Establishment of a stable T lymphoma cell line transduced with HLA-A*24:02-restricted WT1-specific TCR genes and its application to antigen-specific immunomonitoring

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ABSTRACT

Wilms’ tumor gene 1 (WT1) has been proposed as an attractive target for cancer immunotherapy. A natural 9-mer peptide (CYTWNQMNL), which bound to human leukocyte antigen (HLA)-A*24:02, was identified from among WT1-specific cytotoxic T lymphocyte (CTL) epitopes. This natural WT1 CTL epitope peptide was further modified (CMTWNQMNL) to enhance its binding affinity to HLA-A*24:02. This modified WT1 CTL epitope peptide was superior to the natural peptide for inducing HLA-A*24:02-restricted WT1-specific CTLs. Here we induced several WT1 CTLs that reacted with both modified and natural WT1 tetramers from peripheral blood mononuclear cells. Then, T-cell receptor (TCR) genes were isolated from these WT1 CTLs to determine their Vα and Vβ usage. These TCR genes were transduced into human T lymphoma cells to establish a stable cell line, SK37, which expressed a WT1-specific TCR. We confirmed that SK37 cells reacted with both modified and natural WT1 tetramers, which indicated that SK37 cells could be a useful tool for WT1 tetramer reagent quality assurance. One the basis of these findings, we propose that this WT1 tetramer, which was quality-assured using established SK37 cells, will contribute to reliable immunomonitoring of tumor-specific CTL responses of cancer patients who receive WT1-targeted cancer vaccine therapy or TCR-gene therapy.

Cancer vaccine therapy using HLA class I-binding short peptides has been used to induce tumor-specific cytotoxic T lymphocytes (CTLs) in patients with various cancers, because various tumor-associated antigens have been identified in cancer tissues or cells (6, 33). It has been reported that cancer vaccine therapy with short peptides induces increased cancer antigen-specific CTLs and maintains long stable disease in the cancer patients. However, vaccine therapy using short peptides still has numerous issues that need to be resolved for treating cancers (7, 27, 28). Thus, it is necessary to develop more efficient cancer vaccines and establish accurate methods to evaluate cancer antigen-specific immune responses in patients.

It has been reported that Wilms’ tumor gene 1 (WT1), which encodes for a zinc finger transcription factor, is overexpressed in cancer cells or tissues of many tumor types, such as acute myelocytic leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelocytic leukemia (CML), and myelodys-
plastic syndrome (MDS) (2, 10, 13–15, 21, 24, 25). In contrast, WT1 expression is limited in normal cells and tissues of adults (1, 24). Therefore, WT1’s product may be a promising specific target for cancer immunotherapy (26). Indeed, a previous report showed that WT1 was the top ranking from among 75 representative cancer antigens on the basis of nine criteria (4).

To date, phase I cancer immunotherapy clinical studies that targeted the WT1 protein have been conducted for AML, MDS, lung, and breast cancer (22, 23, 31) and phase I/II clinical studies using WT1 peptides have been conducted for patients with various types of cancer (29). It was reported that the increase in the numbers of WT1-specific CTLs in cancer patients after vaccination with WT1 peptides closely correlates with the therapeutic efficacy against cancer (7, 16, 17, 20). Therefore, monitoring antitumor immune responses, particularly the generation of tumor-specific CTLs, is critical for accurate assessments of the efficacy of cancer vaccine immunotherapy.

In general, increases in CTLs have been determined by ELISPOT assays, intracellular IFN-γ assays or tetramer assays. The ELISPOT assay, which was developed 18 years ago, has been used by most investigators to determine the frequency of CTL generation in cancer patients after vaccine therapy. It is very easy to determine CTL frequency using this method. However, it is difficult to accurately identify the effector subsets that are responsible for tumor antigen peptide-specific responses with the ELISPOT assay. This is because the possibility that endogenously produced cytokines nonspecifically activate some antigen-nonspecific T cells, NK cells and NKT cells cannot be excluded.

To overcome this problem, human leukocyte antigen (HLA) tetramers have been used to analyze antigen-specific T-cell immunity, because these reagents provide for the accurate enumeration and efficient immunomagnetic sorting of antigen-specific T cells, regardless of the functional capacity of T cells. Therefore, establishing a WT1 tetramer has been suggested to be a very promising tool for immunomonitoring of cancer immunotherapy that uses WT1 peptides.

HLA-A*24:02 (A24) is the major HLA-A allele in approximately 60% of Japanese. Thus, identifying WT1 epitopes for A24 would be important for clinical immunotherapy applications for Japanese cancer patients. An A24-restricted WT1 epitope was previously identified (amino acids 235–243; CMTWNQMN) (32). In addition, it was demonstrated that a modified 9-mer WT1 epitope (CYTWNQMNL) remarkably increased the binding affinity to A24 molecules and effectively induced WT1-specific CTLs from peripheral blood mononuclear cells (PBMCs) compared with a natural WT1 peptide (A24-natural WT1 peptide). The CTLs that were induced by the modified WT1 peptide (A24-modified WT1 peptide) killed naturally WT1-expressing leukemic cells in an A24-restricted manner. Thus, this modified WT1 peptide is currently being used in clinical studies on cancer immunotherapy using a WT1 peptide vaccine. However, because of the low binding affinity of the A24-natural WT1 peptide to HLA molecules, the A24-natural WT1 tetramer lacks stability compared with the A24-modified WT1 tetramer. Therefore, it was necessary to develop tools that could validate the A24-modified and natural WT1 tetramers. We hypothesized that a stable T lymphoma cell line transduced with A24-restricted WT1-specific T-cell receptor (TCR) genes and had the same binding avidity to both modified and natural WT1-peptide could be a good tool for validating WT1 tetramers.

For this purpose, in the present study, we identified novel WT1-specific TCR genes from A24-modified and natural WT1 tetramer-positive CTLs induced from PBMCs of healthy donors. We used these to establish a TCR gene-transfected T lymphoma cell line, which was designated SK37. Our results suggest that SK37 cells could be used as a positive control in both tetramer-assays and for quality assurance of A24-modified and natural WT1 tetramers.

MATERIALS AND METHODS

**Cells, antibodies, tetramers, and flow cytometry analysis.** The following cells, antibodies, and tetramers were used for staining and cell sorting. Jurkat, J.RT3-T3.5, and Sup-T1 cell lines were purchased from the Riken Cell Bank (Tsukuba, Japan). CD8-FITC, 7-AAD, and IOTest® Beta Mark were purchased from Beckman Coulter Inc. (Miami, FL, USA). A24-modified WT1 tetramer, A24-natural WT1 tetramer, HLA-A*02:01 Mart-1 tetramer (amino acids 26–35; ELAGIGILTV), and A24-HIV negative tetramer (amino acids 584–592; RYLRDQQLL) were purchased from Medical and Biological Laboratories Co., Ltd (MBL, Nagoya, Japan). Cells were first stained with tetramers at 4°C for 15 min, and then stained with an anti-CD8 antibody at 4°C for 15 min. Flow cytometry analysis used a FACScalibur (BD Biosciences, San Diego, CA, USA). Data analysis used CellQuest software (BD...
Biosciences) and FlowJo software (Tree Star, Inc., San Carlos, CA, USA).

**A24-WT1-specific CTL lines.** The research protocols for experiments using human specimens were approved by the medical ethics committees of MBL, Institute for Genetic Medicine, Hokkaido University and the Hokkaido University Graduate School of Medicine. Written informed consent was obtained from each subject. WT1-specific CTL lines were established in our laboratory with A24-positive healthy donors PBMCs purchased from Cellular Technology, Ltd. (Cleveland, Ohio) using a mixed lymphocyte peptide culture (MLPC) method as described (11). Cells were grown in complete RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 12.5 mM HEPES, 5% human AB serum, penicillin/streptomycin, 2 mM L-glutamine (referred to as T-cell medium), and 50 U/mL of human recombinant IL-2 (Shionogi Pharmaceutical Institute Co., Osaka, Japan). The CTL lines were periodically stimulated in the presence of irradiated and peptide-pulsed HLA-A*24:02-positive Epstein-Barr virus-transformed B cells (lymphoblastoid cell lines; LCLs).

**Positive selection of antigen-specific T cells.** CTL lines were stained with A24-modified WT1 tetramer-PE at 4°C for 15 min. After washing with MACS buffer (phosphate-buffered saline supplemented with 0.5% human serum albumin and 2 mM EDTA), the cells were incubated with anti-PE microbeads (Miltenyi Biotech, Auburn, CA, USA) at 4°C for 15 min. AutoMACS (Miltenyi Biotech) was used to prepare separated cells.

**Repertoire analysis of TCR β chains.** An IOTest Beta Mark® TCR Vβ Repertoire kit was used for the analysis of TCR β chains with antibodies against the following TCR Vβ regions: Vβ1, Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2, Vβ5.3, Vβ7.1, Vβ7.2, Vβ8, Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.2, Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ22, and Vβ23.

**PCR cloning and sequencing of WT1 peptide-specific TCR α/β genes.** The TCR α chain composes a TCR alpha chain variable region (TRA V), a joining region (TRAJ), and a constant region (TRAC). The TCR β chain composes a TCR beta chain variable region (TRBV), a diversity region (TRBD), a joining region (TRBJ), and a constant region (TRBC). Total RNA from sorted CTLs was prepared with an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and an aliquot (1 μg) was subjected to reverse transcription using an oligo (dT) primer and SuperScript III (Invitrogen, CA, USA). First strand cDNA was amplified by PCR using a FastStart High Fidelity PCR System (Roche Diagnostics, Rotkreuz, Switzerland) with coding region-specific primers for TRBV5-1 and TRBC1/TRBC2 (WT1 peptide specific TCR β chain), various TRAV primers, and TRAC (TCR α chain) according to the manufacturer’s instructions. These PCR products were separated on a 1% agarose gel. A band of the appropriate size (bp) was excised and extracted from the gel. The recovered DNA fragment was cloned into the vectors pCDNA3.1 and pEF6/Myc-His (Invitrogen, CA, USA), and its DNA sequence was determined using BigDye Terminator reagent and a 3130xl Genetic Analyzer (Applied Biosystems, CA, USA). The confirmed cDNA sequences of each TCR gene were analyzed using the WEB tool IMGT (8). HLA-A*02:01-restricted Mart-1-specific TCR α/β cDNAs (5) were purchased from IDT (Coralville, IA, USA).

**Transduction of TCR genes in lymphoma cells.** To establish stable transfectants, TCR α chain/pCDNA3.1 and TCR β chain/pEF6/Myc-His plasmids (10 μg each) were transduced into lymphoma cells by electroporation, and selection was done in a medium containing 0.5 mg/mL of G418 (Roche) and 5 μg/mL of Blasticidin (Invitrogen). Expression levels of the transduced genes were assessed by flow cytometry with tetramer staining.

**In vitro cytotoxicity assay.** A24-positive and A24-negative LCLs in 100 μL of complete medium were labeled with 3.7 MBq ⁵¹Cr for 1 h at 37°C. For peptide reconstitution assays, 1 μM of a synthetic peptide was added 1 h before introducing effector cells. After 4 h of incubation with effector cells, supernatants were determined with a gamma counter. The percentage specific lysis was determined by: [(experimental release − spontaneous release) / (maximum release − spontaneous release)] × 100.

**Statistical analysis.** Results are given as means and standard deviations. Statistical comparisons were made using two-tailed Student’s t-tests; P-values of 0.05 were considered significant.

**RESULTS**

**Induction of A24-WT1-specific CTL lines by MLPC**

We first attempted to establish WT1-specific CTLs to identify TCR genes that bound to the WT1 peptide-A24 complex. WT1-specific CTLs were induced
Next, we sought to identify the TCR Vβ repertoire of the 37F8 cells using a TCR Vβ Repertoire Kit, which could account for about 70% of the variations in TCR Vβ. We confirmed that the TCR β chains of 37F8 cells were recognized by anti-TCR Vβ5.1 (Fig. 2).

**TCR cloning of the WT1-specific 37F8 cells**

We investigated whole TCR sequences expressed by the 37F8 cells sorted by the AutoMACS system with A24-modified WT1 tetramer. TCR α and β genes were amplified by PCR with coding region-specific primer pairs for TRAC and various TCR α chains, or TRBV5-1 and TRBC1/2. PCR conditions were decided upon using Jurkat cells cDNA (TRA V8-4/TRA J3/TRAC, TRBV12-3/TRBJ1-2/TRBC1) and human PBMCs cDNA that contained many different TCR genes (data not shown). As a result, we found that the TCR Vα chains of the 37F8 cells comprised TRA V12-2, TRA V12-3, and

### Fig. 1

Establishing WT1-specific CTL lines from PBMCs by MLPC. Upper panel (A) shows the CTL epitopes of A24-modified and natural WT1 tetramers used in this study. Lower panel (B) shows representative results for WT1-specific CTLs (37F8) induced by MLPC with A24-modified or natural WT1 peptides. The 37F8 cells were stained with A24-modified or natural WT1 tetramers. HIV tetramer was used as a negative control. The percentages of tetramer+ cells among CD8+ T cells are indicated.
TRAV41 (Fig. 3A). Because of high homology, the sequence for the TRAV12-2 PCR product was the same as for the TRBV12-3 PCR product, and the sequence for the TRBV5-1/TRBC1 PCR product was the same as for TRBV5-1/TRBC2. On the basis of the sequence analyses of these TCR α and β PCR products, we concluded that the 37F8 cells had two types of α chains (A12-3: TRAV12-3/TRAJ52/TRAC, A41: TRAV41/TRAJ47/TRAC) and one β chain (B5-1: TRBV5-1/TRBD2/TRBJ2-5/TRBC2) (Fig. 3B).

Establishing a T lymphoma cell line that expressed WT1-specific TCRs

To evaluate TCR reactivity with A24-modified and natural WT1 tetramers, we transduced the TCR α/β genes from the WT1-specific 37F8 cells into a Sup-T1 T lymphoma cell line by electroporation with expression vectors. Successful transduction of TCR genes (A12-3 and B5-1) into Sup-T1 T lymphoma cells was confirmed by staining with PE-WT1 tetramers (Fig. 4A). However, the A41 and B5-1 gene-transduced cells did not react with either the A24-modified or natural WT1 tetramers. HLA-A*02:01 Mart-1 TCR was used as a positive control for electroporation and tetramer staining. A stable T lymphoma cell line, SK37, was established by drug selection after transducing the 37F8 TCR A12-3 and B5-1 genes. The reactivity of TCRs expressed on SK37 cells was evaluated by flow cytometry with A24-modified or natural WT1 tetramers. This confirmed that the established SK37 cells reacted with both A24-modified and natural WT1 tetramers whereas an A24-HIV-negative control tetramer did not react with SK37 cells (Fig. 4B).

Functional properties of SK37 TCRs from the 37F8 cells

To investigate SK37 TCR function, cytotoxicity of the 37F8 cells was determined by a 51Cr-release assay. The 37F8 cells showed robust, specific cytotoxicity against A24-positive LCLs that were pulsed with A24-modified or natural WT1 peptides (Fig. 5A and 5B). However, the 37F8 cells did not react with peptide-non-pulsed A24-positive LCLs and A24-negative LCLs (Fig. 5B). These results strongly suggested that the transduced WT1-specific TCR α/β genes could recognize the A24-WT1 epitope peptide.

DISCUSSION

To develop better cancer treatments, we established an accurate immunomonitoring system that could be used to demonstrate the mechanisms of antitumor effects of cancer immunotherapy. An HLA-tetramer reagent is one of the important tools used for immunomonitoring to detect antigen-specific CTLs. It was reported that a tetramer assay using peripheral blood
A stable cell line that expresses antigen-specific and monoclonal TCR α/β. In this study, we established a lymphoma cell line, SK37, that expressed WT1-specific TCRs, and this cell line could bind to both A24-modified and natural WT1 tetramers (Fig. 4B). These results suggest that SK37 cells could be useful as a positive control to evaluate the quality assurance of A24-modified and natural WT1 tetramers during immunomonitoring.

We successfully identified novel TCR genes composing two types of α chains (A12-3: TRAV12-3/TRAJ52/TRAC, A41: TRAV41/TRAJ47/TRAC) and one β chain (B5-1).
T lymphoma transduced with WT1-TCR

Fig. 4  HLA-tetramer assay for T lymphoma transduced TCR α and β genes from the 37F8 cells. (A) WT1 TCR (A12-3/B5-1 or A41/B5-1) genes were transduced into Sup-T1 cells. TCR-transduced T lymphoma cells were stained with A24-modified WT1 tetramer. The A24-WT1 tetramer bound to T lymphoma cells with WT1 TCRs composing A12-3/B5-1 but not with WT1 TCRs of A41/B5-1. A T lymphoma cell line with HLA-A*02:01 Mart-1 TCRs was used as a positive control for electroporation and the subsequent assay with Mart-1 tetramer. (B) The SK37 cells were established from T lymphoma cells with WT1 TCRs composing A12-3/B5-1. The TCR reactivity of SK37 cells was evaluated by flow cytometry using A24-modified WT1 (plot at left) or natural WT1 (center plot) tetramer in addition to HIV tetramer as a negative control (plot at right).

a single β chain (B5-1: TRBV5-1/TRBD2/TRBJ2-5/TRBC2) (Fig. 3B) for the WT1-specific 37F8 cells that were established using PBMCs from healthy donors by MLPC (Fig. 1B). It was also important to confirm the various functions of the identified TCR genes that recognized HLA/WT1-peptide complexes. We assessed the cytotoxicity of the established CTLs with A24-restricted WT1 TCRs using a 51Cr release assay. In these experiments, the novel WT1 TCR-transduced CTL line showed specific cytotoxicity against natural WT1 peptide-pulsed A24-positive LCLs (Fig. 5A), although their cytotoxicity against modified WT1 peptide-pulsed LCLs was about four times stronger than natural WT1 peptide-pulsed LCLs. We speculated that the differences in cytotoxicity derived from the different affinities be-
As an innovative cancer vaccine, we also developed an artificially synthesized long peptide, H/K-HELP (helper/killer-hybrid epitope long peptide), which was conjugated to a MAGE-A4 class I-binding epitope and our defined helper epitope (3), and used this for a patient with pulmonary metastatic colon cancer. We found that cancer-specific Th1/Tc1 cells were induced in this cancer patient after vaccination with MAGE-A4-H/K-HELP. Therefore, we are now preparing HLA-class II tetramers for accurate immunomonitoring of MAGE-A4- and Survivin-H/K-HELP cancer vaccine therapy.

In summary, we established WT1-specific CTL lines using PBMCs from healthy donors by MLPC. We cloned TCR genes from these CTLs, which were transduced into T lymphoma cell lines by electroporation. As a result, we successfully established a novel A24-WT1 tetramer-positive lymphoma cell line, designated SK37, which was useful for the quality assurance of A24-modified and natural WT1 tetramers for immunomonitoring. Thus, the present findings clearly indicated that the novel WT1-specific TCR-transduced CTLs could recognize naturally processed WT1 peptides and kill endogenously WT1-expressing cancer cells in a WT1-specific A24-restricted manner. Thus, this may become a promising tool for developing TCR gene immunotherapy.

We recently demonstrated that introducing Th1-dominant immunity is essential for inducing fully activated CTLs and immunological memory in tumor-bearing hosts (9, 18, 19, 30). It has also been demonstrated that a mixture of various synthetic long peptides derived from naturally occurring sequences of HPV-16 oncoproteins was superior to short tumor peptides in terms of inducing complete or partial response in vulvar intraepithelial neoplasia (12). Thus, a long peptide vaccine that contains both helper and killer epitopes appears to be a rational strategy to activate Th1-dependent antitumor immunity (18).

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WT1 tetramer reagent that was validated using the SK37 cells could become a diagnostic product for cancer patients’ antigen-specific immunotherapy.

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REFERENCES


