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## Novel Approach to the Characterization of Melanoma Associated-Peptide-Specific CTL Lines from Melanoma Patients

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### 1. Introduction

Melanoma-associated antigens are categorized as class I human leukocyte antigen (HLA)-restricted cancer/testis antigens (Renkvist et al., 2001) which are considered to be immunogenic to the immune system because they are hardly expressed in normal tissues except testis. However, melanoma is still difficult cancer to treat once it becomes advanced or metastatic. Malignant melanoma is the most well known cancer in which multiple tumor-specific antigens have been defined compared to other solid cancers, and utilized in vaccination strategies as peptide vaccines or peptide-pulsed dendritic cell (DC) vaccines (Nestle et al., 1998; Banchereau et al., 2001). Our group has been running a clinical phase I trial of peptide cocktail-pulsed DC vaccines in metastatic melanoma patients for some years. We reported that almost all cases showed more than 2 peptide-specific cytotoxic T cell (CTL) responses in blood and 2 cases had clinical responses [1 complete remission (CR), 1 partial remissions (PR)] (Akiyama et al., 2005).

We have identified some melanoma peptide-specific CTL lines and determined cDNA sequences of specific TCRs in the clinical trial. However, few studies have focused on the characterization or determination of peptide-oriented single specific CTL clones from melanoma patients treated with DC vaccines. Recently, specific CTLs or tumor-infiltrating lymphocytes (TILs) have been successfully cloned from blood or tumors of melanoma patients (Dudley et al., 2001, 2002, 2005; Yee et al., 2002). In some cases, melanoma peptide-specific CTL clones obtained from the tumor tissue were expanded, and could be utilized for adoptive immunotherapy (Dudley et al., 2002, 2005).

Interestingly, it is also reported that the same TCR repertoire specific to MART-1 peptide was recognized among blood CTLs as TIL clones isolated from tumors.

As to other types of cancers, a very small number of TILs were expanded to isolate tumor-specific clones from a bulk of TILs and utilized to search for novel tumor antigens in a tumor-derived complementary DNA library (Hoshino et al., 1997; Gohara et al., 1997). However, cloning from a bulk of CTLs is time-consuming, and usually very costly.

In the present study, we have established a novel efficient method for the expansion and separation of a very small number of melanoma peptide-specific CTLs using HLA-A2 or A24 peptide tetramer and T cell receptor (TCR)-specific monoclonal antibody (MoAb)-based cell sorting. Through the molecular cloning of melanoma peptide (MART-1, gp100 or MAGE-1)-specific TCRs, the biological characterization of each CTL line was performed in Japanese metastatic melanoma patients given DC vaccines.

## 2. Experimental design

### 2.1 Clinical trial of DC vaccines

Thirty-three cases of metastatic melanoma were enrolled into a phase I/II study of monocyte-derived DC-based immunotherapy. HLA typing showed 7 cases of HLA-A2 and 26 of A24 positive. Briefly, Enriched monocytes were obtained using OptiPrep™ from leukapheresis products, and then incubated with GM-CSF and IL-4 in a closed serum-free system. After pulsing with a cocktail of 5 melanoma-associated synthetic peptides (gp100, tyrosinase, MAGE-2, MAGE-3 and MART-1 or MAGE-1) restricted to HLA-A2 or A24 and keyhole limpet hemocyanin (KLH), cells were cryopreserved until used. Finally, thawed DCs were washed and injected subcutaneously (s.c.) into the inguinal region in a dose-escalation manner.

### 2.2 CTL induction cultures

Peripheral blood mononuclear cells (PBMCs) from 6 cases of HLA-A\*0201<sup>+</sup> and 1 of HLA-A\*2402<sup>+</sup> metastatic melanoma were used for *in vitro* CTL inductions (The clinical research using PBMC from melanoma patients was approved by the Institutional Review Board of Shizuoka Cancer Center, Shizuoka, Japan. All patients gave written informed consent.). All cases of metastatic melanoma were given melanoma-associated peptide-pulsed DC vaccines in clinical trial reported previously (Akiyama et al., 2005). Briefly, after non-adherent PBMCs were stimulated twice with melanoma peptide-pulsed mature DCs (most cells positively stained with CD83 MoAb), cells were boosted in RPMI1640 medium containing L-glutamine (2mM), penicillin (100U/ml), streptomycin (100U/ml) and 5% AB human serum referred to as CTL medium with 2 rounds of stimulation with peptide-pulsed T2 or T2-A24 cells. Finally, expanded peptide-specific CTLs were utilized for various experiments or cell sorting.

### 2.3 TCR repertoire profiling and function analysis

The staining profile of CTLs during the expansion procedure was monitored using a TCR V $\beta$  repertoire kit, major populations positively stained with the specific anti-TCR antibody were determined. For function analysis, CTLs were pre-incubated with melanoma peptide-treated T2 or T2-A24 cells and stained intracellularly with anti-human IFN- $\gamma$  MoAb, peptide-specific tetramer, and/or anti-specific TCR MoAb. The stained cells were analyzed on a flow cytometer.

### 2.4 CTL sorting by TCR-specific MoAb

Melanoma peptide tetramer-based or TCR MoAb-based CTL sorting was performed using the autoMACS (magnetic cell sorting) system (Miltenyi, Germany). Briefly, we used a specific PE-labeled tetramer or FITC-labeled TCR-specific MoAb as primary antibody, and

anti-PE or FITC MoAb microbeads as secondary antibody. The purity of the tetramer<sup>+</sup> or specific TCR<sup>+</sup> CTLs was more than 98% (data not shown). Purified CTLs were sequentially used for PCR cloning of the TCR gene.

### 2.5 PCR cloning and sequencing of melanoma peptide-specific TCRBV cDNA

Total RNA of sorted CTLs was prepared with a kit, Nucleospin RNA II (Machery-Nagel, Germany), and aliquots of 2 µg were subjected to reverse transcription using oligo (dT) primer and SuperScript II (Invitrogen, CA). The first strand cDNA was amplified by PCR using KOD Polymerase (Toyobo, Japan) according to the manufacturer's instructions. Coding region-specific primers for TCRBV28 and TCRBC1 (MART-1 peptide-specific TCR), TCRBV12-4 and TCRBC2 (gp100 peptide-specific TCR) or TCRBV4 and TCRBC1 (MAGE-1 peptide specific TCR) are shown as in Table 1.

**Table 1 TCR-specific primers**

Repertoire	Sequence
TCRBV28;	5'-GCAGCCATGGGAATCAGGCTCCTCTGT-3'
TCRBV12-4;	5'-TCTGCCATGGACTCCTGGACCCTCTGC-3'
TCRBV4;	5'-GCTAGCATGGGCTGCAGGCTGCTCTGC-3'
TCRBC1;	5'-TCAGAAATCCTTTCTCTTGACCATGGC-3'
TCRBC2;	5'-CTAGCCTCTGGAATCCTTTCTCTTGAC-3'

Table 1. TCR-specific primers

The PCR product was separated by electrophoresis 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 plasmid pCR-Blunt (Invitrogen, CA, USA), and its sequence was determined using BigDye Terminator reagent and a 3130xl Genetic Analyzer (Applied Biosystems, CA).

### 2.6 TCRBV gene transduction into primary naive T cells

The plasmid vector pmax was utilized for making the construct containing GFP, cloned specific TCR genes, or vehicle. 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 (2µg/ml) and CD28 MoAb (1µg/ml) for 5 days in GT-T503 medium and collected for the gene transduction procedure. The expression of TCR protein was analyzed on a flow cytometer using anti-TCRBV9 and BV28 (in MART-1) or anti-TCRBV12 (in gp100) MoAbs.

### 2.7 IFN-γ production by specific TCR gene-transduced naïve T cells

Two days after electroporation, naïve T cells transduced with mock, GFP, or a specific TCR gene were harvested and incubated with melanoma peptide-pulsed T2 cells or 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.

### 3. Result

#### 3.1 Tetramer<sup>+</sup> CTL induction and expansion

After the expansion of melanoma peptide-specific CTLs, the frequency of MART-1 tetramer<sup>+</sup> CTLs increased to 46.5 % (mean of 4 cases) compared with before stimulation (less than 1%) (Table.2). The absolute No. of MART-1 tetramer<sup>+</sup> CTLs was shown to increase 187 to 619 fold (average 415 fold) after T2 stimulations compared to prior to the stimulation. Additionally, in case 3 and 5, gp100 A2 and MAGE1 A24 tetramer<sup>+</sup> CTLs were surprisingly expanded up to 1585 and 5068 fold, respectively.

Case No.	Peptide	Total cell No. (x10 <sup>7</sup> )			tetramer (%)		
		Pre	2DC	2DC+2T2 <sup>a</sup>	Pre	2DC	2DC+2T2 <sup>a</sup>
1	MART1 (A2)	2.4	2.5	2.0	0.14 (1)	0.89 (66)	31.7 (186)
2	MART1 (A2)	2.0	3.6	3.9	0.35 (1)	4.64 (24)	78.8 (396)
3	MART1 (A2)	2.0	2.2	4.8	0.12 (1)	1.73 (19)	40.5 (619)
3 <sup>b</sup>	gp100 (A2)	2.0	9.3	-	0.13 (1)	44.3 (1585)	-
4	MART1 (A2)	2.0	1.4	0.62	0.02 (1)	1.32 (46)	35.0 (458)
5	MAGE1 (A24)	2.0	12.3	21.8	0.04 (1)	3.1 (477)	18.6 (5068)

Pre; before starting CTL induction, 2DC; after 2 rounds of peptide-pulsed DC stimulation, 2DC+2T2; after 2 rounds of peptide-pulsed T2 stimulation in addition to 2DC, N.D.; not detected, Each value shows the mean for 2 experiments. <sup>a</sup> In case5, T2-A24 cells treated with MAGE1 A24 peptide were used. <sup>b</sup> Expansion data from gp100 A2 peptide-stimulated CTLs. Values in the parenthesis show fold increase of tetramer<sup>+</sup> CTL No. compared with pre-stimulation.

Table 2. Analysis of peptide-specific CTL production from melanoma patients

#### 3.2 CTL killing activity of expanded melanoma peptide-specific CTLs

Cultured CTLs from 4 melanoma cases showed strong killing activity against MART-1 peptide-pulsed T2 cells and the C32 melanoma cell line (HLA-A\*0201<sup>+</sup>, MART1<sup>+</sup>) (Fig. 1A). In contrast, no significant killing activity was seen in RPMI7951 (HLA-A\*0201<sup>+</sup>, MART1<sup>-</sup>) and NCC-KT (HLA-A\*0201<sup>-</sup>, MART1<sup>+</sup>). The killing activity was shown to be HLA-A2 and antigen (MART-1)-specific. Meanwhile, MAGE-1 A24 peptide-CTLs induced from case 5 were also demonstrated to be HLA-A24 and MAGE-1 antigen-specific in killing against TISI and cancer cell lines (Fig. 1B).

#### 3.3 Intracellular IFN- $\gamma$ staining of expanded tetramer<sup>+</sup> CTLs from melanoma patients

The frequency of both MART-1 tetramer and IFN- $\gamma$ -positive CTLs in 4 melanoma cases after peptide-pulsed T2 stimulation was 7.6%, 34.2%, 25.4%, and 9.8%, respectively. The percentage of IFN- $\gamma$ <sup>+</sup> out of all tetramer<sup>+</sup> cells was 23.9%, 43.4%, 62.7% and 27.9%. CTLs from case 2 and 3 were more efficient in IFN- $\gamma$  production than those from the other two cases (Fig. 2). In the case of MAGE-1 A24 CTLs, 49% of tetramer<sup>+</sup> cells were shown to be IFN- $\gamma$  producer.

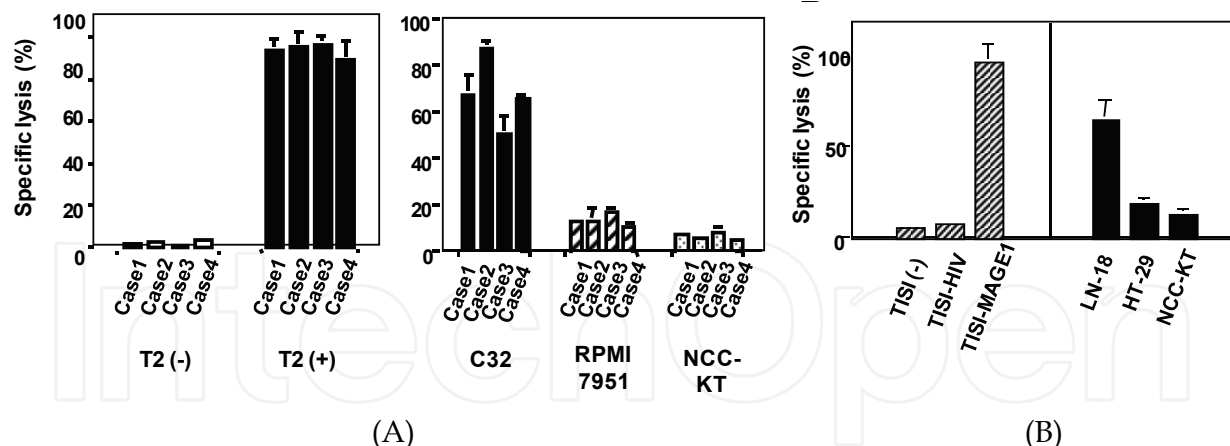


Fig. 1. Cytotoxic activity of expanded melanoma peptide-specific CTL lines from melanoma patients. Target cells were labeled with fluorescence enhancing ligand and co-incubated with CTLs for 3 hrs. (A) MART1-specific CTL lines from 4 cases, T2 (-); untreated, T2 (+); treated with MART1 A2 peptide, melanoma cell lines (C32 : HLA-A\*0201+, MART1+ ; RPMI7951 : HLA-A\*0201+, MART1- ; NCC-KT : HLA-A\*0201-, MART1+). (B) MAGE1-specific CTL line from case 5, LN-18: HLA-A\*2402+, MAGE1+ ; HT-29 : HLA-A\*2402+, MAGE1-. Each column shows the mean  $\pm$  S.D. for triplicate samples

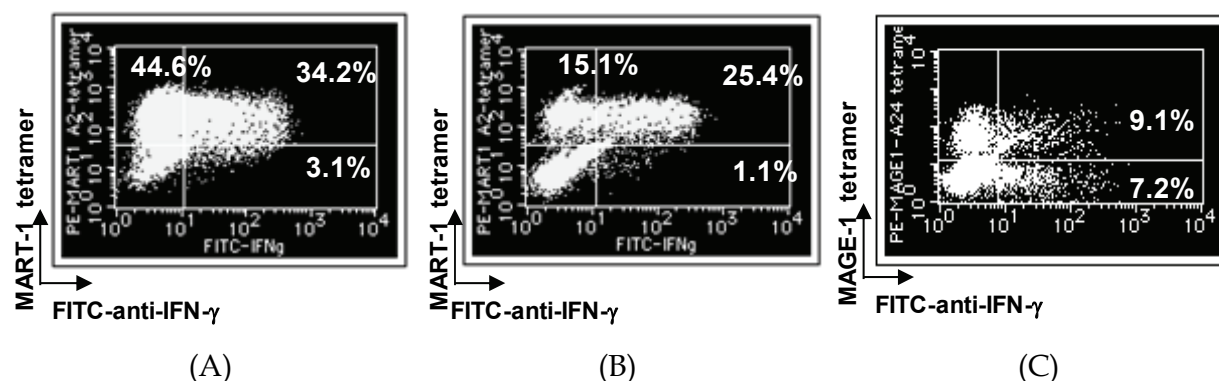


Fig. 2. IFN- $\gamma$  production from melanoma peptide tetramer+ CTL lines stimulated with peptide-pulsed target cells. Each CTL line was stained first with MART-1 A2 or MAGE-1 A24 peptide tetramer and then intracellularly with anti-IFN- $\gamma$  MoAb. (A) case 2, (B) case 3 and (C) case 5

### 3.4 TCR repertoire profiling in melanoma cases and its relation to cytotoxic activity

After the expansion there were 1 major and 3 minor populations with specific TCR repertoires among 78.8 % of MART-1 tetramer+ CTLs in case 2 (Table. 3). Case 3 had a major population in both MART-1 and gp100 A2 tetramer+ CTLs. In the case 5 of MAGE-1 A24 tetramer+ CTLs, 2 major populations were identified. Fig. 3 shows the association of IFN- $\gamma$  production by peptide-stimulated CTLs (cytotoxic activity) with the specific TCR repertoire in cases 2 and 5. TCRBV9+ (MART1) CTL populations alone exhibited a specific killing activity in case 2 (Fig. 3A). TCRBV28+ (MART1) and BV12+ (gp100) in case 3, TCRBV4+ (MAGE1) in case 5 (Fig. 3B), were identified as IFN- $\gamma$  producers. Those populations were specifically sorted (purity >98%) using the autoMACS system, and utilized for TCR gene cloning.

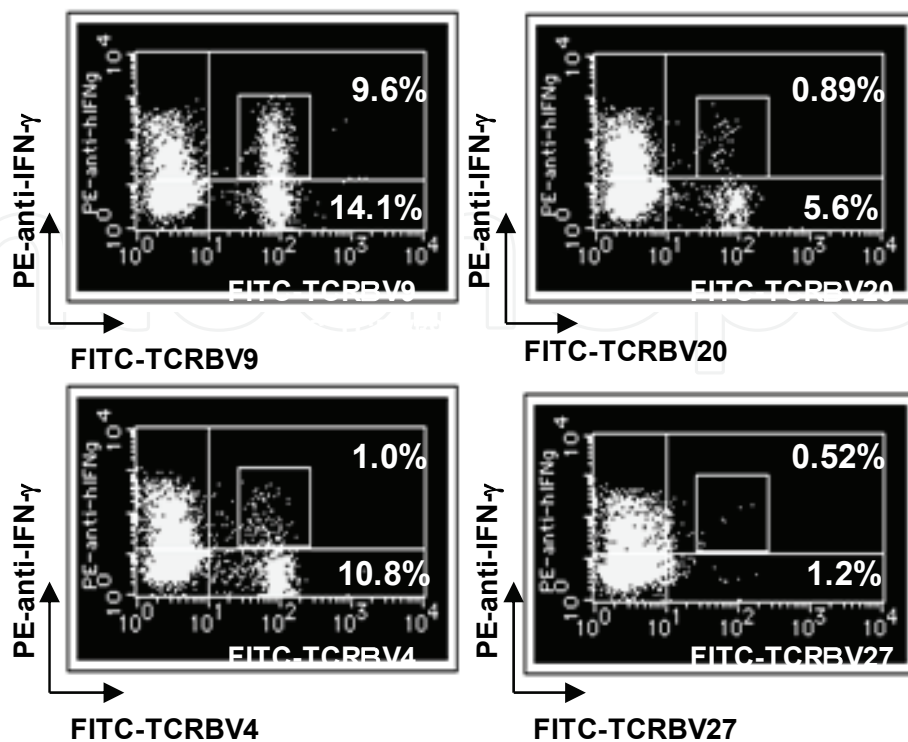
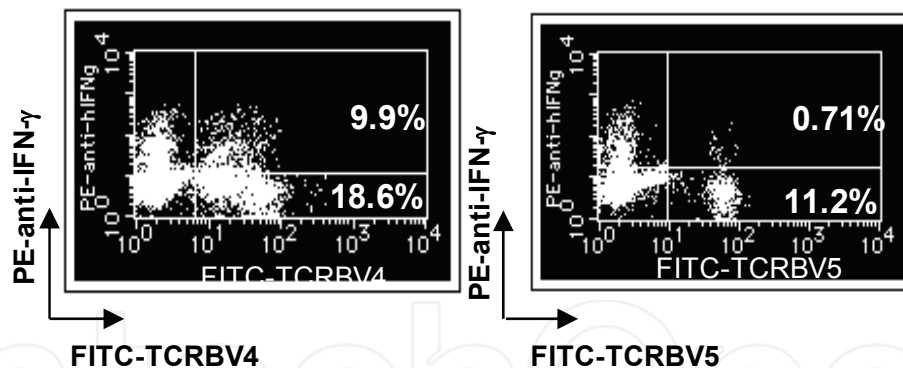
**A: MART-1 A2 CTL****B: MAGE-1 A24 CTL**

Fig. 3. IFN- $\gamma$  production by CTL populations recognized by specific anti-TCR repertoire MoAb. (A) MART-1 A2 peptide-specific CTLs. MoAbs for TCRBV9, TCRBV20, TCRBV4 and TCRBV27 were used. (B) MAGE-1 A24 peptide-specific CTLs. MoAbs for TCRBV4 and TCRBV5 were used. Each CTL was stained first with anti-TCR repertoire MoAb and then intracellularly with anti-IFN- $\gamma$  MoAb after target cell stimulation

**3.5 IFN- $\gamma$  production from peptide-specific CTL line sorted by TCR-specific MoAb**

CTL lines sorted by FITC-labeled anti-TCRBV28, anti-TCRBV12 or anti-TCRBV4 MoAbs showed MRAT-1, gp100 and MAGE-1 peptide specific cytotoxic activity, respectively (Fig. 4). Gp100 A2-peptide specific CTL line exhibited the greater IFN- $\gamma$  production than other peptide-specific lines after the various dose of peptide-pulsed target cell stimulation.

Case No.	Peptide	Tetramer <sup>+</sup> CTLs (%)	Repertoire	Frequency* (%)
2	MART1	78.8	BV9	35.7
			BV20	18.5
			BV4	12.1
			BV27	11.3
3	MART1	40.5	BV28	87.8
3	gp100	44.3	BV12	82.5
5	MAGE1	18.4	BV4	27.1
			BV5	12.8

\*Frequency ; percentage of tetramer<sup>+</sup>/specific TCR repertoire<sup>+</sup> CTLs. A TCR repertoire with a frequency of more than 10% was chosen. Each value shows the mean for 2 experiments.

Table 3. Frequency of specific TCR repertoire<sup>+</sup> CTLs from melanoma patients

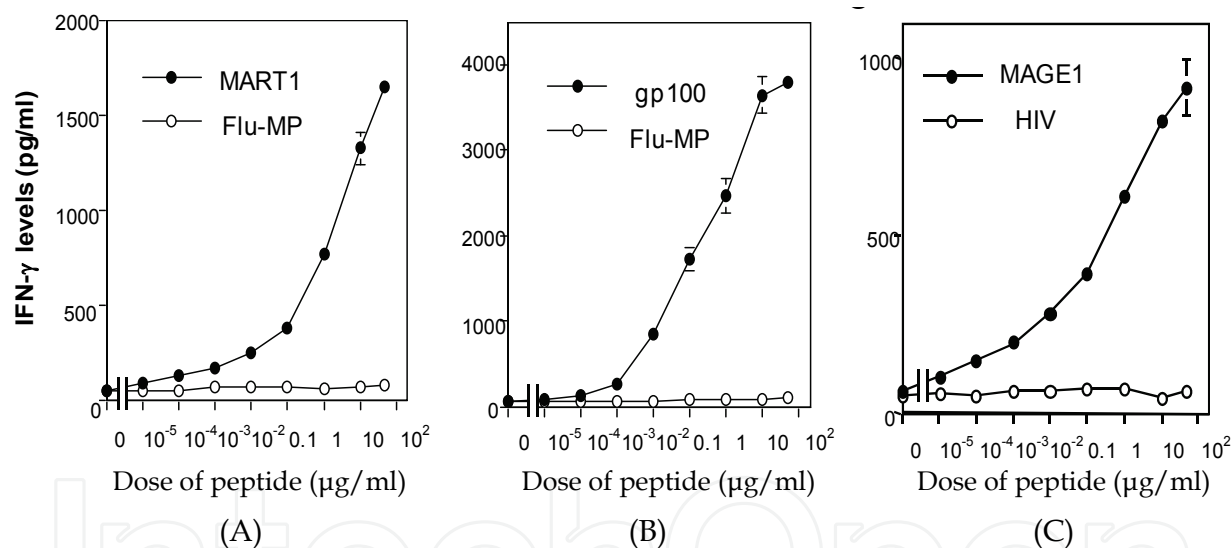


Fig. 4. Peptide dose-dependent IFN- $\gamma$  productions from anti-TCR MoAb-sorted CTL lines. (A) Anti-TCRBV28 MoAb-sorted MART-1-specific CTL line, (B) anti-TCRBV12 MoAb-sorted gp100-specific CTL line, (C) anti-TCRBV4 MoAb-sorted MAGE-1-specific CTL line. These lines were stimulated with peptide-treated target cells. IFN- $\gamma$  levels in the supernatant were measured using human IFN- $\gamma$ -specific ELISA kit. Each point shows the mean  $\pm$  S.D. of triplicate samples

### 3.6 TCR cDNA sequences in melanoma peptide-specific CTL lines

Cloned TCR cDNA sequences are shown in Fig. 5 (MART-1-specific sequence in case2, MART-1, gp100-specific sequence in case3 and MAGE1-specific sequence in case5). The TCR repertoire used was TCRBV9 in case 2, TCRBV28 and TCRBV12 in case 3, and TCRBV4 in case 5, respectively.



**Case2 MART-1 A2 CTL**

Repertoire	TRBV9*01	N1	TRBD2*01	N2	P	TRBJ2-7*01
Nucleotide	tggtccagcagcgtag ... gg ... gcgg ..... tc .. ag...ctcctacgagcagctacttc					
Protein	C A S S V G		A V		S	S Y E Q Y F

**Case3 MART-1 A2 CTL**

Repertoire	TRBV28*01	N1	TRBD1*01	N2	TRBJ1-5*01
Nucleotide	tggtccagcag...CC....cagggggc....ctgggc....cagcccagcatttt				
Protein	C A S S	P	G G	L G	Q P Q H F

**Case3 gp100 A2 CTL**

Repertoire	TRBV12-4*01	TRBD1*01	N2	TRBJ2-2*01
Nucleotide	tggtccagcagtttagc.....aggggg.....tt.....acaccggggagctgtttttt			
Protein	C A S S L A	G G	Y	T G E L F F

**Case5 MAGE-1 A24 CTL**

Repertoire	TRBV4-1*01	N1	P	TRBD1*01	N2	P	TRBJ1-1*01
Nucleotide	tgccagcagccaag...tt ... cc ... gggacag...atg....a...tgaacactgaagctttcttt						
Protein	C A S S Q V	P	G Q	M	M N T E A F F		

Fig. 5. Alignment of cloned TCR cDNA sequences from sorted CTL lines. Segments V, D, J and C were identified using a tool at the IMGT web site (JunctionAnalysis, <http://imgt.cines.fr/>)

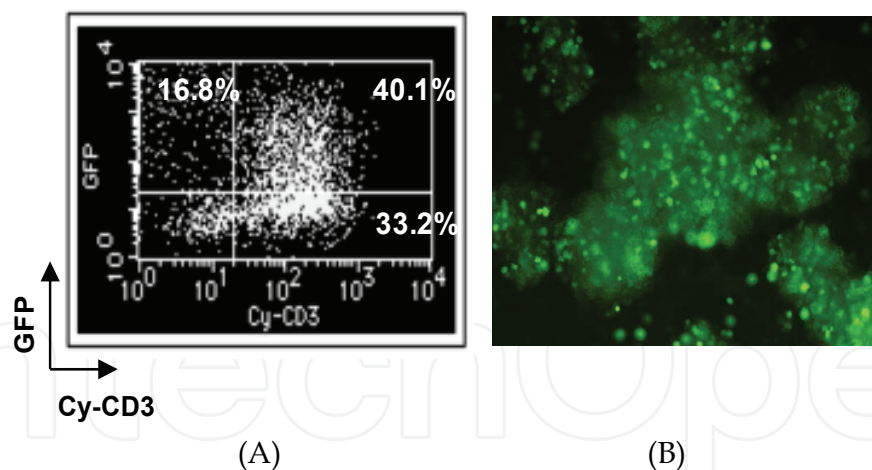


Fig. 6. GFP gene transduction into melanoma patients' PBMCs using electroporation. After electroporation, cells were stained with anti-CD3 MoAb and analyzed on a flow cytometer. (A) Stimulated PBLs, (B) picture of PBLs expressing GFP. PBLs stimulated with anti-CD3 and anti-CD28 MoAb prior to electroporation

### 3.7 TCR cDNA transduction into primary naïve T cells in melanoma cases

The GFP cDNA transduction experiment after antibody-mediated T cell stimulation showed an improved transduction efficiency [unstimulated 25.9% (data not shown) vs stimulated 40.1%] (Fig. 6A). In the case of 4 $\mu$ g of the TCR cDNA for MART-1, gp100 and MAGE-1, the

frequency of TCR-positive T Cells was 23.9% (MART-1, case2), 31.3% (MART-1, case3), 13.3% (gp100, case 3) and 32.4% (MAGE-1, case 5), respectively (data not shown).

### 3.8 IFN- $\gamma$ production by TCR cDNA-transduced naïve T cells on peptide stimulation

PBMCs from melanoma patients were transduced with 4  $\mu$ g of TCR cDNA (MART-1 in case2 and 3, gp100 in case3 and MAGE-1 in case 5) by electroporation and used for co-culture with peptide-pulsed target cells. PBMCs transduced with the MART-1-specific TCR cDNA (case 3) showed specific IFN- $\gamma$  production against MART-1 peptide-pulsed T2 cells in a HLA and antigen-restricted manner (Fig. 7). Additionally, PBMCs transduced with another MART-1-specific (case 2), gp100-specific (case 3) and MAGE1-specific (case 5) TCR cDNAs also demonstrated moderate IFN- $\gamma$  production against each of the peptide-pulsed target cells.

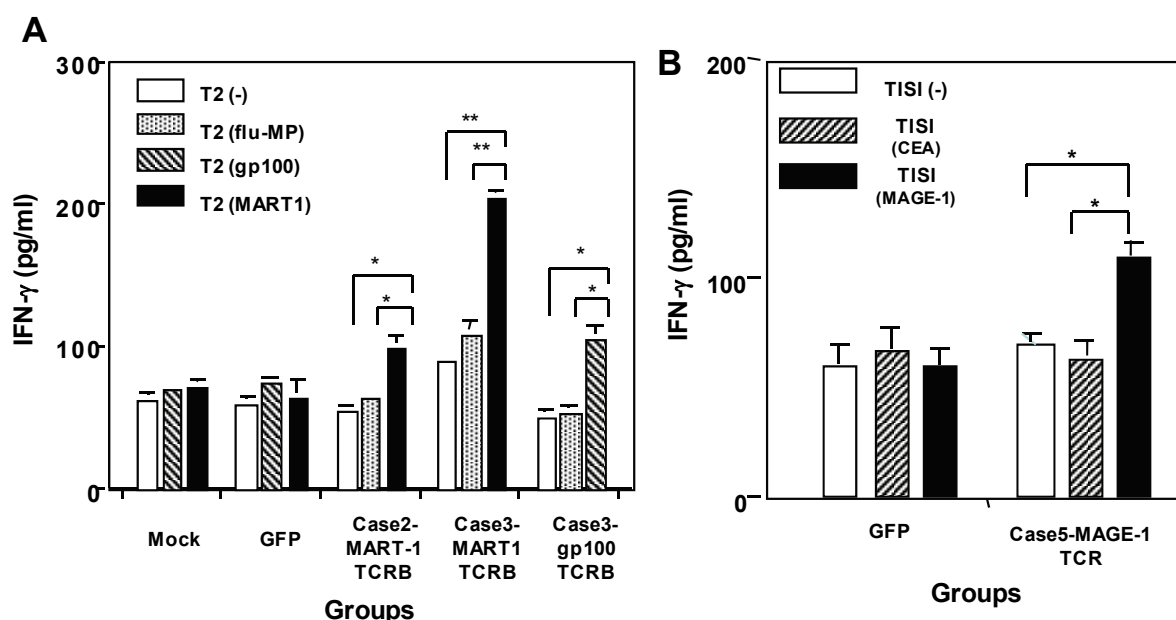


Fig. 7. IFN- $\gamma$  production from specific TCR cDNA-transduced PBLs derived from a melanoma patient against target cells. Mock or TCR gene-transduced PBLs were co-incubated with peptide-treated target cells. (A) MART-1 or gp100 A2 peptide-specific CTLs from melanoma case 2 and 3. (B) MAGE-1 A24 peptide-specific CTL from melanoma case 5. The amount of IFN- $\gamma$  produced in the supernatant was measured using an ELISA kit. T2 (-); untreated, T2 (peptide); treated with various peptides. Each column shows the mean  $\pm$  S.D. for triplicate samples. \*\* ;  $P < 0.01$ , \* ;  $P < 0.05$

## 4. Discussion

It is generally considered that spontaneously immunized CTL clones can be recognized at tumor sites or in peripheral blood without aggressive vaccination, because melanomas are generally immunogenic tumors in terms of the immune response against antigens (Mandrizzato et al., 2002; Sensi et al., 1995). With regard to common melanoma antigens like MART-1, gp100, and tyrosinase and MAGEs, many heterogenous tumor-infiltrating lymphocytes (TILs) or blood CTLs specific to these peptides have been identified using clonal analysis and characterized specifically in terms of antigen avidity and cytotoxic

activity against tumors (Valmori et al., 2000; Sensi et al., 1993; Yee et al., 1999; Hishii et al., 1997).

This time we characterized melanoma antigen-specific CTL lines derived from the blood of patients given DC vaccines and established an *ex vivo* expansion culture method. Finally, our group succeeded in cloning and sequencing melanoma peptide (MART-1, gp100, and MAGE-1)-specific TCR genes. Few clonal CTL analyses after the use of cancer vaccines including DCs and peptides have been performed so far (Valmori et al., 2002; Kan-Mitchell et al., 1993; Powell et al., 2006) Powell *et al.* demonstrated the efficacy of a multiple course peptide-immunization for the generation in high frequencies of tumor antigen-specific T cells, because they recognized vaccine-specific CTLs in blood even one year after the final vaccination (Powell et al., 2004). Additionally, Godelaine *et al.* reported that several potent CTL clones specific to MAGE3 A1 peptide were amplified after the use of a peptide-pulsed DC vaccine and the frequency of tetramer-positive CTLs in blood increased 20-400 fold compared with before the vaccination (Godelaine et al., 2003).

With regard to *ex vivo* CTL expansion, we established our own method to increase number of melanoma-peptide-specific blood CTLs from patients given a DC vaccine several times. Briefly, PBMCs obtained from melanoma patients were stimulated *in vitro* with patient-derived DCs pulsed with the same peptide as used in the DC vaccine, and furthermore activated with peptide-pulsed target cells like T2 and T2-A24. Finally, MART-1 and MAGE-1 tetramer-positive CTLs were able to be expanded up to 620 and 5070 fold, respectively in melanoma patients given the vaccine. In contrast, in the case of CTLs obtained from patients prior to the vaccination, the expansion was much less extensive. This observation demonstrated that utilizing PBMCs from vaccinated patients is a very efficient way of preparing numerous adoptive CTLs for clinical use. More importantly, MAGE-1 A24 peptide-specific CTL line was derived from a DC vaccinated-patient who showed a significant clinical response, and our group succeeded in cloning and sequencing of MAGE-1 A24 peptide-specific T cell receptor (TCR) cDNA for the first time.

Meanwhile, distinguishing these CTLs in terms of tumor-specific avidity and cytotoxicity is important. Generally, tetramer-positive CTLs have polyclonal effectors and the clone responsible for the genuine anti-tumor activity cannot be identified at the expansion stage. We utilized specific staining of CTLs with a combination of anti-TCR MoAb and intracellular IFN- $\gamma$  staining. Using this method, the monoclonal TCR repertoire mediating the anti-tumor cytotoxicity could be elucidated. Furthermore, anti-TCR MoAb-sorted CTL clones were shown to have the very potent melanoma-peptide specific cytotoxic activity. Once the functional TCR repertoire is determined, a functional CTL clone can be purified by MoAb sorting, and finally specific DNA is cloned as we have performed. This might be a novel approach to determine the genuine clone responsible for peptide-specific cytotoxicity at the level of selection of polyclonal CTLs (Fig. 8).

Demonstrating the efficiency and capability of cancer-specific CTLs for clinical application is also important issue. Many studies of cytotoxicity or avidity for tumors comparing TILs with blood CTLs have been reported. Basically, TIL clones tend to be more cytotoxic and have greater affinity for tumor cells and a more limited TCR repertoire than blood CTLs. Cole *et al.* and others showed that the same TCR repertoire specific to MART-1 peptide was recognized among blood CTLs as TIL clones isolated from tumors, which supported the application of vaccine-boosted blood CTLs to adoptive immunotherapy (Cole et al., 1997). In the present study, TILs from melanoma tissue were not analyzed. In future, upcoming resected tumors will be used for TIL expansion according to other researchers' methods.

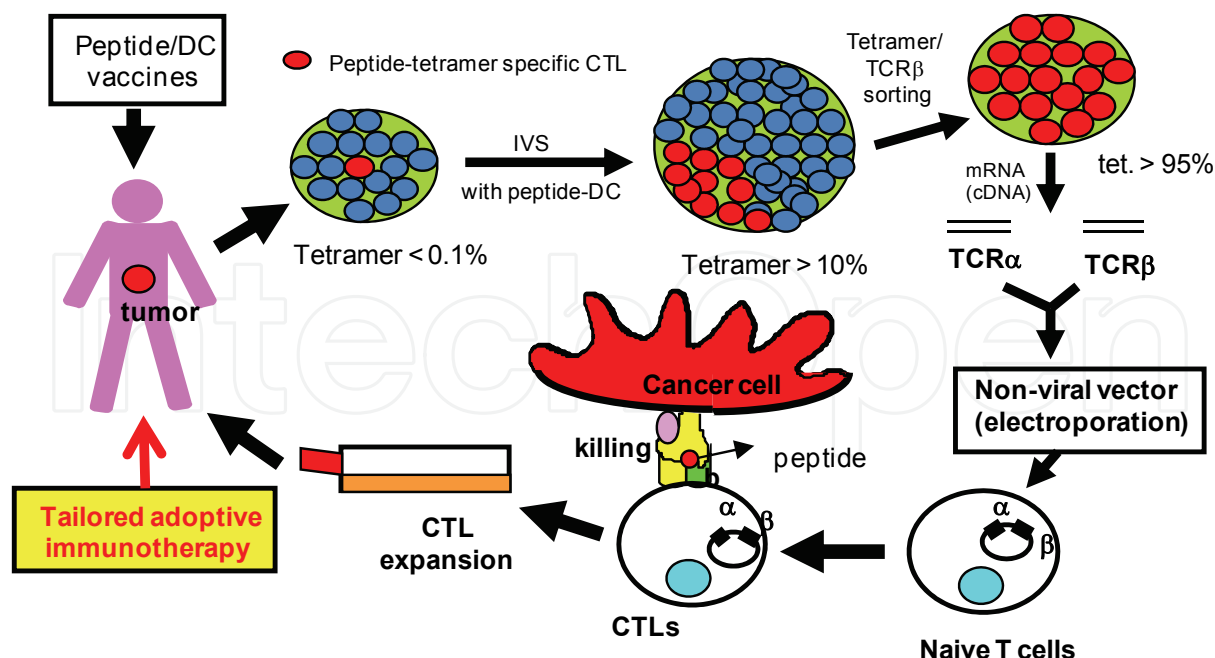


Fig. 8. Strategy for vaccinated patients-based immune-effector cell engineering for developing a novel adoptive CTL therapy

When considering the application of native adoptive CTL therapy, a great number of potent CTLs specific to cancer peptides are needed. The technology of TCR gene-engineering is possibly one efficient tool with which to expand necessary specific effector T cells. Recently, retroviral vector-mediated TCR gene transduction has been utilized in basic research and some clinical trials (Roszkowski et al., 2005; Hughes et al., 2005; Morgan et al., 2006; Tsuji et al., 2005). Recently, the use of a lentiviral vector system was shown to be the optimal way to transduce specific TCR genes into naïve T cells (Van Tendeloo et al., 2007). However, adverse effects such as leukemogenesis in stem cell-based retroviral gene transduction programs cannot be avoided completely. In the present study, a novel electroporation-based TCR gene transduction was performed and the transduction efficiency in naïve T cells derived from melanoma patients was acceptable (56% for GFP gene, 31% for MART-1 TCRBV28 gene, respectively). More importantly, anti-CD3 and anti-CD28 antibody-mediated T cell activation prior to electroporation is needed to reduce the damage to T cells and promote the transduction efficiency as previously reported (Chun et al., 2002). DC vaccine-based efficient CTL expansion using blood CTLs from vaccinated melanoma patients, may be a good immunotherapeutic modality. This novel approach can be employed for adoptive CTL therapy followed by the use of peptide-cocktail pulsed DC vaccines and the administration of a T cell-supporting cytokine like IL-2, IL-7 or IL-15 to maintain and expand infused CTLs *in vivo*.

## 5. Conclusion

We characterized melanoma antigen-specific CTL lines derived from the blood of patients given DC vaccines and established an *ex vivo* expansion culture method. For functional analysis of CTLs, specific staining of CTLs with a combination of anti-TCR MoAb and intracellular IFN- $\gamma$  staining was utilized, and monoclonal TCR repertoire mediating the anti-tumor cytotoxicity was able to be elucidated. Anti-TCR MoAb-sorted CTL clones were

shown to exhibit the very potent melanoma-peptide specific cytotoxic activity. Finally, we succeeded in cloning and sequencing melanoma peptide (MART-1, gp100, and MAGE-1)-specific T cell receptor (TCR) genes. This might be a novel approach to determine the genuine clone responsible for peptide-specific cytotoxicity at the level of selection of polyclonal CTLs.

## 6. Acknowledgement

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