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

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ABSTRACT

Adult T-cell leukemia (ATL) caused by human T-cell leukemia virus type 1 (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 HLA-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 1 (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 autologous 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-leukemia (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
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 corresponding HLA-identical sibling donors, #36, D07, D11, and #98, respectively, donated peripheral blood samples under written informed consent. The patients were participating 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/m^2^ 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 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 10^6 /ml phytohemagglutinin (PHA)-P (Sigma, St. Louis, MO) after depletion of CD8^-^ cells using a Dynabeads M450-CD8 (Dynal, Oslo, Norway) and then maintained in RPMI 1640 (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 autologous 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^-^ cells were analyzed in cytokine production assays (CTLA, IL-2, and IFN-γ). To evaluate the immunotherapeutic efficacy of the generated CTLs, 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 autologous 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^-^ cells of donor #36 were also used in some experiments. In the other ATL cases tested (patients R07, R11, and #97), PHA-stimulated CD8^-^ cells were tested from post-HSCT.

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

<table>
<thead>
<tr>
<th>Patient’s ID</th>
<th>HSCT case</th>
<th>Donor/Recipient</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Status</th>
<th>HTLV-I^ plus infection</th>
<th>HLA</th>
<th>In vitro immune analysis of PBMCs^b</th>
<th>HTLV-I proviral DNA (copies/1000 PBMC)^c</th>
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<tbody>
<tr>
<td>#36</td>
<td>Case 1</td>
<td>Donor</td>
<td>57 M</td>
<td>Healthy</td>
<td>+</td>
<td>A2/-, Cw1/-, DR5/-</td>
<td>0</td>
<td>IRT-#37</td>
<td>Undetectable</td>
</tr>
<tr>
<td>#37</td>
<td>Case 1</td>
<td>Recipient</td>
<td>63 M</td>
<td>Acute ATL</td>
<td>+</td>
<td>A2/−, B46/−, Cw1/−, DR5/−</td>
<td>183</td>
<td>IRT-#37</td>
<td>1150.3 N.T.</td>
</tr>
<tr>
<td>D07</td>
<td>Case 2</td>
<td>Donor</td>
<td>48 F</td>
<td>Healthy</td>
<td>−</td>
<td>A2/−, B35/B60, DRA/−, Cw3/−</td>
<td>N.T.</td>
<td>N.T.</td>
<td>Complete remission for more than 24 months</td>
</tr>
<tr>
<td>R07</td>
<td>Case 2</td>
<td>Recipient</td>
<td>51 M</td>
<td>Acute ATL</td>
<td>+</td>
<td>A2/−, B35/B60, DRA/−, Cw3/−</td>
<td>255</td>
<td>IRT-#17</td>
<td>26.7 N.T.</td>
</tr>
<tr>
<td>D11</td>
<td>Case 3</td>
<td>Donor</td>
<td>52 F</td>
<td>Healthy</td>
<td>−</td>
<td>A2/−, B35/B61, DRA/−, Cw3/−</td>
<td>N.T.</td>
<td>N.T.</td>
<td>Complete remission for more than 23 months</td>
</tr>
<tr>
<td>R11</td>
<td>Case 3</td>
<td>Recipient</td>
<td>54 M</td>
<td>Acute ATL</td>
<td>+</td>
<td>A2/−, B35/B61, DRA/−, Cw3/−</td>
<td>153</td>
<td>IRT-#17</td>
<td>440.8 N.T.</td>
</tr>
<tr>
<td>#98</td>
<td>Case 4</td>
<td>Donor</td>
<td>61 F</td>
<td>Healthy</td>
<td>−</td>
<td>A2/−, B51/−, DRA/−, Cw3/−</td>
<td>N.T.</td>
<td>N.T.</td>
<td>Relapse of lymphoma at 6 months after HSCT</td>
</tr>
<tr>
<td>#97</td>
<td>Case 4</td>
<td>Recipient</td>
<td>66 F</td>
<td>Acute ATL</td>
<td>+</td>
<td>A2/−, B51/−, DRA/−, Cw3/−</td>
<td>104</td>
<td>IRT-#97</td>
<td>3297.2 N.T.</td>
</tr>
</tbody>
</table>

^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 IRT cells established before HSCT, as described in the “Materials and Methods.”

^c: Culture in which CTL specific for autologous IRT 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), 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 μl of supernatant were collected to determine 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 μM 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-p27X (41) containing HTLV-I-pX genes and WR-HA without the HTLV-I gene were kindly provided by Dr. Hisatoki Shida (Hokkaido University, Sapporo, Japan). The recombinant vaccinia virus-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 (MAIPS4510, Millipore, Bedford, MA) was coated overnight at 4°C with 100 μl of 15 μg/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 μg/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 μl of 1 μg/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 × 10⁷) were stained for 30 min at 4°C with Cy-Chrome-conjugated mouse antihuman CD8 mAb (BD Pharmingen) and 7-B6-1 biotin (Mabtech) in 0.1 M carbonate buffer (pH 9.6; Sigma). After six washes with PBS, cells that were cultured 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, were shown in Fig. 1. 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...
periodical stimulations with formalin-fixed ILT-#37 cells, and their IFN-γ/H9253 and LCL-As (H17003 and HLA-mismatched ILT-As-2 (H12135) in), and LCL-Kan (pre-HSCT patient #37 (H11003), HLA-A2- and B46-matched TCL-Kan (A), and donor #36 (E) and donor #36 (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.

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 patient #37. This indicated that the main target antigens of these CTL were those preferably expressed on ILT-#37 as well as ILT-#37 cells (Fig. 2C). Specificity of cytotoxicity against ILT-#37 was 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. 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 targets 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 (□), ILT-#37 (X), and PHA-activated PBMCs of pre-HSCT patient #37 (X). Specificity of cytotoxicity against 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.

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**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 μm 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 31Cr-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 (MAHFPG-GQSSLFGYPVVVFPGDCV) and Tax 11-19 (LGLFYPVVY) 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 1–21 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-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 cytolyis 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.
Proportion of peripheral Tax11-19-specific CTL in the PBMCs of patients R07, R11, and patient #97 is shown in Fig. 5. Of the panel of 15 24-mer oligopeptides of Tax mapping of the CTL from post-HSCT patient R07 was performed. The result was significant higher in post-HSCT PBMCs than in pre-HSCT PBMCs after overnight incubation with various target cells including HTLV-I-specific CTL response to selective Tax epitopes was induced as predicted by a computer program, Tax 301-315 (SFHSLHLLFEEYTNI) and Tax 301-309 (SFHSLHLLFEEYTNI) were selectively reacted with the responder cells. These observations 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. 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 Tax11-19-specific CTL response

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 patient #97

<table>
<thead>
<tr>
<th>Patient ID (days after HSCT)</th>
<th>PBMC culture period</th>
<th>Stimulator</th>
<th>Cell lines</th>
<th>TaxLV-I antigen</th>
<th>Shared HLA alleles with patient</th>
<th>IFN-γ (pg/ml)a</th>
<th>Cytolysis (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>R07 (+255)</td>
<td>Day 21</td>
<td>ILT-R07</td>
<td>IRT-R07</td>
<td>Positive</td>
<td>Identical</td>
<td>1090 ± 67</td>
<td>N.T.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ILT-As-2</td>
<td>Positive</td>
<td>A24</td>
<td>866 ± 57</td>
<td>160 ± 7</td>
<td>N.T.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TOX</td>
<td>Negative</td>
<td>A24</td>
<td>36 ± 12</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ILT-R07</td>
<td>Positive</td>
<td>Identical</td>
<td>778 ± 55</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ILT-Mly-3</td>
<td>Positive</td>
<td>A24, B60, DR4</td>
<td>355 ± 5</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TOX</td>
<td>Negative</td>
<td>A24</td>
<td>62 ± 9</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>Positive</td>
<td>A24</td>
<td>25 ± 19</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td>Day 24</td>
<td>ILT-R07</td>
<td>ILT-R07</td>
<td>Positive</td>
<td>Identical</td>
<td>39 ± 9</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ILT-As-2</td>
<td>Positive</td>
<td>A24</td>
<td>48 ± 5</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TOX</td>
<td>Negative</td>
<td>A24</td>
<td>5 ± 6</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TOX/kp27Xc</td>
<td>pX products</td>
<td>A24</td>
<td>27 ± 11</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TOX/kpEnvd</td>
<td>Envelope</td>
<td>A24</td>
<td>11 ± 4</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TOX/kpGagf</td>
<td>Core</td>
<td>A24</td>
<td>6 ± 10</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td>R11 (+153)</td>
<td>Day 25</td>
<td>ILT-R11</td>
<td>ILT-R11</td>
<td>Positive</td>
<td>Identical</td>
<td>11 ± 1</td>
<td>N.T.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ILT-Nkx-2</td>
<td>Positive</td>
<td>A2, A26</td>
<td>12 ± 2</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LCL-Nkx</td>
<td>Negative</td>
<td>A2, A26</td>
<td>19 ± 6</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>Negative</td>
<td>A24</td>
<td>14 ± 0</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td>#97 (+104)</td>
<td>Day 21</td>
<td>ILT-#97</td>
<td>ILT-#97</td>
<td>Positive</td>
<td>Identical</td>
<td>477 ± 34</td>
<td>N.T.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ILT-Har</td>
<td>Positive</td>
<td>A2, B51, DR4</td>
<td>415 ± 2</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LCL-Har</td>
<td>Negative</td>
<td>A2, B51, DR4</td>
<td>104 ± 15</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>Positive</td>
<td>A24</td>
<td>63 ± 4</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>Positive</td>
<td>A24</td>
<td>63 ± 4</td>
<td>N.T.</td>
<td></td>
</tr>
<tr>
<td>Day 50</td>
<td>ILT-#97</td>
<td>ILT-#97</td>
<td>ILT-#97</td>
<td>Positive</td>
<td>Identical</td>
<td>128 ± 36</td>
<td>29 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ILT-Har</td>
<td>Positive</td>
<td>A2, B51, DR4</td>
<td>111 ± 10</td>
<td>23 ± 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LCL-Har</td>
<td>Negative</td>
<td>A2, B51, DR4</td>
<td>10 ± 1</td>
<td>16 ± 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>Positive</td>
<td>A24</td>
<td>1 ± 0</td>
<td>N.T.</td>
<td></td>
</tr>
</tbody>
</table>

a PBMCs isolated from patients R07, R11, and #97 were cultured for the indicated periods with stimulations from 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 Specific cytolyis at an E:T ratio of 20 measured by 6-h 51Cr-release assays was shown as the mean ± SD of duplicate determinations.
c TOK cells infected with recombinant vaccinia viruses expressing HTLV-I pX products (TOK/kp27X), envelope (TOK/kpEnv), and core (TOK/kpGag) antigens were used as targets.
d Tax-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).

d N.T., not tested.

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 (SFHSLHLLFEETYNIT) 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 chimera 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.
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 (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-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...
(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


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

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