

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),<sup>6</sup> was used as described previously (37-39).

**CTL Assay.** Cytotoxic activities were measured by 6-h <sup>51</sup>Cr-release assay at various E:T cell ratios as described previously (27, 40). Specific cytotoxicity was calculated as [(experimental <sup>51</sup>Cr release - spontaneous <sup>51</sup>Cr release) / (maximum <sup>51</sup>Cr release - spontaneous <sup>51</sup>Cr release)] × 100%. IFN- $\gamma$  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  $\mu$ l of supernatant were collected to measure IFN- $\gamma$  by ELISA (human IFN- $\gamma$  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- $\gamma$  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  $\mu$ M for 1 h at 37°C and then CTLs were added to each well. CTL activity was measured by 6-h <sup>51</sup>Cr-release assay or ELISA assay for IFN- $\gamma$  production in the supernatants after additional incubation for 18 h.

**Recombinant Vaccinia Viruses.** Recombinant vaccinia viruses WR-p27<sup>X</sup> (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- $\gamma$ -producing antigen-specific T cells were counted using IFN- $\gamma$ -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  $\mu$ l of 15  $\mu$ g/ml anti-IFN- $\gamma$  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  $5 \times 10^4$  cells/well in the absence or presence of the same number of stimulator cells or 10  $\mu$ g/ml peptides in RPMI 1640 containing 10% FCS and incubated overnight at 37°C in 5% CO<sub>2</sub>. The next day, cells were removed by washing with PBS/0.05% Tween 20 and biotinylated anti-IFN- $\gamma$  mAb, and 7-B6-1 biotin (Mabtech) was added 100  $\mu$ l of 1  $\mu$ 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 ( $1 \times 10^6$ ) 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  $\beta$ -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- $\gamma$ -producing ability against ILT-#37 and K562 cells at 19 days after initiation of culture. As shown in Fig. 2A, significant levels of IFN- $\gamma$  were produced from post-HSCT #37 in the culture against ILT-#37 but not against K562 cells after an overnight incubation. The PBMCs from

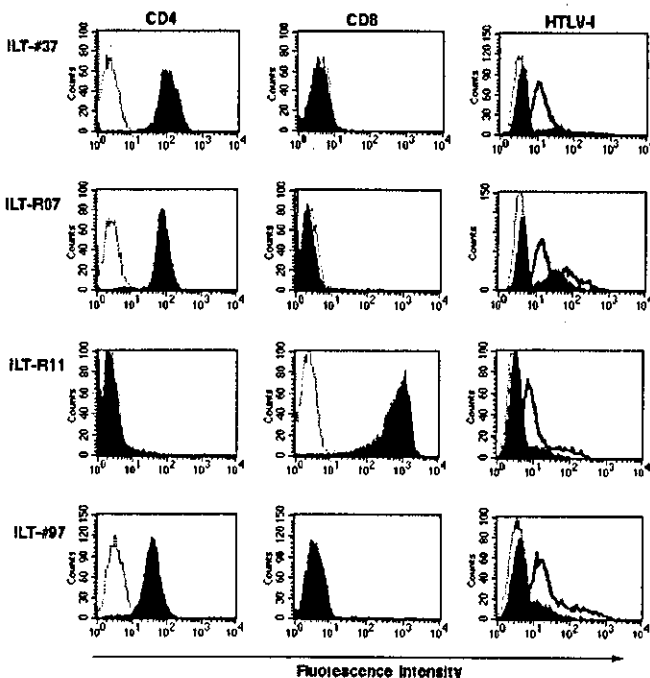


Fig. 1. Surface phenotype and human T-cell leukemia virus type I (HTLV-I) expression in T-cell lines spontaneously established from prehematopoietic stem cell transplantation adult T-cell leukemia (ATL) patients. ILT-#37, ILT-R07, ILT-R11, and ILT-#97 cells that were cultured for >2 months in the presence 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).

<sup>6</sup> Internet address: <http://bimas.dcrf.nih.gov/molbio/hla-bind/>.

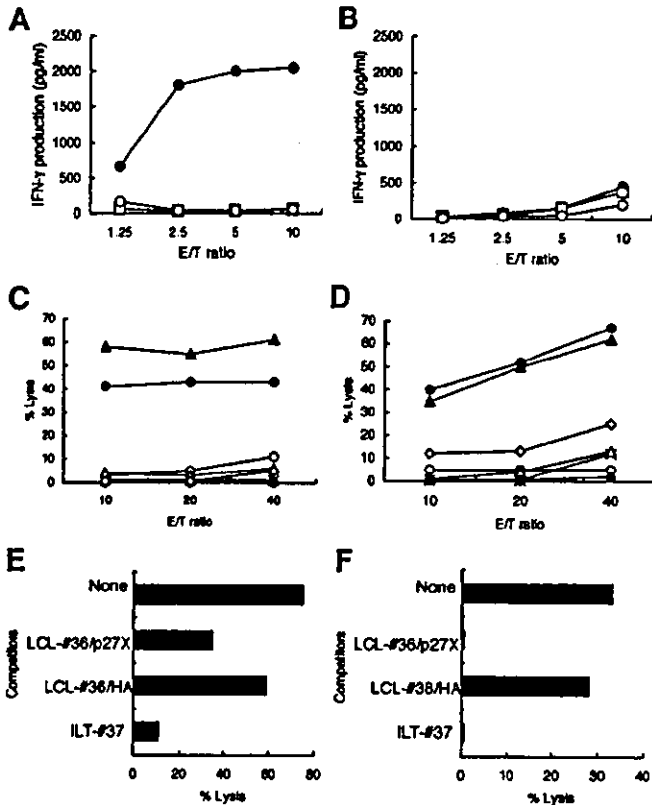


Fig. 2. Induction of and human T-cell leukemia virus type I (HTLV-I)-specific CTLs from post-hematopoietic stem cell transplantation (HSCT) patient #37 and donor #36 in response to ILT-#37 cells. Peripheral blood mononuclear cells (PBMCs) from patient #37 (+183 days post-HSCT) (A, C, and E) and donor #36 (B, D, and F) were cultured with periodical stimulations with formalin-fixed ILT-#37 cells, and their IFN- $\gamma$ -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  $^{51}\text{Cr}$ -release assay, respectively. The target cells used were formalin-fixed ILT-#37 ( $\bullet$ ), K562 ( $\square$ ), or none ( $\circ$ ) in A and B. HLA-identical ILT-#37 ( $\bullet$ ), LCL-#36 ( $\circ$ ), and PHA-activated PBMCs of pre-HSCT patient #37 ( $\times$ ), HLA-A2- and B46-matched TCL-Kan ( $\blacktriangle$ ) and LCL-Kan ( $\triangle$ ), and HLA-mismatched ILT-As-2 ( $\blacklozenge$ ) and LCL-As ( $\lozenge$ ) in C and D. Closed symbols represent HTLV-I-infected while open symbols represent HTLV-I-negative cells. Values represent the mean of IFN- $\gamma$  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  $^{51}\text{Cr}$ -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- $\gamma$  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.

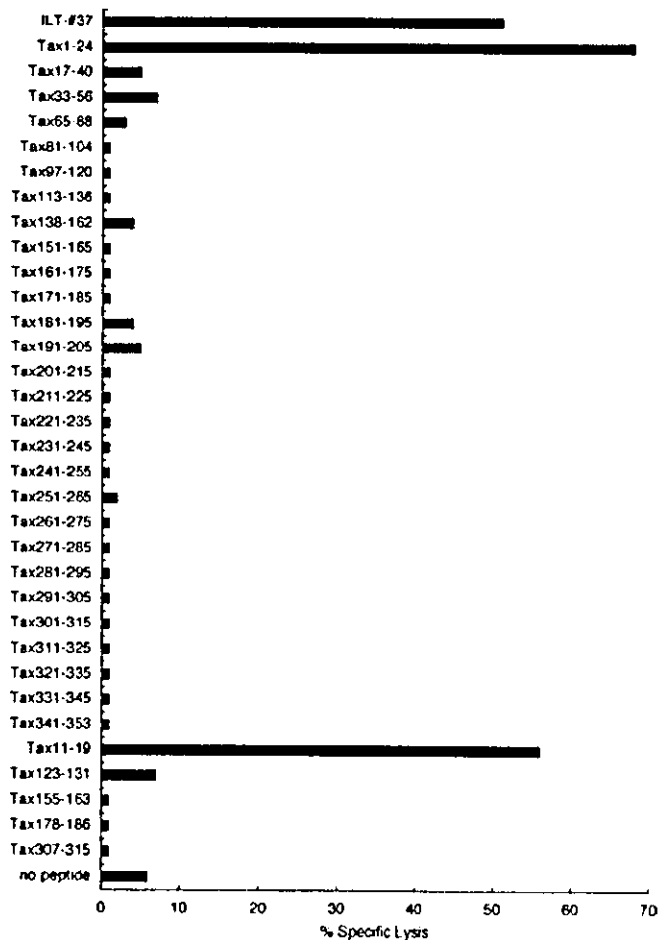


Fig. 3. Mapping of human T-cell leukemia virus type I Tax epitopes recognized by CTLs from post-hematopoietic stem cell transplantation (HSCT) patient #37. LCL-#36 cells were pulsed with 10  $\mu\text{M}$  of 33 kinds of 9-24 mer synthetic oligopeptides corresponding to the Tax amino acid sequence, and their susceptibility to CTLs of post-HSCT patient #37 was measured by  $^{51}\text{Cr}$ -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-GQSLLFGYPVYVFGDCV) 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<sup>-</sup>, tetramer<sup>-</sup> 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<sup>+</sup>, HLA-A\*0201/Tax11-19<sup>+</sup> (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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> PBMCs from patient R07 produced significant levels of IFN- $\gamma$  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-

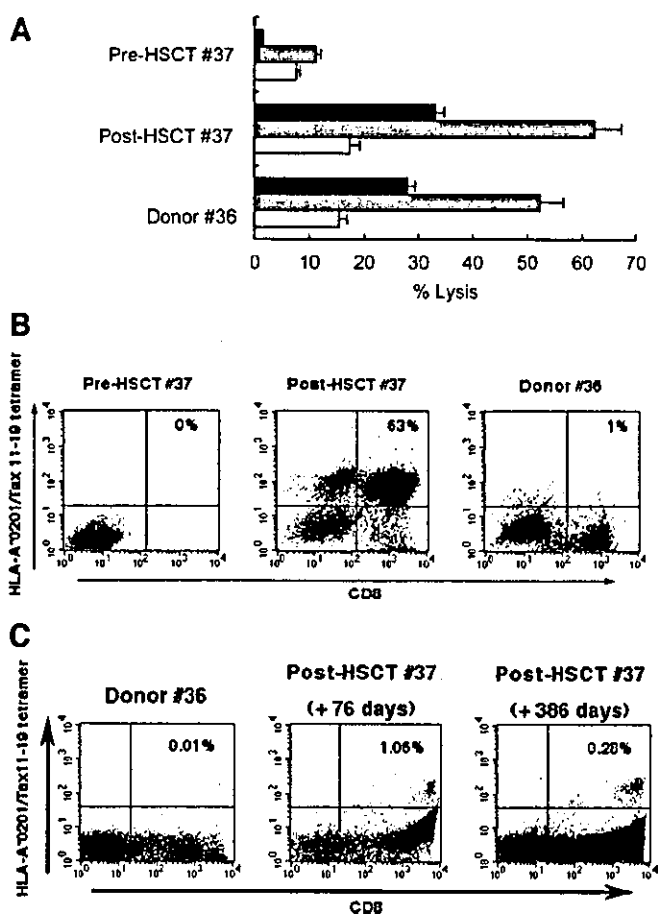


Fig. 4. Selective expansion of Tax11-19-specific CTL in peripheral blood mononuclear cell (PBMC) cultures from patient #37 after hematopoietic stem cell transplantation (HSCT). **A**, PBMCs from pre-HSCT patient #37 at 40 days of culture which were stimulated with formalin-fixed ILT-#37 cells two times, 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<sup>+</sup> 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 1 Tax11-19-specific cell line, Tc-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 1 Tax 11-19-specific CD8<sup>+</sup> 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<sup>+</sup> and HLA-A\*0201/Tax 11-19 tetramer<sup>+</sup> 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<sup>+</sup> PBMC culture from post-HSCT patient #97 stimulated with autologous ILT-#97 cells showed HTLV-I-specific IFN- $\gamma$  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<sup>a</sup>

Patient ID (days after HSCT)	PBMC culture period	Stimulator	Target cells for immunoassays				IFN- $\gamma$ (pg/ml) <sup>b</sup>	Cytolysis (%) <sup>c</sup>		
			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. <sup>d</sup>			
			ILT-As-2	Positive	A24	866 $\pm$ 117	N.T.			
			TOK	Negative	A24	160 $\pm$ 7	N.T.			
			None			36 $\pm$ 12	N.T.			
	Day 24	ILT-R07	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.			
	Day 63	ILT-R07	ILT-R07	Positive	Identical	N.T.	39 $\pm$ 9			
			ILT-As-2	Positive	A24	N.T.	48 $\pm$ 5			
			TOK	Negative	A24	N.T.	5 $\pm$ 6			
			TOK/p27X <sup>e</sup>	pX products	A24	N.T.	27 $\pm$ 11			
			TOK/pEnv <sup>e</sup>	Envelope	A24	N.T.	11 $\pm$ 4			
			TOK/pGag <sup>e</sup>	Core	A24	N.T.	6 $\pm$ 10			
			R11 (+153)	Day 25	ILT-R11	ILT-R11 <sup>f</sup>	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	Negative					14 $\pm$ 0	N.T.			
Day 21	ILT-#97	ILT-#97		Positive	Identical	477 $\pm$ 34	N.T.			
		ILT-Har		Positive	A2, B51, DR4	415 $\pm$ 2	N.T.			
		LCL-Har		Negative	A2, B51, DR4	104 $\pm$ 15	N.T.			
		None				63 $\pm$ 4	N.T.			
Day 50	ILT-#97	ILT-#97		Positive	Identical	128 $\pm$ 36	29 $\pm$ 2			
		ILT-Har		Positive	A2, B51, DR4	111 $\pm$ 10	23 $\pm$ 3			
		LCL-Har		Negative	A2, B51, DR4	10 $\pm$ 1	16 $\pm$ 3			
		None				1 $\pm$ 0	N.T.			
#97 (+104)	Day 21	ILT-#97	ILT-#97	Positive	Identical	477 $\pm$ 34	N.T.			
			ILT-Har	Positive	A2, B51, DR4	415 $\pm$ 2	N.T.			
			LCL-Har	Negative	A2, B51, DR4	104 $\pm$ 15	N.T.			
			None			63 $\pm$ 4	N.T.			
Day 50	ILT-#97	ILT-#97	Positive	Identical	128 $\pm$ 36	29 $\pm$ 2				
		ILT-Har	Positive	A2, B51, DR4	111 $\pm$ 10	23 $\pm$ 3				
		LCL-Har	Negative	A2, B51, DR4	10 $\pm$ 1	16 $\pm$ 3				
		None			1 $\pm$ 0	N.T.				

<sup>a</sup> 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- $\gamma$  production or cytotoxicity of these PBMCs were examined against the various target cells indicated.

<sup>b</sup> IFN- $\gamma$  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.

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

<sup>d</sup> N.T., not tested.

<sup>e</sup> 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.

<sup>f</sup> 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 <sup>51</sup>Cr-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 (SFHSLHLLFEEYTN) 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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- $\gamma$  production after overnight stimulation with ILT-R07 or Tax 301-309 peptide (Table 3). The number of IFN- $\gamma$ -producing cells was significantly higher in post-HSCT PBMCs than in pre-HSCT PBMCs from patient R07. The number of IFN- $\gamma$ -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

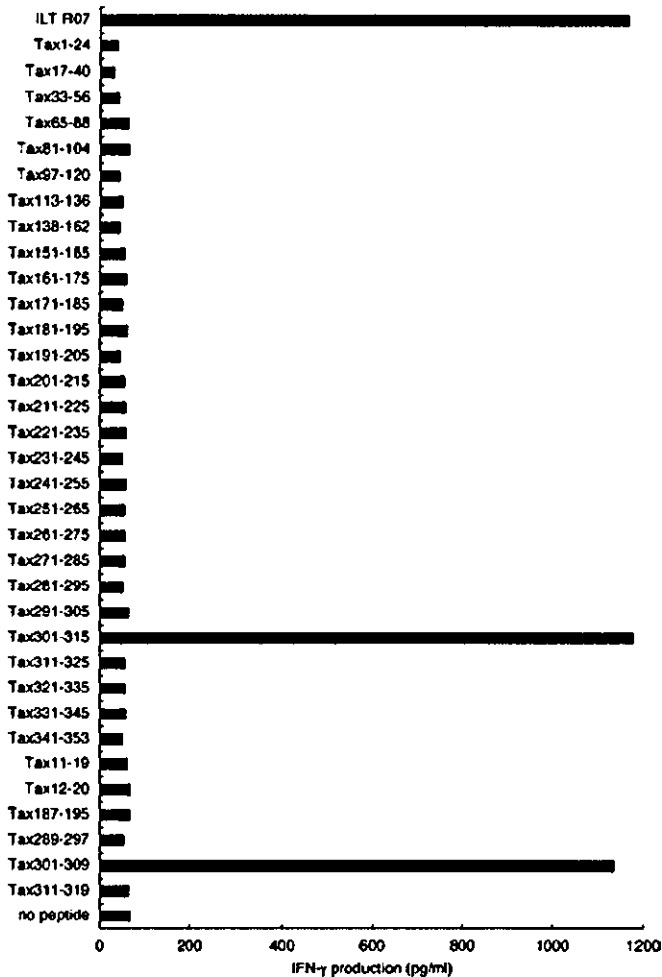


Fig. 5. Mapping of human T-cell leukemia virus type I Tax epitopes recognized by the CTLs induced from post-hematopoietic stem cell transplantation patient R07. CD8<sup>+</sup> cell-enriched peripheral blood mononuclear cell that had been cultured for 32 days with three stimulations with formalin-fixed ILT-R07 cells were mixed with HLA-A24<sup>+</sup> 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- $\gamma$  in the supernatants after 18 h of incubation was measured by ELISA assay. Values represent the mean of duplicate assays.

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-

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- $\gamma$ -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- $\gamma$ -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<sup>a</sup>

Stimulator	IFN- $\gamma$ -producing SFC/5 $\times$ 10 <sup>4</sup> PBMC <sup>b</sup>	
	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

<sup>a</sup> Uncultured PBMCs directly thawed from frozen stocks from patient R07 before and after (+255 days) HSCT were subjected to IFN- $\gamma$ -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  $\times$  10<sup>5</sup>/well as described in "Materials and Methods." Values represent the mean  $\pm$  SD of triplicate assays.

<sup>b</sup> Results of IFN- $\gamma$ -enzyme-linked immunospot assay are expressed as spot-forming cells (SFCs)/5  $\times$  10<sup>4</sup> PBMCs.

(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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## Clinical significance of cytomegalovirus (CMV) antigenemia in the prediction and diagnosis of CMV gastrointestinal disease after allogeneic hematopoietic stem cell transplantation

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### Summary:

To evaluate the clinical significance of a cytomegalovirus (CMV) antigenemia assay in the prediction and diagnosis of CMV gastrointestinal (CMV-GI) disease after hematopoietic stem cell transplantation (HSCT), 19 allogeneic HSCT recipients developing CMV-GI disease were retrospectively reviewed. All patients were monitored by a CMV antigenemia assay, at least once weekly after engraftment. The median onset of CMV-GI disease occurred 31 days post transplant (range: 19–62). Only four of 19 patients (21%) developed a positive CMV antigenemia test before developing CMV-GI diseases. Although all 19 patients subsequently developed positive CMV antigenemia tests during their clinical courses, the values remained at a low-level in nine (47%) patients. Among the 14 patients in whom results of real-time polymerase chain reaction (PCR) were available, seven (50%) yielded positive results of real-time PCR before developing CMV-GI disease. In contrast to the values of CMV antigenemia, all 14 patients exclusively yielded high viral loads (median:  $2.8 \times 10^4$  copies/ml plasma). We conclude that CMV antigenemia testing has limited value in prediction or early diagnosis of CMV-GI disease, and that real-time PCR could have a more diagnostic significance.

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**Keywords:** cytomegalovirus; gastrointestinal disease; CMV antigenemia; real-time PCR; hematopoietic stem cell transplantation

Cytomegalovirus (CMV) disease remains a serious infectious complication that causes morbidity and mortality in recipients of allogeneic hematopoietic stem cell transplantation (HSCT).<sup>1</sup> Although the incidence of CMV disease has decreased over time, prophylactic therapy (the administration of antiviral agents to all HSCT recipients), largely due to its toxicities, resulted in no survival benefit.<sup>2–4</sup> Subsequently, many investigators focused on pre-emptive therapy, treating only patients with proven CMV infection or reactivation.<sup>4–8</sup> Rapid, sensitive, quantitative, and reliable methods, which can detect CMV reactivation before CMV disease develops, are essential for the refinement of pre-emptive therapy. The CMV antigenemia assay is one of the most widely used methods to detect CMV reactivation in a variety of clinical settings,<sup>9,10</sup> and CMV antigenemia-based pre-emptive therapy has been shown to effectively prevent the occurrence of CMV pneumonitis.<sup>4,7,8</sup> However, several reports, including ours, have suggested that pre-emptive therapy based on CMV antigenemia allowed the development of CMV diseases other than pneumonitis, including gastrointestinal (GI) disease, retinitis, and hepatitis, in a small number of patients.<sup>4,7,8</sup> In the present study, we focused on CMV-GI disease in allogeneic HSCT recipients, and evaluated the predictive and diagnostic values of the CMV antigenemia assay in CMV-GI disease in comparison with the efficacy of quantitative real-time polymerase chain reaction (PCR).

### Patients and methods

#### *Patients and transplant procedures*

Among the recipients of allogeneic bone marrow or peripheral blood stem cells at five transplant centers, those who developed histologically diagnosed CMV-GI disease were enrolled into this study. Clinical data from each patient were collected from the medical records and reviewed retrospectively.

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*CMV antigenemia assay*

All patients enrolled into this study were monitored at least once a week for CMV reactivation with a CMV antigenemia assay after engraftment. CMV antigenemia assay using the monoclonal antibodies C10/C11 (Biotest, Dreieich, Germany) or HRP-C7 (Teijin, Tokyo, Japan) was performed as previously reported.<sup>7-10</sup> High-level CMV antigenemia was defined as 10 or more positive cells per applied 150 000 granulocytes for C10/11, and 10 or more positive cells per 50 000 granulocytes for HRP-C7.

*Real-time PCR*

Real-time PCR was performed basically as previously reported.<sup>11,12</sup> In brief, DNA extracted from 100 µl of plasma, using a QIAamp Blood Mini Kit (QIAGEN, Valencia, CA, USA), was subjected to PCR. The sequences of PCR primers and the probe were selected from the US17 region of CMV AD169. The Taqman probe selected between the primers was dual-labeled with 6-carboxyfluorescein (FAM) and with 6-carboxy-teremethyl-rhodamine (TAMRA). PCR reaction was performed by using TaqMan Universal PCR Master Mix (PE Biosystems, Foster City, CA, USA). CMV quantification was carried out with a serially diluted standard ranging from 10 to 1 × 10<sup>7</sup> copies/well, and the gene copy numbers were calculated by Sequence Detection System ver.1.6.3. software (PE Biosystems). The minimum detection level was 20 copies/100 µl of plasma. A high-level copy number was defined as 1000 or more copies of CMV-DNA per ml of plasma.

*Definition of CMV-GI disease*

CMV-GI disease was defined as gastrointestinal symptoms such as diarrhea, nausea, and epigastralgia, accompanied

by histologic demonstration of CMV on biopsy materials obtained by endoscopy.

**Results**

*Study population*

Nineteen patients with histologically diagnosed CMV-GI disease were collected, and then subjected to retrospective review. Patient characteristics are shown in Table 1. Except for one case of aplastic anemia, all these patients had undergone allogeneic HSCT for the treatment of hematological malignancies. Conditioning regimens included total body irradiation (TBI)-based regimens (n = 13), busulfan-based regimens (n = 3), a fludarabine-based regimen (n = 2), and total lymphoid irradiation plus cyclophosphamide (n = 1). A total of 10 patients received a transplant from an unrelated donor. All but one patient developed acute graft-versus-host disease (GVHD) of grade II-IV, and received high-dose glucocorticoids in addition to the ongoing cyclosporin A or tacrolimus. No patient developed CMV disease in any organs outside the gastrointestinal tract.

*Onset of CMV-GI disease and CMV antigenemia/real-time PCR*

The median onset of CMV-GI disease was 31 days post transplant (range: days 19-62). Only four (21%) of the 19 patients yielded positive CMV antigenemia test before developing CMV-GI disease. Although these four patients were pre-emptively treated with ganciclovir, they subsequently developed CMV-GI disease. The remaining 15 patients developed CMV-GI disease before CMV antigenemia was identified, and were therapeutically

**Table 1** Patient characteristics

Case	Gender	Age	Disease	Donor	Conditioning	GVHD prophylaxis	CMV serology recipient/donor	aGVHD
1	Male	26	AA	Related	TLI-regimen	CSA + MTX	Pos/Pos	III
2	Female	54	ALL	Related	TBI-regimen	CSA + MTX	Pos/Pos	I
3	Male	36	ALL	Unrelated	TBI-regimen	CSA + MTX	Pos/Pos	II
4	Male	39	CML	Unrelated	BU-CY-TLI	CSA + MTX	Pos/Pos	II
5	Female	42	MDS	Related	BU-CY	CSA + MTX	Pos/Pos	II
6	Female	54	ATL	Related	TBI-regimen	CSA + MTX	Pos/Pos	III
7	Male	22	ALL	Unrelated	TBI-regimen	CSA	Pos/Pos	III
8	Male	50	CML	Unrelated	BU-CY	Tacrolimus + MTX	Pos/Pos	II
9	Male	50	MDS	Unrelated	TBI-regimen	CSA + MTX	Pos/Pos	IV
10	Male	20	ALL	Unrelated	TBI-regimen	CSA + MTX	Pos/Pos	II
11	Female	44	MDS	Unrelated	TBI-regimen	CSA + MTX	Pos/Pos	II
12	Male	24	AML	Unrelated	TBI-regimen	CSA + MTX	Pos/Pos	III
13	Male	46	CML	Related	TBI-regimen	CSA + MTX	Pos/Pos	II
14	Male	41	AML	Related	TBI-regimen	CSA + MTX	Pos/Pos	II
15	Male	30	ALL	Unrelated	TBI-regimen	Tacrolimus + MTX	Pos/Pos	II
16	Female	25	ALL	Related	TBI-regimen	CSA + MTX	Pos/Pos	II
17	Male	22	ALL	Related	TBI-regimen	CSA + MTX	Pos/Pos	II
18	Female	37	MM	Related	FLU-Melphalan	CSA + MTX	Pos/Pos	III
19	Female	53	NHL	Unrelated	FLU-Melphalan	Tacrolimus + MTX	Pos/Pos	II

CMV = cytomegalovirus; aGVHD = acute graft-versus-host disease; AA = aplastic anemia; ALL = acute lymphoblastic leukemia; CML = chronic myeloid leukemia; MDS = myelodysplastic syndrome; ATL = adult T-cell leukemia; AML = acute myeloid leukemia; MM = multiple myeloma; NHL = non-Hodgkin's lymphoma; TLI = total lymphoid irradiation; TBI = total body irradiation; BU = busulfan; CY = cyclophosphamide; FLU = fludarabine; CSA = cyclosporin A.

treated with ganciclovir. Of these patients, however, all became CMV antigenemia positive after the diagnosis of CMV-GI disease. Among the 14 patients with evaluable real-time PCR results, seven (50%) yielded positive results before the development of CMV-GI disease. As well as positivity for CMV antigenemia, the remaining seven patients yielded positive real-time PCR results after the diagnosis of CMV-GI disease.

*Quantitative significance of CMV antigenemia/real-time PCR in CMV-GI disease*

The quantitative significance of CMV antigenemia and real-time PCR in association with CMV-GI disease was evaluated by the maximum values of each assay (Table 2). The values of CMV antigenemia remained at low levels in nine (47%) of the 19 patients, whereas maximum viral load evaluated by real-time PCR was high in all patients.

**Discussion**

The introduction of the CMV antigenemia assay, a sensitive and quantitative assay to detect viral reactivation, has contributed to the successful outcome of preemptive therapy for CMV disease in allogeneic HSCT recipients. However, we and other investigators have pointed out that the assay has some disadvantages.<sup>7,12,13</sup> For example, it is relatively time-consuming, involves subjective components in the interpretation of slides, and requires sufficient granulocytes. Furthermore, despite its high sensitivity in detecting viral reactivation before the onset of CMV pneumonitis, CMV antigenemia does not necessarily precede the onset of other CMV diseases; thus CMV-GI disease or CMV retinitis can occur in allogeneic HSCT recipients receiving CMV antigenemia-guided pre-emptive

therapy.<sup>4-7</sup> In this retrospective study, we evaluated the clinical significance of CMV antigenemia in predicting and diagnosing CMV-GI disease in allogeneic HSCT recipients. Among 19 cases of histologically diagnosed CMV-GI disease, CMV antigenemia preceded the onset of disease in only four (21%) patients, although the remaining patients subsequently became CMV antigenemia positive after developing CMV-GI disease. These findings strongly suggested that CMV antigenemia is of little clinical value in predicting and diagnosing CMV-GI disease. In addition, these four patients developed CMV-GI disease after starting pre-emptive therapy, suggesting that CMV antigenemia can confirm viral reactivation before CMV-GI disease develops in some cases, but not early enough for therapy to be preventive.

In addition to the CMV antigenemia assay, PCR is a useful technique for detecting CMV reactivation.<sup>13,14</sup> There have been several reports of using PCR for pre-emptive therapy in allogeneic HSCT recipients.<sup>15-17</sup> Although it has been reported that PCR is highly sensitive in the detection of viral reactivation and that PCR-guided pre-emptive therapy is effective in preventing the development of CMV diseases, conventional PCR, as compared to the CMV antigenemia assay, is of less clinical use because of lack of quantification. The quantitative real-time PCR is in clinical use, and has become recognized as one of the standard assays for evaluating viral reactivation both qualitatively and quantitatively.<sup>12,13</sup> In this study, we investigated the clinical significance of real-time PCR and that of the CMV antigenemia assay in assessing viral reactivation in cases of CMV-GI disease. Comparatively, PCR was more effective in predicting and diagnosing CMV-GI disease, although its incidence of preceding disease onset was only 50%. Thus, even real-time PCR, which is considered more sensitive than the CMV antigenemia assay, could not satisfactorily detect CMV reactivation before the onset of CMV-GI

**Table 2** CMV-GI disease and the results of CMV antigenemia/real-time PCR

Case	Onset of CMV-GI disease	Antibodies for CMV Ag	First day of CMV Ag +ive	Maximum value of CMV Ag	First day of PCR +ive	Maximum value or PCR
1	30	HRP-C7	38	96 (H)	ND	ND
2	49	HRP-C7	31 <sup>a</sup>	9 (L)	ND	ND
3	39	HRP-C7	45	52 (H)	ND	ND
4	29	HRP-C7	32	124 (H)	ND	ND
5	62	HRP-C7	69	2 (L)	ND	ND
6	30	HRP-C7	16 <sup>a</sup>	44 (H)	12 <sup>a</sup>	2.0 × 104(H)
7	51	HRP-C7	28 <sup>a</sup>	31 (H)	34 <sup>a</sup>	5.0 × 104(H)
8	26	HRP-C7	26	10 (H)	19 <sup>a</sup>	3.0 × 103(H)
9	34	C 10/11	38	141 (H)	26 <sup>a</sup>	1.9 × 105(H)
10	20	C 10/11	14 <sup>a</sup>	1 (L)	14 <sup>a</sup>	1.1 × 104(H)
11	31	C 10/11	45	22 (H)	31	4.5 × 104(H)
12	27	C 10/11	27	6 (L)	27	1.9 × 104(H)
13	30	C 10/11	34	1 (L)	27 <sup>a</sup>	3.7 × 103(H)
14	31	C 10/11	38	117 (H)	27 <sup>a</sup>	2.6 × 105(H)
15	19	C 10/11	24	7 (L)	19	2.8 × 104(H)
16	42	C 10/11	45	7 (L)	45	1.2 × 105(H)
17	20	C 10/11	27	2 (L)	27	6.0 × 103(H)
18	39	C 10/11	45	54 (H)	39	7.0 × 105(H)
19	31	C 10/11	39	2 (L)	39	3.0 × 103(H)

Maximum value of CMV antigenemia was described as positive cells per 50 000 for HRP-C7 and per one slide for C10/11 (150 000 cells applied). High-level CMV Ag and real-time PCR was defined as 10 or more positive cells and 1 × 103 copies or more, respectively (H = high level; L = low level).

<sup>a</sup>Patients showing positive CMV Ag or PCR before disease occurrence.

disease. This result strongly suggested that real-time PCR-guided pre-emptive therapy could not completely prevent the occurrence of CMV-GI disease. However, it is possible that the incidence of CMV-GI disease might be lower in patients receiving real-time PCR-guided pre-emptive therapy than in those receiving CMV antigenemia-guided pre-emptive therapy, simply due to the higher sensitivity of real-time PCR.

We conclude that, in the setting of allogeneic HSCT, the occurrence of CMV-GI disease is uniformly observed at a particular frequency even in patients receiving strict CMV surveillance and pre-emptive therapy using the CMV antigenemia assay or PCR, and consider that the latter may be more useful. Consequently, the results of these assays should not be wholly relied on in the diagnosis and prediction of CMV-GI disease. Further modification of CMV antigenemia-guided pre-emptive therapy should focus on using more sensitive PCR methods, and take into consideration individual patient CMV-specific immune reconstitution.

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## Reduced intensity conditioning regimens

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

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### 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/m<sup>2</sup>, *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.<sup>1,2</sup> 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.<sup>3</sup> 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.<sup>4</sup> Alleles at HLA-A2, 26, -B39, 61, and 75, which are highly polymorphic in the Japanese population,<sup>5</sup> and DRB1 were routinely identified

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**Table 1** Patient characteristics

No.	Sex	Age	Primary disease	Disease status at transplant	Karyotype	Previous lines of therapy	ANC × 10 <sup>9</sup> /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/ATG	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 4 mg/kg on days -6 and -5,<sup>6</sup> then cladribine was replaced with fludarabine 30 mg/m<sup>2</sup> 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/m<sup>2</sup> 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 × 10<sup>9</sup>/l or an absolute neutrophil count (ANC) of > 0.5 × 10<sup>9</sup>/l for 2 consecutive days, and a platelet count of > 20 × 10<sup>9</sup>/l for 2 consecutive days without transfusions. G-CSF 300 µg/m<sup>2</sup>/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%.<sup>7</sup>

*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.<sup>8</sup> Neutropenic fever was managed as described by Pizzo.<sup>9</sup> Cytomegalovirus (CMV) pp65 antigenemia was monitored weekly with initiation of preemptive ganciclovir at positive results.<sup>8</sup>

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 <sup>a</sup>			
	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

<sup>a</sup>Determined by the NCI-CTC version 2.0.

and pathological findings. Acute and chronic GVHD was graded according to the consensus criteria.<sup>10,11</sup>

### 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  $2.6 \times 10^8$ /recipients' body weight (kg) (range 0.8– $5.2 \times 10^8$ /kg). In all, 19 patients (95%) achieved primary neutrophil engraftment, and 12 (60%) reached more than  $20 \times 10^9$ /l platelets. The median time to recover an ANC of  $0.5 \times 10^9$ /l was 15 days (10–25), while 20.5 days (11–32) were needed to reach a platelet count above  $20 \times 10^9$ /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 <sup>9</sup> /l	Platelets >20 × 10 <sup>9</sup> /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.<sup>12–15</sup> Two studies from Israeli<sup>13</sup> and Texas groups<sup>12</sup> 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.<sup>14</sup> 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.<sup>16</sup> Thus, ATG can deplete both host- and donor-derived T

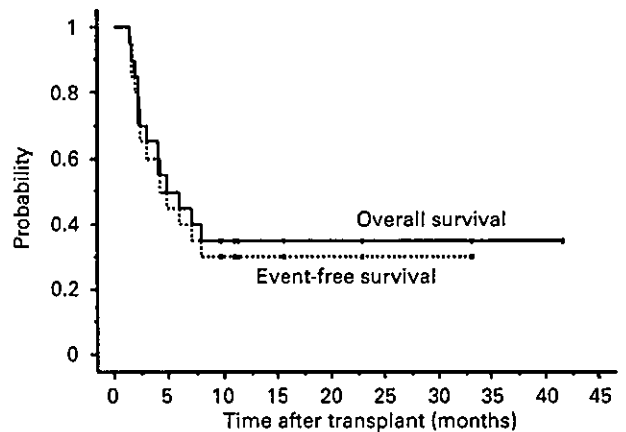


Figure 1 OS and EFS. The estimated 2-year OS (solid lines) and EFS (dotted lines) were, respectively, 35.0% (95% CI 24.7–45.3%) and 30.0% (95% CI 19.8–40.2%).

cells and inhibits GVHD due to its long half-life.<sup>17</sup> 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.<sup>18</sup> 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	Yes/no	3/6	0/11	0.073
Grade II-IV acute GVHD	Yes/no	2/7	5/6	0.37
Reactivation of CMV infection	Yes/no	5/4	6/5	>0.99
Maximal grading of regimen-related toxicity according to NCI-CTC version 2.0	3-4/0-2	4/5	4/7	>0.99
Transplant-related mortality within 100 days of transplant		4	1	0.13
Estimated 1-year OS (95% confidence interval)		33 (17-49)	36 (21-51)	0.52

Another method to enhance engraftment is use of peripheral blood stem cell (PBSC) transplantation instead of BM.<sup>15</sup> 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%).<sup>19</sup> HSCT from unrelated donors achieves immune reconstitution later than HSCT from related donors, which leads to a higher risk for severe infections.<sup>19</sup> 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.<sup>20,21</sup>

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.

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## The mouse natural killer T cell-associated antigen recognized by U5A2-13 monoclonal antibody is intercellular adhesion molecule-1

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### Abstract

Natural Killer T (NKT) cells in mice are generally defined as NK1.1<sup>+</sup> T cells, although NK1.1 antigen is expressed only in C57BL/6 and related strains. This has precluded investigations of other strains. To find a novel NKT cell surface marker, we generated a monoclonal antibody (mAb), U5A2-13, which recognizes phenotypically and functionally similar populations to NKT cells in naïve mice irrespective of strain. Here, by using a COS-7 expressional cloning system, we molecularly cloned a cDNA encoding a protein reactive with the U5A2-13 mAb and then identified it as intercellular adhesion molecule-1 (ICAM-1). Importantly, the U5A2-13 mAb did not stain hepatic mononuclear cells from ICAM-1 gene disrupted mice. Furthermore, Pepscan method disclosed that the discontinuous epitope for U5A2-13 mAb is composed of three loops located in extracellular domain two of ICAM-1. Overall, U5A2-13, a mAb originally established for mouse NKT cells, recognizes a novel conformational epitope of ICAM-1.

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**Keywords:** Natural killer T cell; U5A2-13 monoclonal antibody; Intercellular adhesion molecule-1; Molecular cloning

### 1. Introduction

Natural Killer T (NKT) cells in mice are usually defined as lymphocytes expressing intermediate levels of TCR and NK cell-associated molecules, particularly NK1.1. They predominantly express TCR with invariant V $\alpha$ 14J $\alpha$ 281 and V $\beta$ 8, 7, or 2, and most of them are phenotypically double negative (CD4<sup>-</sup>CD8<sup>-</sup>) or single positive (CD4<sup>+</sup>CD8<sup>-</sup>) T cells [1–6]. These cells produce large amounts of IFN- $\gamma$  and IL-4, suggesting that they play an important role in regulating the Th1/Th2 balance [1,2,7–11]. NKT cells are potentiated by  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) to produce these cytokines in a CD1d dependent manner [7,8,10,12–17].

NK1.1 antigen is expressed on NK and NKT cells in only C57BL/6 and a few related strains. Therefore, studies of NKT cells in NK1.1-negative strains are quite limited by the lack of markers that can reliably enumerate the population [1,2]. Earlier studies have also shown that NK1.1<sup>+</sup>CD4<sup>+</sup> T cells lose NK1.1 expression upon in vitro activation [18].

Several surrogate markers such as CD44, Ly6C, Ly49A and DX5 have been postulated. However, these molecules do not accurately encompass NKT cells in C57BL/6 mice and their specificity is difficult to determine [2]. An antibody (Ab) to V $\alpha$ 14<sup>+</sup> TCR [19] stained less than 20% of V $\alpha$ 14<sup>+</sup>J $\alpha$ 281<sup>+</sup>NK1.1<sup>+</sup> T cells in thymocytes and is no more used [6]. While tetrameric CD1d molecules loaded with  $\alpha$ -GalCer have been used recently to specifically detect V $\alpha$ 14 NKT cells [20,21], NKT cells detected with these tetramers might receive positive signals and become

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activated, making it difficult to ascertain the physiological role of NKT cells *in vivo*. Furthermore, NKT cells are heterogeneous and, regardless of NK1.1 expression, consist of CD1d dependent and independent subsets [22–27].

To overcome these limitations, we established the monoclonal antibody (mAb), U5A2-13, which selectively identifies populations similar to NK1.1<sup>+</sup> T cells in both NK1.1-positive and -negative mouse strains [28]. U5A2-13 mAb was originally obtained by immunizing a Fischer rat with tMK-2U lymphoma cells from a BALB/c nude mouse carrying a xenografted human inflammatory breast tumor [29]. We have shown that U5A2-13<sup>+</sup> T cells use an invariant TCR V $\beta$ 8, 7, or 2 predominantly and that, similar to NK1.1<sup>+</sup> T cells, U5A2-13<sup>+</sup> T cells can produce both IFN- $\gamma$  and IL-4 upon cross-linking with CD3 [30]. We also demonstrated that hepatic U5A2-13<sup>+</sup> T cells recognize the NKT cell ligand,  $\alpha$ -GalCer, presented by CD1d molecules on dendritic cells [31]. These results indicated that U5A2-13 mAb would be a valuable tool in the study of NKT cells in NK1.1-negative mouse strains.

However, the cell surface molecule recognized by U5A2-13 mAb was unknown. To elucidate the novel cell surface marker constitutively expressed on NKT cells, we molecularly cloned the antigen and identified a new epitope of ICAM-1 on NKT cells. The conformational epitope is composed of three loops located in extra-cellular domain two of ICAM-1.

## 2. Materials and methods

### 2.1. Mice

Specific pathogen-free female C57BL/6 wild-type mice (Charles River Japan, Inc., Kanagawa, Japan) and ICAM-1 mutant mice (tm1Bay and tm1Jcgr; Jackson Laboratory, Bar Harbor, ME) maintained in the animal facility of the National Cancer Center Research Institute were studied at 8–12 weeks of age. All animal experiments were conducted in accordance with protocols approved by the institutional review board.

### 2.2. Antibodies and reagents

FITC-conjugated mAbs specific for mouse CD3 (145-2C11) and ICAM-1 (3E2), PE-conjugated control rat IgG2a (R-35-95), PE-conjugated U5A2-13, PE-conjugated anti-ICAM-1 (3E2), PerCP-conjugated streptavidin, biotinylated anti-CD3 (145-2C11) and anti-mouse Fc $\gamma$  II/III receptor (2.4G2) were purchased from BD PharMingen (San Diego, CA) for flow cytometry. PE-conjugated anti-NK1.1 mAb, and PE-conjugated anti-rat IgG2a were obtained from Caltag (Burlingame, CA).

Anti-ICAM-1 Ab (goat polyclonal IgG, M-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and peroxidase-conjugated anti-goat IgG was obtained from

Sigma Aldrich (Saint Louis, MO) for Western blotting and immunoprecipitation.

### 2.3. Cell cultures

The mouse B cell leukemia cell line, BCL1, was cultured in RPMI 1640 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) medium supplemented with 10% heat inactivated FCS (JRH Biosciences, Lenexa, KS), 1 mM sodium pyruvate, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, 50  $\mu$ M 2-ME. COS-7 cells were maintained in DMEM supplemented with 10% heat inactivated FCS.

### 2.4. Construction of cDNA library

We isolated the gene encoding the antigen recognized by U5A2-13 mAb, using an expressional cloning strategy with COS-7 cells (African green monkey kidney cells expressing the T antigen of simian virus 40) [32]. The cloning source was the murine cell line BCL1, which was cultured in RPMI 1640 with 10% heat inactivated FCS. The cells were harvested for total RNA isolation using guanidinium thiocyanate. Poly(A)<sup>+</sup> RNA was selected by oligo(dT)-cellulose column chromatography and converted to double-stranded cDNA using oligo(dT) primers containing a *Not* I site. An *Eco*R I adapter was attached and cDNAs greater than 2.0 kb were fractionated by agarose gel electrophoresis, digested with *Not* I and ligated into the *Eco*R I/*Not* I-cleaved pME18S vector (simian virus 40-based mammalian expression vector; GenBank accession no. AB009864) [33]. Transfection into competent DH10B *E. coli* cells yielded approximately  $1.0 \times 10^6$  independent cDNA clones.

### 2.5. Screening the cDNA library

DH10B cells carrying the plasmids were fused to COS-7 cells as described by Seed and Aruffo [32]. Three days after fusion, COS-7 cells were detached from the plate by incubation with PBS containing 5 mM EDTA. Cells were collected by centrifugation and resuspended in staining buffer (SB) (PBS containing 5% FCS, 0.5 mM EDTA and 0.05% NaN<sub>3</sub>). PE-conjugated U5A2-13 mAb was added and the suspension was incubated at 4 °C for 30 min. The cells were washed three times and resuspended in SB. One percent of the most brightly stained cells were sorted using the fluorescence-activated cell sorter (FACS<sup>®</sup>) Vantage [34]. Plasmid DNA was recovered from the sorted cells and used to transform DH10B *E. coli* cells by electroporation. The transformants were cultured and spheroplast-fused with COS-7 cells for the next round of enrichment. Fourteen colonies were randomly selected after two rounds of enrichment and plasmid DNA was extracted from each colony. Plasmid DNAs purified from these 14 cDNA clones were individually transfected into COS-7 cells using Lipofectamin Plus<sup>®</sup> (Gibco Invitrogen, Carlsbad, CA) and analyzed by flow cytometry. Only one transfectant among the cDNA

clones, BLV-13, bound USA2-13 mAb but not control rat IgG2a.

## 2.6. DNA sequencing

Both strands of the BLV-13 cDNA clone with an insert of 2.8 kb were sequenced with synthetic oligonucleotide primers and analyzed with an automated fluorescent DNA sequencer (Applied Biosystems, Foster City, CA). Nucleotides and deduced amino acids were analyzed by comparison with the GenBank database.

## 2.7. Southern blotting

Plasmid DNA (100 ng) purified from each screened cDNA library was digested with *EcoR I/Not I*, resolved by electrophoresis and blotted onto nitrocellulose membranes by alkaline transfer. A hybridization probe of 2.8 kb prepared by PCR using 100 pg BLV-13 cDNA as a template was labeled and detected using the commercially available DIG High Prime DNA Labeling and Detection Starter Kit<sup>®</sup> (Roche; Mannheim, Germany), according to the manufacturer's instructions.

## 2.8. Western blotting, cell surface biotinylation and immunoprecipitation

The expression of ICAM-1 in COS-7 cells was assessed via immunoblot assay as described [35]. Cell surface proteins of the transfected COS-7 cells were biotinylated using sulfo-succinimidobiotin [36]. After three washes in PBS, cells were suspended at a density of  $25 \times 10^6$ /ml in 0.9% NaCl with 0.01 M HEPES (pH 8.0) and 0.5 mg/ml of Sulfo-NHS-biotin<sup>®</sup> (Pierce, Rockford, IL) and rocked for 30 min at room temperature. The reaction was terminated by adding 1 M Tris-HCl (pH 7.5) and incubated for an additional 15 min. After centrifugation, cells were disrupted in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 1% Triton X-100 in the presence of the protease inhibitors aprotinin (2 µg/ml), leupeptin (2 µg/ml) and phenylmethylsulfonyl fluoride (PMSF) (0.57 mM) at 4 °C for 30 min with occasional mixing. After centrifugation, the supernatant served as the cell lysate. The cell lysate was clarified by an incubation with protein G-Sepharose<sup>®</sup> (Pharmacia, Peapack, NJ), immunoprecipitated with USA2-13 mAb and anti-ICAM-1 polyclonal Ab for 2 h, then incubated again with protein G-Sepharose for 1 h. After five washes with lysis buffer, protein was eluted directly by boiling for 5 min in SDS sample buffer. The immunoprecipitates were resolved by 7.5% of SDS-PAGE and electrophoretically transferred onto Immobilon<sup>®</sup> membranes (Millipore, Bedford, MA). Biotinylated protein was detected using avidin and biotinylated horseradish peroxidase macromolecular complex (Vector, Burlingame, CA) and an Enhanced Chemiluminescence (ECL<sup>®</sup>) kit (Amersham, Aylesbury, UK).

## 2.9. Cell preparations and flow cytometry

Hepatic mononuclear cells (MNCs) were prepared as described [30]. The liver was removed, pressed through 200 µm gauge stainless steel mesh and suspended in RPMI 1640 containing 5% heat inactivated FCS. After washing with the same medium, the cells were resuspended in 30% Percoll<sup>®</sup> (Amersham Pharmacia Biotech, Uppsala, Sweden) and 65 U/ml heparin and centrifuged at  $750 \times g$  for 15 min at room temperature. The cell pellet was collected and erythrocytes in the hepatic MNC suspension were removed using 0.83% ammonium chloride-Tris buffer. The remaining hepatic MNCs were washed in RPMI 1640 and suspended in SB. Except for when anti-rat IgG-PE was used, the cells were incubated beforehand with Fc-block reagent (2.4G2, anti-CD16/32, BD PharMingen). Hepatic MNCs ( $1 \times 10^6$ ) were incubated with FITC or PE conjugated mAbs for 30 min at 4 °C, then washed and suspended in SB for analysis using a FACS Calibur<sup>®</sup> and Cell Quest<sup>®</sup> software (Becton Dickinson, Mountain View, CA). Except for three-color examinations, cells were gated with propidium iodide (PI). Indirect staining proceeded as described [30].

## 2.10. Epitope mapping

Peptides were synthesized to include all overlapping linear 30-mers covering the whole mouse ICAM-1 sequence. We also designed peptides in which loops of the five domains were combined in all kinds of orientations, re-creating discontinuous regions of the Ig-like domains. The binding activities of USA2-13 mAb to these peptides were measured by Pepsican Systems (Lelystad, The Netherlands). Briefly, 2230 overlapping 30-mers, off-set one by one, were synthesized and screened using credit-card format mini-PEPSCAN cards (455 peptide format/card) as described previously [37–39]. The binding of antibody to each peptide was tested in a PEPSCAN-based enzyme-linked immuno assay (ELISA). The 455-well credit-card format polyethylene cards, containing the covalently linked peptides, were incubated with antibody USA2-13 (10 µg/ml, diluted in blocking solution which contains 5% horse-serum (v/v) and 5% ovalbumin (w/v) and 0.05% Tween 20) (4 °C, overnight). After washing the peptides were incubated with anti-rat IgG horseradish peroxidase (dilution 1/1000 in blocking solution) (1 h, 25 °C), and subsequently, after washing the peroxidase substrate 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) and 2 ul/ml 3% H<sub>2</sub>O<sub>2</sub> were added. After 1 h the color development was measured. The color development of the ELISA was quantified with a CCD-camera and an image processing system. The setup consists of a CCD-camera and a 55 mm lens (Sony CCD Video Camera XC-77RR, Nikon micro-nikkor 55 mm f/2.8 lens), a camera adaptor (Sony Camera adaptor DC-77RR) and the Image Processing Software package Optimas, version 6.5 (Media Cybernetics,