Characterization of a MAGE-1-derived HLA-A24 Epitope-specific CTL Line from a Japanese Metastatic Melanoma Patient

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Abstract. *A MAGE-1 HLA-A24 peptide-specific CTL line was characterized using a novel staining approach in the case of a metastatic melanoma patient who exhibited a remarkable clinical response in HLA-A24 peptide cocktail-pulsed dendritic cell (DCs) vaccine therapy. Briefly, pre- or post-vaccine peripheral blood mononuclear cells (PBMCs) from the vaccinated patient were stimulated several times with MAGE-1 A24 peptide-pulsed DCs and T2-A24 cells in vitro. Expanded MAGE-1 A24-specific CTL line was investigated in terms of immunological functions. The proportion of MAGE-1 A24 tetramer$^+$ CTLs increased from 0.04% to 18.6%, and the absolute numbers of MAGE-1 tetramer$^+$ CTLs increased up to 5,068-fold after stimulations. Expanded CTL line exhibited a strong cytotoxic activity against MAGE-1$^+$ cancer cell line in the restriction of HLA. Finally, successful identification of MAGE-1 A24 peptide-specific T-cell receptor (TCR) cDNA from anti-TCR MoAb-sorted CTL was obtained for the first time and the specific cytotoxicity in TCR gene-transduced naive T-cells was confirmed.*

Abbreviations: DC: dendritic cell, HLA: human leukocyte antigen, CTL: cytotoxic T-lymphocyte, TIL: tumor-infiltrating lymphocyte, TCR: T-cell receptor, PBMC: peripheral blood mononuclear cell, APC: antigen-presenting cell, PBL: peripheral blood lymphocyte.

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Malignant melanoma is the best known cancer for which multiple tumor-specific antigens have been identified and utilized in vaccination strategies as peptide vaccines or peptide-pulsed dendritic cell (DC) vaccines (1, 2). The majority of the peptides used in vaccinations against melanoma are human leukocyte antigen (HLA)-A2-restricted because HLA-A2 is the dominant type in Caucasians. However, in Asia, HLA-A24 is more common and several clinical cancer vaccine trials utilizing HLA-A24-restricted peptides, such as CEA, P53, MAGE-3 and VEGFR, have been conducted (3-6). A clinical phase I/II trial of HLA-A24 peptide cocktail-pulsed DC vaccines against metastatic melanoma has been running for some years and it is reported that almost all cases showed more than 2 peptide-specific cytotoxic T-lymphocyte (CTL) responses in blood with 2 patients having clinical responses [1 complete remission (CR), 1 partial remissions (PR)] (7).

To date, few studies have focused on the characterization or determination of HLA-A24 melanoma peptide-oriented specific CTL clones from melanoma patients treated with DC vaccines. Because of the lack of a reliable diagnostic HLA-A24 peptide-oriented tetramer, an appropriate and precise evaluation of CTL responses in vaccinated HLA-A24$^+$ cancer patients has yet to be performed. A number of HLA-A24-restricted CTL or tumor infiltrating lymphocyte (TIL) epitopes from solid cancers have been defined and utilized in vaccine trials (8, 9). However, these studies did not report many tetramer-positive CTL lines and tetramer-oriented T-cell receptor (TCR) cloning.

In the present study, a novel and efficient method for the expansion and separation of a very small number of HLA-A24 MAGE-1 peptide-specific CTLs is established using HLA-A24 MAGE-1 tetramer-based or TCR-specific MoAb-based cell sorting.

Finally, the successful cloning of a MAGE peptide-specific TCR gene from the sorted CTLs was performed.

This is the first study to verify the TCR sequence and immunological function of a MAGE-1 A24 tetramer$^+$ CTL line from a DC-vaccinated melanoma patient. Taking the significance of the vaccinated patient's peripheral blood mononuclear cells (PBMCs) including substantial CTL precursors as the source of adoptive T-cells into consideration, novel *ex vivo* CTL expansion using peptide-pulsed DC or antigen-presenting cell (APC) stimulation might be a good tool for the clinical application of adoptive T-cell therapy.

Materials and Methods

Reagents and cell lines. Recombinant human (rh) granulocyte macrophage colony-stimulating factor (GM-CSF), rh-interleukin (IL)-2, rhIL-4, rhIL-7 and tumor necrosis factor (TNF)-α were purchased from Pepro Tech Inc. (Rocky Hill, NJ, USA). GM-CSF and IL-4 were used at 50 ng/ml for dendritic cell (DC) cultures. FITC and PE-labeled mouse anti-human IFN-γ antibodies were all purchased from Pharmingen (San Diego, CA). HLA*2402 MAGE-1 (NYKHCFPEI) and HIV (RYLRDQQLL) tetramers were supplied from MBL (Nagoya, Japan). The TCR Vβ repertoire kit and FITC-labeled anti-specific TCRVβ repertoire MoAbs were purchased from Beckman Coulter Inc (Fullerton, CA, USA). LN-18 and HT29 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The source of human melanoma cell line, NCC-KT, has been described previously (10). TISI cells, a HLA-A24$^+$ EB virus-transformed B-cell line and T2-A24 cells were kindly supplied by TakaraBio Co., Ltd. and Dr. Kuzushima, Aichi Cancer Center Research Institute, Japan, respectively.

Synthetic peptides. The sequences of melanoma-associated HLA-A24-restricted synthetic peptides used in the present study are MAGE-1 $_{27\text{-}35}$ (NYKHCFPEI), CEA $_{652\text{-}660}$ (TYACFVSNL) and HIV $_{584\text{-}592}$ (RYLRDQQLL).

Generation of DCs from blood monocytes. All PBMCs were derived from a HLA-A*2402$^+$ metastatic melanoma patient. The clinical research using PBMCs from melanoma patients was approved by the Institutional Review Board of National Cancer Center, Tokyo and Shizuoka Cancer Center, Shizuoka, Japan. All patients gave written informed consent. PBMCs were cultured at 4×10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 50 μg/ml gentamycin, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate and 5% AB human serum (Cambrex, MD, USA) (referred to as DC medium) for 90 min at 37˚C. After incubation non-adherent cells were collected and stored at –80˚C until use. The adherent monocyte-enriched population was cultured in the presence of 40 ng/ml of rhGM-CSF and rhIL-4. TNF-α was added to the culture on day 5 at final concentration of 10 ng/ml. After 7-day culture cells were harvested and used for CTL induction cultures (most DCs were positively stained with CD83 MoAb).

CTL induction cultures (stimulation with DC and/or T2-A24 cells). PBMCs (before or after 6 DC vaccinations) were derived from a HLA-A*2402$^+$ metastatic melanoma patient who showed a good clinical response (PR) and remarkable increase in CTLs in blood, especially for HLA-A24-restricted MAGE-1 peptide (Figure 1), after being given DC vaccines in a clinical trial (7). Harvested DCs were incubated with 50 μg/ml of MAGE-1 A24 peptide and 3 μg/ml of β_2-microglobulin (Sigma-Aldrich Co., St. Louis, MO, USA) in PBS with calcium and magnesium containing 1% human serum albumin for 2 h at 37˚C. Thirty Gy-irradiated peptide-pulsed DCs were cultured with non-adherent cells derived from PBMCs at the ratio of 1:10 - 1:100 in DC medium with 10 ng/ml of rhIL-7. On day 2 and 5, rhIL-2 was added to the culture at final concentration of 3 ng/ml. After 7 days' culture CTLs were harvested and re-stimulated with MAGE-1 peptide-pulsed DC for another 7 days. Additionally, cultured CTLs were boosted with 2 rounds of stimulation with MAGE-1 A24 peptide-pulsed T2-A24 cells weekly at the ratio of 1:10. Finally, expanded peptide-specific CTLs were utilized for various experiments including tetramer staining or cell sorting.

Tetramer staining. Cultured CTLs were stained with both FITC-anti-CD8 MoAb and PE-labeled HLA-A24 MAGE1 or HIV tetramer as previously described. Cells were analyzed on a flow cytometer (FACS Calibur, BD science, CA, USA).

TCR repertoire staining by anti-TCR MoAb. The staining profile of CTLs during the expansion procedure was monitored using a TCR Vβ repertoire kit. Following DC plus T2-A24 cell-based expansion, major populations positively stained with the specific anti-TCR antibody were determined.

CTL killing assay. The killing of CTLs was evaluated with the DELFIA non-radioactive cytotoxicity assay (PerkinElmer Inc., Waltham, MA, USA). Briefly, target cells (TISI cells, cancer cell lines) were labeled with a fluorescence-enhancing ligand, BATDA, and mixed with effector cells at an E/T ratio of 10 for 4 hours. The supernatant collected from the CTL culture was incubated with europium solution, and the EuTDA level was measured in real time with a fluorometer, ARVOsx-2 (PerkinElmer Inc.).

Intracellular IFN-γ staining. After cultured CTLs were pre-incubated with MAGE-1 peptide-pulsed or non-pulsed TISI cells for 4 hours, the stimulated CTLs were stained intracellularly with the anti-human IFN-γ MoAb, HLA-A24 MAGE-1 peptide-specific tetramer, and/or anti-specific TCR MoAb. Stained cells were analyzed on a flow cytometer.

CTL sorting by MACS. HLA-A24 MAGE1 tetramer-based or TCR MoAb-based CTL sorting was performed using an autoMACS (magnetic cell sorting) system (Miltenyi, Germany). Briefly, we used a FITC-labeled TCR-specific MoAb as the primary antibody, and anti-FITC MoAb microbeads as secondary antibody. The purity of specific TCR$^+$ CTLs was more than 98% (data not shown). Purified CTLs were sequentially used for PCR cloning of the TCR gene.

TISI-stimulated production of IFN-γ by a MAGE-1 peptide-specific CTL line sorted by TCR-specific MoAb. The expanded CTL line was sorted by FITC-labeled anti-TCRVB4 MoAb using the autoMACS. After an overnight incubation with CTL medium, sorted CTLs (1×10^5) and peptide-pulsed TISI cells (1×10^5) were co-incubated in a round-bottomed 96-well microculture plate for 24 hours. Finally, supernatants were collected and IFN-γ levels were measured using an ELISA kit specific for human IFN-γ (Biosource, Camallilo, CA, USA).

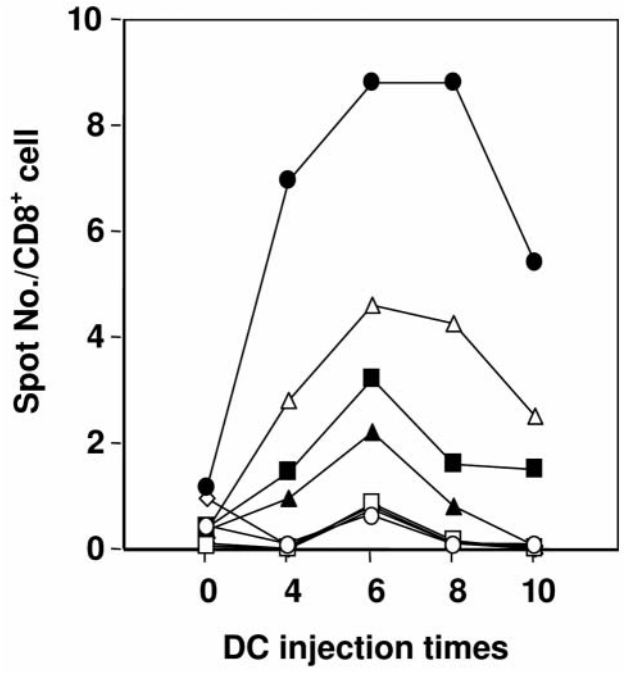


Figure 1. *Frequency of HLA-A24 melanoma-associated peptide-specific CTL precursors in a melanoma patient's blood. An Elispot assay was performed using the patient's peripheral blood lymphocytes before and after DC vaccinations. Each value shows the mean ratio of CTL precursors among CD8+ T-cells from triplicate wells.* ○*: no peptide,* ●*: a cocktail of A24 peptide,* □*: gp100,* ■*: tyrosinase,* △*: MAGE-1,* ▲*: MAGE-2,* ◇*: MAGE-3,* ◆*: EBNA-3 peptide.*

PCR cloning and sequencing of MAGE-1 peptide-specific TCRBV gene. Total RNA of sorted CTLs was prepared with the kit, Nucleospin RNA II (Machery-Nagel, Germany), and an aliquot of 2 μg was subjected to reverse transcription using an oligo (dT) primer and SuperScript II (Invitrogen, CA, USA). The first strand cDNA was amplified by PCR using KOD Polymerase (Toyobo, Japan) according to the manufacturer's instructions and coding region-specific primers for TCRBV4 and TCRBC1 (MAGE-1 peptide specific TCR). The primer sequences was 5'-GCTAGCAT GGGCTGCAGGCTGCTCTGC-3' for TCRBV4 and 5'-TCAGA AATCCTTTCTCTTGACCATGGC-3' for TCRBC1. The PCR product was separated on a 1.5% agarose gel, and the band of appropriate size (bp) was excised and extracted from the gel. The recovered DNA fragment was cloned into the vector pCR-Blunt (Invitrogen, CA, USA), and its DNA sequence was determined using BigDye Terminator reagent and a 3130xl Genetic Analyzer (Applied Biosystems, CA, USA). To confirm DNA sequences more than 12 independent clones were analyzed for each TCR gene. The confirmed cDNA sequences for each TCR gene were analyzed by a WEB tool of IMGT (JunctionAnalysis, http://imgt.cines.fr/).

Construction of expression plasmid. The MAGE-1 peptide-specific TCR gene cloned in pCR-Blunt was digested with *Pst*I and blunted with T4 DNA polymerase (Takara, Japan), and then digested with *Bam*HI. The resultant DNA fragment was cloned into the blunted NheI-BglII site of the expression plasmid pmax (Amaxa, Germany).

TCR gene transduction into primary naive T-cells. Plasmid vector pmax was utilized for making constructs containing GFP cDNA, cloned specific TCR cDNAs or vehicle. A T-cell transfection kit (Nucleofector™ , Amaxa, Cologne, Germany) and a Nucleofector™ device (Amaxa) were used according to the manufacturer's instructions. Prior to electroporation, all lymphocytes including T-cells were usually stimulated with anti-CD3 (2 μg/ml) and CD28 MoAb (1 μg/ml) for 5 days and collected for the gene transduction procedure. The expression of TCR protein was analyzed on a flow cytometer using FITC anti-TCRBV4 MoAb.

Production of IFN-γ by specific TCR gene-transduced naive T-cells. Two days after electroporation, naïve T-cells transduced with GFP or specific TCR cDNA were harvested and incubated with MAGE-1 or other peptide-pulsed TISI cells for 24 hours. The supernatant was collected and the IFN-γ level was measured using an ELISA kit specific for human IFN-γ (Biosource, Camallilo, CA, USA).

Results

Tetramer+ CTL induction and expansion. After expansion of the MAGE-1 A24 peptide-specific CTLs from the vaccinated patient, the frequency of MAGE-1 tetramer+ CTLs increased to 18.6% compared to before the stimulation (less than 0.1%) (Table I). However, the expansion of CTLs from pre-vaccine peripheral blood lymphocytes (PBLs) was not successful. The increase of MAGE-1 A24 peptide tetramer+ CTLs was 5,068-fold in post-vaccine PBLs and 87.5-fold in pre-vaccine PBLs. The frequency of HIV A24 peptide tetramer as a negative control was less than 0.1% (data not shown).

CTL killing activity of expanded MAGE-1-specific CTLs. Cultured MAGE-1 A24 peptide-specific CTLs had a potent killing effect on the MAGE-1 peptide-pulsed TISI cells and LN-18, human glioblastoma cell line (HLA-A*2402+, MAGE-1+) (Figure 2). In contrast, no significant killing was seen in HT-29 (HLA-A*2402+, MAGE-1−) and NCC-KT (HLA-A*2402−, MAGE-1+) cells. The killing activity was demonstrated to be both HLA-A24 and antigen (MAGE-1) specific.

TCR repertoire profiling in a melanoma patient and its relation to the IFN-γ production. There were 2 major populations (27.1% for TCRVB4, 12.8% for TCRVB5) with a specific TCR repertoire among expanded CTLs (Table II). TCRVB4+ CTLs were more efficiently expanded after the stimulation. Figure 3 shows the association of MAGE-1 A24 peptide tetramer staining with specific TCR repertoire. MAGE-1 A24 peptide tetramer+ CTLs were shown to be mainly TCRBV4 positive. In contrast, there were no significant HIV A24 peptide tetramer+ CTLs. Figure 4 demonstrates the correlation between the TCR repertoire profile and IFN-γ production on a MAGE-1 peptide stimulation. The CTLs with the TCRVB4 repertoire were

Table I. *Analysis of MAGE-1 A24 peptide-specific CTL production from HLA-A*2402-positive melanoma patient.*

PBLs	Total cell no. ($\times 10^7$)			HLA-A24 MAGE-1 tetramer (%)		
	Pre	2DC	2DC+2T2-A24	Pre	2DC	2DC+2T2-A24
Pre-DC	2.0	4.1	5.3	0.01 (1)	0.2 (41)	0.35 (87.5)
Post-6DCs	2.0	12.3	21.8	0.04 (1)	3.1 (476.6)	18.6 (5068)

Pre: Before starting CTL induction; 2DC: after 2 peptide-pulsed DC stimulations; 2DC+2T2-A24: after 2 peptide-pulsed T2-A24 stimulations in addition to 2DC. Each value shows the mean for 2 experiments. Values in parentheses show fold increase of tetramer$^+$ CTL no. compared with pre-vaccine.

Table II. *Expansion of major anti-TCR repertoire MoAb$^+$ CTL populations in HLA-A*2402-positive melanoma patient.*

Repertoire MoAb	MoAb$^+$ CTL rate (%)			MoAb$^+$ CTL No. ($\times 10^7$)		
	Pre	2DC	2DC+2T2-A24	Pre	2DC	2DC+2T2-A24
TCRVB4	2.4	16.5	27.1	0.05	2.01	5.91
TCRVB5	4.3	11.7	12.8	0.09	1.44	2.79

Pre: Before starting CTL induction; 2DC: after 2 peptide-pulsed DC stimulations; 2DC+2T2-A24: after 2 peptide-pulsed T2-A24 stimulations in addition to 2DC. Each value shows the mean of 2 experiments.

shown to exhibit MAGE-1-specific IFN-γ production (TCRVB4 9.9% *vs*. TCRVB5 0.71%). Additionally, approximately 60% of MAGE-1 tetramer$^+$ CTLs produced IFN-γ. Finally, TCRBV4$^+$ CTLs were specifically sorted (purity >98%) using the autoMACS system and all utilized for TCR gene cloning.

TISI-stimulated production of IFN-γ by a MAGE-1 peptide-specific CTL line sorted by TCR-specific MoAb. The CTL line sorted with the FITC-labeled anti-TCRBV4 MoAb showed MAGE-1 A24-peptide-specific cytotoxic activity (Figure 5). The MAGE-1 A24-peptide-specific CTL line exhibited a very strong response to peptide-treated TISI cells at a dose as low as 10 pg/ml. In contrast, there was no significant response to HIV A24 peptide-treated TISI cells.

TCR cDNA sequences in MAGE-1 A24 peptide-specific CTLs. Two clones of MAGE-1 A24 peptide-specific TCR cDNA sequences are shown in Figure 6 (sequence A and B). The TCR repertoire was TCRBV4-1*01 and sequence B was the major clone (frequency 80%). The complementarity-determining region (CDR)3 sequence was CASSARQVDEAFF and CASSQVPGQMMNTEAFF in sequence A and B, respectively.

TCR cDNA transduction into primary naive T-cells of a melanoma patient. The GFP gene transduction experiment after antibody-mediated T-cell stimulation showed that

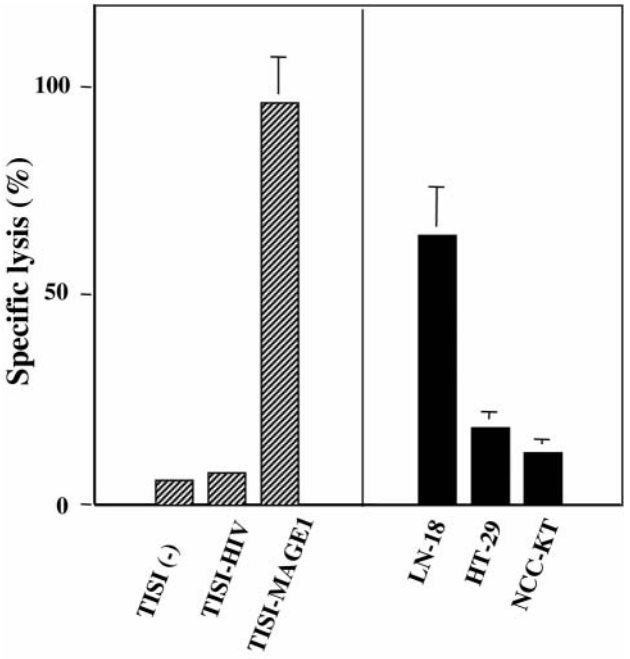


Figure 2. *Cytotoxic activity of expanded MAGE-1 peptide-specific CTLs. Target cells were labeled with fluorescence-enhancing ligand and co-incubated with CTLs for 3 h. TISI (–): untreated; TISI-HIV or MAGE-1: treated with HIV or MAGE-1 A24 peptide. Cancer cell lines (LN-18: HLA-A*2402$^+$, MAGE-1$^+$; HT-29: HLA-A*2402$^+$, MAGE-1$^-$; NCC-KT: HLA-A*2402$^-$, MAGE-1$^+$). Each column shows the mean±SD of triplicate samples.*

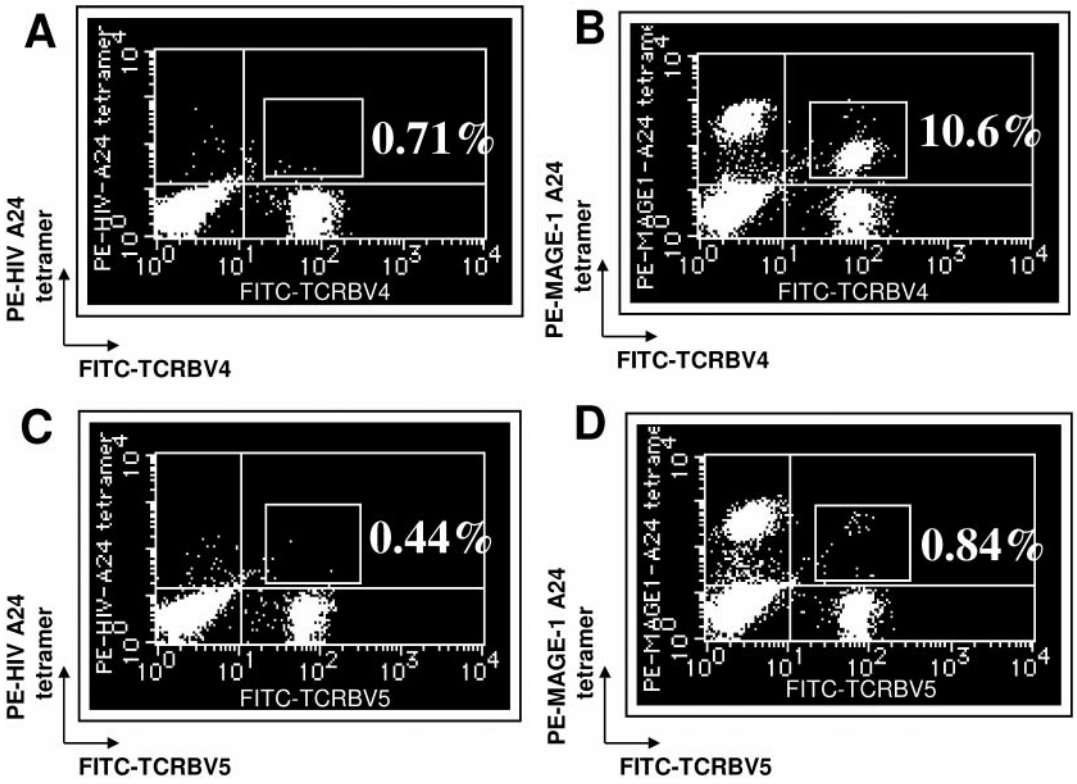


Figure 3. *Staining profile of the MAGE-1 A24 peptide-specific CTL line using MAGE-1 A24 peptide tetramer and anti-TCRBV4 or anti-TCRBV5 MoAb. As a negative control, HIV A24 peptide tetramer was used. Staining by both tetramers and anti-TCR MoAbs was performed. A, B: Anti-TCRBV4 MoAb and HIV or MAGE-1 A24 tetramer. C, D: Anti-TCRBV5 MoAb and HIV or MAGE-1 A24 tetramer. The result is representative of three experiments.*

approximately 60% of $CD3^+$ T-cells were positive for GFP. In the case of gene transduction with 4 μg of MAGE-1-specific TCR cDNA for sequence A and B, the frequency of TCR-positive T-cells was 29.4% and 32.4%, respectively (Figure 7).

Production of IFN-γ by TCR gene-transduced naive T-cells on antigen stimulation. PBMCs from a melanoma patient were transduced with 4 μg of MAGE-1-specific TCR cDNA by electroporation and used for co-culture with peptide-pulsed TISI cells. PMBCs transduced with MAGE-1-specific TCR cDNA (sequence B) showed specific IFN-γ production against MAGE-1 peptide-pulsed TISI cells (Figure 8). In contrast, PBMCs transduced with sequence A cDNA did not show significant activity.

Discussion

Since MAGE-1 was demonstrated to be the first cancer antigen recognized by specific T-cells, intensive and collective research into the molecular identification and characterization of tumor antigens has been performed using clinical specimens from melanoma patients. Melanoma-associated antigens are allocated to the cancer/testis antigen (MAGE, GAGE family, NY-ESO) and melanoma differentiation antigen (MART-1, gp100, tyrosinase) groups according to tumor antigen categories reported by Renkvist *et al*. (11). Based on immunological observations regarding melanoma-associated antigens, many HLA-class I (mainly A*0201)-restricted peptide-specific CTLs have been cloned and characterized (1, 2). Some peptides including MART-1, gp100, tyrosinase and MAGE3 with or without dendritic cells have been subjected to clinical immunotherapeutic trials in metastatic melanoma patients (12) and substantial amounts of clinical data have been accumulated. However, so far HLA-A24 (A*2402)-restricted melanoma-associated peptides have not been investigated enough because the HLA-A24 allele is not common in Caucasians.

Peptide-cocktail-pulsed DC-based immunotherapy has been previously performed in Japanese metastatic melanoma patients (mainly $HLA\text{-}A24^+$) as a phase I/II study (7). In the present paper a MAGE-1 A24 peptide-specific CTL line derived from a DC vaccinated-patient who showed a significant clinical response (shown in Figure 1) was

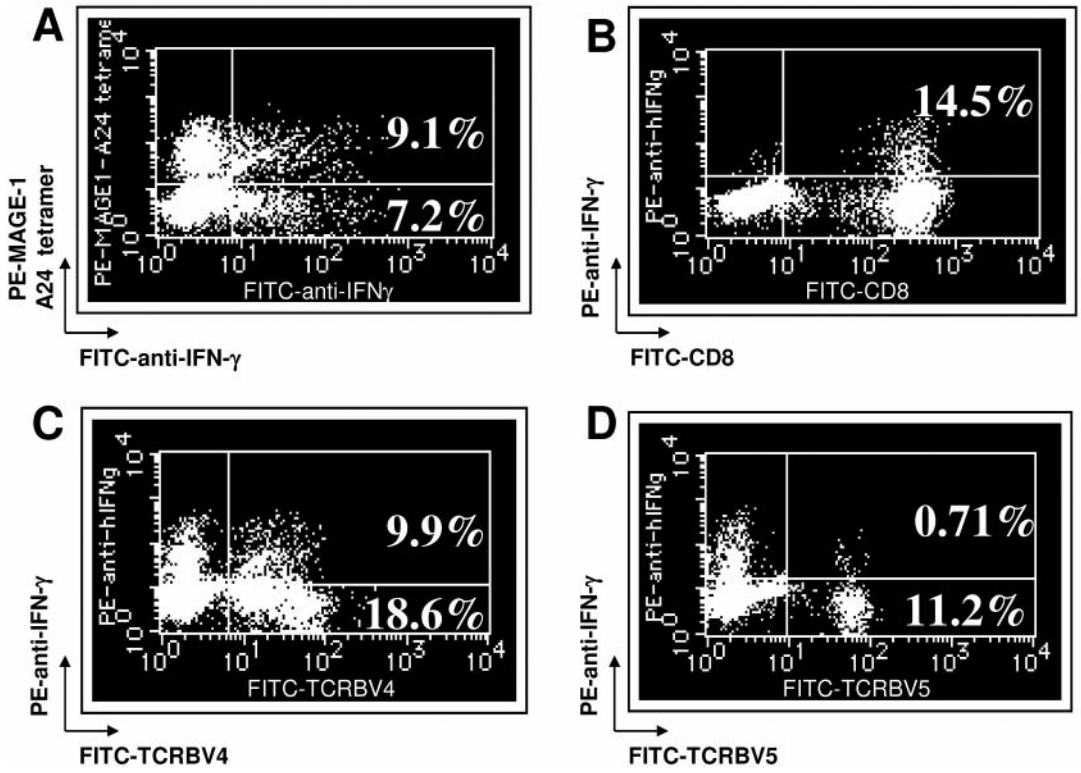


Figure 4. *TCR repertoire profiling and its relation to IFN-γ production. After the MAGE-1 A24 peptide-specific CTL line was stimulated with MAGE-1 peptide-treated TISI cells, cells were stained with MAGE-1 A24 peptide tetramer, anti-CD8 MoAb or anti-TCR MoAbs, and then intracellularly with anti-IFN-γ MoAb. A, MAGE-1 A24 peptide tetramer; B, anti-CD8 MoAb; C, anti-TCRBV4 MoAb; and D, anti-TCRBV5 MoAb.*

characterized and an *ex vivo* expansion culture method was established. More importantly, successful cloning and sequencing of MAGE-1 A24 peptide-specific T-cell receptor (TCR) cDNA is reported for the first time. Clonal CTL analyses after the use of cancer vaccines including DC and peptides have not been frequently performed (13). Powell *et al*. (14) demonstrated the efficacy of a multiple course peptide-immunization strategy for the generation of high frequencies of tumor antigen-specific T-cells because they recognized circulating vaccine-specific CTLs in blood with effector memory phenotype even one year after the final vaccine. Karanikas *et al*. (15) also reported that MAGE-3 HLA-A1-specific CTL clones were identified in peripheral blood using MAGE3-A1-specific tetramer staining in the course of a recombinant canarypox virus-based vaccination. As to HLA-A24 peptide-specific CTL clones, Ikuta *et al*. (16) established HER2-specific CTL clones from PBLs of a cancer patient given several HER2 peptide-pulsed DC vaccines and characterized the cytotoxicity of the CTL clones.

In the present study, *ex vivo* expansion of CTLs using 6DC-vaccinated PBLs resulted in up to a 5,068-fold increase in MAGE-1 A24 tetramer$^+$ cells (Table I). In contrast, in the case of PBLs prior to DC vaccination the expansion was not remarkable. This demonstrated that utilizing PBMCs from DC-vaccinated patients is a very efficient and capable way of preparing a large amounts of adoptive CTLs for clinical use. Different approaches to the expansion of CTLs including the use of autologous DCs, anti-CD3, CD28 antibody (equipped with beads) or EB virus-transformed B-cells (17) have been tried, however autologous DCs with immunogenic peptides might be the best in terms of safety. Additionally, it is important to distinguish between these CTLs in terms of tumor-specific avidity and cytotoxicity. Generally, tetramer-positive CTLs have polyclonal effectors and the clone responsible for the genuine antitumor activity cannot be identified at the expansion stage. A specific staining of CTLs using a combination of anti-TCR MoAb and intracellular IFN-γ staining was utilized as shown in Figure 4. Using this method, the monoclonal TCR repertoire which mediates the antitumor cytotoxicity could be identified. Once the functional TCR repertoire is determined, a functional CTL clone can be purified by MoAb sorting, and finally specific DNA can be cloned. This is a novel approach for the determination of the genuine clone responsible for the peptide-specific cytotoxicity during the selection of polyclonal CTLs.

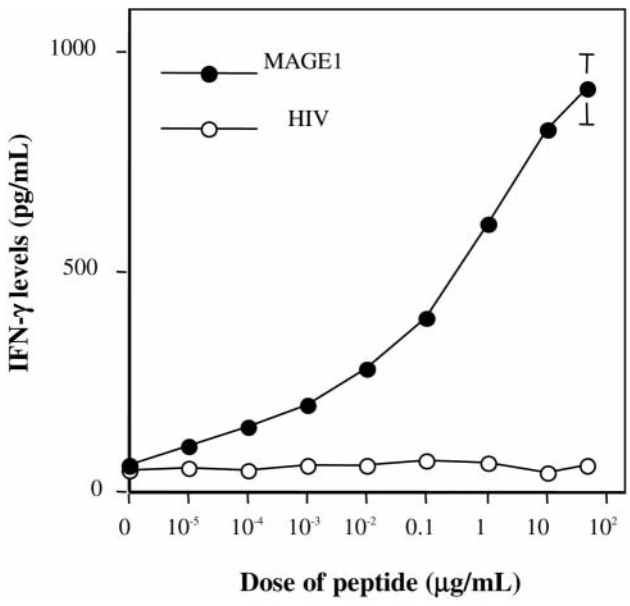
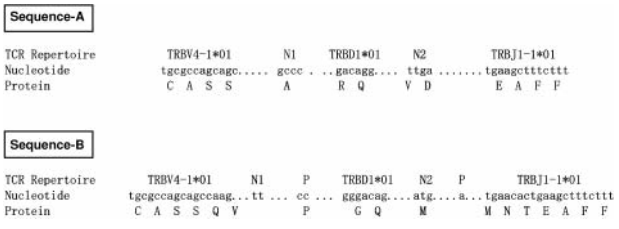


Figure 5. *Peptide dose-dependent IFN-γ productions by the anti-TCR MoAb-sorted CTL line. Purified TCRBV4+ CTLs were stimulated with MAGE-1 or HIV A24 peptide-pulsed TISI cells. IFN-γ levels in the supernatant of the culture were measured using an ELISA kit. The dose of peptide ranged from 10 pg/ml to 50 μg/ml. Each point shows the mean±SD for triplicate samples.*



Firure 6. *Cloned TCR cDNA-derived CDR3 sequence A and B from purified TCRBV4+ CTLs. The TCR repertoire used was TCRBV4, TCRBD1 and TCRBJ-1. Identification of V, D, and J segments was performed using a tool of the IMTG web site (JunctionAnalysis, http://imgt.cines.fr/).*

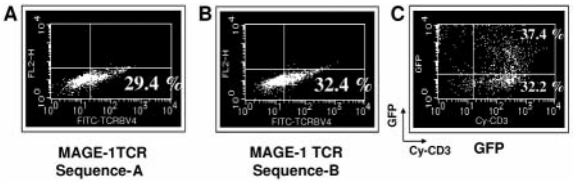


Figure 7. *MAGE-1 A24 peptide-specific TCR cDNA transduction into a HLA-A24+ melanoma patient's PBLs. All PBMCs were stimulated with a cocktail of anti-CD3 and anti-CD28 MoAb prior to electroporation. Specific TCR cDNA or the GFP gene was transduced: A, sequence A; B, sequence B; and C, GFP. After electroporation, all cells were stained with specific anti-TCR repertoire MoAb or anti-CD3 antibody and analyzed on a flow cytometer.*

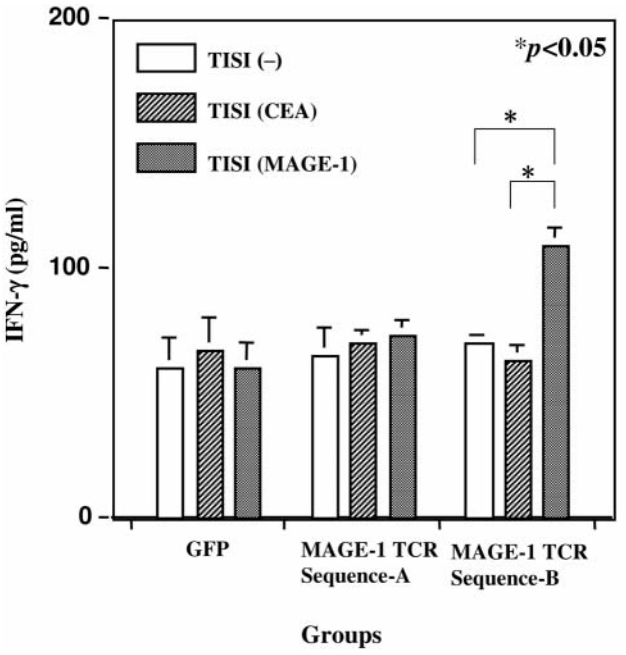


Figure 8. *IFN-γ production by MAGE-1 A24 specific TCR cDNA-transduced lymphocytes derived from a HLA-A24+ melanoma patient. GFP or TCR cDNA-transduced lymphocytes were co-incubated with peptide-treated TISI cells for 24 hours. IFN-γ produced in the supernatant was measured using an ELISA kit. Each column shows the mean±SD of triplicate samples.*

Very recently, as intensive clinical studies targeting HLA-A24-restricted immunogenic epitopes from various tumor antigens have been performed, many novel epitopes have been found to be effective vaccines *in vivo* (18). In particular, in Asian countries including Japan, many HLA-A24-restricted peptides (such as Glypican, SART, LCK, WT1, OY-TES, CA9, HPV16-E6, TTK, LY6K, KIF, Tara and SCC) have been identified from gastrointestinal, lung, prostate, liver and brain tumors (19-21). Yajima *et al*. demonstrated that a specific combination of HLA-A24-restricted peptides suitable to individual patients was effective in prolonging the overall survival time of lung and brain tumor patients (18). On the other hand, MAGE antigen-specific CTL lines recognizing HLA-A24-restricted peptides (MAGE-1 NYKCRFPEI, MAGE-3 195-203 IMPKAGLLI) have been characterized *in vitro* from many patients with solid cancer (22-24). So *et al*. (22) reported that they identified a MAGE-3 A24 peptide-specific CTL clone from a vaccinated patient with significant tumor regression and noticed that the clone recognized peptide-treated HLA-A24+ EBV-transformed B-cells, but lacked killing activity against MAGE-3+ tumor cells. Lu *et al*. (23) demonstrated that CTL activity against MAGE-1 peptide (NYKCRFPEI)-pulsed target cells could be induced

from hepatic cancer PBLs by rough stimulation with peptide-treated PBMCs. In this case, the killing activity against MAGE-1$^+$ tumor cells was also shown to be weak. However, our expanded CTL line was demonstrated to exhibit antitumor killing against a tumor cell line in the restriction of HLA and MAGE-1.

Finally, considering the application of native adoptive CTL therapy, a great number of potent CTLs specific for cancer peptides will be needed. Recently, the great success by Dudley *et al*. with adoptive CTL therapy against metastatic melanoma using a prior lympho-depletion protocol is promoting the therapeutic possibilities of adoptive immunotherapy (25). The technology of TCR gene-engineering is possibly an efficient way to expand necessary specific effector T-cells. In the present study, an electroporation-based TCR gene transduction was performed and the efficiency in naive T-cells derived from melanoma patients was acceptable (transduction rate of 69.6% for the GFP gene). Additionally, our data showed that CTLs after TCR gene transduction seemed to be functionally active and exhibited HLA-restricted cytotoxicity against TISI cells pulsed with peptides. More importantly, anti-CD3 and anti-CD28 antibody-mediated T-cell activation prior to electroporation should be recommended to reduce the damage to T-cells and promote the transduction efficiency as previously reported.

The novel approach used in this study, namely DC vaccine-based expansion using blood CTLs from several time vaccinated melanoma patients, may be a good immunotherapeutic modality. This approach can be employed as adoptive CTL therapy with peptide-cocktail pulsed DC vaccines and the administration of a T-cell-supporting cytokine, such as IL-2, IL-7 or IL-15, to maintain and expand infused CTLs *in vivo*.

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