

Reduced intensity conditioning regimens

Feasibility of reduced intensity hematopoietic stem cell transplantation from an HLA-matched unrelated donor

E Kusumi¹, M Kami², K Yuji¹, T Hamaki², N Murashige², A Hori², R Kojima², Y Kishi², S-W Kim², J Ueyama¹, S Miyakoshi¹, R Tanosaki², S Morinaga¹, S Mori², Y Heike², Y Muto¹, S Masuo³, S Taniguchi¹, Y Takaue², for the Tokyo SCT Consortium

¹Department of Hematology, Toranomon Hospital, Tokyo, Japan; ²Hematopoietic Stem Cell Transplantation Unit, The National Cancer Center Hospital, Tokyo, Japan; and ³Department of Rheumatology and Hematology, The JR Tokyo General Hospital, Tokyo, Japan

Summary:

To evaluate the feasibility of reduced intensity stem cell transplantation (RIST) with bone marrow from a matched unrelated donor (MUD), we retrospectively investigated 20 patients with hematological disorders who received RIST in the Tokyo SCT consortium from January 2000 to October 2002. The preparative regimens were fludarabine-based (150–180 mg/m², *n* = 18) or cladribine-based (0.77 mg/kg, *n* = 2). To enhance engraftment, antithymocyte globulin (ATG) and 4 or 8 Gy total body irradiation (TBI) were added to these regimens in nine and 11 patients, respectively. GVHD prophylaxis was cyclosporine with or without methotrexate. In all, 19 achieved primary engraftment. Three developed graft failure (one primary, two secondary), and five died of treatment-related mortality within 100 days of transplant. Seven of the 19 patients who achieved initial engraftment developed grade II–IV acute GVHD, and seven of 13 patients who survived >100 days developed chronic GVHD. At a median follow-up of 5.5 months, estimated 1-year overall survival was 35%. Compared with a TBI-containing regimen, an ATG-containing regimen was associated with a high risk of graft failure (30 vs 0%, *P* = 0.0737). This study supports the feasibility of RIST from MUD; however, procedure-related toxicities remain significant in its application to patients.

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the treatment of choice for patients with

refractory hematological malignancies; however, it has been restricted to young patients without comorbidity. The introduction of reduced intensity stem cell transplantation (RIST) has expanded the treatment option to older, medically infirm patients.^{1,2} Another limitation of the treatment is the stem cell source; HLA-matched related donors are available to only 30% of the patients who require this procedure. An HLA-matched unrelated donor (MUD) is an important alternative donor source.³ The feasibility of RIST from a MUD has not been extensively studied, leaving an optimal conditioning regimen to be determined.

As of October 2002, we had treated 191 patients with hematological diseases or solid tumors with RIST, 20 of whom underwent RIST with unrelated bone marrow (BM). This study retrospectively examined the feasibility of RIST from a MUD.

Patients and methods

Patients and donors

We studied 20 consecutive patients who underwent RIST from a MUD following either antithymocyte globulin (ATG)- or total body irradiation (TBI)-containing conditioning regimens at the Tokyo Stem Cell Transplant Consortium between January 2000 and October 2002. They were eligible for RIST due to age > 50 years and/or organ dysfunction. Of the 20 patients, 17 had high-risk hematological malignancies (progressive diseases or those in > 2nd remission) (Table 1). The other three patients were classified as having low-risk diseases. All of the patients and donors gave their written informed consent in accordance with the requirements of the Institutional Review Board.

HLA typing and donor matching

An HLA-A, -B, and -DR antigen-matched donor was sought through the Japan Marrow Donation Program (JMDP) as reported previously.⁴ Alleles at HLA-A2, 26, -B39, 61, and 75, which are highly polymorphic in the Japanese population,⁵ and DRB1 were routinely identified

Correspondence: Dr M Kami, Hematopoietic Stem Cell Transplantation Unit, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; E-mail: mkami@ncc.go.jp

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Table 1 Patient characteristics

No.	Sex	Age	Primary disease	Disease status at transplant	Karyotype	Previous lines of therapy	ANC × 10 ⁸ /kg	Conditioning regimens	HLA allele mismatch	GVHD prophylaxis
1	F	29	MDS RA	RA	8 tetrasomy	2	2.8	2-CdA/Bu/ATG	1	CsA + PSL
2	F	55	AML M2	Second CR	Normal	1	2.8	2-CdA/Bu/ATG	0	CsA
3	M	52	CML BC	Refractory	t(9;22)	2	1.8	Flu/Bu/ATG	0	CsA
4	F	59	AML M2	First RL	Complex del(5)	1	2.4	Flu/Bu/ATG	0	CsA
5	M	59	ATLL	First RL	—	3	2.5	Flu/CY/ATG	0	CsA/PSL
6	F	71	AML M1	Third CR	Normal	3	0.8	Flu/Bu/ATG	0	CsA
7	M	38	MM	Refractory	Normal	Auto SCT/2	2.5	Flu/Mel/ATG	0	CsA
8	F	35	SAA	—	Normal	3	3.3	Flu/Mel/ATG/TLI4Gy	0	CsA + sMTX
9	M	52	MDS RAEB	Refractory	17(q10)	1	2.5	Flu/BU/TBI4Gy	1	CsA
10	M	60	AML	Overt leukemia	Complex	1	2.9	Flu/BU/TBI4Gy	1	CsA + MMF
11	M	65	AML M3	Third CR	t(15;17)	2	2.2	Flu/Mel/TBI8Gy	0	CsA + sMTX
12	F	58	NK lymphoma	First RL	—	2	2.5	Flu/BU/TBI8Gy	0	CsA
13	F	53	MDS	Graft rejection	Normal	Allo SCT/1	3.3	Flu/BU/TBI4Gy	0	CsA
14	M	58	PCL	Refractory	47,XY,+Y	5	1.0	Flu/BU/TBI4Gy	0	CsA
15	F	55	AML M4	Third CR	inv(16)	3	1.8	Flu/BU/TBI4Gy	0	CsA + sMTX
16	M	52	MDS RA	RA	Normal	3	0.8	Flu/BU/TBI4Gy	0	CsA
17	F	50	AML M4	Second CR	inv(16)	4	5.2	Flu/BU/TBI8Gy	0	CsA + sMTX
18	F	50	FL	Refractory	—	2	1.1	Flu/BU/TBI4Gy	0	CsA + sMTX
19	F	52	MDS	Overt leukemia	-7	2	2.2	Flu/BU/TBI4Gy	1	CsA + sMTX
20	F	57	AML M4	Second RL	Normal	4	1.5	Flu/BU/TBI4Gy	0	CsA + sMTX

MDS=myelodysplastic syndrome; RA=refractory anemia; RAEB=RA with excess blasts; ATLL=adult T-cell leukemia lymphoma; AML=acute myeloid leukemia; CML=chronic myeloid leukemia; SAA=severe aplastic anemia; DLBCL=diffuse large B-cell lymphoma; FL=follicular lymphoma; MM=multiple myeloma; PCL=plasma cell leukemia; 2CdA=cladribine; Flu=fludarabine; BU=busulfan; TBI=total body irradiation; TLI=total lymphoid irradiation; ATG=antithymocyte globulin; 2CdA=cladribine; CSP=cyclosporine; PSL=prednisolone; sMTX=short-term methotrexate; MMF=mycophenolate mofetil; CR=complete remission; RL=relapse; Auto=autologous transplantation with high-dose chemotherapy; Allo=allogeneic myeloablative hematopoietic stem cell transplantation; ANC=all nucleated cell.

by high-resolution DNA typing. BM was collected by a standardized technique on the day of infusion.

Preparative regimens

In the National Cancer Center Hospital, the preparative regimens used were cladribine 0.11 mg/kg on days -10 to -4 and busulfan 4mg/kg on days -6 and -5,⁶ then cladribine was replaced with fludarabine 30 mg/m² on days -8 to -3 as the supply of cladribine was suspended (Table 1). In the Toranomon Hospital, fludarabine was administered in the same schedule and cyclophosphamide was 60 mg/kg on days -3 to -2. Cyclophosphamide was switched to melphalan 140 mg/m² on day -1 because of the number of patients with cardiac dysfunction.

To enhance engraftment, rabbit ATG (thymoglobulin; IMTIX-SANGSTAT, Lyons, France) 2.5 mg/kg/day was administered for 2 (n=3), 2 (n=3), and 4 (n=3) consecutive days, finishing on day -1. ATG was replaced by 4 or 8 Gy fractionated TBI because of the observed high rate of graft failure. TBI was administered on day -1 in two fractions. Case 8 received ATG and total lymph node irradiation, and was classified in the ATG group in this study.

Engraftment and chimerism analysis

Engraftment was defined as a white blood cell (WBC) count of >1.0 × 10⁹/l or an absolute neutrophil count (ANC) of >0.5 × 10⁹/l for 2 consecutive days, and a platelet count of >20 × 10⁹/l for 2 consecutive days without transfusions. G-CSF 300 μg/m²/day was administered intravenously from day 5 till neutrophil engraftment. Secondary graft failure

was defined as peripheral cytopenia and marrow hypoplasia occurring later than day 21 without detection of donor markers by cytogenetic and molecular techniques.

Donor-recipient chimerism was assessed using CD3-positive cells by fluorescent *in situ* hybridization (FISH) in sex-mismatched donor-recipient pairs. In sex-matched pairs, multiplex amplification of short tandem repeats (STR) was used with donor cells detected at a sensitivity of 10%.⁷

Regimen-related toxicity

Regimen-related toxicity (RRT) was defined as all non-hematological organ dysfunctions from day 0 to day 28, and was graded according to the toxicity criteria developed by the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2.0 (Table 2). Transplant-related mortality (TRM) was nonrelapse mortality.

Supportive cares and management of GVHD

All of the patients were managed in laminar airflow-equipped rooms, and received prophylaxis with trimethoprim/sulfamethoxazole or pentamidine inhalation, ciprofloxacin, fluconazole, and acyclovir.⁸ Neutropenic fever was managed as described by Pizzo.⁹ Cytomegalovirus (CMV) pp65 antigenemia was monitored weekly with initiation of preemptive ganciclovir at positive results.⁸

GVHD prophylaxis was cyclosporin alone (n=10), or with short-term methotrexate (n=7), prednisolone 0.5 mg/kg (n=2), or mycophenolate mofetil (n=1) (Table 1). In the absence of GVHD, cyclosporin was tapered from day 100 until day 180. GVHD was diagnosed based on clinical

Table 2 Regimen-related toxicity

	Maximal grade ^a			
	1	2	3	4
Mucosa	6	4	3	0
Gut	4	5	2	1
Liver	2	3	3	2
Lung	2	1	3	1
Kidney	4	2	2	0
Heart	0	0	2	0
CNS	0	0	2	0
Bladder	4	1	0	0

^aDetermined by the NCI-CTC version 2.0.

and pathological findings. Acute and chronic GVHD was graded according to the consensus criteria.^{10,11}

End points and statistical analysis

The primary end points were durable engraftment and TRM at day 100. The secondary end points were RRT, incidence of acute and chronic GVHD, event-free survival (EFS), and overall survival (OS). These end points were compared between ATG- and TBI-conditioned groups. EFS was defined as the post-RIST survival duration without disease progression, relapse, graft failure, or death. Probabilities of OS and EFS were calculated as a function of time by the Kaplan–Meier method. Surviving patients were censored on the last day of follow-up.

A univariate analysis using Fisher's exact test and the Mann–Whitney test was performed to compare the clinical characteristics of ATG- and TBI-conditioned groups. A Cox regression analysis was used to determine the effects of several variables on OS. Significant factors were identified based on a forward stepwise procedure. The variables entered in each stepwise analysis were sex, age, disease (acute leukemia vs other, and mature B-cell neoplasms and others), risk of primary disease (high vs low), preparative regimen (TBI-containing vs ATG-containing), HLA-allele matching, time-to-RIST from diagnosis, history of autologous HSCT with high-dose chemotherapy, ABO mismatch, and the number of infused nucleated cells. *P*-values <0.05 were considered significant.

Results

Engraftment

The median number of nucleated cells infused was 2.6×10^8 /recipients' body weight (kg) (range 0.8– 5.2×10^8 /kg). In all, 19 patients (95%) achieved primary neutrophil engraftment, and 12 (60%) reached more than 20×10^9 /l platelets. The median time to recover an ANC of 0.5×10^9 /l was 15 days (10–25), while 20.5 days (11–32) were needed to reach a platelet count above 20×10^9 /l. The median number of transfused red blood cell and platelet products, within 60 days post transplant, was 12 (0–104) and 125 (10–835) units, respectively.

Graft failure

Three patients, all of whom received an ATG-containing preparative regimen, developed graft failure (primary in one and secondary in two). One patient with AML (case 4) who was refractory to conventional chemotherapy did not recover her blood cell counts, and finally died of sustained disease progression on day 48. Two patients (cases 2 and 9) developed secondary graft failure following preemptive use of ganciclovir. These patients did not respond to G-CSF therapy.

Statistical analysis showed a significant association between graft failure and the use of an ATG-containing regimen ($P=0.0491$). Two of the five patients who received an allele-mismatched graft developed graft failure, whereas one of the 15 patients who received a matched graft developed graft failure, and this difference was not statistically significant ($P=0.071$).

Chimerism analysis

Chimerism analysis was available in all but four patients who showed early disease progression (cases 4 and 20) and cytopenia (cases 1 and 2). Of these 16 patients, 15 (94%) achieved full donor T-cell chimerism by day 100. The remaining patient (case 9) had 68% donor chimerism on day 30 and subsequently developed secondary graft failure on day 38.

Toxicity

NCI-CTC grade III–IV toxicity within 28 days post transplantation was observed in seven patients (Table 2). Five patients (25%) died of TRM within 100 days of transplant. The causes of death were pulmonary bleeding due to acute GVHD, interstitial pneumonitis, gastrointestinal bleeding, graft failure, and liver failure.

GVHD

Grade II–IV acute GVHD developed in 7/18 evaluable patients on a median of day 24 (range 19–70; Table 3). Three patients died of acute GVHD. Given the high risk of relapse of the underlying diseases, immunosuppressants were tapered rapidly in two patients (cases 6 and 15) to induce GVHD; case 15 died of acute GVHD and case 6 died of invasive pulmonary aspergillosis following steroid treatment for mild GVHD. Seven of the 11 patients (64%), who survived longer than 100 days, developed chronic GVHD. Chronic GVHD was preceded by acute GVHD in six patients.

Infection

Reactivation of CMV infection was documented in 8/20 (40%), while none of them developed CMV disease. One (5%) developed fungal infection, which led to TRM (Table 3). Case 16 developed hemorrhagic cystitis attributable to adenovirus infection (serotype 11) on day 87, which responded to hydration.

Table 3 Clinical course, graft failure, and GVHD

No.	Graft failure	Neutrophils >0.5 × 10 ⁹ /l	Platelets >20 × 10 ⁹ /l	Chimerism (donor %)	Acute GVHD	Chronic GVHD	CMV reactivation	Best response	Current status (months)	Causes of death
1		14	13	—	0	—	Yes	NC	Dead (2)	Pulmonary bleeding
2	Day 49	14	15	—	0	0	Yes	CR	CCR (41+)	
3		12	16	70	0	Extensive	No	CR	CCR (32+)	
4	Primary	Not evaluable	Not reached	—	—	—	No	PD	DEAD (2)	Disease progression
5		10	Not reached	100	0	—	Yes	CR	Dead (2)	Liver failure
6		12	21	100	II skin/gut	Extensive	Yes	CR	Dead (7)	Invasive aspergillosis
7		13	Not reached	100	0	—	No	PR	Dead (2)	Interstitial pneumonitis
8		19	22	100	II skin	0	Yes	CR	CCR (22+)	
9	Day 38	20	Not reached	68	0	—	No	NC	Dead (2)	Graft failure
10		11	Not reached	100	I skin	Extensive	No	CR	Dead (6)	Pneumonia
11		25	Not reached	100	III skin/gut	Limited	No	CR	Dead (8)	Interstitial pneumonitis
12		11	Not reached	100	I skin	0	No	PR	Dead (5)	Disease progression
13		19	32	100	0	0	Yes	CR	CCR (15+)	
14		23	Not reached	100	III skin	—	Yes	CR	Dead (3)	Acute GVHD
15		98	Not reached	100	II skin/gut	—	Yes	CR	Dead (4)	Acute GVHD
16		12	Not reached	100	I skin	Extensive	Yes	CR	CCR (11+)	
17		15	24	100	II skin	Extensive	Yes	CR	CCR (10+)	
18		16	16	100	III skin/liver/gut	Extensive	Yes	PR	CCR (9+)	
19		20	23	100	0	—	No	NC	Dead (4)	Disease progression
20		17	22	—	—	—	No	PD	Dead (2)	Disease progression

NE = not evaluable; CR = complete remission; PR = partial remission; NC = no change; PD = progressive disease; CCR = continuous CR; — = no data.

Survival

As of August 2003, the median follow-up was 5.5 months (range 2.0–41 months). The estimated 2-year OS and EFS were, respectively, 35.0% (95% confidence interval (CI) 24.7–45.3%) and 30.0% (95% CI 19.8–40.2%) (Figure 1).

Comparison of ATG- and TBI-containing regimens

These two treatment groups are compared in Table 4. While GVHD prophylaxis tended to be more intense in the TBI group than in the ATG group, graft failure developed more frequently in the ATG group than in the TBI group (P=0.074). There was no difference in TRM between TBI and ATG groups.

Discussion

Few studies have been reported on the feasibility of RIST from a MUD.^{12–15} Two studies from Israeli¹³ and Texas groups¹² suggest its feasibility for durable engraftment (25/29 vs 15/16, respectively), while the German study reported that 41/42 patients engrafted followed by 8/41 with secondary graft failure.¹⁴ However, the variety of the conditioning regimens, GVHD prophylaxis, and patient characteristics make the risk factors for graft failure difficult to determine. The present study showed that all the 20 patients engrafted, supporting the feasibility of RIST from MUD. However, two of them developed secondary graft failure, both of whom had received ATG as part of the conditioning regimen. This may suggest a negative effect of ATG on engraftment.

ATG is comprised of polyclonal serum immunoglobulin cultivated in rabbits against T-cell lines. It has observable effects on T cells for up to 4 days after administration.¹⁶ Thus, ATG can deplete both host- and donor-derived T

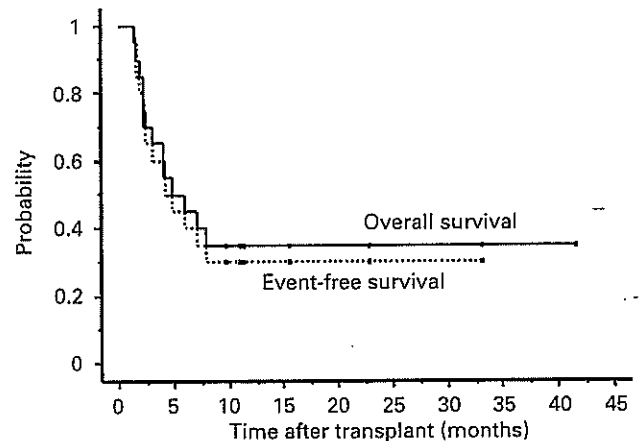


Figure 1 OS and EFS. The estimated 2-year OS (solid lines) and EFS (dotted lines) were, respectively, 35.0% (95% CI 24.7–45.3%) and 30.0% (95% CI 19.8–40.2%).

cells and inhibits GVHD due to its long half-life.¹⁷ In contrast, TBI has no effect on donor-derived T cells. These findings support the contention that the use of ATG in RIST from MUD is associated with a high rate of graft failure, while TBI-containing regimens might enhance engraftment. Since we replaced ATG with TBI, we have not experienced any graft failure. This is consistent with a previous report that TBI-containing RIST from MUD has attained engraftment in patients with aplastic anemia.¹⁸ However, it is to be noted that our purine analog-based regimens using intermediate-dose TBI cause considerable myelosuppression. Since regimen-related toxicities were moderate and acceptable in this study, we consider that our regimens are classified as reduced intensity regimens. Further investigation is warranted to investigate optimal preparative regimens for RIST.

Table 4 Patient characteristics according to conditioning regimen

	ATG group (n=9)	TBI group (n=11)	P-value
Secondary graft failure			
Grade II-IV acute GVHD	Yes/no 3/6	0/11	0.073
Reactivation of CMV infection	Yes/no 2/7	5/6	0.37
Maximal grading of regimen-related toxicity according to NCI-CTC version 2.0	Yes/no 5/4	6/5	>0.99
Transplant-related mortality within 100 days of transplant	3-4/0-2 4/5	4/7	>0.99
Estimated 1-year OS (95% confidence interval)	4 33 (17-49)	1 36 (21-51)	0.13 0.52

Another method to enhance engraftment is use of peripheral blood stem cell (PBSC) transplantation instead of BM.¹⁵ Since PBSC collections contain more CD34-positive and T cells than BM, they may be advantageous in achieving engraftment in RIST from MUD.

GVHD is the most important problem in RIST. In the present study, there were more patients with grade II-IV GVHD in the TBI group (n=5) than in the ATG group (n=2) despite additional GVHD prophylaxis with MTX. This may be because ATG reduces the frequency and severity of GVHD by suppressing T cells in the graft. However, day 100 TRM tended to be lower in the TBI group (n=1) than in the ATG group (n=4). These results suggest that TBI-containing regimens are safer in RIST from MUD than ATG-containing regimens; however, further studies are required to improve management of GVHD following RIST from MUD.

The incidences of CMV reactivation and disease have been reported to be higher in conventional SCT from MUD (87 and 73%) than conventional SCT from matched siblings (53 and 14%).¹⁹ HSCT from unrelated donors achieves immune reconstitution later than HSCT from related donors, which leads to a higher risk for severe infections.¹⁹ Our study revealed that the incidences of fungal infection and CMV reactivation were, respectively, 15 and 54%, which are comparable to the reports in conventional SCT recipients.^{20,21}

Our results suggest that RIST with BM from MUD is feasible. However, we should comment on some limitations of this study. First, patients who had been enrolled on some pilot studies of RIST were analyzed in this study, and several different preparative regimens and GVHD prophylaxis were utilized. Second, the two comparison groups (ATG vs TBI) had different follow-up periods. This might complicate the interpretation of OS and EFS data when one attempts to compare the two groups. While this study suggests that RIST from MUD is feasible, further studies are required to improve its safety and efficacy. The use of intermediate-dose TBI as an alternative to ATG may enhance engraftment, although the optimal dose of TBI and GVHD prophylaxis regimen remains to be defined.

References

1 Khouri IF, Keating M, Korbling M et al. Transplant-lite: induction of graft-versus-malignancy using fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. *J Clin Oncol* 1998; 16: 2817-2824.

2 Slavin S, Nagler A, Naparstek E et al. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood* 1998; 91: 756-763.

3 Grewal SS, Barker JN, Davies SM, Wagner JE. Unrelated donor hematopoietic cell transplantation: marrow or umbilical cord blood? *Blood* 2003; 101: 4233-4244.

4 Kodera Y, Morishima Y, Kato S et al. Analysis of 500 bone marrow transplants from unrelated donors (UR-BMT) facilitated by the Japan Marrow Donor Program: confirmation of UR-BMT as a standard therapy for patients with leukemia and aplastic anemia. *Bone Marrow Transplant* 1999; 24: 995-1003.

5 Sasazuki T, Juji T, Morishima Y et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. *Japan Marrow Donor Program. N Engl J Med* 1998; 339: 1177-1185.

6 Saito T, Kanda Y, Kami M et al. Therapeutic potential of a reduced intensity preparative regimen for allogeneic transplantation with cladribine, busulfan, and antithymocyte globulin against advanced/refractory acute leukemia/lymphoma. *Clin Cancer Res* 2002; 8: 1014-1020.

7 Thiede C, Florek M, Bornhauser M et al. Rapid quantification of mixed chimerism using multiplex amplification of short tandem repeat markers and fluorescence detection. *Bone Marrow Transplant* 1999; 23: 1055-1060.

8 Kanda Y, Mineishi S, Saito T et al. Long-term low-dose acyclovir against varicella-zoster virus reactivation after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2001; 28: 689-692.

9 Pizzo PA. Management of fever in patients with cancer and treatment-induced neutropenia. *N Engl J Med* 1993; 328: 1323-1332.

10 Przepiorka D, Weisdorf D, Martin P et al. 1994 consensus conference on acute GVHD grading. *Bone Marrow Transplant* 1995; 15: 825-828.

11 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.

12 Wong R, Giralt SA, Martin T et al. Reduced intensity conditioning for unrelated donor hematopoietic stem cell transplantation as treatment of myeloid malignancies in patients older than 55 years of age. *Blood* 2003; 102: 3052-3059.

13 Nagler A, Aker M, Or R et al. Low-intensity conditioning is sufficient to ensure engraftment in matched unrelated bone marrow transplantation. *Exp Hematol* 2001; 29: 362-370.

14 Bornhauser M, Thiede C, Platzbecker U et al. Dose-reduced conditioning and allogeneic hematopoietic stem cell transplantation from unrelated donors in 42 patients. *Clin Cancer Res* 2001; 7: 2254-2262.

15 Maris MB, Niederwieser D, Sandmaier BM et al. HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative conditioning for patients with hematologic malignancies. *Blood* 2003; 102: 2021-2030.

- 16 Eiermann TH, Lambrecht P, Zander AR. Monitoring anti-thymocyte globulin (ATG) in bone marrow recipients. *Bone Marrow Transplant* 1999; 23: 779-781.
- 17 Bunn D, Lea CK, Bevan DJ et al. The pharmacokinetics of anti-thymocyte globulin (ATG) following intravenous infusion in man. *Clin Nephrol* 1996; 45: 29-32.
- 18 Deeg HJ, Amylon ID, Harris RE et al. Marrow transplants from unrelated donors for patients with aplastic anemia: minimum effective dose of total body irradiation. *Biol Blood Marrow Transplant* 2001; 7: 208-215.
- 19 Takenaka K, Gondo H, Tanimoto K et al. Increased incidence of cytomegalovirus (CMV) infection and CMV-associated disease after allogeneic bone marrow transplantation from unrelated donors. The Fukuoka Bone Marrow Transplantation Group. *Bone Marrow Transplant* 1997; 19: 241-248.
- 20 Kanda Y, Mineishi S, Nakai K et al. Frequent detection of rising cytomegalovirus antigenemia after allogeneic stem cell transplantation following a regimen containing antithymocyte globulin. *Blood* 2001; 97: 3676-3677.
- 21 Fukuda T, Boeckh M, Carter RA et al. Risks and outcomes of invasive fungal infections in recipients of allogeneic hematopoietic stem cell transplants after nonmyeloablative conditioning. *Blood* 2003; 102: 827-833.

Graft-versus-Tax Response in Adult T-Cell Leukemia Patients after Hematopoietic Stem Cell Transplantation

Nanae Harashima,¹ Kiyoshi Kurihara,¹ Atae Utsunomiya,² Ryuji Tanosaki,³ Shino Hanabuchi,¹ Masato Masuda,⁴ Takashi Ohashi,¹ Fumiyo Fukui,¹ Atsuhiko Hasegawa,¹ Takao Masuda,¹ Yoichi Takaue,³ Jun Okamura,⁵ and Mari Kannagi¹

¹Department of Immunotherapeutics, Tokyo Medical and Dental University, Medical Research Division, Tokyo; ²Department of Hematology, Imamura Bun-in Hospital, Kagoshima; ³National Cancer Center Hospital, Tokyo; ⁴Second Department of Internal Medicine, University of the Ryukyus, Okinawa; and ⁵National Kyushu Cancer Center, Fukuoka, Japan

ABSTRACT

Adult T-cell leukemia (ATL) caused by human T-cell leukemia virus type I (HTLV-I) is characterized by poor prognosis after chemotherapy. Recent clinical trials have indicated, however, that allogeneic but not autologous hematopoietic stem cell transplantation (HSCT) for ATL can yield better clinical outcomes. In the present study, we investigated cellular immune responses of ATL patients who obtained complete remission after nonmyeloablative allogeneic peripheral blood HSCT from HLA-identical sibling donors. In the culture of peripheral blood mononuclear cells (PBMCs) from a post-HSCT but not pre-HSCT ATL patient, CD8⁺ CTLs proliferated vigorously in response to stimulation with autologous HTLV-I-infected T cells that had been established before HSCT *in vitro*. These CTLs contained a large number of monospecific CTL population directed to a HLA-A2-restricted HTLV-I Tax 11-19 epitope. The frequency of Tax 11-19-specific CD8⁺ CTLs in this patient markedly increased also *in vivo* after HSCT, as determined by staining with HLA-A2/Tax 11-19 tetramers. Similar clonal expansion of HTLV-I Tax-specific CTLs exclusively directed to a HLA-A24-restricted Tax 301-309 epitope was observed in the PBMCs from another ATL patient after HSCT from a HTLV-I-negative donor. Among four post-HSCT ATL patients tested, HTLV-I-specific CTLs were induced in the PBMC culture from three patients but not from the remaining one who had later recurrence of ATL. These observations suggested that reconstituted immunity against antigen presentation in ATL patients after HSCT resulted in strong and selective graft-versus-HTLV-I response, which might contribute to graft-versus-leukemia effects.

INTRODUCTION

Adult T-cell leukemia (ATL) is a T-cell malignancy that develops in ~5% of human T-cell leukemia virus type I (HTLV-I)-infected individuals and is characterized by mostly CD4⁺ and CD25⁺ mature T-lymphocyte phenotypes, onset at middle age or later, immune suppression, and poor prognosis (1-3). Clinical use of combination chemotherapy for ATL brought the 4-year overall survival rate up to 8 to 12%, which is still lower than those of other types of leukemia (4, 5). Recently, hematopoietic stem cell transplantation (HSCT) has been applied to a limited number of ATL patients. Initial studies of autologous HSCT revealed frequent recurrence of ATL (6). However, more recent studies have revealed that allogeneic HSCT could produce better results, although there was also a risk of graft-versus-host-disease (GVHD; Ref. 7). This strongly suggests that the cellular immune responses of donor against recipient, *i.e.*, graft-versus-leuke-

mia (GVL) effects, contribute to eradicating ATL cells, as observed in other types of leukemia.

It has been demonstrated that allogeneic HSCT from HLA-identical siblings can cause GVHD to some degree, and the minor histocompatibility antigen (mHA) in the recipient has been referred to as the target antigen of GVHD (8). Several mHA, including the male-specific H-Y transplantation antigen (9), HA-1 antigen (10), CD31 molecule (11, 12), and human platelet antigens (12, 13), have been suggested to be involved in GVHD. It is known that the probability of recurrence of leukemia after allogeneic HSCT increases when the graft has been depleted of T cells or the donor is a genetically identical twin, indicating that GVL effects are important in preventing the recurrence of leukemia (14). Therefore, an augmentation of the donor T-cell response specific for mHA expressed in the recipient's hematopoietic cells but not in the nonhematopoietic cells has been proposed as one strategy for inducing GVL effects without causing GVHD (15). Tumor antigens such as bcr/abl fusion protein and WT-1, which are specific for or overexpressed in tumor cells, are also candidates for the target antigens of GVL effects (16, 17).

Host cellular immune responses against HTLV-I, especially outgrowth of cytotoxic T cells, are frequently found in peripheral blood mononuclear cell (PBMC) culture of asymptomatic HTLV-I carriers and HTLV-I-associated myelopathy/Tropical spastic paraparesis patients but infrequently in ATL patients (18, 19). Of the HTLV-I antigens such as *env*, *gag*, *pol*, and *pX* gene products, it has been shown that HTLV-I Tax is a dominant target antigen of HTLV-I-specific CTL (20, 21). Tax is also known to play a critical role in HTLV-I leukemogenesis by accelerating cell growth and inhibiting apoptosis (22, 23). These findings suggest that Tax-specific CTL may play a role in immune surveillance for HTLV-I leukemogenesis.

In a recently established animal model for HTLV-I-infected T-cell tumors, we demonstrated an antitumor effect of Tax-specific CTL *in vivo* (24, 25). In this model, otherwise fatal T-cell lymphomas in nude rats inoculated with syngeneic HTLV-I-infected cells could be eradicated by transferring fresh T cells from syngeneic immunocompetent rats vaccinated with either Tax-encoded DNA or peptides corresponding to a CTL epitope (26, 27). However, it is unclear whether such observations in experimental models apply to humans because HTLV-I expression is extremely low in human ATL cells in the periphery (28-30).

In the present study, we investigated the cellular immune responses of ATL patients after HSCT against spontaneously HTLV-I-infected T cells derived from the same patient before HSCT. These HTLV-I-infected cells were thought to possess antigens originating from the recipients, including targets for GVL effects. We found that in response to the recipient-origin cells, the PBMCs from post-HSCT patients exhibited vigorous HTLV-I-specific CTL responses that were directed to a limited number of Tax epitopes. Furthermore, such oligoclonal expansion of HTLV-I-specific CTL in post-HSCT PBMCs was observed also *in vivo*. These observations indicated that

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Requests for reprints: Mari Kannagi, Department of Immunotherapeutics, Tokyo Medical and Dental University, Medical Research Division, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Phone: 81-3-5803-5798; Fax: 81-3-5803-0235; E-mail: kann.impr@tmd.ac.jp.

a strong graft-versus-HTLV-I response occurred in ATL patients after HSCT.

MATERIALS AND METHODS

Recipient/Donor Pairs and Blood Samples. Four acute type ATL patients, #37 (case 1), R07 (case 2), R11 (case 3), and #97 (case 4) and their corresponding HLA-identical sibling donors, #36, D07, D11, and #98, respectively, donated peripheral blood samples under written informed consent. The patients were participants in the clinical trial protocol for allogeneic HSCT for ATL with a reduced-intensity conditioning regimen that was supported by the Ministry of Health, Welfare, and Labor of Japan. After cyclophosphamide, doxorubicin, vincristine, prednisolone therapy, patient #37 at the beginning of recurrence, patients R07 and #97 in partial remission, and patient R11 in complete remission received conditioning treatment consisting of fludarabine (30 mg/m² i.v. days -8 to -3), busulfan (4 mg/kg p.o. days -6 and -5), and ATG (2.5 mg/kg days -2 and -1) before the infusion of granulocyte-colony stimulating factor-mobilized peripheral blood stem cells from the donors. Prophylaxis for GVHD was cyclosporine A alone starting from day -1. Although patients #37, R07, and #97 obtained complete remission within 2 months after HSCT, R11 had recurrence of ATL lymphoma in the neck 6 months after HSCT. Donor #36 was a HTLV-I carrier, but the other donors were not. The HLA and other clinical characteristics of the patients and donors are summarized in Table 1.

Cell Lines. PBMCs from the donors and recipients isolated on a Ficoll-Hypaque PLUS (Amersham Biosciences, Piscataway, NJ) gradient were partially stored in liquid nitrogen until use and partially used to obtain HTLV-I-infected IL-2-dependent T-cell (ILT) lines and EBV-transformed lymphoblastoid B-cell lines LCL. ILT-#37, ILT-R07, ILT-R11, and ILT-#97 were spontaneously HTLV-I-infected T-cell lines originating from pre-HSCT recipients #37, R07, R11, and #97, respectively. To establish these ILT lines, PBMCs were stimulated with 1 µg/ml phytohemagglutinin (PHA)-P (Sigma, St. Louis, MO) after depletion of CD8⁺ cells using a Dynabeads M450-CD8 (Dyna, Oslo, Norway) and then maintained in RPMI 1640 (Invitrogen-Life Technologies, Inc., Grand Island, NY) containing 10% FCS (Sigma), 10 units/ml recombinant human interleukin (IL)-2 (Shionogi, Osaka, Japan), or 10 ng/ml recombinant human IL-15 (Sigma) at 37°C with 5% CO₂ for over 2 months. An EBV-transformed B-cell line, LCL-#36, was established by main-

taining positively separated CD19⁺ PBMCs from donor #36 in RPMI 1640 with 10% FCS after infection with an EBV-containing culture supernatant of the B95-8 cell line (31). TCL-Kan (HLA-A2/A11, B7/Bw46, Cw1/Cw3/Cw7, and DR2/DR9; Ref. 32), ILT-As-2 (HLA-A24/A31, B7/B51, C3/C7, and DR1/DR5), ILT-Myj-3 (HLA-A2/A24, B54/B60, Cw1/Cw3, and DR4/DR5; Ref. 21), ILT-Nkz-2 (HLA-A2/A26, B51/B54, and Cw1/-; Ref. 21), and ILT-Har (HLA-A2/-, B51/B62, Cw3/-, and DR4/-) are HTLV-I-infected T-cell lines, and LCL-Kan (HLA-A2/A11, B7/Bw46, Cw1/Cw3/Cw7, and DR2/DR9), LCL-As (HLA-A24/A31, B7/B51, C3/C7, and DR1/DR5), TOK (HLA-A24/-, B52/-, and DR2/-; Ref. 33), LCL-Nkz (HLA-A2/A26, B51/B54, and Cw1/-; Ref. 21), and LCL-Har (HLA-A2/-, B51/B62, Cw3/-, and DR4/-) are EBV-transformed B-cell lines. An erythroblastoid cell line, K562 (34), was also used.

Flow Cytometry for Phenotyping and HTLV-I Expression. Cell surface phenotypes were determined using directly FITC-conjugated murine antihuman monoclonal antibodies (mAbs) followed by analysis on a FACSCalibur (Becton Dickinson, San Jose, CA), and data were analyzed using CellQuest software (Becton Dickinson). The mAbs used were anti-CD4 (clone: RPA-T4; BD PharMingen), anti-CD8 (clone: RPA-T8; BD PharMingen), anti-CD19 (clone: HIB19; BD PharMingen), and for isotype controls, antimouse IgG1. For detection of intracellular HTLV-I proteins, cells were stained with anti-Tax mAb (Lt-4; Ref. 35) and anti-Gag mAb (GIN-7; Ref. 36) after cell membrane permeabilization. These mAbs were kindly provided by Dr. Yuetsu Tanaka (University of the Ryukyus, Okinawa, Japan).

Induction of HTLV-I-Specific CTL. One million whole PBMCs from post-HSCT patient #37 were stimulated with 1 µg/ml PHA-P and then mixed with the same number of ILT-#37 cells, derived from pre-HSCT patient #37, and pretreated with 1% formaldehyde/PBS. These T cells were maintained in AIM-V medium (Invitrogen-Life Technologies, Inc.) supplemented with 100 units/ml penicillin, 0.5 mg/ml streptomycin, 10% heat-inactivated FCS, and 100 units/ml recombinant human IL-2 with periodic stimulation with formaldehyde-fixed respective ILT cells at 10-14-day intervals. PBMCs from donor #36 and pre-HSCT patient #37 were similarly stimulated with PHA and subsequently with formaldehyde-fixed ILT-#37 in cultures for CTL induction. CTL induced from CD8⁺ cell-enriched PBMCs of donor #36 were also used in some experiments. In the other ATL cases tested (patients R07, R11, and #97), PHA-stimulated CD8⁺ cell-enriched PBMCs from each post-HSCT

Table 1 Summary of clinical status and T-cell immune response of the participants in hematopoietic stem cell transplantation (HSCT)

Patient's ID	HSCT case	Donor/recipient	Age (yrs)	Sex	Status	HTLV-I ^a infection	HLA	In vitro immune analysis of PBMC ^b			HTLV-I proviral DNA (copies/1000 PBMC) ^d		Clinical outcome after HSCT
								Sampling date (days after HSCT)	Stimulated with	Induction of CTL ^c	Pre-HSCT	Post-HSCT	
#36	Case 1	Donor	57	M	Healthy	+	A2/-, B46/-, Cw1/-, DR8/-	0	ILT-#37	+	Undetectable	N.T.	
#37	Case 1	Recipient	63	M	Acute ATL	+	A2/-, B46/-, Cw1/-, DR8/-	+183	ILT-#37	+	1150.3	Undetectable	Complete remission for more than 24 months
D07	Case 2	Donor	48	F	Healthy	-	A24/A32, B35/B60, DR4/-	N.T.	N.T.	N.T.	N.T.	N.T.	
R07	Case 2	Recipient	51	M	Acute ATL	+	A24/A32, B35/B60, DR4/-	+255	ILT-R07	+	26.7	Undetectable	Complete remission for more than 23 months
D11	Case 3	Donor	52	F	Healthy	-	A2/A26, B35/B61, DR4/6	N.T.	N.T.	N.T.	N.T.	N.T.	
R11	Case 3	Recipient	54	M	Acute ATL	+	A2/A26, B35/B61, DR4/6	+153	ILT-R11	-	440.8	Undetectable	Relapse of lymphoma at 6 months after HSCT
#98	Case 4	Donor	61	M	Healthy	-	A2/A26, B51/, DR4/5	N.T.	N.T.	N.T.	N.T.	N.T.	
#97	Case 4	Recipient	66	F	Acute ATL	+	A2/A26, B51/, DR4/5	+104	ILT-#97	+	3297.2	Undetectable	Died of GVHD at 9 months after HSCT

^a HTLV-I, human T-cell leukemia virus type I; ATL, adult T-cell leukemia; PBMC, peripheral blood mononuclear cell; N.T., not tested; GVHD, graft-versus-host disease; ILT, IL-2-dependent T-cell line.

^b PBMCs isolated from patients #37, R07, R11, and #97 at the indicated days after HSCT were repeatedly stimulated in culture with formalin-fixed autologous ILT cells established before HSCT, as described in the "Materials and Methods."

^c Culture in which CTL specific for autologous ILT cells grew is indicated as (+) and that without CTL induction is indicated as (-).

^d HTLV-I proviruses in the peripheral blood were measured just before and after HSCT at similar dates when *in vitro* immune responses were analysed. A level less than 0.5 copies/1000 cells was undetectable.

patient were stimulated with formaldehyde-fixed autologous ILT cells established before HSCT and otherwise similarly maintained.

Synthetic Peptides. We prepared a total of 38 peptides (9 to 24-mer) to cover the entire sequence of the HTLV-I Tax protein. Some of the peptides were synthesized as described previously (27, 33). All 9-mer peptides were purchased from Hokudo Co. (Hokkaido, Japan). To identify potential HLA-A2- or A24-binding peptides within HTLV-I Tax, a computer-based program, BioInformatics and Molecular Analysis Section (BIMAS),⁶ was used as described previously (37-39).

CTL Assay. Cytotoxic activities were measured by 6-h ⁵¹Cr-release assay at various E:T cell ratios as described previously (27, 40). Specific cytotoxicity was calculated as [(experimental ⁵¹Cr release - spontaneous ⁵¹Cr release)/(maximum ⁵¹Cr release - spontaneous ⁵¹Cr release)] × 100%. IFN- γ production by the effector cells was also measured in response to specific antigens. Briefly, effector cells were added to microtiter wells containing some target cells in RPMI 1640 containing 10% FCS at various E:T ratios. After incubation for 18 h at 37°C, 50 μ l of supernatant were collected to measure IFN- γ by ELISA (human IFN- γ ELISA kit; Endogen, Woburn, MA) in duplicate assay. Absorbances were detected at 450 nm using a microplate reader (Bio-Rad, Hercules, CA), and data were analyzed with Microplate Manager III software. The limit of detection of the IFN- γ production ELISA assay was 3 pg/ml.

Mapping of CTL Epitopes. HLA-matched EBV-transformed B-cell lines were pulsed with a series of peptides at a final concentration of 10 μ M for 1 h at 37°C and then CTLs were added to each well. CTL activity was measured by 6-h ⁵¹Cr-release assay or ELISA assay for IFN- γ production in the supernatants after additional incubation for 18 h.

Recombinant Vaccinia Viruses. Recombinant vaccinia viruses WR-p27^X (41) containing HTLV-I pX genes and WR-HA without the HTLV-I gene were kindly provided by Dr. Hisatoshi Shida (Hokkaido University, Sapporo, Japan). The recombinant vaccinia viruses-infected cells were used as targets for CTL assay after 16 h infection at a multiplicity of infection of 50 as described previously (21, 27).

Enzyme-Linked Immunospot (ELISPOT) Assays. IFN- γ -producing antigen-specific T cells were counted using IFN- γ -specific ELISPOT assays as described previously (42, 43). A 96-well polyvinylidene difluoride plate (MAIP54510; Millipore, Bedford, MA) was coated overnight at 4°C with 100 μ l of 15 μ g/ml anti-IFN- γ mAb, 1-D1K (Mabtech, Nacka, Sweden) in 0.1 M carbonate-bicarbonate buffer (pH 9.6; Sigma). After six washes with PBS, PBMCs were added in triplicate at 5 × 10⁴ cells/well in the absence or presence of the same number of stimulator cells or 10 μ g/ml peptides in RPMI 1640 containing 10% FCS and incubated overnight at 37°C in 5% CO₂. The next day, cells were removed by washing with PBS/0.05% Tween 20 and biotinylated anti-IFN- γ mAb, and 7-B6-1 biotin (Mabtech) was added 100 μ l of 1 μ g/ml and left for 2 h at room temperature, followed by incubation with streptavidin-alkaline phosphatase (Mabtech) for an additional 1 h. Individual cytokine-producing cells were detected as dark purple spots after 10-min reaction with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium alkaline phosphatase substrate (Sigma). After washing in tap water to stop color development, colored spots on the dried membranes were counted using a KS-ELISPOT microscopy system (Carl Zeiss, Jena, Germany).

Tetramer Staining. Phycoerythrin-conjugated HLA-A*0201/Tax11-19 (LLFGYPVYV) tetramer was provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility, Emory University Vaccine Center at Yerkes (Atlanta, GA). Lymphocytes (1 × 10⁶) were stained for 30 min at 4°C with Cy-Chrome-conjugated mouse antihuman CD8 mAb (BD Pharmingen) and then for an additional 60 min with tetramer (1:100 diluted) at 4°C. After washing in PBS, the cells were fixed in 1% formaldehyde/PBS, and the samples were subjected to two-color analysis on a FACScalibur using CellQuest software (Becton Dickinson; Ref. 44).

Quantification of HTLV-I Provirus. HTLV-I proviral load in peripheral blood was quantified by real-time PCR on a LightCycler PCR Instrument (Roche Diagnostics, Mannheim, Germany) using primers specific for HTLV-I pX region and β -globin as described elsewhere (7).

RESULTS

Establishment of HTLV-I-Infected T-Cell Lines from Pre-HSCT ATL Patients. To examine the immune responses of the post-HSCT recipients to hematopoietic cells of pre-HSCT-recipients origin, we established T-cell lines from PHA-stimulated PBMCs from patients #37, R07, R11, and #97 before HSCT by maintaining them for longer than 2 months in the presence of IL-2 or IL-15. The phenotypes of the resulting IL-15-dependent T-cell lines, ILT-#37, ILT-R07, ILT-R11, and ILT-#97, are shown in Fig. 1. ILT-#37, ILT-R07, and ILT-#97 were positive for surface CD4, whereas ILT-R11 was positive for CD8. All of these ILT lines expressed intracellular HTLV-I antigens such as HTLV-I Tax and p19 (Fig. 1). Thus, spontaneously HTLV-I-infected T-cell lines of the pre-HSCT ATL patients were obtained.

Induction of CTL from a Post-HSCT Recipient Reacting with Pre-HSCT HTLV-I-Infected Cells. The T-cell response in the PBMCs of post-HSCT patient #37 to ILT-#37 cells was examined at +183 days after HSCT. The hematopoietic cells of this patient had been completely replaced by those of donor origin in the first 2 months after HSCT as determined by short tandem repeat polymorphism in the genome. Because donor #36 was a HTLV-I carrier, we also examined the T-cell response of donor #36 to ILT-#37. The PBMCs from post-HSCT patient #37 and donor #36 stimulated *in vitro* with 1% formaldehyde/PBS-treated ILT-#37 twice with a 10-day interval in the presence of IL-2 were examined for IFN- γ -producing ability against ILT-#37 and K562 cells at 19 days after initiation of culture. As shown in Fig. 2A, significant levels of IFN- γ were produced from post-HSCT #37 in the culture against ILT-#37 but not against K562 cells after an overnight incubation. The PBMCs from

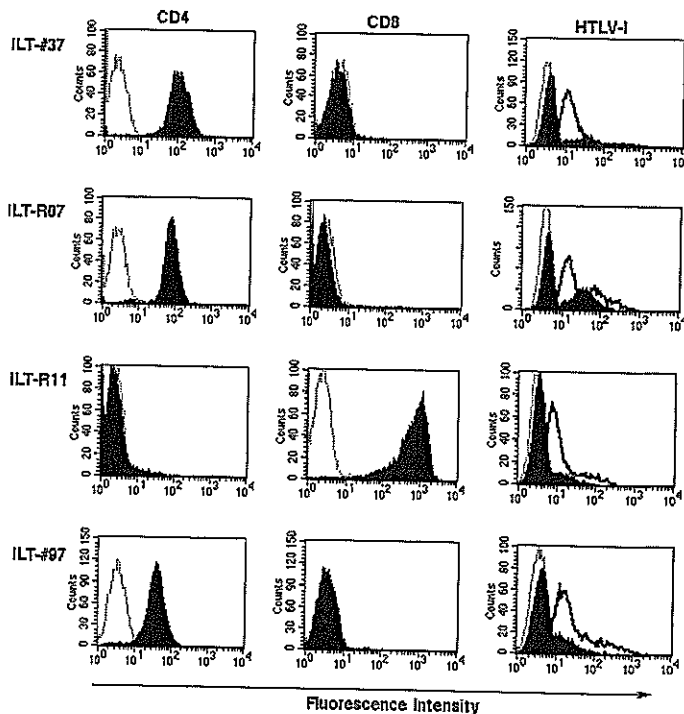


Fig. 1. Surface phenotype and human T-cell leukemia virus type I (HTLV-I) expression in T-cell lines spontaneously established from prehematopoietic stem cell transplantation adult T-cell leukemia (ATL) patients. ILT-#37, ILT-R07, ILT-R11, and ILT-#97 cells that were cultured for >2 months in the presence of 10 ng/ml recombinant human interleukin 15 were stained with FITC-conjugated antibodies to CD4 and CD8 (closed histogram) on the cell surface or intracellularly stained with monoclonal antibodies to HTLV-I Tax (closed histogram), Gag p19 (solid line), or control antibody (broken line) followed by FITC-conjugated second antibodies and analyzed by flow cytometry. Histograms represent the log of fluorescence (x axis) versus relative cell number (y axis).

⁶ Internet address: http://bimas.dcrn.nih.gov/molbio/hla_bind/.

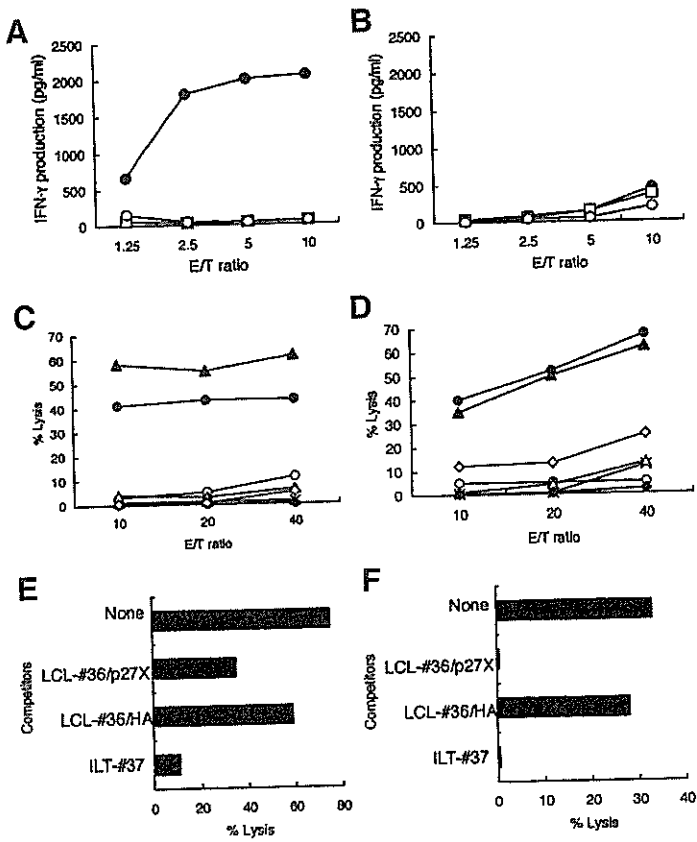


Fig. 2. Induction of and human T-cell leukemia virus type I (HTLV-I)-specific CTLs from post-hematopoietic stem cell transplantation (HSCT) patient #37 and donor #36 in response to ILT-#37 cells. Peripheral blood mononuclear cells (PBMCs) from patient #37 (+183 days post-HSCT) (A, C, and E) and donor #36 (B, D, and F) were cultured with periodical stimulations with formalin-fixed ILT-#37 cells, and their IFN- γ -producing ability at 19 days (A and B) and cytotoxicity at 53 days (C and D) after initiation of culture were evaluated against various target cells by ELISA and 6-h ^{51}Cr -release assay, respectively. The target cells used were formalin-fixed ILT-#37 (●), K562 (□), or none (○) in A and B, HLA-identical ILT-#37 (●), LCL-#36 (○), and PHA-activated PBMCs of pre-HSCT patient #37 (×), HLA-A2- and B46-matched TCL-Kan (▲) and LCL-Kan (△), and HLA-mismatched ILT-As-2 (◆) and LCL-As (◇) in C and D. Closed symbols represent HTLV-I-infected while open symbols represent HTLV-I-negative cells. Values represent the mean of IFN- γ concentrations of duplicate assays (A and B) and percentage of specific lysis of triplicate assays (C and D). Specificity to HTLV-I Tax of the PBMCs from post-HSCT patient #37 (E) and donor #36 (F), which had been stimulated five times with formalin-fixed ILT-#37 cells in culture for 90 days, was examined by ^{51}Cr -release assay against radiolabeled ILT-#37 in the presence of unlabelled LCL-#36 cells infected with vaccinia recombinants expressing HTLV-I pX gene products (LCL-#36/p27X) or control vaccinia vector (LCL-#36/HA) or ILT-#37 cells. Both the E:T and competitor-to-target ratios were 30 to 1.

donor #36 cultured for the same period grew more slowly than those from patient #37 and did not significantly produce IFN- γ against stimulation with ILT-#37 (Fig. 2B), although the activities increased at later time points of culture (Fig. 2D).

HTLV-I Specificity of CTL Induced from a Post-HSCT Recipient and Donor. We then assessed cytotoxicity and specificity of the responder cells expanding in response to stimulation with ILT-#37 cells in the PBMCs from post-HSCT patient #37 and donor #36 at 53 days after initiation of culture. The responder PBMCs from both patient #37 (Fig. 2C) and donor #36 (Fig. 2D) showed significant levels of cytotoxicity against ILT-#37 but not against PHA-stimulated PBMCs of pre-HSCT #37 patient. This indicated that the main target antigens of these CTL were those preferably expressed on ILT-#37 but not on PHA-stimulated PBMCs, although both of these target cells originated from pre-HSCT #37 patient. Furthermore, these CTLs efficiently killed allogeneic HTLV-I-infected TCL-Kan cells sharing HLA-A2 and B46 but not HLA-mismatched HTLV-I-infected ILT-As-2, EBV-infected LCL-#36 derived from HLA-identical donor #36,

LCL-Kan nor LCL-As cells. These results strongly indicated that the CTL line established from post-HSCT #37 patient (CTL-post-HSCT-#37) and donor #36 (CTL-donor-#36) in response to ILT-#37 was specific for HTLV-I antigens.

Recognition of HTLV-I Tax by CTL-Post-HSCT-#37. The target antigens of CTL-post-HSCT-#37 were then analyzed. Because it is known that HTLV-I Tax is a major target antigen for HTLV-I-specific CTLs in HTLV-I-infected individuals, we examined whether HTLV-I Tax was recognized by CTL-post-HSCT-#37. A CTL line similarly induced from donor #36 (CTL-#36) with stimulation by ILT-#37 cells was also examined. The results are shown in Fig. 2, E and F. Unlabelled LCL-#36 infected with vaccinia recombinants expressing HTLV-I pX gene products including Tax (LCL-#36/p27X) significantly inhibited the cytotoxicity of CTL-post-HSCT-#37 against ILT-#37, compared with unlabelled LCL-#36/HA that was infected with control vaccinia vector. Unlabelled ILT-#37 cells more efficiently competed with the cytotoxicity of CTL-post-HSCT-#37 for radiolabeled ILT-#37 (Fig. 2E). The cytotoxicity of CTL-#36 for ILT-#37 was almost completely inhibited by unlabelled LCL-#36/p27X as well as ILT-#37 cells (Fig. 2F). These findings suggest that the majority of CTL-#36 and a substantial part of CTL-post-HSCT-#37 consisted of HTLV-I Tax-specific CD8⁺ CTL capable of lysing ILT-#37 cells.

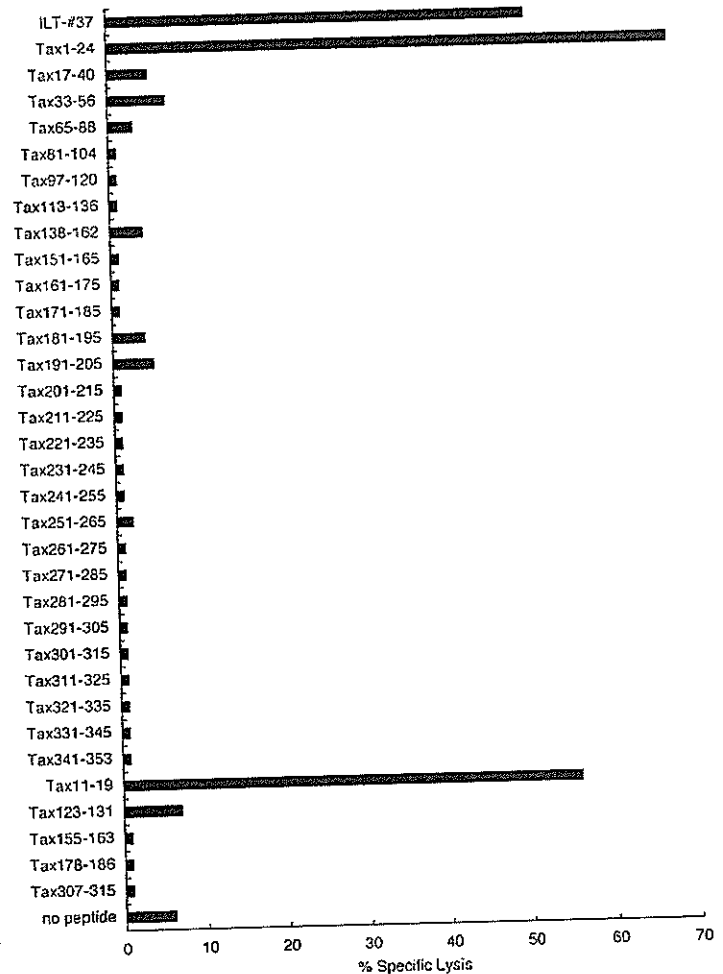


Fig. 3. Mapping of human T-cell leukemia virus type I Tax epitopes recognized by CTLs from post-hematopoietic stem cell transplantation (HSCT) patient #37. LCL-#36 cells were pulsed with 10 mM of 33 kinds of 9–24 mer synthetic oligopeptides corresponding to the Tax amino acid sequence, and their susceptibility to CTLs of post-HSCT patient #37 was measured by ^{51}Cr -release assays at an E:T ratio of 10. Values represent the mean of percentage specific lysis of triplicate assays.

Mapping of HTLV-I Tax-Specific CTL Epitope. We additionally examined the epitopes in HTLV-I Tax recognized by CTL-post-HSCT-#37 with a panel of 15–24-mer oligopeptides corresponding to the Tax amino acid sequence, and five 9-mer peptides that were the most probable HLA-A2-restricted Tax epitopes as predicted by a computer program based on the anchor motifs. As shown in Fig. 3, LCL-#36 cells pulsed with the oligopeptides Tax 1-24 (MAHFPGF-GQSLFGYPVYVFGDCV) and Tax 11-19 (LLFGYPVYV) were selectively killed by CTL-post-HSCT-#37, indicating that the major population of HTLV-I Tax-specific CTL in the CTL-post-HSCT-#37 culture was directed to a HLA-A2-restricted Tax 11-19 epitope.

Different HTLV-I-Specific Responses among Pre-HSCT Patient, Post-HSCT Patient, and Donor. We next investigated whether there are any qualitative or quantitative differences in HTLV-I-specific CTL responses among pre-HSCT #37, post-HSCT #37, and donor #36. Unlike the PBMCs of post-HSCT #37, pre-HSCT PBMCs failed to multiply in culture when stimulated with ILT-#37 cells in the presence of IL-2 and could not be maintained for >7 weeks. The cytotoxic ability of this cell line at 40 days after initiation of culture was examined and compared with similarly cultured PBMCs from post-HSCT #37 and donor #36 at 41 days of culture. As shown in Fig. 4A, the PBMC cultures from post-HSCT #37 and donor #36, but not from pre-HSCT #37 patient, exhibited significant levels of HTLV-I-specific cytotoxicity.

We also stained these cultured PBMCs with phycoerythrin-conjugated HLA-A*0201/Tax11-19 tetramer and Cy-chrome-conjugated mAb to CD8. As shown in Fig. 4B, the PBMC culture from pre-HSCT #37 patient mostly consisted of CD8⁻, tetramer⁻ cells, consistent with the results of cytotoxicity assay shown above. In contrast, in the PBMC culture from post-HSCT #37 patient, 63% of the cells were CD8⁺, HLA-A*0201/Tax11-19⁺ (Fig. 4B), indicating oligoclonal expansion of CTLs directed to the single epitope in this culture. In the PBMCs from donor #36, which were cultured for the same period, the proportion of HLA-A*0201/Tax11-19⁺ cells was 1%. These observations indicated that HTLV-I-specific CTL response in the patient #37 was strongly activated after HSCT in an extremely selective manner distinct from that in the HTLV-I-carrying donor #36.

Induction of HTLV-I-Specific CTL after HSCT from HTLV-I-Negative Donors. T-cell immune responses of the other three ATL patients, R07, R11, and #97, after HSCT from HTLV-I-negative HLA-identical sibling donors were similarly investigated *in vitro* against 1% formaldehyde/PBS-treated autologous ILT cells established before HSCT. Although all of these post-HSCT patients were in complete remission when tested, patient R11 had recurrence of ATL 6 months after HSCT (Table 1). Patient #97 had chronic GVHD. In response to stimulation with autologous ILT cells, PBMCs isolated from patients R07 and #97 at +255 and +104 days after HSCT, respectively, proliferated well in culture as was similarly observed in PBMCs of post-HSCT patient #37. However, PBMCs isolated from patient R11 at +153 days after HSCT neither grew *in vitro* nor exhibited cytotoxicity against stimulation with autologous ILT-R11 cells. Because ILT-R11 cells expressed HTLV-I antigens (Fig. 1) and were susceptible to HLA-A2-restricted Tax-specific CTL (data not shown), the unresponsiveness of the PBMCs from post-HSCT patient R11 could not be due to the absence of specific antigens.

The specificities of the responding CD8⁺ PBMC from post-HSCT patients R07, R11, and #97, in cultures that were stimulated with ILT-R07, ILT-R11, or ILT-#97, respectively, at 14-day intervals, are shown in Table 2. At 21–24 days after initiation of culture, CD8⁺ PBMCs from patient R07 produced significant levels of IFN- γ in response to overnight incubation with autologous ILT-R07 cells, allogeneic HTLV-I-infected ILT-Myj-3 cells that shared HLA-A24, B60, and DR4, ILT-As-2 that shared HLA-A24 but not to EBV-

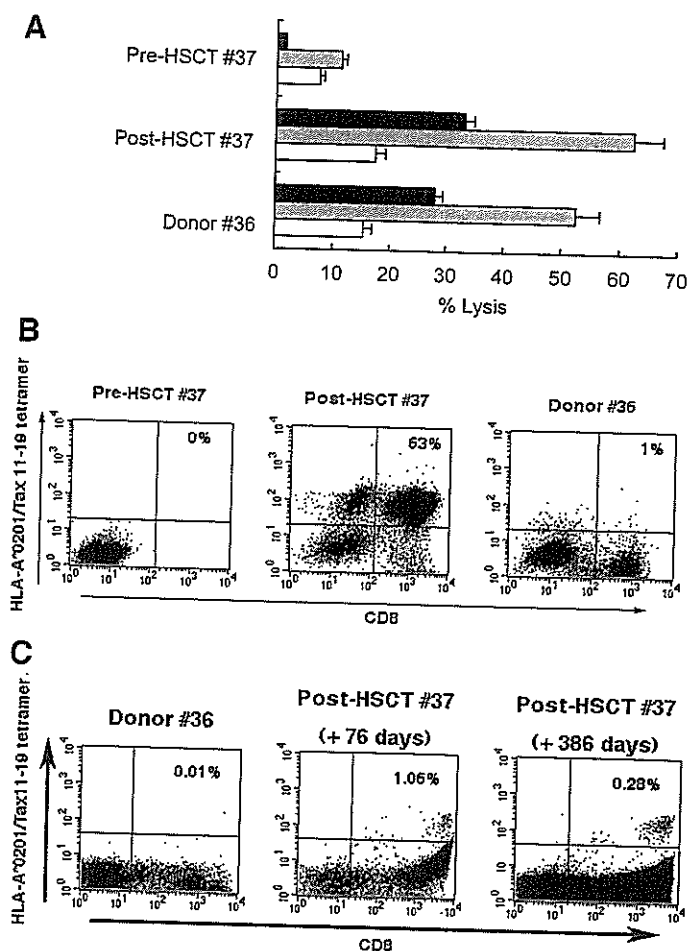


Fig. 4. Selective expansion of Tax11-19-specific CTL in peripheral blood mononuclear cell (PBMC) cultures from patient #37 after hematopoietic stem cell transplantation (HSCT). **A**, PBMCs from pre-HSCT patient #37 at 40 days of culture which were stimulated with formalin-fixed ILT-#37 cells two times, and those from post-HSCT patient #37 and donor #36 at day 41 after three stimulations were examined for cytotoxicity against ILT-#37 (■), TCL-Kan (□), and LCL-Kan (□) cells at an E:T ratio of 30. Values represent the mean of triplicate assays. **B**, flow cytometric analysis of HLA-A*0201/Tax11-19 tetramer-binding CD8⁺ T cells in the indicated PBMC cultures stimulated with ILT-#37. The PBMC cultures from post-HSCT patient #37 (middle) and donor #36 (right) were used at day 46 of culture, whereas those from pre-HSCT patient #37 (left) were used at day 36 because they failed to grow long term. Tetramer specificity was confirmed by staining a human T-cell leukemia virus type I Tax11-19-specific cell line, Te-Myj (Ref. 21; data not shown). Numbers in top right corners indicate percentages of PBMCs bound to the tetramer. A total of 100,000 events was collected in each case. **C**, kinetics of human T-cell leukemia virus type I Tax 11-19-specific CD8⁺ cells in uncultured PBMCs in post-HSCT patient #37. Uncultured cryopreserved PBMCs isolated from donor #36 (left) or from post-HSCT patient #37 at +76 days (middle) and +386 days (right) after HSCT were stained with phycoerythrin-conjugated HLA-A*0201/Tax 11-19 tetramer. The percentages of CD8⁺ and HLA-A*0201/Tax 11-19 tetramer⁺ cells in the PBMCs are indicated in the top right quadrants.

infected TOK cells that shared HLA-A24. Cytotoxicity assays at later time points (63 days of culture) confirmed that this culture contained HLA-A24-restricted HTLV-I-specific CTL mainly recognizing HTLV-I *pX* gene products as they killed EBV-transformed TOK cells infected with vaccinia recombinants expressing HTLV-I *pX* gene products (TOK/p27X) more significantly than the ones expressing the HTLV-I envelope (TOK/pEnv) or HTLV-I core (TOK/pGag). CD8⁺ PBMC culture from post-HSCT patient #97 stimulated with autologous ILT-#97 cells showed HTLV-I-specific IFN- γ production and cytotoxicity against ILT-#97 and allogeneic HTLV-I-infected ILT-Har cells sharing HLA-A2 and B51 but not with EBV-transformed LCL-Har cells from the same donor (Table 2). This HTLV-I-specific cell population present in the PBMC culture from post-HSCT patient #97 was, however, lost during a longer period of culture.

Table 2. Human T-cell leukemia virus (HTLV-I)-specific CTL responses of peripheral blood mononuclear cell cultures from post-hematopoietic stem cell transplantation (HSCT) patients R07, R11, and #97^a

Patient ID (days after HSCT)	PBMC culture period	Stimulator	Target cells for immunoassays			IFN- γ (pg/ml) ^b	Cytolysis (%) ^c
			Cell lines	HTLV-I antigen	Shared HLA alleles with patient		
R07 (+255)	Day 21	ILT-R07	IRT-R07	Positive	Identical	1090 \pm 67	N.T. ^d
			ILT-As-2	Positive	A24	866 \pm 117	N.T.
			TOK	Negative	A24	160 \pm 7	N.T.
	Day 24	ILT-R07	None			36 \pm 12	N.T.
			ILT-R07	Positive	Identical	778 \pm 55	N.T.
			ILT-Myj-3	Positive	A24, B60, DR4	355 \pm 0	N.T.
			TOK	Negative	A24	62 \pm 9	N.T.
			None			25 \pm 19	N.T.
			None			N.T.	39 \pm 9
	Day 63	ILT-R07	ILT-R07	Positive	Identical	N.T.	48 \pm 5
			ILT-As-2	Positive	A24	N.T.	5 \pm 6
			TOK	Negative	A24	N.T.	27 \pm 11
TOK/p27X ^e			pX products	A24	N.T.	11 \pm 4	
TOK/pEnv ^e			Envelope	A24	N.T.	6 \pm 10	
TOK/pGag ^e			Core	A24	N.T.	6 \pm 10	
R11 (+153)	Day 25	ILT-R11	ILT-R11 ^f	Positive	Identical	11 \pm 1	N.T.
			ILT-Nkz-2	Positive	A2, A26	12 \pm 2	N.T.
			LCL-Nkz	Negative	A2, A26	19 \pm 6	N.T.
			None			14 \pm 0	N.T.
			None			477 \pm 34	N.T.
			None			415 \pm 2	N.T.
#97 (+104)	Day 21	ILT-#97	ILT-#97	Positive	Identical	104 \pm 15	N.T.
			ILT-Har	Positive	A2, B51, DR4	104 \pm 15	N.T.
			LCL-Har	Negative	A2, B51, DR4	63 \pm 4	N.T.
			None			63 \pm 4	N.T.
			None			128 \pm 36	29 \pm 2
			None			111 \pm 10	23 \pm 3
	Day 50	ILT-#97	ILT-#97	Positive	Identical	10 \pm 1	16 \pm 3
			ILT-Har	Positive	A2, B51, DR4	10 \pm 1	16 \pm 3
			LCL-Har	Negative	A2, B51, DR4	1 \pm 0	N.T.
			None			1 \pm 0	N.T.
			None				
			None				

^a PBMCs isolated from patients R07, R11, and #97 were cultured for the indicated periods with stimulations with formalin-fixed autologous ILT-R07, ILT-R11, or ILT-#97 cells, respectively; then IFN- γ production or cytotoxicity of these PBMCs were examined against the various target cells indicated.

^b IFN- γ producing responses after overnight incubation with various target cells indicated at an E:T ratio of 5 was shown as the mean \pm SD of duplicate determinations by ELISA.

^c Specific cytolysis at an E:T ratio of 40 measured by 6-h ⁵¹Cr-release assays was shown as the mean \pm SD of triplicate determinations.

^d N.T., not tested.

^e TOK cells infected with recombinant vaccinia viruses expressing HTLV-I pX products (TOK/p27X), envelope (TOK/pEnv), and core (TOK/pGag) antigens were used as targets.

^f ILT-R11 target cells used were confirmed for their susceptibility to HLA-A2-restricted Tax-specific CTL induced from post-HSCT patient #37 by 6-h ⁵¹Cr-release assay (53% lysis at an E:T ratio of 20).

Recognition of a Single HLA-A24-Restricted Tax Epitope by CTL Induced from Post-HSCT Patient R07. Subsequently, epitope mapping of the CTL from post-HSCT R07 was performed. The result is shown in Fig. 5. Of the panel of 15-24-mer oligopeptides of Tax and five 9-mer oligopeptides, the most probable HLA-A24-restricted epitopes as predicted by a computer program, Tax 301-315 (SFHSLHLLFEEYTNI) and Tax 301-309 (SFHSLHLLF), were selectively reacted with the responder cells. These observations indicated that HTLV-I-specific CTL response to selective Tax epitopes was induced from patient R07 after HSCT as similarly observed in the case of post-HSCT patient #37.

Ex Vivo Analysis on Tax-Specific CTL Expansion in Post-HSCT Patients. The almost exclusive expansions of Tax 11-19-specific CTL and Tax 301-309-specific CTL in PBMCs from post-HSCT patients 337 and R07, respectively, were observed in *in vitro* culture stimulated with pre-HSCT autologous ILT cells as shown above. Finally, we examined whether Tax-specific CTL response was also augmented *in vivo* by using uncultured PBMCs from these patients.

For patient #37, fluorescence-activated cell sorting analysis on frozen stored uncultured PBMCs was performed after staining with the HLA-A*0201/Tax11-19⁺ tetramer. Because the hematopoietic chimerism in patient #37 had shifted completely to the donor type by 2 months after HSCT, we compared the number of Tax11-19-specific CTL in uncultured PBMCs taken from patient #37 at +76 days after HSCT to that from donor #36. As shown in Fig. 4C, >1% of CD8⁺ cells in uncultured PBMCs from patient #37 (+76 days after HSCT) were stained with the HLA-A*0201/Tax11-19 tetramer, whereas only 0.01% of CD8⁺ PBMCs from donor #36 bound the tetramer. The proportion of peripheral Tax11-19-specific CTL in the PBMCs of

post-HSCT patient #37 decreased to 0.28% at a later time point (+386 days after HSCT) but was still much higher than that in donor #36. This indicated that the Tax11-19-specific CTL population of donor-origin was activated and expanded to a high level in the recipient after HSCT and then gradually decreased.

For patient R07, uncultured PBMCs before and after HSCT (+255 days) that had been stored frozen were subjected to ELISPOT assay for IFN- γ production after overnight stimulation with ILT-R07 or Tax 301-309 peptide (Table 3). The number of IFN- γ -producing cells was significantly higher in post-HSCT PBMCs than in pre-HSCT PBMCs from patient R07. The number of IFN- γ -producing cells responding to Tax 301-309 peptide was also elevated in the post-HSCT PBMCs in a lesser degree than ILT-R07-responding cells. These PBMCs did not react with control peptide Tax 11-19. Although PBMC samples from patient R07 at any earlier date after HSCT or the donor were not available, these results suggested that CTLs reacting with ILT-R07 and Tax 301-309 in patient R07 were activated *in vivo* after HSCT and were still detectable at +255 days after HSCT.

DISCUSSION

In the present study, the cellular immune responses in ATL patients after nonmyeloablative HSCT from HLA-identical siblings against pre-HSCT T-cell lines spontaneously infected with HTLV-I were investigated. We demonstrated that HTLV-I-specific CTLs were induced in the PBMCs from three patients with complete remission and that CTLs induced from two of these patients showed strong activity directed against a limited number of Tax epitopes. The donor in the first case of HSCT was an HTLV-I carrier, but the other donors were negative for HTLV-I. Therefore, the HTLV-I-specific CTL response

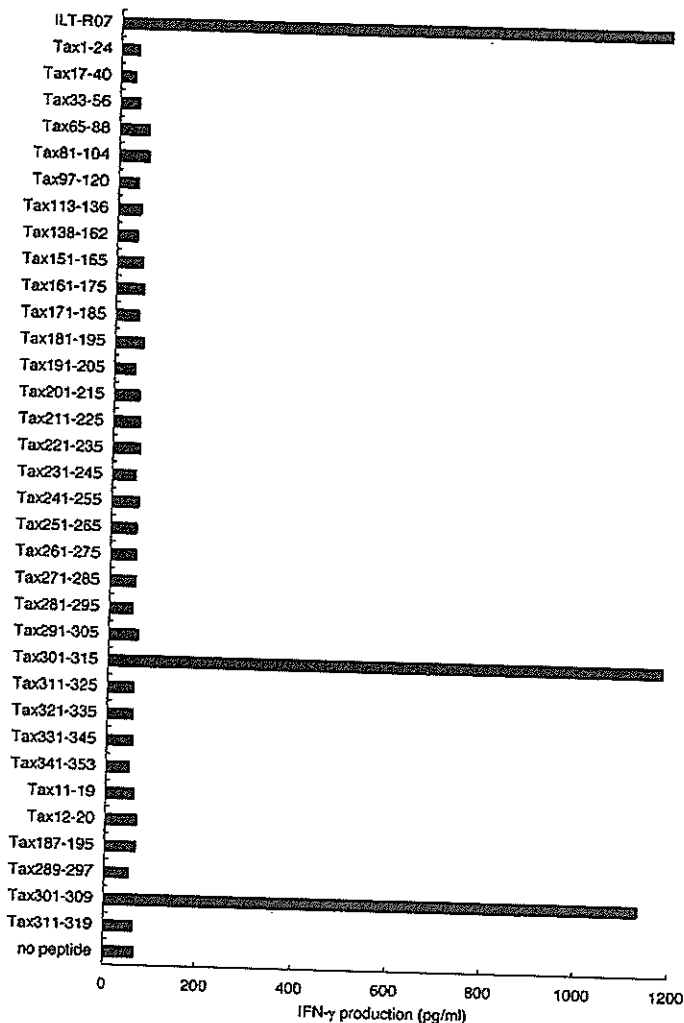


Fig. 5. Mapping of human T-cell leukemia virus type I Tax epitopes recognized by the CTLs induced from post-hematopoietic stem cell transplantation patient R07. CD8⁺ cell-enriched peripheral blood mononuclear cell that had been cultured for 32 days with three stimulations with formalin-fixed ILT-R07 cells were mixed with HLA-A24⁺ EBV-transformed B-cell line, TOK, pulsed with a series of 33 synthetic oligo-peptides for Tax at an E:T ratio of 8, and IFN- γ in the supernatants after 18 h of incubation was measured by ELISA assay. Values represent the mean of duplicate assays.

A24, supporting the notion that the selective CTL responses are more likely to be influenced by the *in vivo* conditions of the recipient rather than those of the donor.

Ex vivo analyses revealed that the number of cells stained with the HLA-A*0201/Tax11-19 tetramer in uncultured PBMCs was 100 times greater in post-HSCT patient #37 at +76 days after HSCT than in donor #36 (Fig. 4C). The number of tetramer-bound cells in the PBMCs of this patient then gradually decreased, presumably as the viral load decreased. Similarly, in uncultured PBMCs from patient R07, elevation of the number of IFN- γ -producing cells against ILT-R07 or Tax 301-309 peptide was shown by ELISPOT assay, although the only sample available from this patient was taken a long time after HSCT (+255 days; Table 3). These findings indicated that the Tax-specific CTLs of these ATL patients selectively activated not only *in vitro* but also *in vivo* after HSCT.

Proviral loads before HSCT varied among patients and decreased down to undetectable levels after HSCT in all patients tested, including R11 who did not show a CTL response. This suggests that the spread of HTLV-I *in vivo* in the reconstituted immune system after HSCT may not be as effective as primary HTLV-I-infection, even in the absence of CTL. The reason for the unresponsiveness of patient R11 against ILT-R11 remains to be clarified.

It has been reported that most ATL cases who obtained complete remission after HSCT were associated with GVHD (7), indicating that graft-versus-host (GVH) responses contribute to GVL effects for ATL. In patient #37 in the present study, ATL cells increased at +4 weeks after HSCT but decreased again on withdrawing cyclosporine A, although some episodes of grade 2 GVHD occurred. In patient R07, acute GVHD of the stomach (grade 2) and no chronic GVHD were observed after transplantation, but the clinical course was essentially uneventful without recurrence of ATL. Acute and chronic GVHD in patients #37 and R07 were eventually controlled. In patient R11, who was in complete remission before HSCT and later had recurrence of ATL 6 months after HSCT, a transient acute GVHD of the stomach but no chronic GVHD was observed. In patient #97, no acute but some chronic GVHD in the skin, liver, and lungs have been observed. Complete remission has been sustained for >18 months for patients #37 and R07 and 7 months for patient #97. Successful induction of HTLV-I-specific CTL only from the patients who sustained complete remission implies that HTLV-I-specific CTL as well as effector cells for GVHD might contribute to the GVL effects. Previous findings such as cytotoxicity of HTLV-I Tax-specific CTL against ATL cells *in vitro* (19, 29) and antitumor effects demonstrated in animal models of ATL (27, 40) support this notion. The contribution of Tax-specific T-cell responses to the anti-ATL effects remains to be clarified, most likely by a clinical trial of Tax-directed immunotherapy for ATL in the future.

Several mHA that have been suggested to be involved in GVHD

Table 3 *Ex vivo* IFN- γ -producing response of peripheral blood mononuclear cells (PBMCs) from patient R07 before and after hematopoietic stem cell transplantation (HSCT) in response to ILT-R07 or Tax peptides^a

Stimulator	IFN- γ -producing SFC/5 \times 10 ⁴ PBMC ^b	
	Pre-HSCT R07	Post-HSCT R07
ILT-R07	5 \pm 6	44 \pm 16
Tax301-309 (SFHSLHLLF)	1 \pm 1	5 \pm 1
Tax11-19 (LLFGYPVYV)	1 \pm 1	0 \pm 1
Medium	0 \pm 0	0 \pm 0

^a Uncultured PBMCs directly thawed from frozen stocks from patient R07 before and after (+255 days) HSCT were subjected to IFN- γ -enzyme-linked immunospot assay after overnight incubation with formalin-fixed ILT-R07, synthetic oligopeptides Tax 301-309, and Tax 11-19, or control medium at a concentration of 5 \times 10⁴/well as described in "Materials and Methods." Values represent the mean \pm SD of triplicate assays.

^b Results of IFN- γ -enzyme-linked immunospot assay are expressed as spot-forming cells (SFCs)/5 \times 10⁴ PBMCs.

in post-HSCT ATL patients cannot be explained by the transfer of memory CTLs from the donor but must be a result of the new immune response by reconstituted donor-derived T cells after HSCT against HTLV-I antigen-presenting cells present in the ATL patients.

It is a striking phenomenon that >60% of CTL induced from post-HSCT patient #37 were stained with the HLA-A*0201/Tax11-19 tetramer. Such an almost exclusive expansion of Tax11-19-specific CTL was not merely caused by *in vitro* selection because a similarly cultured CTL line from HTLV-I-carrying HLA-identical donor #36 contained a much smaller number of Tax11-19-specific cells (Fig. 4B). Although CTLs induced from donor #36 also recognize Tax as shown in Fig. 2F, they were directed to multiple heterologous Tax epitopes, including Tax 11-19 (data not shown). It is intriguing that oligoclonal expansion of the HTLV-I Tax11-19-specific CTL response has been observed in HTLV-I-associated myelopathy/Tropical spastic paraparesis patients whose viral load is generally high (33, 45, 46), suggesting that the pattern of HTLV-I-specific response observed in the post-HSCT ATL patients in the present study might be due to abundant antigen presentation *in vivo*. CTLs induced from patient R07 who received HSCT from an uninfected donor also exhibited preference for a limited epitope, Tax 301-309 epitope restricted by HLA-

(8-10, 12, 13) are candidates for GVL targets. In the present study, the T-cell lines used as stimulators (ILT-#37, ILT-R07, ILT-R11, and ILT-#97) originating from the ATL patients before HSCT possessed antigens of recipient-origin as well as HTLV-I antigens. Therefore, it is likely that these cells express GVH target antigens to induce GVH-effector cells *in vitro* from the PBMCs of post-HSCT patients when used as a stimulator. In fact, the cytotoxicity of post-HSCT-#37 CTL against ILT-#37 was not completely competed by Tax-expressing cells (Fig. 2E), indicating the presence of CTL populations recognizing other antigens such as mHA. In the PBMC culture from post-HSCT patient #97 with chronic GVHD, HTLV-I-specific CTLs were induced at an early culture period (Table 2), but additional culture resulted in expansion of another CTL population capable of killing ILT-#97 but not directed against HTLV-I Tax. The target antigens of these CTL are currently under investigation. These observations suggest that HTLV-I and some other antigens associated with GVH responses could act as strong target antigens for post-HSCT CTL responses.

We and others (33, 47) previously demonstrated that Tax 11-19 is one of the major target epitopes of HLA-A2-restricted HTLV-I-specific CTLs in HTLV-I-infected individuals. In the present study, Tax 11-19 was also the major CTL epitope, especially in post-HSCT patient #37. In the second HSCT case, the CTL line of post-HSCT patient R07, induced by only two stimulations with ILT-R07 cells, almost exclusively recognized the Tax 301-309 epitope restricted by HLA-A24, which is one of the most common HLA alleles in Japanese individuals. We assume that Tax 301-309 is one of the major epitopes for HLA-A24. These major CTL epitopes could be candidate antigens for potential immunotherapy for ATL.

In conclusion, a new balance between host immunity and HTLV-I-infected cells was established after allogeneic HSCT into ATL patients from HLA-identical donors. The phenomenon of *in vitro* induction and proliferation of CTL specific for selected Tax epitopes observed in these recipients is very similar to that in HTLV-I-associated myelopathy/Tropical spastic paraparesis patients. In this sense, allogeneic HSCT converted HTLV-I-specific T-cell immunity in the recipients from one extreme to the other in which host immunity could control the malignant expansion of HTLV-I-infected cells *in vivo*.

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REFERENCES

- Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K. I., Shirakawa, S., and Miyoshi, I. Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc. Natl. Acad. Sci. USA*, 78: 6476-6480, 1981.
- Tajima, K. The 4th nation-wide study of adult T-cell leukemia/lymphoma (ATL) in Japan: estimates of risk of, A. T. L., and its geographical and clinical features. The T- and B-cell Malignancy Study Group. *Int. J. Cancer*, 45: 237-243, 1990.
- Uchiyama, T., Yodoi, J., Sagawa, K., Takatsuki, K., and Uchino, H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood*, 50: 481-492, 1977.
- Shimoyama, M., Ota, K., Kikuchi, M., Yunoki, K., Konda, S., Takatsuki, K., Ichimaru, M., Ogawa, M., Kimura, I., Tominaga, S., *et al.* Chemotherapeutic results and prognostic factors of patients with advanced non-Hodgkin's lymphoma treated with VEPA or VEPA-M. *J. Clin. Oncol.*, 6: 128-141, 1988.
- Shimoyama, M., Ota, K., Kikuchi, M., Yunoki, K., Konda, S., Takatsuki, K., Ichimaru, M., Tominaga, S., Tsugane, S., Minato, K., *et al.* Major prognostic factors of adult patients with advanced T-cell lymphoma/leukemia. *J. Clin. Oncol.*, 6: 1088-1097, 1988.
- Tsukasaki, K., Maeda, T., Arimura, K., Taguchi, J., Fukushima, T., Miyazaki, Y., Moriuchi, Y., Kuriyama, K., Yamada, Y., and Tomonaga, M. Poor outcome of autologous stem cell transplantation for adult T-cell leukemia/lymphoma: a case report and review of the literature. *Bone Marrow Transplant.*, 23: 87-89, 1999.
- Utsunomiya, A., Miyazaki, Y., Takatsuka, Y., Hanada, S., Uozumi, K., Yashiki, S., Tara, M., Kawano, F., Saburi, Y., Kikuchi, H., Hara, M., Sao, H., Morishima, Y., Kodaera, Y., Sonoda, S., and Tomonaga, M. Improved outcome of adult T-cell leukemia/lymphoma with allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant.*, 27: 15-20, 2001.
- Goulmy, E., Schipper, R., Pool, J., Blokland, E., Falkenburg, J. H., Vossen, J., Grathwohl, A., Vogelsang, G. B., van Houwelingen, H. C., and van Rood, J. J. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N. Engl. J. Med.*, 334: 281-285, 1996.
- Wang, W., Meadows, L. R., den Haan, J. M., Sherman, N. E., Chen, Y., Blokland, E., Shabanowitz, J., Agulnik, A. I., Hendrickson, R. C., Bishop, C. E., *et al.* Human H-Y: a male-specific histocompatibility antigen derived from the SMCY protein. *Science (Wash. DC)*, 269: 1588-1590, 1995.
- den Haan, J. M., Meadows, L. M., Wang, W., Pool, J., Blokland, E., Bishop, T. L., Reinhardus, C., Shabanowitz, J., Offringa, R., Hunt, D. F., Engelhard, V. H., and Goulmy, E. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. *Science (Wash. DC)*, 279: 1054-1057, 1998.
- Behar, E., Chao, N. J., Hiraki, D. D., Krishnaswamy, S., Brown, B. W., Zehnder, J. L., and Grumet, F. C. Polymorphism of adhesion molecule CD31 and its role in acute graft-versus-host disease. *N. Engl. J. Med.*, 334: 286-291, 1996.
- Balduini, C. L., Noris, P., Giorgiani, G., Martinetti, M., Klersy, C., Spedini, P., Belletti, S., MacCario, R., Gusberti, L., and Locatelli, F. Incompatibility for CD31 and human platelet antigens and acute graft-versus-host disease after bone marrow transplantation. *Br. J. Haematol.*, 106: 723-729, 1999.
- Maruya, E., Saji, H., Seki, S., Fujii, Y., Kato, K., Kai, S., Hiraoka, A., Kawa, K., Hoshi, Y., Ito, K., Yokoyama, S., and Juji, T. Evidence that CD31, CD49b, and CD62L are immunodominant minor histocompatibility antigens in HLA identical sibling bone marrow transplants. *Blood*, 92: 2169-2176, 1998.
- Horowitz, M. M., Gale, R. P., Sondel, P. M., Goldman, J. M., Kersey, J., Kolb, H. J., Rimm, A. A., Ringden, O., Rozman, C., Speck, B., *et al.* Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*, 75: 555-562, 1990.
- Mutis, T., Verdijk, R., Schrama, E., Esendam, B., Brand, A., and Goulmy, E. Feasibility of immunotherapy of relapsed leukemia with *ex vivo*-generated cytotoxic T lymphocytes specific for hematopoietic system-restricted minor histocompatibility antigens. *Blood*, 93: 2336-2341, 1999.
- Pinilla-Ibarz, J., Cathcart, K., Korontsvit, T., Soignet, S., Bocchia, M., Caggiano, J., Lai, L., Jimenez, J., Kolitz, J., and Scheinberg, D. A. Vaccination of patients with chronic myelogenous leukemia with bcr-abl oncogene breakpoint fusion peptides generates specific immune responses. *Blood*, 95: 1781-1787, 2000.
- Gaiger, A., Reese, V., Disis, M. L., and Cheever, M. A. Immunity to WT1 in the animal model and in patients with acute myeloid leukemia. *Blood*, 96: 1480-1489, 2000.
- Kannagi, M., Matsushita, S., Shida, H., and Harada, S. Cytotoxic T cell response and expression of the target antigen in HTLV-I infection. *Leukemia (Baltimore)*, 8 (Suppl. 1): S54-S59, 1994.
- Kannagi, M., Sugamura, K., Kinoshita, K., Uchino, H., and Hinuma, Y. Specific cytotoxicity of fresh tumor cells by an autologous killer T cell line derived from an adult T cell leukemia/lymphoma patient. *J. Immunol.*, 133: 1037-1041, 1984.
- Jacobson, S., Shida, H., McFarlin, D. E., Fauci, A. S., and Koenig, S. Circulating CD8+ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I-associated neurological disease. *Nature (Lond.)*, 348: 245-248, 1990.
- Kannagi, M., Harada, S., Maruyama, I., Inoko, H., Igarashi, H., Kuwashima, G., Sato, S., Morita, M., Kidokoro, M., Sugimoto, M., *et al.* Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX gene products by human CD8+ cytotoxic T cells directed against HTLV-I-infected cells. *Int. Immunol.*, 3: 761-767, 1991.
- Yoshida, M., Osame, M., Usuku, K., Matsumoto, M., and Igata, A. Viruses detected in HTLV-I-associated myelopathy and adult T-cell leukaemia are identical on DNA blotting. *Lancet*, 1: 1085-1086, 1987.
- Tsukahara, T., Kannagi, M., Ohashi, T., Kato, H., Arai, M., Nunez, G., Iwanaga, Y., Yamamoto, N., Ohtani, K., Nakamura, M., and Fujii, M. Induction of Bcl-x(L) expression by human T-cell leukemia virus type 1 Tax through NF- κ B in apoptosis-resistant T-cell transfectants with Tax. *J. Virol.*, 73: 7981-7987, 1999.
- Hanabuchi, S., Ohashi, T., Koya, Y., Kato, H., Takemura, F., Hirokawa, K., Yoshiki, T., Yagita, H., Okumura, K., and Kannagi, M. Development of human T-cell leukemia virus type 1-transformed tumors in rats following suppression of T-cell immunity by CD80 and CD86 blockade. *J. Virol.*, 74: 428-435, 2000.
- Ohashi, T., Hanabuchi, S., Kato, H., Koya, Y., Takemura, F., Hirokawa, K., Yoshiki, T., Tanaka, Y., Fujii, M., and Kannagi, M. Induction of adult T-cell leukemia-like T-cell lymphoproliferative disease and its inhibition by adoptive immunotherapy in T-cell-deficient nude rats inoculated with syngeneic human T-cell leukemia virus type 1-immortalized cells. *J. Virol.*, 73: 6031-6040, 1999.
- Ohashi, T., Hanabuchi, S., Kato, H., Tateno, H., Takemura, F., Tsukahara, T., Koya, Y., Hasegawa, A., Masuda, T., and Kannagi, M. Prevention of adult T-cell leukemia-like lymphoproliferative disease in rats by adoptively transferred T cells from a donor immunized with human T-cell leukemia virus type 1 Tax-coding DNA vaccine. *J. Virol.*, 74: 9610-9616, 2000.
- Hanabuchi, S., Ohashi, T., Koya, Y., Kato, H., Hasegawa, A., Takemura, F., Masuda, T., and Kannagi, M. Regression of human T-cell leukemia virus type I (HTLV-I)-associated lymphomas in a rat model: peptide-induced T-cell immunity. *J. Natl. Cancer Inst. (Bethesda)*, 93: 1775-1783, 2001.
- Hinuma, Y., Gotoh, Y., Sugamura, K., Nagata, K., Goto, T., Nakai, M., Kamada, N., Matsumoto, T., and Kinoshita, K. A retrovirus associated with human adult T-cell leukemia: *in vitro* activation. *Gann*, 73: 341-344, 1982.

29. Kannagi, M., Matsushita, S., and Harada, S. Expression of the target antigen for cytotoxic T lymphocytes on adult T-cell leukemia cells. *Int. J. Cancer*, *54*: 582-588, 1993.
30. Kinoshita, T., Shimoyama, M., Tobinai, K., Ito, M., Ito, S., Ikeda, S., Tajima, K., Shimotohno, K., and Sugimura, T. Detection of mRNA for the *tax1/rex1* gene of human T-cell leukemia virus type I in fresh peripheral blood mononuclear cells of adult T-cell leukemia patients and viral carriers by using the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA*, *86*: 5620-5624, 1989.
31. Sugamura, K., and Hinuma, Y. *In vitro* induction of cytotoxic T lymphocytes specific for Epstein-Barr virus-transformed cells: kinetics of autologous restimulation. *J. Immunol.*, *124*: 1045-1049, 1980.
32. Sugamura, K., Fujii, M., Kannagi, M., Sakitani, M., Takeuchi, M., and Hinuma, Y. Cell surface phenotypes and expression of viral antigens of various human cell lines carrying human T-cell leukemia virus. *Int. J. Cancer*, *34*: 221-228, 1984.
33. Kannagi, M., Shida, H., Igarashi, H., Kuruma, K., Murai, H., Aono, Y., Maruyama, I., Osame, M., Hattori, T., Inoko, H., *et al.* Target epitope in the Tax protein of human T-cell leukemia virus type I recognized by class I major histocompatibility complex-restricted cytotoxic T cells. *J. Virol.*, *66*: 2928-2933, 1992.
34. Lozzio, C. B., and Lozzio, B. B. Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. *Blood*, *45*: 321-334, 1975.
35. Tanaka, Y., Yoshida, A., Takayama, Y., Tsujimoto, H., Tsujimoto, A., Hayami, M., and Tozawa, H. Heterogeneity of antigen molecules recognized by anti-tax1 monoclonal antibody Lt-4 in cell lines bearing human T cell leukemia virus type I and related retroviruses. *Jpn. J. Cancer Res.*, *81*: 225-231, 1990.
36. Tanaka, Y., Lee, B., Inoi, T., Tozawa, H., Yamamoto, N., and Hinuma, Y. Antigens related to three core proteins of HTLV-I (p24, p19, and p15) and their intracellular localizations, as defined by monoclonal antibodies. *Int. J. Cancer*, *37*: 35-42, 1986.
37. Parker, K. C., Bednarek, M. A., Hull, L. K., Utz, U., Cunningham, B., Zweerink, H. J., Biddison, W. E., and Coligan, J. E. Sequence motifs important for peptide binding to the human MHC class I molecule, HLA-A2. *J. Immunol.*, *149*: 3580-3587, 1992.
38. Parker, K. C., Bednarek, M. A., and Coligan, J. E. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J. Immunol.*, *152*: 163-175, 1994.
39. Kubo, R. T., Sette, A., Grey, H. M., Appella, E., Sakaguchi, K., Zhu, N. Z., Arnott, D., Sherman, N., Shabanowitz, J., Michel, H., *et al.* Definition of specific peptide motifs for four major HLA-A alleles. *J. Immunol.*, *152*: 3913-3924, 1994.
40. Ohashi, T., Hanabuchi, S., Suzuki, R., Kato, H., Masuda, T., and Kannagi, M. Correlation of major histocompatibility complex class I down-regulation with resistance of human T-cell leukemia virus type I-infected T cells to cytotoxic T-lymphocyte killing in a rat model. *J. Virol.*, *76*: 7010-7019, 2002.
41. Siomi, H., Shida, H., Nam, S. H., Nosaka, T., Maki, M., and Hatanaka, M. Sequence requirements for nucleolar localization of human T cell leukemia virus type I pX protein, which regulates viral RNA processing. *Cell*, *55*: 197-209, 1988.
42. Herr, W., Schneider, J., Lohse, A. W., Meyer zum Buschenfelde, K. H., and Wolfel, T. Detection and quantification of blood-derived CD8+ T lymphocytes secreting tumor necrosis factor α in response to HLA-A2.1-binding melanoma and viral peptide antigens. *J. Immunol. Methods*, *191*: 131-142, 1996.
43. Lalvani, A., Brookes, R., Hambleton, S., Britton, W. J., Hill, A. V., and McMichael, A. J. Rapid effector function in CD8+ memory T cells. *J. Exp. Med.*, *186*: 859-865, 1997.
44. Bieganowska, K., Hollsberg, P., Buckle, G. J., Lim, D. G., Greten, T. F., Schneck, J., Altman, J. D., Jacobson, S., Ledis, S. L., Hanchard, B., Chin, J., Morgan, O., Roth, P. A., and Hafler, D. A. Direct analysis of viral-specific CD8+ T cells with soluble HLA-A2/Tax1-19 tetramer complexes in patients with human T cell lymphotropic virus-associated myelopathy. *J. Immunol.*, *162*: 1765-1771, 1999.
45. Daenks, S., Kermodé, A. G., Hall, S. E., Taylor, G., Weber, J., Nightingale, S., and Baughman, C. R. High activated and memory cytotoxic T-cell responses to HTLV-I in healthy carriers and patients with tropical spastic paraparesis. *Virology*, *217*: 139-146, 1996.
46. Furukawa, K., Mori, M., Ohta, N., Ikeda, H., Shida, H., and Shiku, H. Clonal expansion of CD8+ cytotoxic T lymphocytes against human T cell lymphotropic virus type I (HTLV-I) genome products in HTLV-I-associated myelopathy/tropical spastic paraparesis patients. *J. Clin. Investig.*, *94*: 1830-1839, 1994.
47. Elovaara, I., Koenig, S., Brewah, A. Y., Woods, R. M., Lehky, T., and Jacobson, S. High human T-cell lymphotropic virus type I (HTLV-1)-specific precursor cytotoxic T lymphocyte frequencies in patients with HTLV-1-associated neurological disease. *J. Exp. Med.*, *177*: 1567-1573, 1993.

Adult T-cell leukemia: future prophylaxis and immunotherapy

Mari Kannagi[†], Nanae Harashima, Kiyoshi Kurihara, Atae Utsunomiya, Ryuji Tanosaki and Masato Masuda

A small population of human T-cell leukemia virus Type I (HTLV-I) carriers develop adult T-cell leukemia after a long incubation period. The results of a series of experiments using animal models suggest that insufficiency of HTLV-I-specific T-cell response induced by vertical HTLV-I infection allows enlargement of the HTLV-I-infected cell reservoir *in vivo*, a crucial risk factor of adult T-cell leukemia. In this review it is proposed that prophylactic Tax-targeted vaccines for the high-risk group of adult T-cell leukemia, which is characterized by low HTLV-I-specific T-cell response and high proviral load, can reduce the risk. Immunological studies on adult T-cell leukemia patients after hematopoietic stem cell transplantation also suggest that Tax-targeted immunotherapy may be effective against full-blown disease, although its indication may be limited.

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Human T-cell leukemia virus Type I (HTLV-I) is etiologically linked to adult T-cell leukemia (ATL) [1–3]. Endemic areas of HTLV-I infection include Japan, the Caribbean, South America, Africa, Island Melanesia and the Middle East [4]. It is estimated that around 1 million people are infected with HTLV-I in Japan and between 1 and 5% of those infected develop ATL [5,6]. Most HTLV-I carriers are asymptomatic throughout their lives and another small fraction develop a chronic progressive neurological disorder termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and other inflammatory disorders [7,8]. Monoclonal integration of HTLV-I provirus in ATL cells indicates that ATL arises from one HTLV-I-infected cell that has undergone malignant phenotypic progression [9]. However, oligoclonal expansion of HTLV-I-infected cells *in vivo* is also observed in HAM/TSP patients and some asymptomatic HTLV-I carriers [10]. HTLV-1-infected cells appear to have either a greater proliferative potential than normal T-cells, or possibly a survival advantage due to a slower death rate *in vivo*, or both.

The HTLV-I viral protein Tax transactivates and interacts with many cellular proteins

related to cell growth and apoptosis via activation of transcription factors, such as nuclear factor (NF)κB, cAMP response element binding protein (CREB), serum response factor (SRF) and activator protein (AP)-1, or inactivation of p53, partly accounting for the mechanisms of HTLV-I-induced leukemogenesis [3,11–13].

However, HTLV-I expression in freshly isolated peripheral ATL cells is hardly detectable and can be induced following several hours of *in vitro* cultivation [14–16]. Similar silencing of HTLV-I expression is observed not only in ATL but also in the peripheral blood mononuclear cells (PBMCs) of HAM/TSP patients and HTLV-I carriers [17,18]. Interestingly, in fresh ATL cells, NFκB is constitutively activated [19], which can be induced by Tax, while Tax is undetectable in these cells. This paradoxical observation creates controversy regarding the role of Tax in HTLV-I leukemogenesis. HTLV-I Tax expression in ATL cells might be transient before they appear in the periphery, where the level of Tax expression decreases. Alternatively, Tax might be required for the early steps, but dispensable at the late stages of HTLV-I leukemogenesis because of accumulation of multiple additional phenotypic changes.

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Affiliations

[†]Author for correspondence
Department of Immunotherapeutics,
Tokyo Medical & Dental University,
Medical Research Division,
1-5-45 Yushima, Bunkyo-ku,
Tokyo 113-8519, Japan
Tel.: +81 358 035 798
Fax: +81 358 030 235
kannim@cmd.ac.jp

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ATL is characterized by tumor cells with mainly CD4⁺ and CD25⁺ mature T-lymphocyte phenotypes, onset during middle age or later, immunosuppression and poor prognosis [20]. The clinical use of combination chemotherapy in ATL patients elevated the 4-year overall survival rate to 8–12%, which is still lower than that of other types of leukemia [21]. Some, but not all, ATL patients respond to intravenous administration of anti-CD25 monoclonal antibody [22]. The combination therapy of zidovudine (Retrovir®, GlaxoSmithKline) and interferon (IFN)- α achieved a high response rate in ATL patients [23,24], but does not prevent relapse after cessation of the therapy. Combination of arsenic and IFN [25] and other NF κ B-targeted therapies have been proposed.

Recently, hematopoietic stem cell transplantation (HSCT) was applied to a limited number of ATL patients. Initial studies of autologous HSCT revealed a frequent recurrence of ATL [26]. However, more recent reports revealed that allogeneic HSCT could produce better results [27]. HSCT appears to achieve long-lasting complete remission, although there is a risk of graft-versus-host (GVH) disease that is sometimes lethal. Further improvements or new approaches are therefore required for ATL treatment.

Antitumor immunity in HTLV-I leukemogenesis

Although no consistent differences have been observed among HTLV-I strains isolated from ATL and HAM/TSP patients [28,29], immunological studies have found a clear difference in HTLV-I-specific T-cell immune responses among these two diseases. HTLV-I-specific cytotoxic T-lymphocytes (CTLs) can be induced in *in vitro* cultures of PBMCs from HAM/TSP patients and asymptomatic HTLV-I carriers, but rarely from ATL patients [30–33]. Among HTLV-I antigens, Tax, a critical viral protein for T-cell immortalization, is a main target for HTLV-I-specific CTLs found in HTLV-I-infected individuals [32,34]. The HTLV-I envelope is also a popular target, especially of CD4⁺ CTLs [35]. Despite the high pressure of Tax-specific CTL response, the frequency of escape mutations in the Tax gene in HAM/TSP patients is less than that in asymptomatic

HTLV-I carriers [36], demonstrating the complexity of the mechanism of viral persistence. Escape mutants are sporadically found in ATL cells [37]. The nature of HTLV-I-specific T-cell insufficiency in ATL patients is not fully understood. A recent report indicates that PBMCs from patients usually fail to respond to HTLV-I antigens *ex vivo*, although CD8⁺ HTLV-I-specific CTLs are inducible from these PBMCs when fully activated *in vitro* [38], suggesting the presence of some immunosuppressive or tolerating mechanisms *in vivo*. HTLV-I Tax-specific CTLs are capable of lysing short-term-cultured ATL cells *in vitro* [39], indicating that HTLV-I-specific CTLs might contribute to antitumor surveillance in HTLV-I-infected individuals.

To understand the influence of host immunity to HTLV-I leukemogenesis *in vivo*, a series of experiments using rat models of T-cell lymphomas were conducted. In these models, a syngeneic HTLV-I-transformed T-cell line could cause fatal lymphomas in T-cell-deficient or immunosuppressed but not in immunocompetent rats (TABLE 1) [40,41]. The antitumor effects in immunocompetent rats was mediated by T-cells directed to HTLV-I Tax [42]. This is consistent with the importance of Tax-specific CTLs in human cases. It is interesting that the major target of CD8⁺ HTLV-I-specific CTLs is Tax both in rats and humans, implying that HTLV-I Tax was most efficiently presented by major histocompatibility complex I molecules among viral proteins *in vivo*. Furthermore, in the rat model, Tax-directed vaccination eradicated ATL-like lymphomas [42,43]. The rat model, however, differs from the human disease, as human ATL develops in immunocompetent individuals following 40 years or more of incubation, while in the rat model the host is immunosuppressed and the lymphoma is derived from a HTLV-I-transformed cell line.

Risk factors of ATL

Epidemiological risk factors

In cohort studies of HTLV-I carriers, it appears that the risk factors for ATL might include vertical HTLV-I infection, gender (male > female) and increasing numbers of abnormal lymphocytes [44,45]. The higher incidence of ATL in males is

Table 1. Summary of experiments using rat models of human T-cell leukemia virus Type I-infected lymphoma.

Rat strain	Cells inoculated	Route	Other treatment	HTLV-I-specific T-cell response	Outcome	Ref.
F344/hetero	FPM1-V1AX	Subcutaneous	None	+	Tumor eradication	[40]
F344/nude	FPM1-V1AX	Subcutaneous	None	-	Lymphomas	[40]
F344/nude	FPM1-V1AX	Subcutaneous	Adoptive T-cell transfer (intraperitoneal) from Tax DNA vaccinated rats	+	Tumor eradication	[43]
F344/nude	FPM1-V1AX	Subcutaneous	Adoptive T-cell transfer (intraperitoneal) from Tax peptide vaccinated rats	+	Tumor eradication	[42]
WKAH	TARS-1	Subcutaneous	Anti-CD80/CD86 mAb (intraperitoneal)	-	Lymphomas	[41]

Subcutaneous inoculation of human T-cell leukemia virus Type I (HTLV-I)-transformed FPM1-V1AX cells caused fatal systemic lymphomas in syngeneic athymic F344 nude but not immunocompetent F344 hetero rats. Transfer of the spleen T-cells from hetero rats vaccinated with Tax DNA or peptides eradicated tumors in nude rats. WKAH rats treated with anti-CD80/CD86 monoclonal antibodies (mAbs) also developed lymphomas by inoculation of TARS-1, syngeneic HTLV-I-infected rat cells.

Table 2. Summary of experiments using rat models of human T-cell leukemia virus Type I infection [48,49].

Rat strain	Cells inoculated	Route	Other treatment	HTLV-I-specific T-cell response	Proviral load
F344/hetero	MMC-treated MT-2	Intraperitoneal	None	+	Low
F344/hetero	MMC-treated MT-2	Intravenous	None	+	Low
F344/hetero	MMC-treated MT-2	Oral	None	-	Sometimes high
F344/hetero	MMC-treated MT-2	Oral	Inoculation (subcutaneous) of FPM1-V1AX cells 2 months later	+	Not tested

HTLV-I proviral load was higher in oral infection than in other routes, despite the absence of HTLV-I-specific T-cell response. F344/hetero is an immunocompetent rat strain. HTLV-I: Human T-cell leukemia virus Type I; MMC: Mitomycin C; FPM1-V1AX: HTLV-I-infected rat T-cell line derived from F344/hetero.

attributed to vertical infection because horizontal transmission from husbands to wives increases the number of female HTLV-I carriers, resulting in the reduced incidence of ATL in females [46]. Genetic analysis indicated the presence of typical human leukocyte antigen (HLA) haplotypes for ATL in an endemic area [47], also implying that vertical infection might transmit some determinants of HTLV-I leukemogenesis.

Although immunological studies have highlighted clear differences in the magnitude of HTLV-I-specific CTL responses between ATL and HAM/TSP patients, such a study has not been conducted in HTLV-I carriers who subsequently develop ATL. Therefore, it is not known whether the insufficiency in HTLV-I-specific T-cell responses in ATL patients is spontaneously associated or acquired after the onset of ATL. The reasons for the wide variety of immune responses to HTLV-I among HTLV-I carriers are also unknown. One possibility is the influence of vertical HTLV-I infection.

Experimental risk factors

In another series with rat models of HTLV-I infection, it was found that the routes of infection strongly affected HTLV-I-specific immunity [48,49]. Immunocompetent adult rats orally inoculated with HTLV-I-infected cells showed very low levels of HTLV-I-specific T-cell proliferation or IFN- γ production, which were indistinguishable from those of naive rats, whereas significant levels of response were detected in the rats infected intraperitoneally or intravenously. In contrast, HTLV-I proviral load in the spleen cells, examined several months after infection, was significantly higher in orally infected rats than in intraperitoneally infected rats (TABLE 2).

There was an inverse correlation between HTLV-I proviral load and HTLV-I-specific T-cell proliferation in the rats infected through various routes [49]. This suggests that HTLV-I-specific T-cell immune responses might contribute to limited expansion of HTLV-I-infected cells *in vivo*, and the magnitude of the host immune response at primary infection might be a critical determinant of persistent HTLV-I levels thereafter. Since HTLV-I proviruses are associated with infected cells, the increase in proviral load means that the increase in the infected cell number is due to self proliferation of the infected cells or dynamic cell-to-cell infections.

Since oral infection via mother's milk is a major route of vertical HTLV-I infection in humans [50], the results of the rat experiments strongly suggest that the three risks of ATL noted above (i.e., vertical HTLV-I infection, high viral load and low T-cell responses to HTLV-I) are interlinked. Immune unresponsiveness to HTLV-I and subsequent enlargement of the HTLV-I-infected cell population may prove to be conditions that favor the evolution of infected cells towards more malignant phenotypes.

Potential reduction of ATL risk

Infants born to HTLV-I-carrying mothers are fed approximately 1×10^8 HTLV-I-infected cells before weaning [51] and a number of infantile carriers stay seronegative for HTLV-I for a certain period of time [52], suggesting that immunological tolerance to HTLV-I infection might be established during this period. Not all but most of these HTLV-I-infected individuals exhibit seroconversion within a few years [53]. Although T-cell immune responses to HTLV-I in children have not been investigated, many adult HTLV-I carriers exhibit HTLV-I-specific CTL responses, suggesting that the T-cell response might also recover spontaneously later in life, just as occurs in vertical hepatitis B virus infection.

In orally HTLV-I-inoculated rats, the otherwise very low HTLV-I-specific T-cell immune response could be fully recovered by subcutaneous reimmunization with syngeneic HTLV-I-infected cells [49]. Usually, immunological tolerance established against orally administered protein antigens, such as albumin, is not reversed by reimmunization of the fed antigen [54]. It is not clear why immune tolerance in oral HTLV-I infection is reversible. It may be due to the presence of persistent virus *in vivo*.

FIGURE 1 schematically demonstrates the authors' current hypothesis on immunological risk factors for ATL in the natural course of HTLV-I infection. Vertically infected HTLV-I carriers harbor risks of ATL (i.e., insufficient HTLV-I-specific T-cell response and expansion of infected cells). However, such risks may be reduced in many HTLV-I carriers by spontaneous recovery of HTLV-I-specific T-cell response. Once T-cell immunity to HTLV-I is activated, the magnitude of the immune response would positively correlate with pre-existing viral load *in vivo*, as shown in adult HTLV-I carriers and

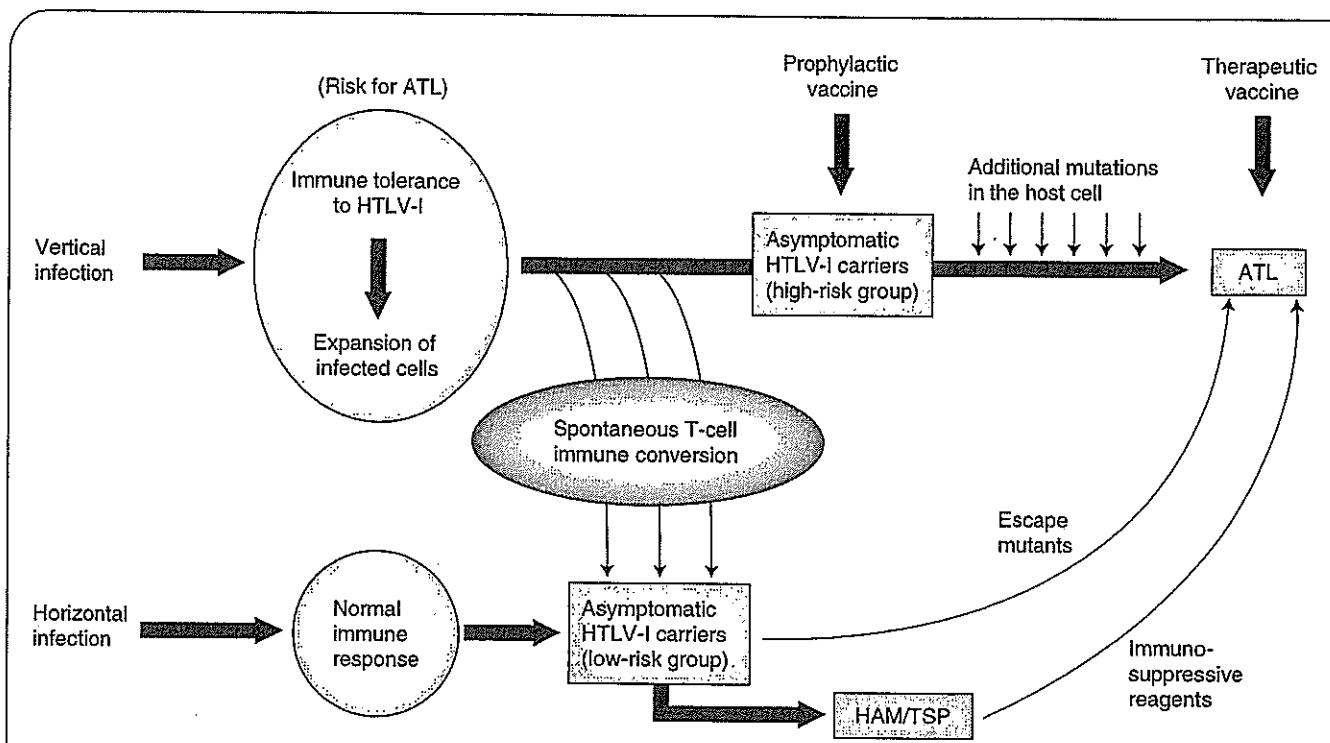


Figure 1. Hypothesis on immunological risk factors for ATL in the natural course of HTLV-I infection. Vertically infected HTLV-I carriers harbor various risks of ATL (i.e., insufficient HTLV-I-specific T-cell response and expansion of infected cells). Sooner or later, HTLV-I-specific T-cell responses spontaneously occur in most of these carriers and the risk of ATL decreases. However, a small population remains in the high-risk group. Under insufficient T-cell immunity, HTLV-I-infected cells accumulate additional mutations towards ATL. HTLV-I carriers infected through other routes have a lower risk of ATL. ATL: Adult T-cell leukemia; HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis; HTLV-I: Human T-cell leukemia virus Type I.

HAM/TSP patients [55,56]. Nevertheless, a small population of adult HTLV-I-infected individuals still exhibit insufficient T-cell responses to HTLV-I despite an abundant viral load, implying that these populations might be the true high-risk group for ATL, to whom prophylactic vaccines targeting Tax can be beneficial.

Graft-versus-leukemia response in ATL patients following HSCT

The rat models previously described mimic the process between HTLV-I infection and the early steps of leukemogenesis. However, there is not a suitable animal model of full-blown ATL. To investigate antitumor immunity in ATL, the T-cell responses in ATL patients who obtained complete remission following nonmyeloablative allogeneic peripheral blood HSCT from HLA-identical sibling donors was analyzed [57]. These patients are presumed to have raised GVH and graft-versus-leukemia (GVL) responses. Minor histocompatibility antigens have been referred to as the target of GVH response in HSCT [58]. Tumor-specific antigens are also possible targets of the GVL response. To detect GVH or GVL response *in vitro*, a phytohemagglutinin-stimulated interleukin (IL)-2-dependent T-cell line (ILT) derived from the same ATL patient before HSCT was first established. ILT cells expressed the antigens originating from the recipient, which may act as the target of GVH response. These cells were also spontaneously infected with HTLV-I and expressed viral

antigens. When the PBMCs from the same patient after HSCT were stimulated in culture with formalin-treated ILT cells *in vitro*, CD8⁺ T-cells proliferated vigorously. These cells were CTLs capable of killing autologous ILT cells. Further analysis revealed that most of these CTLs predominantly recognized a single epitope, Tax11-19, restricted by HLA-A2 (FIGURE 2). The culture also contained a minor population probably related to the GVH response.

Oligoclonal expansion of Tax 11-19-specific CTLs was previously reported in HLA-A2⁺ HAM/TSP patients [59] and also some asymptomatic HTLV-I carriers [60]. This was explained by a highly activated host CTL response against an abundance of HTLV-I antigens in these individuals. Therefore, similar Tax-specific CTL expansion observed in the post-HSCT ATL patient implies the presence of Tax antigen presentation and reactive T-cell response in this patient. Significant reduction in the proviral load occurs following HSCT, which might be partly due to such a strong anti-Tax CTL response.

Thus, a strong graft-versus-HTLV-I response occurred in the post-HSCT ATL patients. Similar reactivation of Tax-specific CTLs was also observed in other post-HSCT ATL patients who obtained complete remission, supporting the notion that these CTLs, as well as the GVH effectors, might participate in the maintenance of remission from ATL. Contribution of the graft-versus-HTLV-I response to GVL effects remains to be clarified by increasing the number of subjects.

Relationship between ATL & HAM/TSP

In the study of post-HSCT immunity, only post-HSCT but not pre-HSCT PBMCs from the ATL patient showed proliferation of HTLV-I Tax-specific CTLs [57]. Since the post-HSCT PBMCs were derived from the HLA-identical donor, it is clear that the insufficiency of HTLV-I-specific CTLs in the pre-HSCT ATL patient was not related to HLA.

The new balance between host immunity and HTLV-I, established in the post-HSCT ATL patients, is very similar to that in HAM/TSP patients. In this sense, allogeneic HSCT converted HTLV-I-specific T-cell immunity in the recipient from one extreme to the other. Here, one concern arises that extreme conversion of CTL response might induce an immunopathogenic reaction in individuals who have a risk of HAM/TSP.

It is still unclear what determines the risk of HAM/TSP. Coexistence of Tax messenger RNA and activated HTLV-I-specific T-cell response in the CNS strongly indicates the involvement of immunopathological mechanisms [61,62]. In addition, host genetic factors must also be involved [63]. Therefore, the post-HSCT patients should be carefully followed up for a long period. To date, post-HSCT ATL patients in remission have shown reduction in the viral load and no symptoms of HAM/TSP.

It is not clear why HAM/TSP patients have a large amount of viral load despite the highly activated HTLV-I-specific T-cell immunity. This may be partly explained by the elevated levels of inflammatory cytokines or mitosis of infected cells [36]. Oligoclonal expansion of HTLV-I-infected cell clones often seen in HAM/TSP patients indicates that some process of leukemogenesis might also be going on in these patients [9]. Nevertheless, ATL and HAM/TSP occur independently, suggesting that activated host HTLV-I-specific T-cell immunity in HAM/TSP patients still appears to make contributions to tumor surveillance. In this respect, administration of immunosuppressive reagents to HAM/TSP patients might increase the risk of ATL development.

Expert opinion

HTLV-I infection affects various aspects of the balance between host and virus during the long-term course of the infection. From

the results of animal models it is concluded that insufficiency of the T-cell response to HTLV-I Tax is a crucial risk factor of ATL development. For prophylactic approaches, a wide survey among HTLV-I carriers is required to identify the high-risk group of ATL patients, which is characterized by low HTLV-I-specific T-cell response and high proviral load. Tax-targeted vaccines to the high-risk group might be beneficial in reducing risk.

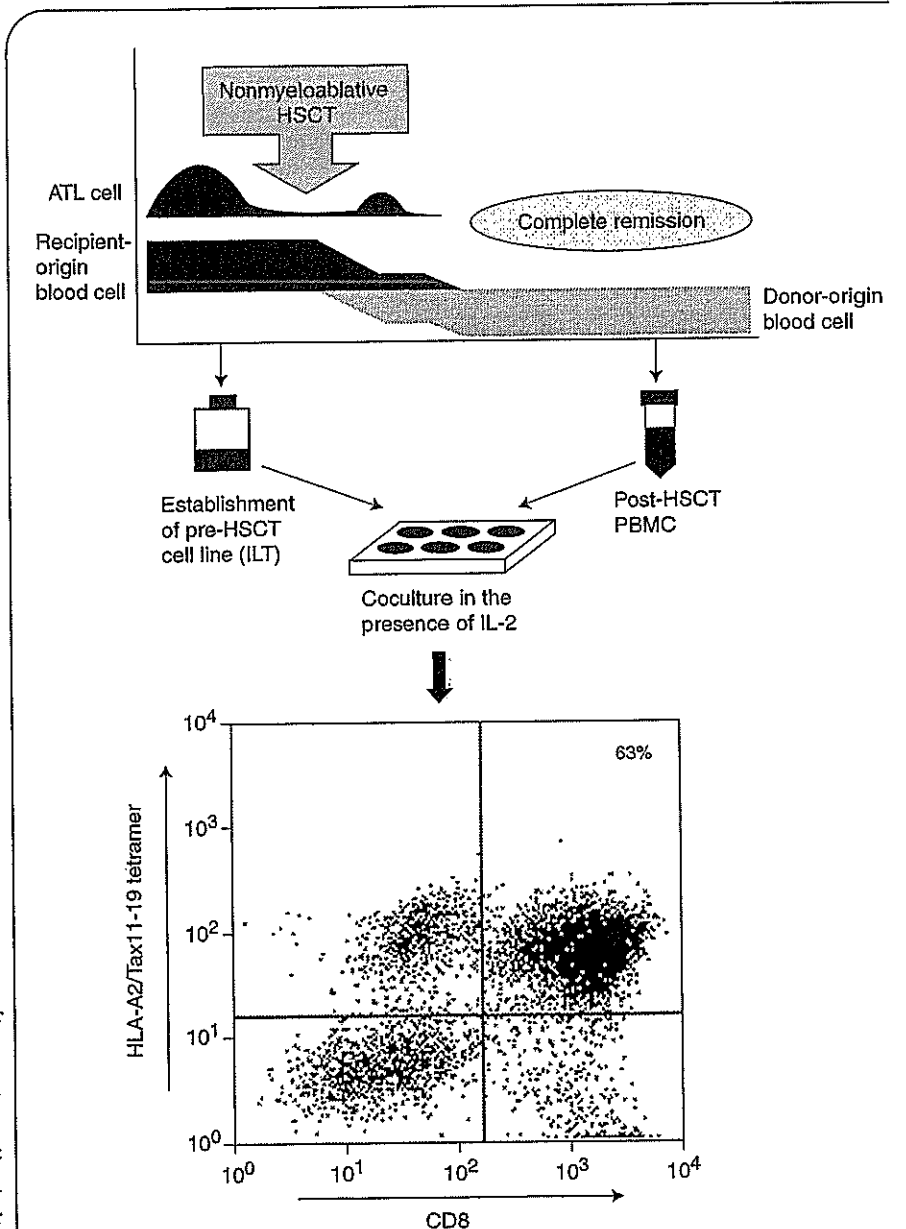


Figure 2. Analysis of T-cell immunity of ATL patients following HSCT. Top panel indicates clinical course of an ATL patient who received HSCT from an HLA-identical sibling and obtained complete remission. The pre-HSCT cell line (ILT) spontaneously infected with HTLV-I was established first, then PBMCs from this ATL patient after HSCT were cocultured with formalin-treated ILT cells. Fluorescence-activated cell sorter analysis using tetramer (lower panel) indicated that HLA-A2-restricted Tax11-19-specific CD8⁺ cytotoxic T-lymphocytes capable of killing ILT cells predominantly proliferated in several weeks of culture [57].

ATL: Adult T-cell leukemia; HLA: Human leukocyte antigen; HSCT: Hematopoietic stem cell transplantation; IL: Interleukin; ILT: IL-2-dependent T-cell line; PBMC: Peripheral blood mononuclear cells.