] The graft-versus-ATL effect observed in ATL patients treated successfully with allo-HSCT[5] strongly suggests that a cellular immune-mediated approach for ATL can be clinically effective. With regard to cellular immunotherapy for ATL (unlike Epstein-Barr virus [EBV]-associated malignancy[6]), targeting of antigens associated with HTLV-I (the causative virus of ATL) such as Tax[7] and HBZ[8] still remains controversial, and the recently proposed NY-ESO-1[9] (a cancer-testis antigen) still awaits clinical validation. Thus, at this time, no effective therapeutic target antigen for anti-ATL immunotherapy has been clinically defined.

Human telomerase reverse transcriptase (hTERT), which is a component of human telomerase and a catalytic subunit for telomere elongation, is activated in almost all cancer cells, including hematologic malignancies, but not in normal cells.[10] In HTLV-I–infected cells and ATL tumor cells, Tax or interleukin-2 (IL-2) signaling strongly activates the *hTERT* promoter through the nuclear factor-κB or PI3K pathway,[11-13] suggesting that expression of hTERT protein would be upregulated in ATL tumor cells. Clinical trials of anticancer immunotherapy targeting hTERT have already been conducted, and both the safety and induction of immune responses to hTERT have been reproducibly confirmed.[10,14-17] In our previous studies, we defined a [human leukocyte antigen] HLA-A*24:02-restricted $hTERT_{461-469}$ nonameric peptide (VYGFVRACL) that was capable of inducing antileukemia cytotoxic T lymphocytes (CTLs),[18] and we subsequently established a CTL clone, K3-1, specific for this

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epitope.[19] We previously conducted a phase I/II clinical trial of hTERT peptide vaccine for treatment of HLA-A*24:02$^+$ patients with lung cancer and metastatic renal cell cancer.[20] These achievements strongly encouraged us to further explore cellular immune-mediated treatment of ATL targeting hTERT. Because of concern over the potential regulatory T-cell function of ATL tumor cells,[21] in this study we focused on developing a redirected T-cell–based immunotherapy targeting hTERT rather than using an hTERT$_{461\text{-}469}$ peptide vaccine. Recently developed forms of anticancer immunotherapy using gene-modified T cells that redirect defined tumor-associated antigens have been shown to have clinical promise.[22-25] To this end, therefore, we first cloned the rearranged HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$-specific T-cell receptor α/β (*TCR-α/β*) genes from K3-1 and inserted them into a novel *TCR* gene expression vector carrying silencers for endogenous TCRs (*siTCR* vector)[26] in redirected T cells (hTERT-*siTCR* vector). Notably, we used a souped-up second-generation 2A peptide-based *siTCR* vector that achieved an increased level of expression of the introduced TCR.[27]

In this study, we used the newly established hTERT-*siTCR* vector to examine the feasibility of a novel redirected T-cell–based adoptive immunotherapy targeting hTERT for treatment of ATL.

Patients and methods

Cell lines, freshly isolated leukemia cells, and normal cells

Approval for this study was obtained from the institutional review board of Ehime University Hospital. Written informed consent was obtained from all patients, healthy volunteers, and parents of cord blood donors in accordance with the Declaration of Helsinki.

B-lymphoblastoid cell lines (B-LCLs) were established by transformation of peripheral blood B lymphocytes with EBV. ATN-1,[28] TL-Om1,[29] HUT102[29], and TL-MAT[30] were human T-cell lines established from ATL patients, and TL-Su,[31] MT-1,[32] MT-2[32], and MT-4[33] were human T-cell lines transformed by HTLV-I infection. LCLs, T2-A24,[19] K562 (American Type Culture Collection [ATCC]), and human T-cell lines (except TL-Om1), maintenance of which requires 10 U/mL recombinant human IL-2 (rhIL-2) (R&D Systems), were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The HLA-A*24:02 gene-transduced K562 (K562-A24) was maintained in culture medium supplemented with 1.0 μg/mL puromycin (Sigma-Aldrich). Peripheral blood mononuclear cells (PBMCs) from ATL patients and healthy donors and cord blood mononuclear cells (CBMCs) from healthy donors were isolated by density gradient centrifugation and stored in liquid nitrogen until use. All samples from ATL patients contained more than 90% ATL cells. CD4$^+$ T cells, CD14$^+$ cells from PBMCs, and CD34$^+$ cells from CBMCs were isolated by using CD4$^+$ cell-, CD14$^+$ cell-, or CD34$^+$ cell-isolating immunomagnetic beads (MACS beads; Miltenyi Biotec), respectively. IL-2–dependent CD4$^+$ cell lines induced by HTLV-I infection were generated as reported previously.[8]

Cloning of full-length *TCR* α and β chain genes and construction of hTERT-*siTCR* retroviral vector

HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$ nonameric peptide (VYGFVRACL)-specific *TCR-α/β* genes were cloned from our previously established CTL clone, K3-1,[19] by using the 5′ rapid amplification of complementary DNA ends method (Clontech). The rearranged *TCR-α/β* genes of K3-1 expressed the germ line gene segments *TRAV29DV5/TRAJ34/TRAC* and *TRBV20-1/TRBJ2-1/TRBC2*, respectively. The retroviral vector expressing K3-1–derived *TCR* genes was constructed as reported previously.[26,27,34] Briefly, the constant regions of the hTERT-specific *TCR-α/β* genes were codon optimized and then integrated into a novel Splice-b2Aa-siTCR–based retroviral vector encoding small interfering RNAs that complementarily bind to the constant regions of the endogenous *TCR-α/β* genes (hTERT-*siTCR* vector).[27]

Establishment of hTERT-*siTCR*–transduced CD8$^+$ T-cell lines

Isolated CD8$^+$ T cells from PBMCs of healthy volunteers or ATL patients using CD8$^+$ cell-isolating MACS beads and stimulation with 1 μg/mL anti-CD3 monoclonal antibody (mAb; OKT-3; BioLegend) were cultured in GT-T503 (Takara Bio) supplemented with 5% human serum, 0.2% human albumin, 50 U/mL rhIL-2, 5 ng/mL rhIL-7 (R&D Systems), 10 ng/mL rhIL-15 (PeproTech), and 10 ng/mL rhIL-21 (Shenandoah Biotechnology). Then, CD8$^+$ T cells were transfected with the hTERT-*siTCR* retroviral vector using RetroNectin (Takara Bio) -coated plates as described previously.[34] In some experiments, because TRBV20-1 is specifically labeled with anti-Vβ2 mAb (IMGT Web resources: http://www.imgt.org/), Vβ2-positive cells among hTERT-*siTCR*–transduced CD8$^+$ T cells (hTERT-*siTCR*/CD8) were further isolated by using fluorescein isothiocyanate (FITC) –conjugated Vβ2 mAb (Beckman Coulter) and anti-FITC–conjugated MACS beads. To measure the expression levels of the introduced hTERT-specific TCR in gene-modified CD8$^+$ T cells, the cells were labeled with anti-CD8 (BD Biosciences) and anti-Vβ2 mAbs and phycoerythrin-conjugated HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer or HLA-A*24:02/HIV-1 Env$_{584\text{-}592}$ (RYLRDQQLL) tetramer, as a negative control.[19] Labeled cells were analyzed by using a Gallios flow cytometer (Beckman Coulter) and FlowJo Version 7.2.2 software (TreeStar). To expand the hTERT-*siTCR*/CD8 cells, they were stimulated weekly with mitomycin-C (Kyowa Hakko) –treated and hTERT$_{461\text{-}469}$ peptide–pulsed HLA-A*24:02$^+$ LCLs.

Cytotoxicity assays

Standard ^{51}Cr-release assays were performed as described previously.[35] Briefly, 5 × 10^3 unpulsed or peptide-pulsed target cells were labeled with ^{51}Cr ($Na_2{}^{51}CrO_4$; MP Bio Japan) and incubated at various ratios with effector cells in 200 μL of culture medium in 96-well round-bottomed plates. To assess HLA class I restriction, target cells were incubated with 10 μg/μL anti-HLA class I framework mAb (clone w6/32; ATCC) or a control anti-HLA -DR mAb (clone L243; ATCC) for 1 hour, then incubated with effector cells for 5 hours. The percentage of specific lysis was calculated as (experimental release cpm – spontaneous release cpm)/(maximal release cpm – spontaneous release cpm) × 100 (%). In some experiments, time-lapse imaging was used. Ten thousand ATL cells lentivirally gene-modified to express monomeric Azami-Green (Amalgaam) were cocultivated with 5 × 10^4 effector cells expressing hTERT-specific TCR (at an effector:target ratio of 5:1) for 12 hours in culture medium supplemented with 10 μg/mL propidium iodide (Sigma) to label dead cells red by using a glass dish for microscopic observation of live cells (iBIDI-dish1 Hi-Q4; Nikon). Images were acquired by using a systemic bio-imaging tool (BioStation IM; Nikon). To examine the cytotoxicity of these effector cells against early-differentiated and highly proliferating subsets of hematopoietic progenitor cells, CB-CD34$^+$ cells cultured by using a hematopoietic cell expansion medium (StemSpan CC100 and StemSpan SFEM; Stem Cell) for 7 days were subjected to flow-based cytotoxicity assay. 7-Aminoactinomycin D (7-AAD) –positive dead cells in each subset were examined by flow cytometry.

Quantitative analysis of hTERT mRNA expression

Quantitative real-time PCR (qRT-PCR) for *hTERT* messenger RNA (mRNA) was performed as described previously.[36] Briefly, after complementary DNA was synthesized, qRT-PCR for *hTERT* mRNA (NM_198253) was performed by using the QuantiTect SYBR green PCR Kit (QIAGEN) and primers as follows: forward, 5′-TTCTTGTTGGTGACACCTCACCTC-3′; reverse, 5′-CAGCCATACTCAGGGACACCTC-3′ (Takara Bio). Human hypoxanthine phosphoribosyltransferase 1 (*hHPRT1*) mRNA (NM_000194) was prepared and used as an internal control. Samples were analyzed by using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The expression level of *hTERT* mRNA was corrected by reference to that of *hHPRT1* mRNA, and the amount of *hTERT* mRNA relative to that in PBMCs was calculated by the comparative threshold cycle method. K562, which strongly expresses *hTERT* mRNA, was used as an internal control.

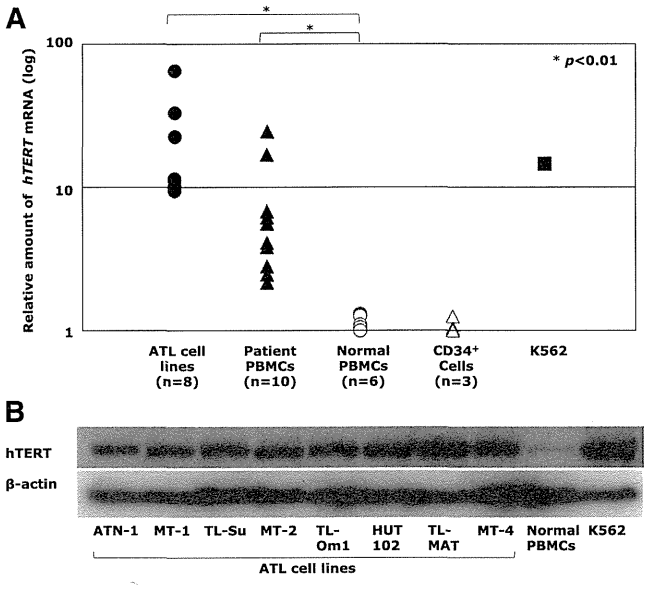

Figure 1. Abundant expression of hTERT in ATL tumor cells. (A) Expression of *hTERT* mRNA in ATL/HTLV-I infected cell lines (■), freshly isolated ATL tumor cells from patients (▲), normal PBMCs (○), and CB-CD34$^+$ cells (△) were examined by qRT-PCR. The level of *hTERT* mRNA expression in the K562 leukemia cell line (●) was used as an internal control. The expression level of *hTERT* mRNA in each sample was calculated relative to that of PBMCs. *hTERT* mRNA expression relative to normal PBMCs was 21.3 ± 17.9 for the ATL/HTLV-I–infected cell line, 7.48 ± 6.89 for freshly isolated ATL tumor cells, and 1.10 ± 0.12 for CB-CD34$^+$ (mean ± standard deviation [SD]). The ATL/HTLV-I–infected cell line and freshly isolated ATL tumor cells expressed *hTERT* mRNA abundantly and significantly ($^*P < .01$). (B) Expression of hTERT protein in ATL cell lines and normal PBMCs was confirmed by western blotting.

Western blotting of hTERT protein

For analysis of protein expression, western blotting was performed as described previously.[35] Briefly, cell lysates were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (e-PAGEL, ATTO) and blotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). The blots were incubated first with anti-hTERT rabbit mAb (Millipore), then with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G Ab (GE Healthcare). The probed proteins were visualized by using an enhanced chemiluminescence system (GE Healthcare). Subsequently, the blotted membranes were stripped and reprobed with anti–β-actin mouse mAb (Sigma-Aldrich) to confirm equivalent protein loading between samples.

Detection of hTERT$_{461\text{-}469}$–specific CTL precursors in the periphery of ATL patients

PBMCs from HLA-A*24:02$^+$, HLA-A*24:02$^-$ ATL patients, or HLA-A*24:02$^+$ healthy individuals were seeded in 24-well plates at 1.5 × 10^6 per well in the presence of the hTERT$_{461\text{-}469}$ peptide at a concentration of 1 μM in GT-T503 medium supplemented with 5% human serum and 10 U/mL IL-2. After culturing for 14 days, cultured PBMCs were stained with FITC-conjugated anti-CD8 mAb and HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer or control tetramer at a concentration of 20 μg/mL at 4°C for 20 minutes. Subsequently, the stained cells were analyzed by flow cytometry.

IFN-γ secretion assay

hTERT-*siTCR*/CD8 or K3-1 (2 × 10^4) cells were incubated with 2 × 10^4 hTERT$_{461\text{-}469}$ peptide-pulsed (1 μM) or unpulsed K562-A24 or K562 cells for 24 hours. Interferon gamma (IFN-γ) in the culture supernatant was measured by using an enzyme-linked immunosorbent assay kit (Pierce). Enzyme-linked immunospot assays were used to detect the epitope-responsive IFN-γ production mediated by hTERT$_{461\text{-}469}$-specific CTL precursors in the periphery of ATL patients as described previously.[34]

Anti-ATL tumor effect of hTERT-*siTCR*–transduced CD8$^+$ T cells in xenografted mouse models

To assess the in vivo anti-ATL tumor effect mediated by hTERT-*siTCR*/CD8, a bioluminescence assay using a xenografted mouse model was used. First, we lentivirally generated a luciferase gene–transduced HLA-A*24:02$^+$ ATL cell line, ATN-1 (ATN-1/luc). For measurement, anesthetized xenografted mice were given an intraperitoneal injection of 2.5 mg/body VivoGlo luciferin (Caliper Life Science), and images were acquired for 5 to 10 minutes by using an AEQUORIA luminescence imaging system (Hamamatsu Photonics). The acquired photon counts were analyzed by using AQUACOSMOS software (Hamamatsu Photonics).

Six-week-old NOD/scid/γc^{null} (NOG) female mice[37] were purchased from the Central Institute for Experimental Animals and maintained in the institutional animal facility at Ehime University. All in vivo experiments were approved by the Ehime University animal care committee. For the Winn assay, 5 × 10^5 ATN-1/luc cells and 2.5 × 10^6 hTERT-*siTCR*/CD8 or non–gene-modified CD8$^+$ T cells (NGM/CD8) were subcutaneously inoculated into the abdominal wall of NOG mice that had been pretreated with 1 Gy irradiation. Thereafter, 2.5 × 10^6 effector cells of each type were administered weekly to the corresponding mice, respectively, via the tail vein for a total of 3 times. For the adoptive transfer experiments, similarly pretreated mice were intravenously inoculated with 5 × 10^5 ATN-1/luc cells. After 4 days, mice started to receive intravenously infused 5 × 10^6 hTERT-*siTCR*/CD8 or NGM/CD8, respectively, for a total of 5 times. These mice were serially monitored for tumor growth determined by photon counts acquired every 7 days until they were euthanized owing to disease progression.

Statistical analysis

The Mann-Whitney *U* test was used to assess differences between two groups; a *P* value of < .05 was considered significant.

Results

ATL tumor cells abundantly express *hTERT* mRNA and hTERT protein

The expression level of *hTERT* mRNA in the ATL/HTLV-I–infected cell line (n = 8), freshly isolated tumor cells from ATL patients (n = 10), normal PBMCs from healthy individuals (n = 6), and CD34$^+$ cells from normal CBMCs (CB-CD34$^+$) (n = 3) were measured by using the qRT-PCR method. *hTERT* mRNA expression relative to normal PBMCs was 21.3 ± 17.9 for the ATL/HTLV-I–infected cell line, 7.48 ± 6.89 for freshly isolated ATL tumor cells, and 1.10 ± 0.12 for CB-CD34$^+$ cells (mean ± standard deviation). In Figure 1A, the ATL/HTLV-I–infected cell line and freshly isolated ATL tumor cells, but not CB-CD34$^+$, abundantly produced *hTERT* mRNA in comparison with normal PBMCs, the difference being statistically significant. The *P* value was .002 for the ATL/HTLV-I–infected cell line, .001 for freshly isolated ATL tumor cells, and .243 for CB-CD34$^+$ cells. Similarly, western blotting demonstrated abundant expression of hTERT protein in the ATL tumor cells (Figure 1B).

Circulatory hTERT$_{461\text{-}469}$-specific CTL precursors were exclusively detectable in the periphery of HLA-A*24:02$^+$ ATL patients

Next, by using the tetramer assay, we examined circulatory hTERT$_{461\text{-}469}$-specific CTL precursors in PBMCs from HLA-A*24:02$^+$ ATL patients (n = 7), HLA-A*24:02$^-$ ATL patients (n = 3) before chemotherapy, and HLA-A*24:02$^+$ healthy individuals as controls (n = 6). Since freshly isolated PB lymphocytes were almost

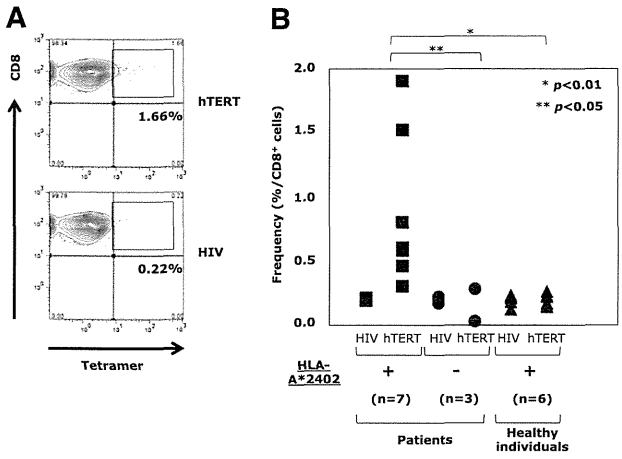

Figure 2. Detection of circulatory hTERT$_{461-469}$-specific CTL precursors in the periphery of ATL patients. (A) hTERT$_{461-469}$-specific CTL precursors in PBMCs repetitively stimulated with hTERT$_{461-469}$ peptide from HLA-A*24:02$^+$ ATL patients were detected by using HLA-A*24:02/hTERT$_{461-469}$ tetramer. A representative case is shown. HLA-A*24:02/HIV tetramer was used as a negative control. (B) In comparison with HLA-A*24:02$^-$ ATL patients (●) (n = 3) and HLA-A*24:02$^+$ healthy individuals (▲) (n = 6), the frequency of hTERT$_{461-469}$-specific CTL precursors in HLA-A*24:02$^+$ ATL patients (■) (n = 7) was significantly high (*$P < .01$; **$P < .05$). The frequency was 0.88% ± 0.55% for HLA-A*24:02$^+$ ATL patients, 0.11% ± 0.1% for HLA-A*24:02$^-$ ATL patients, and 0.2% ± 0.04% for HLA-A*24:02$^+$ healthy individuals (mean ± SD).

negative for tetramer staining, PBMCs stimulated with hTERT$_{461-469}$ peptide were analyzed. A representative example of an HLA-A*24:02$^+$ ATL patient is shown in Figure 2A. The frequencies of hTERT$_{461-469}$-specific CTL precursors in HLA-A*24:02$^+$ and HLA-A*24:02$^-$ ATL patients and HLA-A*24:02$^+$ healthy individuals are summarized in Figure 2B. hTERT$_{461-469}$-specific CTL precursors were detected at 0.88% ± 0.55% in HLA-A*24:02$^+$ ATL patients, being significantly more frequent than in HLA-A*24:02$^-$ ATL patients (0.11% ± 0.1%; $P < .05$) or HLA-A*24:02$^+$ healthy individuals (0.2% ± 0.04%; $P < .01$). These observations confirmed the presence of primed memory CD8$^+$ T cells with hTERT$_{461-469}$ epitope/HLA-A*24:02 complex (ie, that the hTERT$_{461-469}$ epitope must be naturally immunogenic) in HLA-A*24:02$^+$ ATL patients.

hTERT-*siTCR*–transduced CD8$^+$ T cells exert anti-ATL reactivity in vitro

The hTERT-*siTCR* gene was retrovirally introduced into normal CD8$^+$ T cells. Transduction efficiency determined by expression of Vβ2 on the gene-modified T cells was 85% to 95% (data not shown), and almost 50% of the transfectants were positive for HLA-A*24:02/hTERT$_{461-469}$ tetramer (Figure 3A). The cognate epitope specificity and HLA-A*24:02 restriction were examined by using standard ^{51}Cr-release assays (Figure 3B). Because expression of *hTERT* mRNA in LCLs was upregulated (supplemental Figure 2C), hTERT peptide-unpulsed HLA-A*24:02$^+$ LCLs were killed to some extent, reflecting the presence of endogenously processed hTERT (Figure 3B). Such epitope-specific cytotoxicity mediated by hTERT-*siTCR*/CD8 was obviously attenuated by anti-HLA class I mAb, but not by anti-HLA-DR mAb (Figure 3C). The antigen sensitivity to cognate hTERT$_{461-469}$ peptide mediated by hTERT-*siTCR*/CD8 (shown in Figure 3D) was similar to that of the parental CTL clone, K3-1 (Figure 3E-F).

hTERT-*siTCR*/CD8 dose-dependently killed the HLA-A*24:02$^+$ ATL/HTLV-I–infected cell lines ATN-1, TL-Su, and MT-2, but not the HLA-A*24:02$^-$ TL-Om1, HUT102, and MT-4 (Figure 4A). Additionally, the tumoricidal effect mediated by hTERT-*siTCR*/CD8 was abrogated by anti-HLA class I mAb, but not by anti-HLA-DR mAb (Figure 4B). Furthermore, time-lapse imaging directly demonstrated this tumoricidal activity of hTERT-*siTCR*/CD8 against HLA-A*24:02$^+$ ATN-1, but not that against HLA-A*24:02$^-$ HUT102 or K562 (negative control) (supplemental Fig 1-(1)). We then examined the tumoricidal activity against freshly isolated ATL tumor cells and found that these transfectants also dose-dependently killed HLA-A*24:02$^+$, but not -A*24:02$^-$ freshly isolated ATL tumor cells (Figure 5A).

Conversely, as shown in Figure 5B, neither HLA-A*24:02$^+$ normal CD4$^+$ T cells (the normal counterpart of ATL tumor cells)

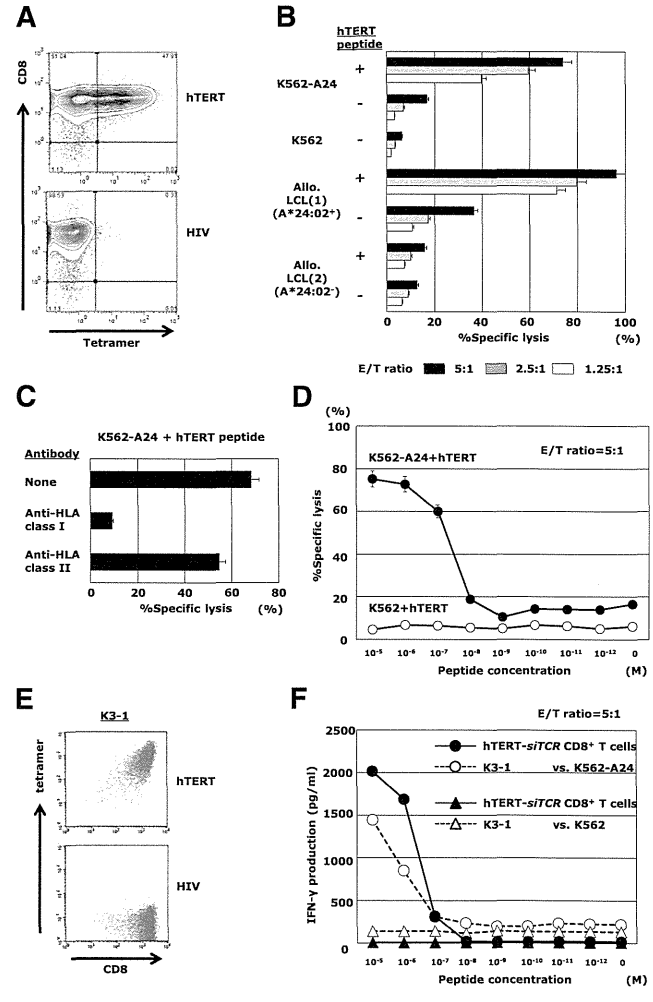

Figure 3. hTERT-*siTCR*–transduced CD8$^+$ T cells display epitope-specific responsiveness. (A) Representative flow cytometry plots showing staining of hTERT-*siTCR*–transduced CD8$^+$ T cells with HLA-A*24:02/hTERT$_{461-469}$ tetramer. HLA-A*24:02/HIV tetramer was used as a negative control. (B) ^{51}Cr-release assays were conducted by using hTERT-*siTCR*–transduced CD8$^+$ T cells with unpulsed or hTERT$_{461-469}$ peptide-loaded (1 μM) K562-A24, K562, HLA-A*24:02$^+$, or HLA-A*24:02$^-$ allogeneic B-LCLs at the indicated effector:target (E/T) ratios. (C) Effect of HLA class I and class II blockade on the cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against the cognate peptide-pulsed (1 μM) K562-A24 was determined by ^{51}Cr-release assays at an E/T ratio of 5:1. (D) hTERT-*siTCR*–transduced CD8$^+$ T cells were tested in ^{51}Cr release assays against K562 (negative control) and K562-A24 cells pulsed with the indicated concentrations of hTERT$_{461-469}$ peptide at an E/T ratio of 5:1. Error bars represent SDs. (E) Representative flow cytometry plots showing staining of K3-1 with the HLA-A*24:02/hTERT$_{461-469}$ tetramer (upper) and the irrelevant HLA-A*24:02/HIV-1 Env$_{584-592}$ tetramer (negative control; bottom). (F) IFN-γ production by hTERT-*siTCR*–transduced CD8$^+$ T cells was measured by using a format similar to that described for panel D. The parental K3-1 CTL clone was tested in parallel.

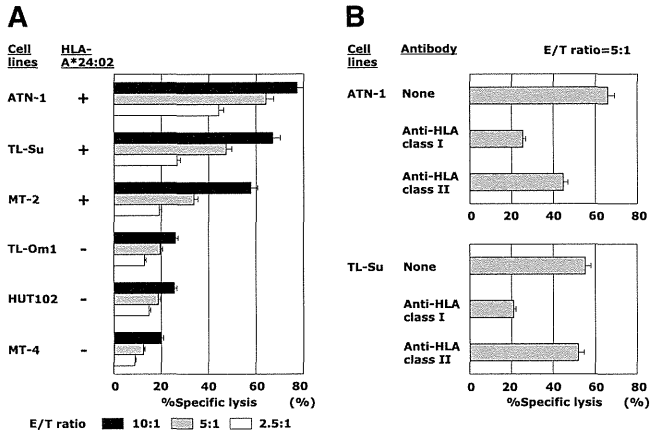

Figure 4. Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against ATL/HTLV-I–infected cell lines. (A) Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against HLA-A*24:02$^+$ or HLA-A*24:02$^-$ ATL/HTLV-I–infected cell lines was tested in ^{51}Cr-release assays at the indicated E/T ratios. All tested ATL/HTLV-I–infected cell lines overexpressed *hTERT* mRNA and protein, as shown in Figure 1. (B) Effect of HLA class I and class II blockade on the cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against ATN-1 and TL-Su was tested in ^{51}Cr-release assays at an E/T ratio of 5:1.

nor HLA-A*24:02$^+$ normal CB-CD34$^+$ cells as normal hematopoietic progenitor cells were killed. In the same experiment, newly established IL-2–dependent HTLV-I–infected CD4$^+$ T cells (Patient #1 and Patient #2), but not the corresponding original normal/HTLV-I$^-$ CD4$^+$ T cells (Patient #1 and Patient #2), became to some extent sensitive to the same transfectants as the level of *hTERT* mRNA expression increased (Figure 5B). This observation confirmed that not only ATL tumor cells, but also HTLV-I–infected cells from which ATL tumor cells were derived could be killed by these hTERT-specific effector cells.

Next, because the majority of ATL patients were of an advanced age and were therefore ineligible for allo-HSCT, we examined the tumoricidal activity against autologous ATL tumor cells mediated by gene-modified PB-CD8$^+$ T cells from the patient (Figure 6). Although PB-CD8$^+$ T cells from heavily pretreated ATL patients were sometimes difficult to subject to *TCR* gene modification and ex vivo expansion, hTERT-*siTCR*/CD8 cells generated from HLA-A*24:02$^+$ patients (n = 3) were able to substantially lyse autologous ATL tumor cells in proportion to the corresponding level of *hTERT* mRNA expression. Autologous CD14$^+$ PB monocytes were used as a negative control because they lacked expression of *hTERT* mRNA. These results demonstrated that hTERT-*siTCR*/CD8 cells were able to exert tumoricidal activity against ATL tumor cells through recognition of the hTERT$_{461\text{-}469}$ epitope/HLA-A*24:02 complex, which is naturally presented on the surface of ATL tumor cells.

hTERT-*siTCR*–transduced CD8$^+$ T cells display in vivo anti-ATL reactivity

In vivo anti-ATL reactivity mediated by hTERT-*siTCR*/CD8 cells was assessed by using a xenografted mouse model and bioluminescence assay. Serial bioluminescence assay images were simultaneously acquired.

In the Winn assay (Figure 7A), tumor cell growth in NOG mice treated with hTERT-*siTCR*/CD8 (n = 2) was completely inhibited for longer than 6 months. In contrast, when compared with nontreated NOG mice (n = 2) in which the inoculated ATL tumor mass rapidly enlarged, activated NGM/CD8 (n = 2) did suppress ATL tumor growth to some degree, but eventually huge tumor masses developed within 2 months. In a therapeutic adoptive transfer model (Figure 7B), the tumor cell growth in mice treated with hTERT-*siTCR*/CD8 (n = 2) was obviously suppressed within the 8-week observation period, in contrast to that in mice treated with NGM/CD8 (n = 2) and that in control mice (n = 2).

Discussion

Although ATL still has a poor prognosis, the clinical presence of the graft-versus-ATL in patients treated successfully by allo-HSCT has encouraged the search for a novel cellular immune-mediated treatment of ATL. Unlike EBV-related malignancy,[6] the feasibility of HTLV-I–associated Tax[7] and HBZ[8] proteins as therapeutic targets of anti-ATL immunotherapy still remains controversial. Therefore, in this study, we explored the feasibility of a novel therapeutic target other than one associated with HTLV-I. Consequently, we demonstrated for the first time that hTERT was a promising therapeutic target for anti-ATL adoptive immunotherapy. Freshly isolated ATL tumor cells produced *hTERT* mRNA abundantly, and HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$-specific CTL precursors were detected in the periphery of HLA-A*24:02$^+$ ATL patients. These findings suggested that naturally processed and presented hTERT$_{461\text{-}469}$/HLA-A*24:02 complex on the surface of ATL tumor cells was sufficiently immunogenic to be recognized by the target-specific CTLs in HLA-A*24:02$^+$ ATL patients. Additionally, *hTERT* mRNA expression in newly generated HTLV-I–infected CD4$^+$ T cells was upregulated, and these cells became sensitive to gene-modified hTERT-specific CTLs (Figure 5B). The involvement of Tax[12] and HBZ[38] in upregulation of the *hTERT* gene in

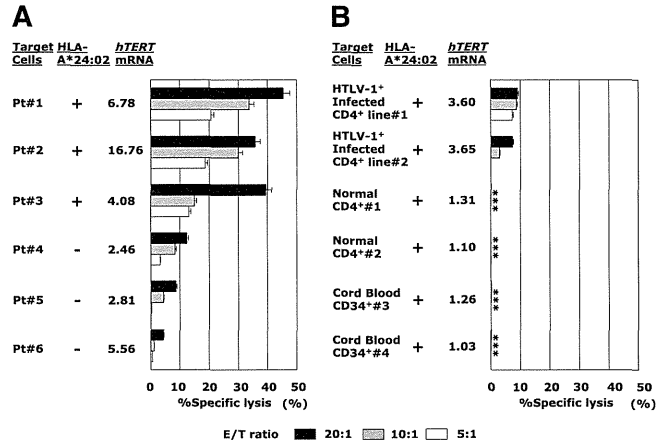

Figure 5. hTERT-*siTCR*–transduced CD8$^+$ T cells kill freshly isolated ATL cells and newly HTLV-I–infected CD4$^+$ T cells, but not normal cells, in vitro. (A) Freshly isolated HLA-A*24:02$^+$ (n = 3) or HLA-A*24:02$^-$ (n = 3) ATL tumor cells overexpressing *hTERT* mRNA were used as targets in ^{51}Cr-release assays with hTERT-*siTCR*–transduced CD8$^+$ T cells at the indicated E/T ratios. (B) The same hTERT-*siTCR*–transduced CD8$^+$ T cells used in panel A at the same E/T ratios were tested in ^{51}Cr-release assays against newly generated HLA-A*24:02$^+$ HTLV-I–infected CD4$^+$ T cells (n = 2) representing HTLV-I carrier CD4$^+$ T cells, original HLA-A*24:02$^+$ normal CD4$^+$ T cells (n = 2) representing the normal counterpart ATL tumor cells (corresponding number indicating cells from the identical donor), and HLA-A*24:02$^+$ normal CB-CD34$^+$ cells (n = 2) encompassing steady-state normal hematopoietic progenitor cells. Listed levels of expression of *hTERT* mRNA are those relative to the mean levels of expression across 6 PBMC samples from healthy donors determined by qRT-PCR and calculated by using the comparative threshold cycle method. Error bars represent SDs (* indicates less than detectable).

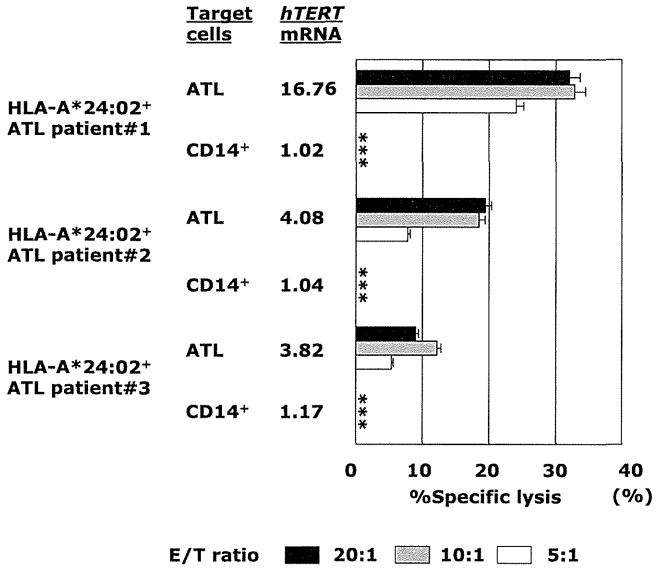

Figure 6. hTERT-*siTCR*–transduced CD8$^+$ T cells kill freshly isolated autologous ATL tumor cells on the basis of hTERT expression levels. Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells obtained from HLA-A*24:02$^+$ ATL patients (n = 3) against autologous freshly isolated ATL tumor cells and autologous peripheral CD14$^+$ cells (negative control) was tested in ^{51}Cr-release assays at the indicated E/T ratios. *hTERT* mRNA in each patient's ATL tumor cells is listed using a format similar to that used in Figure 5. Error bars represent SDs (* indicates less than detectable).

HTLV-I–infected immortalized CD4$^+$ T cells and ATL tumor cells has been reported previously. Initially, it might seem more realistic to develop an hTERT$_{461\text{-}469}$ peptide vaccine for treatment of HLA-A*24:02$^+$ ATL patients. However, because we were concerned that CTL induction of hTERT peptide vaccine might have a tendency to be impeded by the regulatory T-cell function of ATL tumor cells,[21] we focused on developing a redirected T-cell–based adoptive immunotherapy targeting hTERT to allow administration of a number of hTERT-specific CTLs directly.

To this end, we cloned the full-length rearranged *TCR-α/β* genes from K3-1, the HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$-specific CTL clone.[19] With codon optimization of the constant regions, we inserted them into our new souped-up second-generation 2A peptide-based *siTCR* vector to accomplish an increased expression level of the introduced TCR, carrying small interfering RNAs for the endogenous *TCR-α/β* genes in the redirected T cells (hTERT-*siTCR* vector).[26,27,34] The *siTCR* vector system makes it possible to simultaneously accomplish profound suppression of endogenous *TCR* genes and markedly increase the cell-surface expression of the introduced TCR, resulting in upregulated antitumor reactivity,[34] thus leading to inhibition of mispaired TCR formation between the endogenous and introduced TCR-α and -β chains, and lowering the potential risk of lethal graft-versus-host disease.[39] We found that both allogeneic and autologous gene-modified CD8$^+$ T cells using the hTERT-*siTCR* vector successfully killed ATL tumor cells both in vitro and in vivo (Figures 4-7), but not normal cells, including steady-state hematopoietic progenitor cells (Figure 5B). The introduced cytocidal activity against ATL tumor cells mediated by these gene-modified CTLs was actually accomplished through recognition of the HLA-A*24:02/hTERT$_{461\text{-}469}$ complex on the surface of ATL tumor cells (Figures 3 and 4).

Clinical studies of anticancer immunotherapy targeting hTERT have not demonstrated any significant adverse events so far.[14-17,20] However, for clinical application, because a number of activated gene-modified hTERT-specific CTLs would be administered at once, it would again be necessary to be mindful of on-target adverse events against normal tissues that constitutively express the *hTERT* gene.[10,40] Notably, any impairment of hematopoiesis would be the major concern. In this study, both allogeneic and autologous gene-modified effector CD8$^+$ T cells expressing hTERT-specific TCR from adult peripheral lymphocytes, and CB lymphocytes did not kill CB-CD34$^+$ cells representing steady-state hematopoietic progenitors (Figure 5B). By using cytokine-driven myeloid differentiation with CB-CD34$^+$ cells, gene-modified CTLs targeting hTERT showed a slight cytocidal effect against differentiated and highly proliferating subsets of CD34$^+$CD33$^+$ and CD34$^-$CD33$^+$ cells but spared CD34$^+$CD33^{dim} cells (supplemental Fig 2A). Additionally, contrary to resting CD4$^+$ cells and CD19$^+$ cells, highly mitotic polyhydroxic acid–stimulated CD4$^+$ cells and CD19$^+$ EBV LCLs became sensitive to effector CTLs because of increased expression of *hTERT* mRNA, the latter being more salient (Figure 5B and supplemental Fig 2B). Taken together, our findings suggest that gene-modified hTERT-specific CTLs will

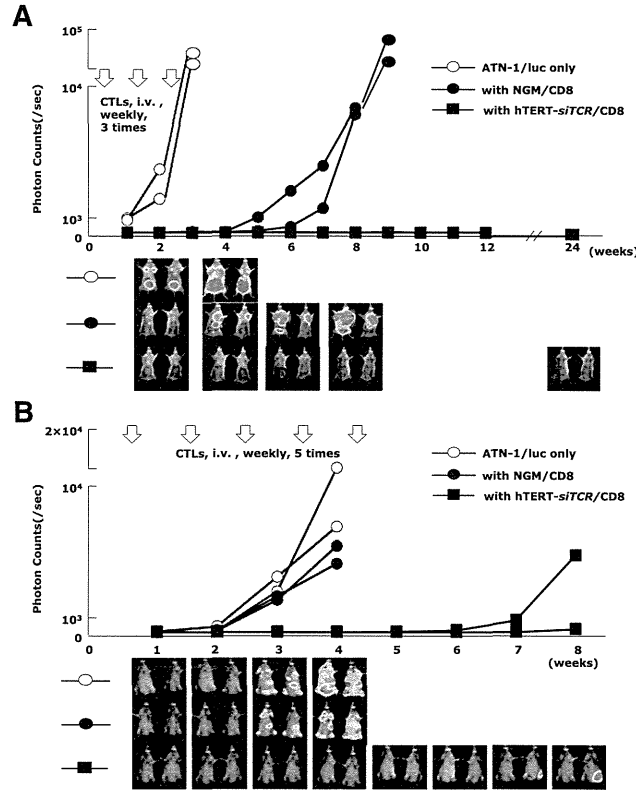

Figure 7. Anti-ATL reactivity of hTERT-*siTCR*–transduced CD8$^+$ T cells in vivo. (A) Winn assay. NOG mice were coinjected with a luciferase-transduced HLA-A*24:02$^+$ ATL cell line (ATN-1/luc) (5×10^5) and either 2.5×10^6 hTERT-*siTCR*–transduced (hTERT-*siTCR*/CD8) or NGM/CD8$^+$ T cells (n = 2 per group). Subsequently, 3 weekly infusions of the respective CD8$^+$ T-cell populations (2.5×10^6 cells per infusion) were administered intravenously (i.v.). Tumor growth was monitored every 7 days by using bioluminescence assay. Nontreated ATN-1/luc cells were similarly inoculated into NOG mice (n = 2) as a control. Although NGM/CD8 activated using OKT-3 and rhIL-2 suppressed tumor growth to some extent, hTERT-*siTCR*/CD8 durably suppressed tumor growth for longer than 6 months. (B) Therapeutic adaptive transfer model. NOG mice were intravenously inoculated with 5×10^5 ATN-1/luc cells. Four days later, intravenous administration of either 5×10^6 hTERT-*siTCR*/CD8 or NGM/CD8 (n = 2 per group) was started once a week for a total of 5 infusions. NOG mice given only ATN-1/luc cells (n = 2) were used as a control. In comparison with NGM/CD8, therapeutically infused hTERT-*siTCR*/CD8 also obviously suppressed the tumor cell growth within the 8-week observation period. Serial images of the bioluminescence assay demonstrate tumor growth in each group.

spare steady-state hematopoietic progenitor cells. However, to ensure safety, it would be better to avoid the active recovery phase of bone marrow after chemotherapy, notably under granulocyte colony-stimulating factor support, and also the acute infectious period in which immune-cell components are stimulated.

Another likely problem in clinical practice is that heavily pretreated peripheral lymphocytes from ATL patients might fail to proliferate. Proliferative activity of therapeutically infused gene-modified T cells in vivo is an important prerequisite for a successful outcome.[41] In this connection, although the control of treatment-related graft-versus-host disease still remains unsolved, use of CB lymphocytes has been investigated.[42] In this study, gene-modified CB-CD8$^+$ T cells from 2 donors successfully killed ATL tumor cells but spared autologous steady-state CB-CD34$^+$ cells (supplemental Figure 1-(2)). Compelling lack of suitable allo-HSCT donors for patients of advance age with ATL will encourage the application of CB transplantation using reduced-intensity preconditioning in the near future. Genetic redirection of CB lymphocytes using tumor antigen–specific *TCR* gene transfer will also play a considerable role.

Conversely, because hTERT is overexpressed in various kinds of cancer,[10] this approach may have widespread potential clinical application. Furthermore, the clinical availability of a new defucosylated anti-CCR4 mAb for treatment of ATL[43] can be reasonably anticipated to diminish regulatory T cells, the key player in the immunosuppressive microenvironment in patients with cancer,[44] because CCR4 is also expressed on regulatory T cells.[45] Therefore, hTERT-targeting immunotherapy after preconditioning with this anti-CCR4 mAb may become a realistically promising treatment option not only for ATL, but also for other malignancies.

In summary, using a newly established hTERT-*siTCR* vector, we have demonstrated the feasibility of anti-ATL redirected T-cell–based adoptive immunotherapy targeting hTERT, notably for patients who are ineligible for allo-HSCT. Further studies will be needed to investigate the clinical safety and utility of this novel therapy.

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Authorship

Contribution: Y.M. performed the research and wrote the paper; H.F. designed and performed the research, wrote and edited the paper and provided financial support; H.A., F.O., and T.O. performed the research and discussed the experimental results; T.A. interpreted the experimental results and provided financial support; T.I., S.O., J.M., K.K., and H.S. provided materials and discussed the experimental results; and M.Y. discussed and interpreted the experimental results and provided financial support.

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

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References

1. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood.* 1977; 50(3):481-492.
2. Matsuoka M, Jeang KT. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat Rev Cancer.* 2007;7(4): 270-280.
3. Tsukasaki K, Utsunomiya A, Fukuda H, et al; Japan Clinical Oncology Group Study JCOG9801. VCAP-AMP-VECP compared with biweekly CHOP for adult T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study JCOG9801. *J Clin Oncol.* 2007;25(34):5458-5464.
4. 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(8): 1369-1376.
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(9):2141-2148.
6. Louis CU, Straathof K, Bollard CM, et al. Adoptive transfer of EBV-specific T cells results in sustained clinical responses in patients with locoregional nasopharyngeal carcinoma. *J Immunother.* 2010;33(9):983-990.
7. Kannagi M. Immunologic control of human T-cell leukemia virus type I and adult T-cell leukemia. *Int J Hematol.* 2007;86(2):113-117.
8. Suemori K, Fujiwara H, Ochi T, et al. HBZ is an immunogenic protein, but not a target antigen for human T-cell leukemia virus type 1-specific cytotoxic T lymphocytes. *J Gen Virol.* 2009; 90(Pt 8):1806-1811.
9. Nishikawa H, Maeda Y, Ishida T, et al. Cancer/testis antigens are novel targets of immunotherapy for adult T-cell leukemia/ lymphoma. *Blood.* 2012;119(13):3097-3104.
10. Patel KP, Vonderheide RH. Telomerase as a tumor-associated antigen for cancer immunotherapy. *Cytotechnology.* 2004;45(1-2): 91-99.
11. Sinha-Datta U, Horikawa I, Michishita E, et al. Transcriptional activation of hTERT through the NF-kappaB pathway in HTLV-I-transformed cells. *Blood.* 2004;104(8):2523-2531.
12. Hara T, Matsumura-Arioka Y, Ohtani K, Nakamura M. Role of human T-cell leukemia virus type I Tax in expression of the human telomerase reverse transcriptase (hTERT) gene in human T-cells. *Cancer Sci.* 2008;99(6):1155-1163.
13. Bellon M, Nicot C. Central role of PI3K in transcriptional activation of hTERT in HTLV-I-infected cells. *Blood.* 2008;112(7):2946-2955.
14. Brunsvig PF, Aamdal S, Gjertsen MK, et al. Telomerase peptide vaccination: a phase I/II study in patients with non-small cell lung cancer. *Cancer Immunol Immunother.* 2006;55(12): 1553-1564.
15. Domchek SM, Recio A, Mick R, et al. Telomerase-specific T-cell immunity in breast cancer: effect of vaccination on tumor immunosurveillance. *Cancer Res.* 2007;67(21):10546-10555.
16. Suso EM, Dueland S, Rasmussen AM, Vetrhus T, Aamdal S, Kvalheim G, Gaudernack G. hTERT mRNA dendritic cell vaccination: complete response in a pancreatic cancer patient associated with response against several hTERT epitopes. *Cancer Immunol Immunother.* 2011; 60(6):809-818.
17. Rapoport AP, Aqui NA, Stadtmauer EA, et al. Combination immunotherapy using adoptive T-cell transfer and tumor antigen vaccination on the basis of hTERT and survivin after ASCT for myeloma. *Blood.* 2011;117(3):788-797.
18. Arai J, Yasukawa M, Ohminami H, Kakimoto M, Hasegawa A, Fujita S. Identification of human telomerase reverse transcriptase-derived peptides that induce HLA-A24-restricted antileukemia cytotoxic T lymphocytes. *Blood.* 2001;97(9):2903-2907.
19. Tajima K, Ito Y, Demachi A, et al. Interferon-γ differentially regulates susceptibility of lung cancer cells to telomerase-specific cytotoxic