Single-Cell Analysis of T-Cell Receptor Repertoire of HTLV-1 Tax-Specific Cytotoxic T Cells in Allogeneic Transplant Recipients with Adult T-Cell Leukemia/Lymphoma

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Abstract

Adult T-cell leukemia (ATL) is a lymphoproliferative malignancy associated with human T-cell lymphotropic virus type 1 (HTLV-1) infection. Recently, it has been shown that allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for ATL, and that HTLV-1 Tax-specific CD8+ cytotoxic T cells (CTL) contribute to the graft-versus-ATL effect. In the present study, we, for the first time, analyzed the T-cell receptor (TCR) repertoire of isolated Tax301–309 (SFHSLHLLF)-specific CTLs in HLA-A*2402+ ATL patients before and after allo-HSCT by single-cell reverse transcription-PCR. The Tax301–309-specific CTLs in bone marrow and peripheral blood showed highly restricted oligoclonal diversity. In addition, a unique conserved amino acid motif of “P-D/P-R” in TCR-β complementarity-determining region 3 in either BV7- or BV18-expressing CTLs was observed not only in all of the samples from ATL patients, but also in samples from the same patient before and after HSCT. Furthermore, the P-D/P-R motif-bearing CTL clones established from peripheral blood samples after HSCT exhibited strong killing activity against the HTLV-1–infected T cells of the patient. CTL clones were not established in vitro from samples prior to allo-HSCT. In addition, CTL clones with a strong killing activity were enriched in vivo after HSCT in the patient. Hence, Tax301–309-specific CTLs in ATL patients might have a preference for TCR construction and induce strong immune responses against the HTLV-1–infected T cells of patients, which contribute to the graft-versus-ATL effects after allo-HSCT. However, further analyses with a larger number of patients and more frequent sampling after allo-HSCT is required to confirm these findings. Cancer Res; 70(15): 6181–92. ©2010 AACR.

Introduction

Adult T-cell leukemia (ATL) is an aggressive lymphoproliferative malignancy caused by infection with human T-cell lymphotropic virus type 1 (HTLV-1). Although most HTLV-1–infected individuals behave as asymptomatic carriers during their lifetime, a few develop ATL and show a lifterative malignancy caused by infection with human T-cell lymphotropic virus type 1 (HTLV-1) infection. Recently, it has been shown that allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for ATL, and that HTLV-1 Tax-specific CD8+ cytotoxic T cells (CTL) contribute to the graft-versus-ATL effect. In the present study, we, for the first time, analyzed the T-cell receptor (TCR) repertoire of isolated Tax301–309 (SFHSLHLLF)-specific CTLs in HLA-A*2402+ ATL patients before and after allo-HSCT by single-cell reverse transcription-PCR. The Tax301–309-specific CTLs in bone marrow and peripheral blood showed highly restricted oligoclonal diversity. In addition, a unique conserved amino acid motif of “P-D/P-R” in TCR-β complementarity-determining region 3 in either BV7- or BV18-expressing CTLs was observed not only in all of the samples from ATL patients, but also in samples from the same patient before and after HSCT. Furthermore, the P-D/P-R motif-bearing CTL clones established from peripheral blood samples after HSCT exhibited strong killing activity against the HTLV-1–infected T cells of the patient. CTL clones were not established in vitro from samples prior to allo-HSCT. In addition, CTL clones with a strong killing activity were enriched in vivo after HSCT in the patient. Hence, Tax301–309-specific CTLs in ATL patients might have a preference for TCR construction and induce strong immune responses against the HTLV-1–infected T cells of patients, which contribute to the graft-versus-ATL effects after allo-HSCT. However, further analyses with a larger number of patients and more frequent sampling after allo-HSCT is required to confirm these findings. Cancer Res; 70(15): 6181–92. ©2010 AACR.

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diversity, by using a specific peptide-loading HLA tetramer and a PCR technique, Bourcier and colleagues reported that A*0201 restricted Tax11-19 (LFGLYTVK)-specific CTLs in a patient with HTLV-1–associated myelopathy (HAM/TSP) exhibited diverse TCR usages with the conserved amino acid motif "PG-G" in the CDR3 of TCR-β (20). Saito and colleagues also reported that A*0201 restricted Tax11-19-specific CTLs in patients with HAM/TSP (21) and HTLV-1 polyomavirus (22) exhibited diverse TCR usages with the conserved amino acid motif "GLAG" in the CDR3 of TCR-β. In addition, without using HLA tetramer, Hara and colleagues reported that Tax-specific T cells in patients with HAM/TSP exhibited diverse TCR usages with the conserved amino acid motif “L-G(G)” in CDR3 of TCR-β (15). However, other investigators reported only diverse TCR usages of Tax11-19-specific CTLs without any conserved amino acid motifs in the CDR3 of TCR-β (17, 19, 23). Thus, Tax-specific CTLs in HLA-A*0201+ patients with HTLV-1–associated disease, especially HAM/TSP, have been shown to exhibit a more diverse TCR repertoire than those with some other retrovirus; e.g., HIV 1–specific CTLs (26) and several kinds of conserved amino acid motifs in CDR3 of TCR-β have been observed. Furthermore, the mechanism of TCR-MHC/Tax-peptide interaction has been more precisely understood by the analysis of the crystal structure of conserved CDR3 amino acid motif–bearing TCR specific CTL clones (27–29). However, these previous studies of TCR repertoire analyses did not include patients with ATL nor those with HLA-A*2402. Furthermore, the TCR repertoire was not analyzed at a single-cell level, which could not be avoided.

In this study, we investigated the TCR repertoire of HLA-A*2402–restricted Tax301-309-specific CTLs in ATL patients because even though A*2402 is the most common HLA-A allele in Japanese (~60%), the TCR features of HLA-A*2402–restricted Tax-specific CTLs have not been clarified. In addition, a single-cell reverse transcription-PCR (RT-PCR) technique for direct TCR gene amplification from each isolated tetramer+ cell was used to analyze the TCR repertoire of the Tax301-309-specific CTLs to avoid bias during in vitro culture or PCR. Consequently, we, for the first time, revealed the natural in vivo status of the TCR repertoire of Tax301-309-specific CTLs in HLA-A*2402+ ATL patients before and after allo-HSCT. In this study, we show that Tax301-309+ specific CTLs in ATL patients show a highly restricted TCR repertoire and a particular amino acid motif was conserved in TCR-β CDR3 among different ATL patients and also within the same patient before and after allo-HSCT.

Materials and Methods

Patients and cells

PB or bone marrow (BM) samples were obtained from four HLA-A*2402+ ATL patients (Pt-1, -2, -3, and -4) before and/or after HSCT. This study was approved by the Institutional Review Board of Jichi Medical University and written informed consent was obtained from all patients. The stem cell source was BM from an unrelated donor in Pt-1 and Pt-2, whereas Pt-3 and Pt-4 received PB graft from a family donor. HLA was matched for HLA-A, HLA-B, and HLA-DRB1 in all patients except that Pt-3 received HLA-B and HLA-DRB1 mismatched grafts. The conditioning regimen was the myeloablative regimen containing cyclophosphamide and total body irradiation in Pt-3, but other patients received fludarabine-based reduced-intensity regimens. Three patients (Pt-1, -2, and -3) were alive without relapse of ATL at 540, 583, and 291 days after HSCT, respectively, but Pt-4 died of graft-versus-host disease on day 120. Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells were isolated by Ficoll-Paque Plus (Pharmacia) and cryopreserved in liquid nitrogen until use. Samples obtained from Pt-1 were tested before and after HSCT, a sample obtained from Pt-3 was tested only before HSCT, and samples obtained from the two remaining patients (Pt-2 and Pt-4) were tested only after HSCT due to the limited availability of enough cells for the following analyses.

Tetramer staining and single cell sorting of Tax tetramer+ cells by flow cytometry

Cells were incubated with phycoerythrin-conjugated HLA-A*2402 HTLV-1 Tax301-309 peptide (SFHSLHLLFL) binding HLA tetramer (MBL, Tokyo, Japan) for 30 minutes at room temperature, and then stained with FITC- or PerCP-Cy5.5–conjugated murine anti-human CD3 and CD8 monoclonal antibodies (mAbs; BD Biosciences) for an additional 25 minutes on ice. After staining, cells were washed twice with PBS containing 5% fetal bovine serum, and individual CD3+ CD8+ Tax301-309 tetramer+ T cells (Tax tetramer+ cells) were directly single cell–sorted into PCR tubes or microplates using FACS Aria (BD Biosciences). Data were analyzed by FACS Diva software (BD Biosciences).

Direct TCR repertoire analysis of individual Tax tetramer+ cells

The TCR-β CDR3 amino acid sequence from each isolated Tax tetramer+ cell was directly analyzed after single-cell RT-PCR for TCR-β gene amplification as described previously (30). In brief, individual Tax tetramer+ cells were single cell–sorted into PCR tubes and complementary DNAs (cDNA) of TCR-β were synthesized by direct cell lysate followed by reverse transcription using a TCR-β gene constant region–specific primer. The synthesized cDNAs of TCR-β were used for two steps of semi-nested PCR using 24 kinds of TCR-β variable region (BV) gene family–specific primers and two kinds of TCR-β constant region–specific primers to identify the usage of the BV gene family in each Tax tetramer+ cell. After identifying the BV usage in the cells, we performed direct sequencing of the V-D-J CDR3 region of the T cells with BV gene family–specific primers. TCR-β CDR3 amino acid sequence sequences were analyzed from a total of 160 Tax tetramer+ cells from two patients (Pt-1, 50 cells; Pt-3, 110 cells) before HSCT and from a total of 282 tetramer+ cells from three patients (Pt-1, 80 cells; Pt-2, 102 cells; and Pt-4, 100 cells) after HSCT. In this study, TCR clones with a
frequency of >10% of all the analyzed cells of each sample were arbitrarily defined as "preferred repertoires" of the sample.

Recipient/donor chimerism of Tax tetramer+ cells after HSCT

Genomic DNAs from approximately 50 and 400 sorted tetramer+ cells, respectively, in the PBMCs of two patients (Pt-1 and Pt-2) after HSCT were collected using a QIA Amp DNA Micro kit (Qiagen). PCR short tandem repeat (STR) polymorphism analysis was performed to evaluate the donor/recipient chimerism of Tax tetramer+ cells using a PowerPlex 16 system (Promega) according to the instructions of the manufacturer on an ABI 3100 genetic analyzer (Applied Biosystems). Data analysis was performed using GeneMapper v4.0 software (Applied Biosystems).

Induction of an HTLV-1–infected T-cell line

An interleukin 2–dependent HTLV-1–infected T-cell line was induced as described previously (12). In brief, the CD8-negative fraction in PBMCs of Pt-1 before HSCT was enriched by magnetic-activated cell sorting (Miltenyi Biotec) and cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (Sigma), 10 units/mL of interleukin 2 (Shionogi), and 1 μg/mL of PHA-P (Sigma) for ~2 months. First, the induced HTLV-1–infected T-cell line was stained with PerCP-conjugated anti-human CD3 (BD Biosciences), APC-conjugated anti-human CD4 (BD Pharmingen), FITC-conjugated anti-human CD70, and phycoerythrin-conjugated anti-human CCR4 mAbs (BD PharMingen). Second, infection of the cells by HTLV-1 virus was checked by RT-PCR using extracted total RNA from the cells. The PCR primers used were RPX3 (5′-ATCCCCGTG-GAGACTCCTCAA-3′), RPX4 (5′-AACACGTAGACTGGG-TATCC-3′), RENV1 (5′-AGCGCGGTTGAGTCGCGTTCT-3′), and RENV4 (5′-CAGCGGAGATGAGGGGCAGA-3′). The PCR primers and PCR cycle conditions were described previously (31). RPX3 and RPX4 were used for the amplification of 145 bp fragments of Tax/rev mRNA, and RENV1 and RENV4 were used for the amplification of 316 bp fragments of env mRNA. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also amplified by GAPDH1A (5′-CCCAGTCACCATCTTCCAGGAG-3′) and GAPDH1S (5′-CCTTTTGTTGCTATACCCAGGA-3′), and the primer sets for GAPDH mRNA amplified 740 bp fragments.

Induction of Tax301–309 tetramer+ CTL clones

Tax tetramer+ cells in PBMCs of Pt-1 at day 196 after HSCT were seeded at one cell per well in round-bottomed microplates using FACS Aria, and the sorted individual Tax tetramer+ cells were stimulated weekly by the addition of feeder cells and peptide-pulsed EBV-B cells. After expansion, the cells were analyzed for their reaction to Tax tetramer, and the CD3+ sequences of their TCR-β chain were analyzed by RT-PCR. Finally, four kinds of Tax tetramer+ CTL clones were induced (clones 4-5B, 1-3C, 2-5G, and 1-11E).

HLA-A*2402/Tax301–309 tetramer binding assay of Tax tetramer+ CTL clones

The specific binding of HLA-A*2402/Tax301–309 tetramer of the four Tax tetramer+ CTL clones (4-5B, 1-3C, 2-5G, and 1-11E) was evaluated in terms of the mean fluorescence intensity of tetramer-phycoerythrin on the cell surfaces after staining with the tetramer at dilutions of 1:100, 1:50, and 1:10 for 30 minutes at room temperature. As a control, the expression level of TCR-α/β on the cell surface was also evaluated by phycoerythrin-conjugated TCR-α/β mAbs (BD Pharmingen) at 1:100 density of the mAbs.

IFN-γ production of Tax tetramer+ CTL clones

IFN-γ production by each of the four Tax tetramer+ CTL clones (4-5B, 1-3C, 2-5G, and 1-11E) after 24 hours of coculture with 1 × 10^5 target cells (HTLV-1–infected T-cell line or HLA-A*2402+ EBV-B cells) at 37°C was measured by ELISA for human IFN-γ (OptiELA kit, BD Biosciences) according to the protocols of the manufacturer. The CTL clone 1-3C was also cocultured with HLA-A*2402–restricted Tax301–309 peptide or HIV env gp160 (RYLRDQQLL)–pulsed EBV-B cells.

Cytotoxicity of Tax tetramer+ CTL clones

The cytotoxicity of Tax tetramer+ CTL clones was measured by FACScalibur (BD Biosciences) using an IMMUNOCYTO Cytotoxicity Detection Kit (MBL). Induced Tax tetramer+ CTL clones (4-5B, 1-3C, 2-5G, and 1-11E) were cocultured with 2 × 10^6 carboxyfluorescein diacetate succinimidyl ester–labeled target cells (HTLV-1–infected T-cell line, Tax301–309 peptide–pulsed or irrelevant HIV env gp160 peptide-pulsed HLA-A*2402 EBV-B cells) at various effector/target ratios at 37°C for 5 hours. The specific killing activity of the CTL clones against each target was evaluated in terms of the expression of Annexin V on the surface of target cells after coculture (32), and calculated as [(experimental Annexin V expression on the surface of target cells – spontaneous Annexin V expression on the surface of target cells) / (100 – spontaneous Annexin V expression on the surface of target cells)] × 100%.

Statistical analysis

The continuous variables were compared using one-way ANOVA or t test. Two-sided P < 0.05 values were considered significant.

Results

Experimental efficiency of TCR analysis for a single Tax tetramer+ cell

The efficiencies of direct TCR-β gene amplification at the single-cell level for individual Tax tetramer+ cells in each sample were 78.1% (50 of 64) in Pt-1 before HSCT, 76.2% (80 of 105) in Pt-1 after HSCT, 71.8% (102 of 142) in Pt-2 after

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HSCT, 88.7% (110 of 124) in Pt-3 before HSCT, and 80.6% (100 of 124) in Pt-4 after HSCT. To reflect the natural in vivo status of the TCR repertoire of the Tax tetramer+ cells in each sample, TCR-β gene amplification with high efficiency was required. As a result, the mean experimental efficiency of five samples from four patients was sufficient (~80%). Therefore, we considered that these experiments reflected the in vivo TCR repertoire of Tax tetramer+ cells of the patients at the sampling points.

**Oligoclonal diversity and a conserved amino acid motif in TCR-β CDR3 of Tax tetramer+ cells before HSCT**

BM samples from two patients (Pt-1 and Pt-3) were used for the single-cell analysis of the TCR repertoire of Tax tetramer+ cells before HSCT because Tax tetramer+ cells were more frequently observed in BM than in PB samples. As shown in Fig. 1 (left), we detected only a limited number of Tax tetramer+ cells in the BM of Pt-1 (0.08% in CD8+ T cells), whereas Pt-3 showed a large number of Tax tetramer+ cells in BM (2.1% in CD8+ T cells). Table 1 summarizes the TCR-β CDR3 amino acid sequence information of detected CTL clones (TCR clones) in the analyzed Tax tetramer+ cells from the two patients.

Pt-1 showed oligoclonal diversity consisting of a total of 17 kinds of TCR clones and three kinds of preferred repertoire in a total of 50 analyzed cells. Eight BV gene families were used (BV 4, 5, 6, 7, 10, 12, 20, and 23). Eight of the 17 TCR clones (35 of 50, 70.0% of analyzed tetramer+ cells) used the BV7 gene family, and the three preferred repertoires (TCR-clones 7, 11, and 13) exclusively used the BV7 gene family.

Pt-3 also showed oligoclonal diversity of Tax tetramer+ cells consisting of a total of 14 kinds of TCR clones and

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**Figure 1.** Identification of Tax301–309 peptide-specific CTLs. Tax301–309 peptide-specific CTLs (Tax tetramer+ cells) in bone marrow mononuclear cells before HSCT (left column) and peripheral blood mononuclear cells after HSCT (right column) of four ATL patients. Tax tetramer+ cells were defined as CD8- and HLA-A24/Tax301–309 tetramer-positive cells in the CD3-positive lymphocyte population. The numbers in the top-right corners of the dot plots represent the percentage of Tax tetramer+ cells in CD8+ T cells. Individual Tax tetramer+ cells were single cell–sorted and subjected to RT-PCR for TCR-β gene amplification. These procedures were performed only once. N.T., not tested.
<table>
<thead>
<tr>
<th>Pt/sample</th>
<th>Clone ID</th>
<th>BV</th>
<th>CDR3 amino acid sequences</th>
<th>BJ</th>
<th>Frequency (%)</th>
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<td>Pt-1/BM, before</td>
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<td>TRBJ2-3</td>
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NOTE: TCR-β CDR3 amino acid sequences of individual HLA-A*2402-Tax301–309 tetramer+ CTL clones (TCR clones) of two ATL patients before HSCT. Bold type indicates CDR3 amino acid sequences of the dominantly existing TCR-clones preferred repertoire in the sample (>10% among analysis cells in each analyzed sample). Thick underlined type represents conserved CDR3 amino acid sequence, which is PDR-motif or PPR-motif in CDR3 region of BV7 or BV18 bearing TCR clones of each patient. Underlined type represents a conserved amino acid sequence, which has a -DR motif in the CDR3 region of TCR clones of each patient.
two kinds of preferred repertoire in a total of 110 analyzed cells. Only three BV gene families were used (BV 7, 18, and 19). The BV7 gene family was the most frequently observed in Pt-3 (as in Pt-1), and 9 of the 14 kinds of TCR clones (91 of 110, 82.7% of the analyzed tetramer+ cells) used the BV7 gene family. TCR-clones 3 and 5, which were considered to be part of the preferred repertoire, also used a BV7-9 gene segment. In addition, the BV18 gene family was also frequently used by 4 of the 14 TCR clones (18 of 110, 16.4% of analyzed tetramer+ cells), although we did not observe any preferred repertoire in them. Interestingly, when CDR3 amino acid sequences of TCR clones of Pt-3 were compared, 8 of the 14 kinds of TCR clones commonly expressed a particular CDR3 amino acid motif “P-D/P-R” at position 108 to 110, with either the BV7 or BV18 gene family (thick underlining in Table 1) and 3 of the remaining 6 kinds of TCR clones commonly expressed a ~DR motif at position 109 to 110 of TCR-β CDR3 (underlining in Table 1). Furthermore, the P-D/P-R motif was also observed in CDR3 of TCR-clone 10 and the preferred repertoire TCR-clone 11 of Pt-1.

The P-D/P-R amino acid motif in TCR-β CDR3 of Tax tetramer+ cells was conserved after HSCT

PB samples from three patients (Pt-1, -2, and -4) were used for single-cell analysis of the TCR repertoire of Tax tetramer+ cells after HSCT. Samples that were obtained when Tax tetramer+ cells became detectable for the first time after HSCT (Pt-1, day 175; Pt-2, day 260; and Pt-4, day 97) were chosen because we wanted to analyze the TCR repertoire of Tax tetramer+ cells before clonal selection in vivo. Therefore, the frequencies of Tax tetramer+ cells were very limited (0.03–0.09% in CD8+ T cells; Fig. 1, right). TCR-β CDR3 amino acid sequences (TCR clones) in analyzed Tax tetramer+ cells from these three patients after HSCT are shown in Table 2.

Pt-1 showed highly restricted oligoclonal diversity consisting of a total of six kinds of TCR clones and two kinds of preferred repertoire in a total of 80 analyzed cells. Five BV gene families were used (BV 3, 7, 9, 12, and 30) without bias. Surprisingly, the preferred repertoire TCR-clone 2 that existed at position 108 to 110 of CDR3, and another preferred repertoire TCR-clone 4 (11 of 100, 11.0% of analyzed tetramer+ cells), and the remaining TCR clones (TCR-clones 2, 3, 5, and 6) were detected at very low frequencies (1 of 100, 1.0%). The preferred repertoire TCR-clone 4 also used the BV7-9 gene segment and had a PDR motif at position 108 to 110 of CDR3, as observed in the other patients.

Chimerism of Tax tetramer+ cells after HSCT

To evaluate the donor/recipient chimerism of Tax tetramer+ cells at the time of TCR analysis after HSCT, PCR amplification for amelogenin, which displays a 106-base X-specific band and a 112-base Y-specific band, was performed in Pt-1 (on day 175) because of a donor/recipient sex mismatch, and FGA and TPOX loci STR PCR amplification was performed in Pt-2 (on day 260) because of a donor/recipient sex match. Chimerism in Pt-4 could not be analyzed because samples were not available before/after HSCT. The Tax tetramer+ cells of both patients after HSCT showed complete donor type chimerism (Fig. 2). This observation strongly implied that Tax tetramer+ TCR clones, including those bearing a P-D/P-R motif, that were detected after HSCT, might be newly activated/expanded from infused donor-naïve T cells or derived from donors’ hematopoietic stem cells, but not from the remaining recipient Tax tetramer+ cells.

HTLV-1–infected T-cell recognition of Tax tetramer+ CTL clones

An HTLV-1–infected T-cell line was induced from the PBMCs of Pt-1 before HSCT as a representative case. The cells fully expressed CD3, CD4, CD70 (33), and chemokine receptor CCR4 (34) molecules on the cell surfaces, whereas donor lymphocytes expressed CCR4 at low levels and did not express CD70 molecules (Fig. 3A). Additionally, induced T-cell lines were shown to be infected with HTLV-1 by RT-PCR for tax/rex mRNA and env mRNA (Fig. 3B).

To address whether Tax tetramer+ CTL clones in ATL patients after HSCT had sufficient cytotoxicity against the HTLV-1–infected T cells of a patient, we performed a functional assay for four kinds of the Tax tetramer+ CTL clones (4-5B, 1-3C and 2-5G, and 1-11E) induced from the PB sample of Pt-1 obtained on day 196 after HSCT. The CTL clones 4-5B,
Table 2. TCR-β CDR3 amino acid sequences and frequencies of Tax_{301–309} tetramer^+ CTL clones after HSCT

<table>
<thead>
<tr>
<th>Pt/sample</th>
<th>Clone ID</th>
<th>BV</th>
<th>CDR3 amino acid sequences</th>
<th>BJ</th>
<th>Frequency (%)</th>
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</thead>
<tbody>
<tr>
<td>Pt 1/PB, after TCR-clone 1</td>
<td>TRBV3-1</td>
<td>CAS</td>
<td>S Q R G E N T E A F F</td>
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<td>TCR-clone 2</td>
<td>TRBV7-9</td>
<td>CAS</td>
<td>S P D R E Q T Q Y F</td>
<td>TRBJ2-5</td>
<td>8/80 (10.0)</td>
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<tr>
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<td>TRBV9</td>
<td>CAS</td>
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<td>TRBJ2-1</td>
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</tr>
<tr>
<td>TCR-clone 4</td>
<td>TRBV9</td>
<td>CAS</td>
<td>V G F S A N Y G Y T F</td>
<td>TRBJ1-2</td>
<td>3/80 (3.8)</td>
</tr>
<tr>
<td>TCR-clone 5</td>
<td>TRBV12-3</td>
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<td>TCR-clone 6</td>
<td>TRBV30</td>
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<td>60/80 (75.0)</td>
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<tr>
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NOTE: TCR-β CDR3 amino acid sequences of individual HLA-A*2402-Tax_{301–309} tetramer^+ CTL clones (TCR clones) of three ATL patients after HSCT. Thick underlined and underlined type, see details in Table 1.
1-3C, and 2-5G corresponded to TCR-clones 1, 2, and 6, respectively, of Pt-1 after HSCT (Table 2), but 1-11E (TCR-clone X, BV7-9, BJ2-1, and CDR3 amino acid V-D-J sequence: CASSSGTSGGMHYNEQFF) did not correspond with any of the TCR clones of Pt-1 in Table 2. Therefore, only clone 1-3C had the PDR motif, whereas clone 2-5G had the -DR motif.

These four kinds of CTL clones showed three different binding properties with the Tax tetramer reagents at various densities (Fig. 4A). The PDR motif–bearing clone (1-3C) and 1-11E showed the strongest binding of the tetramer reagents at all densities of tetramer reagents. 2-5G showed moderate binding of the tetramer reagents. In contrast, 4-5B showed weak binding of the tetramer reagents.

Figure 4B shows the IFN-γ production by each CTL clone after 24 hours of coculture with the HTLV-1–infected T cells of patients. All of the CTL clones showed the specific production of IFN-γ against the patient’s HTLV-1–infected T cells. In particular, 1-3C and 2-5G strongly produced IFN-γ against the patient’s HTLV-1–infected T cells. In addition, 1-3C also specifically produced IFN-γ against Tax301–309 peptide–pulsed EBV-B cells (Fig. 4C).

Finally, these CTL clones, especially 1-3C and 2-5G, showed specific and sufficient killing activity against not only Tax301–309 peptide–pulsed EBV-B cells but also the patient’s HTLV-1–infected T cells (Fig. 4D).

These results strongly suggested that Tax tetramer+ CTL clones, especially those which used a P-D/P-R motif, in the PB of ATL patients after allo-HSCT could exert strong killing activity against HTLV-1–infected cells. We intensively tried to induce CTL clones from Tax tetramer+ cells of Pt-1 and Pt-3 before HSCT, but failed. Therefore, we could not compare the functional activities of Tax tetramer+ cells before and after HCST.

Discussion

Recent studies on human CTLs specific for viral (35–37) or tumor antigens (38) have suggested that it is important to evaluate the quality rather than the quantity (frequency) of CTLs to determine the in vivo efficiency of CTLs. In this study, to evaluate the quality of CTLs specific for HTLV-1 Tax antigen in ATL patients undergoing allo-HSCT, we, for the first time, tried to analyze the TCR repertoire of the HLA-A*2402–restricted HTLV-1 Tax301–309 peptide–specific CTLs in PB or BM samples from ATL patients at a single-cell level before and after allo-HSCT.

To date, numerous studies on the TCR repertoire of antigen-specific T cells have been performed by conventional analysis methods such as FACS-based methods with a panel of mAbs directed against TCR-BV gene family products (18) or PCR-based methods with a panel of TCR-BV–specific primers (15, 21, 24) and immunoscope profiles of the CDR3 length distribution (19). Especially for PCR-based methods, they often used whole lymphocytes (15) or bulky cells [isolated CD8+ T cells (refs. 19, 24), antigen-specific tetramer+ cells (refs. 21, 22)] and antigen-specific tetramer+ cells expanded by in vitro culture (20) as samples. Recently, however, several investigators have raised concerns about these experimental methods: (a) mAbs using a FACS-based analysis or CDR3 length-based molecular analysis of the TCR-BV repertoire does not allow for analysis of the TCR repertoire at the clonal...
level (30), (b) analyzed cells might not target antigen-specific T cells without the use of specific HLA tetramers, (c) in vitro culture might induce biases in the TCR repertoire by clonal selection affected by rapid or slow proliferation and high or low sensitivity to activation-induced cell death (39, 40), and (d) PCR using cDNAs derived from bulky cells might also induce biases due to intrinsic differences in the amplification efficiency of the templates (41). In this study, TCR-β CDR3 was directly sequenced using individually sorted Tax tetramer+ cells without in vitro culture because we aimed to analyze the TCR repertoire of HTLV-1 Tax301–309 peptide–specific CTLs in vivo without any technical bias in vitro. As a result, the experimental efficiency was sufficiently high, and technical biases could be minimized.

This sensitive analysis, using a single-cell RT-PCR technique, has provided novel findings regarding Tax-specific CTL responses in ATL patients before and after allo-HSCT. HLA-A*2402-restricted Tax301–309-specific CTLs exhibited a highly restricted TCR repertoire, especially after HSCT with two or three kinds of dominant TCR clones, regardless of the number of analyzed cells, and a particular amino acid motif P-D/P-R was conserved in TCR-β CDR3 of either BV7- or BV18-bearing Tax-specific CTL clones among unrelated ATL patients, and before and after HSCT in the same patient. The presence of a particular conserved amino acid motif in TCR-β CDR3 of Tax301–309 peptide–specific CTLs among unrelated individuals agreed with a similar finding in HLA-A*0201–restricted Tax11–19-specific CTLs in HAM/TSP patients (15, 20–22). We might have missed these findings if we had used conventional TCR analysis methods rather than single-cell analysis because the frequencies of some P-D/P-R motif-bearing T cells was relatively low.

Some recent studies on viral antigen–specific human T cells (42, 43) or CTL clones in patients with large granular lymphocyte leukemia (44) and paroxysmal nocturnal hemoglobinuria (45) showed the existence of TCR-β CDR3 amino acid sequences that were completely shared by T cells between unrelated individuals. A previous study on the TCR repertoire of HLA-A*0201–restricted Tax11–19-specific CTLs also showed the existence of a TCR-β CDR3 amino acid sequence that was completely shared between unrelated HAM/TSP patients, even though limited numbers of patients and T cell clones were analyzed (21). However, in this study, we did not find any TCR-β CDR3 amino acid sequences that were completely shared by HLA-A*2402–restricted Tax301–309-specific CTLs among unrelated ATL patients. Instead, a few very similar CDR3 amino acid sequences were observed in PDR motif–bearing preferred repertoires after HSCT, including TCR-clone 2 in Pt-1, TCR-clone 13 in Pt-2, and TCR-clone 4 in Pt-4. These clones expressed almost the same CDR3 amino acid sequences with only one different amino acid, E, V, or T at position 111, respectively (Table 2).

Furthermore, when we identified the “P-P/D-R” motif–bearing TCR clones before and after HSCT in the same patient, a question was raised as to whether the identified P-P/D-R motif–bearing CTLs after HSCT were derived from the

Figure 3. Induction of an HTLV-1–infected T-cell line. A, an HTLV-1–infected T-cell line induced from a peripheral blood sample of Pt-1 before HSCT fully expressed (left upper panel) CD3, CD4, (left lower panel) CD70, and chemokine receptor CCR4, whereas control lymphocytes from a peripheral blood sample of a healthy donor expressed (right upper panel) CD3, CD4, and (right lower panel) CCR4 at low levels and did not express CD70. B, infection by HTLV-1 was confirmed by the RT-PCR technique for fragments of tax/rex and env mRNA. As an internal control, GAPDH (740 bp) was amplified simultaneously. These procedures were performed only once.
To address this issue, we confirmed the origin of Tax tetramer+ cells after HSCT by a Tax tetramer+ cell-specific chimera analysis. The results showed that the Tax tetramer+ cells in ATL patients after HSCT were derived from the donor’s reconstituted T cells. Consistent with these results, Harashima and colleagues induced Tax-specific CTLs from an HLA-A*1101+ ATL patient at day 145 after HSCT and showed that the cells were derived from donor cells by STR polymorphisms (14). Our results strongly suggest that Tax301-309-specific TCRs of (at least) ATL patients exhibit selective pressure on V-D-J gene recombination of CDR3 of TCR-β, which results in the conservation of the P-P/D-R motif. However, at present, the biological mechanism is unknown. Furthermore, it is of interest whether the P-P/D-R motif-bearing Tax301-309-specific TCRs are more preferentially observed in asymptomatic carriers because we hypothesized that (a) selection of the P-P/D-R motif in CDR3 of TCR-β is independent of the disease status, and (b) proliferation of P-P/D-R motif-bearing CTLs might be depressed in the course of ATL development.

Cognate peptide/MHC complex recognition by TCRs is the initial event for CTL responses. After a suitable stimulation by TCRs in contact with antigen-presenting cells such as dendritic cells, CTLs are activated and expanded to attack the target cells. Based on the functional analysis of CTLs specific...
for virus antigens, recent studies have suggested that CTL populations in infected patients could be classified into two groups; "driver" CTLs that exert strong antiviral pressure with efficient lytic activity and "passenger" CTLs that exert only weak antiviral pressure with inefficient lytic activity (37, 46, 47). We considered that P-P/D-R motif-expressing CTL clones behaved as driver CTL clones in vivo because P-P/D-R motif-bearing CTLs (TCR-clone 2) of Pt-1 showed strong binding activity of Tax301–309 peptide-loaded HLA tetramers even at a low density of tetramer reagents (Fig. 4A), and they produced sufficient IFN-γ and exerted killing activity against not only Tax301–309 peptide-pulsed target cells but also the patient’s HTLV-1-infected T cells in vitro. In addition, the P-P/D-R motif-bearing CTL clone (TCR-clone 2), the second-most frequent CTL clone on day 175 after HSCT in Pt-1 (10%), further expanded on day 287 after HSCT (23%), whereas TCR-clone 6, the most predominant clone on day 175 (75%), decreased later on day 287 after HSCT (54.1%; data not shown). This observation also implied that P-P/D-R motif-bearing CTL clones could expand through selective expansion of a best-fit to the TCR-Tax301–309 peptide/MHC complex, and might play a significant role in the graft-versus-ATL effect as a driver CTL clone after HSCT.

In conclusion, for the first time, we have shown the highly restricted oligoclonal diversity of Tax301–309 peptide-specific CTLs of ATL patients before and after allo-HSCT at a single-cell level, and that a particular CDR3 amino acid motif P-P/D-R in the CDR3 of TCR-β was conserved between unrelated ATL patients and in the same ATL patient before and after allo-HSCT. Further investigations are needed to understand this correlation between the features of TCR and the clinical course to contribute to the development of a novel immunologic therapy for ATL patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Single-Cell Analysis of T-Cell Receptor Repertoire of HTLV-1 Tax-Specific Cytotoxic T Cells in Allogeneic Transplant Recipients with Adult T-Cell Leukemia/Lymphoma

Yukie Tanaka, Hideki Nakasone, Rie Yamazaki, et al.

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