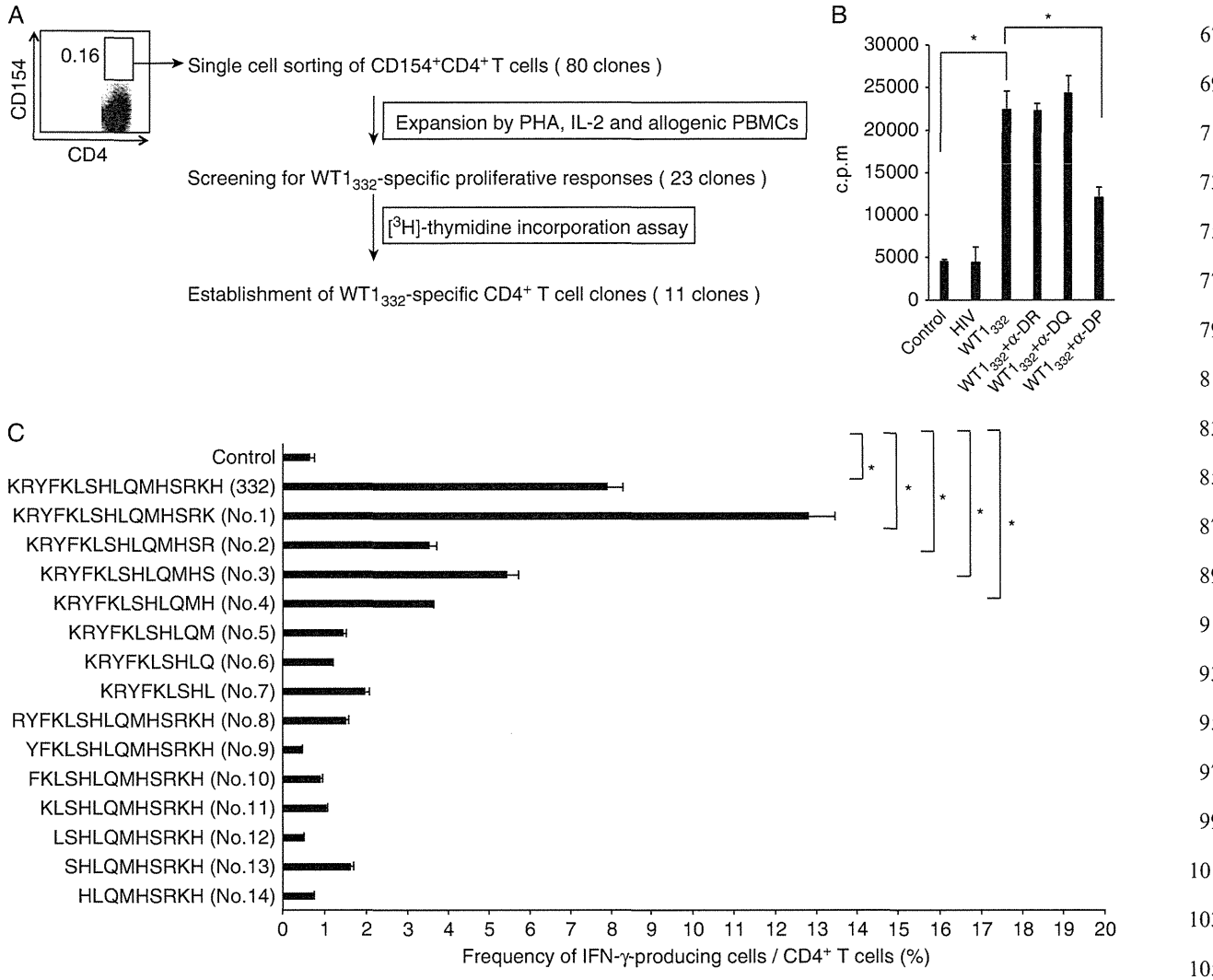

FIGURE 1. Establishment of HLA-DPB1*05:01-restricted, Wilms tumor gene 1 $(WT1)_{332}$-specific $CD4^+$ T cell clone. A, Establishment of HLA-DPB1*05:01-restricted, $WT1_{332}$-specific $CD4^+$ T-cell clones. $WT1_{332}$ peptide-primed PBMCs were restimulated with $WT1_{332}$ peptide in the presence of anti-CD154-APC mAb for 6 hours, and $CD154^+$ $CD4^+$ T cells were single-cell sorted and expanded in the presence of irradiated allogeneic PBMCs and IL-2 for 1–2 weeks. The expanded clones were screened for $WT1_{332}$-specific proliferative response by $[^3H]$-thymidine incorporation as described in the Materials and methods section. B, A $WT1_{332}$-specific CD4 T-cell clone, clone 10, was cocultured with irradiated homozygous DPB1*05:01-expressing autologous PBMCs pulsed or unpulsed with $WT1_{332}$ peptide in the presence of HLA-DR-blocking, HLA-DQ-blocking, or HLA-DP-blocking mAb and tested for proliferative responses by $[^3H]$-thymidine incorporation. Columns represent mean values ± SEM from triplicated wells. Asterisks (*) indicate significant difference ($P<0.05$). C, Epitope mapping of clone 10. Clone 10 was stimulated with the indicated WT1 peptides (20 μg/mL) in the presence of CD28/CD49d Costimulatory Reagent and Brefeldin A for 4 hours and intracellular interferon (IFN)γ staining assay was performed. Columns represent mean values ± SEM from triplicated wells. Asterisks (*) indicate significant difference ($P<0.05$). These experiments were repeated several times and similar results were obtained. Representative data are shown.

with CSII-EF-MCS-IRES2-Venus encoding $WT1_{332}$-specific TCR gene or empty plasmid, pCAG-HIVgp, and pCMV-VSUG-RSV-Rev (kindly provided by Dr H Miyoshi) using Polyethyleneimine "Max" (Polyscience Inc., Warrington, PA). After 12 hours of transfection, the medium was changed and the cells were further cultured for 48 hours. The supernatant containing the recombinant lentiviruses were collected, filtered through 0.45-μm filters and concentrated by using PEG-it Virus Concentration Solution (System Biosciences, Mountain View, CA) according to the manufacture's procedures. The concentrated viruses were dissolved in Hanks' balanced salt solution and stored at −80°C.

Cloning of HLA-DPA1*01/DPB1*05:01 (HLA-DP5) Gene and Establishment of HLA-DP5-positive TF-1 Cells

Total RNA was obtained from PBMCs from a healthy donor with homozygous HLA-DPB1*05:01 and reverse-transcribed into cDNA by Super Script III (Invitrogen Life Technologies). HLA-DPA1 and HLA-DPB1 were amplified and linked with p2A sequence by PCR with primers as follows: DPA1 primer Forward; 5′-CAGGGTCCCCTG GGCCCGGGGGTC-3′, DPA1 primer Reverse; 5′-GGG ACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTA ACAGAGAGAAGTTCGTGGCTCCGGAACCCAGGG TCCCCTGGGCCCGGGGGTC-3′, DPB1 primer Forward;

5′-GCCACGAACTTCTCTCTGTTAAAGCAAGCAGGA GACGTGGAAGAAAACCCCGGTCCCATGATGGTTC TGCAGGTTTCTGCG-3′, DPB1 primer Reverse; 5′-ATG ATGGTTCTGCAGGTTTCTGCG-3′. PCR conditions were as follows: 94°C for 2 minutes and 25 cycles (98°C for 10 s, 60°C for 30 s, and 68°C for 90 s). Amplified HLA-DPA1-p2A-HLA-DPB1 cassette was cloned into the *Eco*RI and *Xho*I site of pcDNA3.1 (+) cloning vector (Invitrogen) then sequenced by BigDye Terminator v3.1 cycle sequencing kit.

The HLA-DP5-encoding pcDNA3.1 (+) was electroporated into TF-1 cells with Neon Microporation System (Invitrogen Life Technologies) and then the HLA-DP5-positive TF-1 cells were selected with G418 (Nacalai Tesque corp.)

Transduction of $WT1_{332}$-specific TCR Gene into Mouse TG40 Cells

Thirty thousand TG40 cells were added to a 48-well plate and incubated with $WT1_{332}$-specific *TCR* genes-encoding ($WT1_{332}$-TCR) or control lentiviruses (Mock) in the presence of 8 μg/mL polybrene (Sigma, St Louis, MO). After 16 hours of incubation, the medium was changed and the transduced cells were further cultured and analyzed for the expression of CD3 molecules on their cell surface.

Generation of $WT1_{332}$-specific TCR Gene-transduced $CD4^+$ T Cells

PBMCs were stimulated with plate-bound anti-CD3 (5 μg/mL) and anti-CD28 (1 μg/mL) mAbs in the presence of 40 IU/mL IL-2 for 2 days. Thirty thousand activated cells were incubated in the presence of recombinant lentiviruses and 8 μg/mL polybrene on a RetroNectin-coated (TaKaRa Bio Co., Shiga, Japan) 48-well plate. The plate containing the cells was centrifuged at 2000 rpm at 33°C for 1 hour. After 12 hours of incubation, medium change was carried out and the cells were further incubated for 48–72 hour. Then the transduced cells, $Venus^+CD4^+$ T cells, were sorted by FACSAria and restimulated with irradiated, $WT1_{332}$ peptide-pulsed autologous PBMCs. Mock-transduced $CD4^+$ T cells were stimulated with 3 μg/mL PHA in the presence of irradiated, autologous PBMCs. One week later, the established $CD4^+$ T cells were used for various experiments as described later or stored. To investigate the stability of the established $CD4^+$ T cells, they were weekly restimulated with irradiated, $WT1_{332}$-pulsed autologous PBMCs.

Intracellular Cytokine Staining Assay and CD107a Mobilization Assay

For intracellular cytokine staining assays, 1×10^5 $CD4^+$ T cells were incubated with respective peptides in the presence of 2 μg/mL CD28/CD49d Costimulatory Reagent and 10 μg/mL Brefeldin A (Sigma) for 4 hours. Intracellular staining for cytokines was performed using BD Cytofix/Cytoperm Buffer (BD Biosciences) according to the manufacturer's procedures after surface staining of CD3 and one each of CD4 and CD8 molecules. The cells were analyzed with FACSAria. The data were analyzed with FlowJo software (TreeStar, San Carlos, CA).

For CD107a mobilization assay, 1×10^5 $CD4^+$ T cells were incubated with 1×10^5 $WT1_{332}$ peptide-pulsed or peptide-nonpulsed HLA-DP5-positive TF-1 in the presence of 2 μ BD GolgiStopTM and anti-CD107a-APC mAb for 5 hours. Then, the cells were harvested and intracellular cytokine staining was performed as described earlier.

Proliferation Assay and Enzyme-Linked Immunosorbent Assay (ELISA)

Proliferative capacity was assessed using a standard [^{3}H]-thymidine incorporation assay. In brief, $CD4^+$ T cells were plated at a concentration of 1×10^4/well (U-bottomed 96-well plate), and cultured with 1×10^5 irradiated (30 Gy) PBMCs pulsed or unpulsed with tumor lysate, WT1 protein (100 μg/mL), or WT1 peptides (20 μg/mL). [^{3}H]-thymidine (Amersham Biosciences, NJ) was added after culture for 2 days, and the cells were harvested onto glass-fiber filters 18 hours after the addition of [^{3}H]-thymidine. Radioactivity was then measured on a β-scintillation counter in triplicate wells. For the blocking assays, W6/32, L243, SPVL3, and B7/21 mAbs were added to the proliferation assays at their optimal concentrations for blocking of HLA class I, HLA-DR, HLA-DQ, and HLA-DP, respectively, and cell proliferation was measured as described earlier.

For evaluation of interferon (IFN)γ-release from $CD4^+$ T cells, culture supernatants were collected before the addition of [^{3}H]-thymidine and frozen at −80°C until use. IFNγ in the supernatants was measured by double sandwich ELISA using the Quantikine provided by R&D Systems (Minneapolis, MN).

^{51}Cr release assay

^{51}Cr release assays were performed as described previously with minor modification. Briefly, target cells (1×10^4 cells) labeled with ^{51}Cr were added to wells containing varying numbers of effector cells in 96-well plates. After 18 hours of incubation at 37°C, the supernatant was collected and measured for radioactivity. The percentage of specific lysis (% specific lysis) was calculated as follows: percentage of specific lysis = (cpm of experimental release − cpm of spontaneous release)/(cpm of maximal release − cpm of spontaneous release) × 100. Maximal release and spontaneous release were determined from supernatants of target cells incubated with 1% Triton X-100 and those incubated without effector cells, respectively. For granzyme B inhibition, target cells were pretreated with 100 μM Ac-IETD-Cho or DMSO as a control at 37°C for 2 hours, washed extensively, and used for the ^{51}Cr release assays.

Enhancement of the Induction of WT1-specific CTL by $WT1_{332}$-TCR-transduced $CD4^+$ T Cells

Three million of PBMCs from an HLA-DPB1*05:01-positive and HLA-DPB1-A*24:02-positive healthy donor were freshly isolated and cocultured with $WT1_{332}$-TCR-transduced or mock-transduced $CD4^+$ T cells at the indicated ratios in the presence of 20 μg/mL $WT1_{332}$ peptide and 10 μg/mL modified $WT1_{235}$ peptide ($WT1_{235m}$: CYTWNQMNL), which is an HLA-A*24:02-restricted CTL epitope. The X-VIVO 15 medium supplemented with 10% AB serum but not with exogenous IL-2 was used to evaluate a helper activity of $CD4^+$ T cells. One week later, the cells were restimulated with 2×10^6 irradiated, $WT1_{235m}$ peptide-pulsed autologous PBMCs, and cultured for further 1 week. Then, the frequencies of WT1-specific $CD8^+$ T cells were determined using $WT1_{235m}$ tetramer-staining and $WT1_{235m}$-specific IFN-γ expression by flow cytometry.

Statistical Analysis

The paired *t* test was used to assess differences between groups. *P*-value < 0.05 was considered significant.

RESULTS

Identification of a novel HLA-DPB1*05:01-restricted $CD4^+$ T-cell epitope in $WT1_{332}$ helper peptide.

We previously identified $WT1_{332}$ helper peptide (332-347: KRYFKLSHLQMHSRKH) that could promiscuously bind to multiple HLA class II molecules including HLA-DRB1*04:05, 15:02, 15:01, and HLA-DPB1*09:01 and induce the peptide-specific $CD4^+$ T cells.[22,23] First, whether or not $WT1_{332}$ helper peptide could bind to HLA-DPB1*05:01 (DP5), which was most popular in Japanese, and induce $WT1_{332}$-specific $CD4^+$ T cells was examined. PBMCs obtained from an HLA-DP5$^+$ donor were stimulated with $WT1_{332}$ for a week, and then CD154-expressing cells, which were $CD4^+$ T cells specifically activated by $WT1_{332}$, were sorted for cloning. As shown in Figure 1A, the CD154-expressing $CD4^+$ T cells that were detected at the frequency of 0.16% after the stimulation with $WT1_{332}$ were single-cell sorted by FACSAria. Twenty-three clones were obtained from 80 single cells and 11 of 23 expanded clones (47.8%) were examined for the $WT1_{332}$-specific proliferation (data not shown). To screen HLA-DP5-restricted $CD4^+$ T-cell clones, blocking assays against their proliferative responses to $WT1_{332}$ were performed (Fig. 1B). As the proliferative responses of clone 10 to $WT1_{332}$ were strongly inhibited by addition of anti-HLA-DP antibody, clone 10 was restricted to HLA-DP molecules. As the donor used here had homozygous HLA-DP5, clone 10 recognized $WT1_{332}$ in an HLA-DP5-restriction manner. Thus, clone 10 had been established as a $WT1_{332}$-specific, HLA-DP5-restricted $CD4^+$ T-cell clone.

To confirm the $WT1_{332}$-specific response of clone 10, it was stimulated with various deletion peptides and the frequencies of IFNγ-producing cells were examined (Fig. 1C). The response of clone 10 to WT1 peptide (No.1) that was deleted by 1 amino acid at carboxyl terminus was higher, but the response to the remaining 13 that were deleted WT1 peptides was less or nothing, compared to that to original $WT1_{332}$ peptide. These results confirmed that clone 10 specifically responded to $WT1_{332}$ peptide whose core amino acid sequence was KRYFKLSHLQMHSRK.

Cloning of HLA-DP5-resticited, $WT1_{332}$-specific TCR Genes

Full-length TCR α- chain and β-chain genes of clone 10 were cloned and identified by using 5′-RACE technique. Then, they were linked to both ends of p2A peptide to ensure simultaneous expression of both α and β chains, and the resultant TCR α 8.2-p2A-TCR β 13.3 cassette was cloned into lentiviral vector (Fig. 2A). To verify that the cloned TCR could be correctly expressed on cell surface, the TCR α 8.2-p2A-TCR β 13.3 or empty vector (mock)-expressing lentivirus were transfected into mouse TG40 hybridoma cell line which cannot express CD3 molecules on their cell surface because of deficiency in their TCR α/β expression.[26] Both TCR α 8.2-p2A-TCR β 13.3-transduced and mock-transduced TG40 cells showed high Venus fluorescence protein expression compared to parental TG40 cells (Fig. 2B), whereas only TCR α 8.2-p2A-TCR β 13.3-transduced TG40 cells showed CD3ε expression in

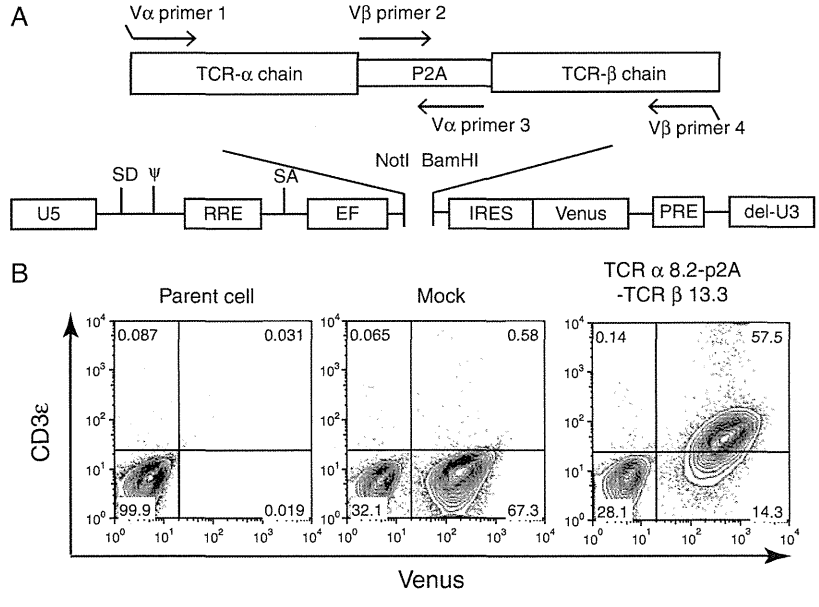

FIGURE 2. Cloning and expression of *TCR α 8.2/TCR β 13.3* gene using lentiviral vector. A, Construction of a lentiviral vector encoding full-length *TCR α 8.2* and *β 13.3* genes derived from clone 10, and primer positions for cloning of *TCR*. Lentiviral vector constructions are as follows: SD, splicing donor site; Ψ, packaging signal; RRE, Rev responsive element; SA, splicing acceptor site; EF, human elongation factor 1-α subunit promoter; MCS, multiple cutting site; IRES, encephalomyocarditis virus internal ribosomal entry site; Venus, a variant of yellow fluorescent protein (YFP) gene; PRE, Woodchuck hepatitis virus posttranscriptional regulatory element; and del-U3′, deletion of enhancer and promoter sequences in the U3 region. B, Mouse TG40 cells were transduced with T-cell receptor (TCR) α 8.2-p2A-TCR β 13.3 cassette-encoding lentiviral vector (TCR α 8.2-p2A-TCR β 13.3) or empty lentiviral vector (Mock). After 3 days of transduction, parental TG40 and lentiviral vector-transduced cells were stained with anti-mouse CD3ε-PE mAb and analyzed with flow cytometry.

the Venus$^+$ cell population. Thus, these results clearly indicated that the TCR α 8.2-p2A-TCR β 13.3 could be correctly expressed on the surface of the TG40 cells.

Functional Expression of TCR α 8.2-p2A-TCR β 13.3 in human CD4$^+$ T Cells

Next, whether or not TCR α 8.2-p2A-TCR β 13.3 was functional in human CD4$^+$ T cells was investigated. To establish TCR α 8.2-p2A-TCR β 13.3-transduced CD4$^+$ T cells, PBMCs with homozygous HLA-DP5 were transfected with TCR α 8.2-p2A-TCR β 13.3-encoding lentiviral vector. After 72 hours of transfection, the Venus$^+$ CD4$^+$ T cells were sorted and cocultured with irradiated, and $WT1_{332}$-pulsed autologous PBMCs. After 1 week of culture, intracellular cytokine assay was performed to investigate $WT1_{332}$ specificity of the expanded CD4$^+$ T cells. As expected, the expanded TCR α 8.2-p2A-TCR β 13.3-transduced CD4$^+$ T cells expressed IFN-γ and IL-2 only by the stimulation with $WT1_{332}$ (Fig. 3A), whereas mock-transduced CD4$^+$ T cells did not show any cytokine expression in response to $WT1_{332}$. In addition, the cytokine expression of the TCR α 8.2-p2A-TCR β 13.3-transduced CD4$^+$ T cells was dependent on the concentration of $WT1_{332}$ peptide (Fig. 3B). Furthermore, proliferative response to $WT1_{332}$ of the TCR α 8.2-p2A-TCR β 13.3-transduced CD4$^+$ T cells was remarkably inhibited by addition of an anti-HLA-DP antibody, but not by addition of anti-HLA class I, anti-HLA-DR, or anti-HLA-DQ antibody (Fig. 3C). As PBMCs with homozygous HLA-DP5 were used as a stimulator, it was concluded that the TCR α 8.2-p2A-TCR β 13.3-transduced CD4$^+$ T cells recognized $WT1_{332}$ in an HLA-DP5-restriction manner. Importantly, $WT1_{332}$-specific cytokine expression in the TCR α 8.2-p2A-TCR β 13.3-transduced CD4$^+$ T cells was observed even after 3 months of culture (Fig. 3D), indicating that function of TCR α 8.2-p2A-TCR β 13.3-transduced CD4$^+$ T cells were stable for long-term culture.

In our previous studies, it was demonstrated that $WT1_{332}$ ($WT1_{332-347}$, KRYFKLSHLQMHSRKH) was a natural epitope for CD4$^+$ T cells with the restriction of HLA-DRB1*04:05, 15:01, 15:02, and HLA-DPB1*09:01. To examine whether or not TCR α 8.2-p2A-TCR β 13.3-transduced CD4$^+$ T cells could recognize the natural epitope of WT1 protein in an HLA-DP5-restriction manner, the CD4$^+$ T cells were cocultured with WT1 peptide-pulsed, WT1 protein-pulsed, or WT1-expressing tumor lysate-pulsed autologous PBMCs that were used as a stimulator and the proliferative responses of the CD4$^+$ T cells were measured. Consequently, the TCR α 8.2-p2A-TCR β 13.3-transduced CD4$^+$ T cells showed proliferative responses to $WT1_{332}$ peptide-pulsed or full-length WT1 protein (HWT1)-pulsed autologous PBMCs, but not to those pulsed with the truncated WT1 protein not containing $WT1_{332}$ peptide sequences (HWT3). Furthermore, the CD4$^+$ T cells could strongly proliferate (Fig. 3E) and produce IFN-γ (Fig. 3F) in response to the PBMCs pulsed with the lysate of WT1-expressing leukemia cell line (TF-1 and K562).

Taken together, these results clearly demonstrated that cloned TCR α 8.2-p2A-TCR β 13.3 really encoded $WT1_{332}$-specific, HLA-DP5-restriced TCR of clone 10. Thus, in the following chapters, "$WT1_{332}$ TCR-transduced' was used in exchange for 'TCR α 8.2-p2A-TCR β 13.3-transduced."

$WT1_{332}$ TCR-transduced CD4$^+$ T Cells are Th1 Type-Cytokine Profile

It was previously reported that $WT1_{332}$ could dominantly induce Th1-type CD4$^+$ T cells. Therefore, whether or not $WT1_{332}$ TCR-transduced CD4$^+$ T cells displayed Th1 dominant cytokine profile was examined. $WT1_{332}$ TCR-transduced CD4$^+$ T cells from 3 healthy donors with HLA-DP5 were established as shown in Figure 3 and examined for cytokine production by intracellular cytokine staining assay (Fig. 4). As expected, all the 3 established CD4$^+$ T-cell lines expressed at high frequencies the Th1-type cytokines such as IL-2, IFN-γ, TNF-α, and GM-CSF in response to $WT1_{332}$ stimulation. However, the expressions of Th2-type cytokines (IL-5 and IL-10) or Th17-type cytokine (IL-17) were at low frequencies or undetectable in all the 2 CD4$^+$ T-cell lines.

Thus, the results that the cloned $WT1_{332}$ TCR let CD4$^+$ T cells endow Th1-type were consistent with those that $WT1_{332}$-specific CD4$^+$ T-cell clones established from PBMCs from healthy donors were dominantly Th1-type.

$WT1_{332}$ TCR-transduced CD4$^+$ T Cells can Directly Recognize and Kill WT1-expressing Leukemia Cell Lines through Perforin/Granzyme B Pathway

Next, whether $WT1_{332}$ TCR-transduced CD4$^+$ T cells could recognize and kill WT1-expressing leukemia cell line in HLA-DP5-restriction manner was examined.

HLA-DPA1*-01-DPB1*05:01-expression vector was transduced into TF-1 cells and HLA-DP5-positive TF-1 cells were established. The HLA-DP5-positive TF-1 cells established could present $WT1_{332}$ peptide to the $WT1_{332}$ TCR-transduced CD4$^+$ T cells and were useful as target cells in killing assay (data not shown). $WT1_{332}$-TCR-transduced CD4$^+$ T cells showed strong cytotoxic activity against HLA-DP5-positive TF-1 cells, but not against parental TF-1 cells (Fig. 5A). In contrast, empty lentiviral vector-transduced CD4$^+$ T cells did not show cytotoxicity against both target cells. To confirm WT1-specific cytotoxicity of the $WT1_{332}$ TCR-transduced CD4$^+$ T cells, WT1-overexpressing autologous B-LCL (B-LCL(+)) and its parental B-LCL [B-LCL(−)] were used as target cells (Fig. 5B). As expected, the $WT1_{332}$ TCR-transduced CD4$^+$ T cells effectively lysed B-LCL(+) cells compared to B-LCL(−) cells. Furthermore, the $WT1_{332}$ TCR-transduced CD4$^+$ T cells could lyse endogenously WT1-expressing and HLA-DP5-positive C2F8 leukemia cells (Fig. 5C). Thus, these results clearly demonstrated that the $WT1_{332}$ TCR-transduced CD4$^+$ T cells had a potent cytotoxic activity against WT1-expressing, HLA-DP5-positive malignant cells such as leukemia cells.

Next, whether or not the $WT1_{332}$ TCR-transduced CD4$^+$ T cells exerted the cytotoxic activity through a granzyme B and perforin pathway was investigated. High expression of granzyme B and perforin was observed in the $WT1_{332}$ TCR-transduced CD4$^+$ T cells (Fig. 5D). Furthermore, the simultaneous expression of IFN-γ and CD107a, which reflected degranulation, was observed only when the $WT1_{332}$ TCR-transduced CD4$^+$ T cells were incubated with $WT1_{332}$-pulsed HLA-DP5-positive TF-1 cells (Fig. 5E). Finally, in order to confirm that the cytotoxicity of the $WT1_{332}$ TCR-transduced CD4$^+$ T cells was dependent on granzyme B/perforin pathway, HLA-DP5-

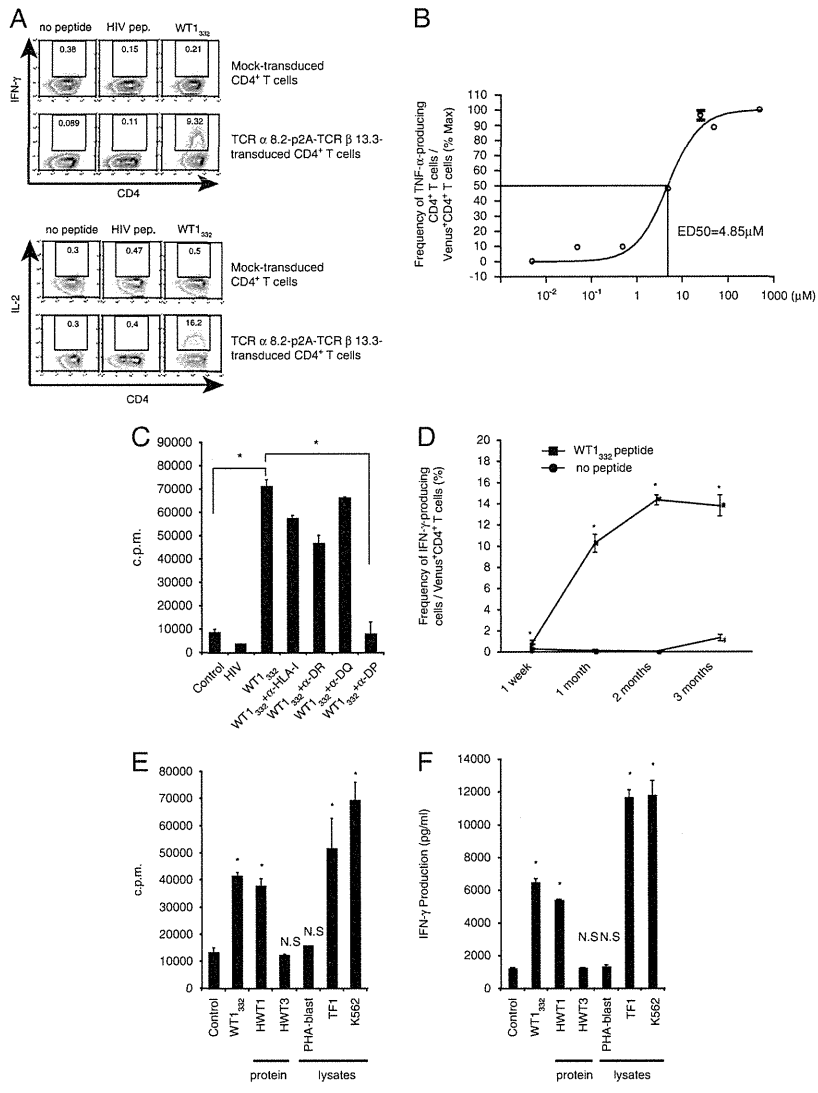

FIGURE 3. T-cell receptor (TCR) α 8.2-p2A-TCR β 13.3-transduced CD4+ T cells displays antigen-specific T-cell responses. PBMCs from a healthy donor were transduced with TCR α 8.2-p2A-TCR β 13.3 cassette-encoding lentiviral vector (TCR α 8.2-p2A-TCR β 13.3) or empty lentiviral vector (Mock) and then the transduced CD4+ T cells were sorted and expanded for a week as described in the Materials and methods section. A, The transduced CD4+ T cells were stimulated with or without the indicated peptides for 4 hours and then intracellular interferon (IFN)γ (upper) and IL-2 (lower) were analyzed by flow cytometry. Representative data from 3 independent experiments are shown. B, The TCR α 8.2-p2A-TCR β 13.3-transduced CD4+ T cells were stimulated with various concentrations of Wilms tumor gene 1 $(WT1)_{332}$ for 4 hours and intracellular cytokine staining assay was performed. Each plots represent mean values ± SEM from duplicated wells. A half maximum effective dose (ED50) calculated is shown. Data are representative of several independent experiments. C, The TCR α 8.2-p2A-TCR β 13.3-transduced CD4+ T cells were cocultured with irradiated homozygous DPB1*05:01-expressing autologous PBMCs pulsed or unpulsed with Wilms tumor gene 1 $(WT1)_{332}$ peptide in the presence of HLA class I-blocking, HLA-DR-blocking, HLA-DQ-blocking, or HLA-DP-blocking mAb and tested for proliferative responses by [^{3}H]-thymidine incorporation. Columns represent mean values ± SEM from triplicated wells. Asterisks (*) indicate significant difference ($P<0.05$). Representative data from 3 independent experiments are shown. D, The TCR α 8.2-p2A-TCR β 13.3-transduced CD4+ T cells that were cultured with weekly $WT1_{332}$ peptide stimulation were tested for the capacity of $WT1_{332}$-specific IFNγ production by intracellular cytokine staining in response to $WT1_{332}$ peptide stimulation at the indicated time points. Data represent mean values ± SEM from triplicated assays. The TCR α 8.2-p2A-TCR β 13.3-transduced CD4+ T cells were cocultured with autologous irradiated PBMCs pulsed or unpulsed with $WT1_{332}$ peptide (20 μg/mL), HWT1 (full-length WT1 protein, 100 μg/mL), HWT3 (truncated WT1 protein, 1–294 amino acids, 100 μg/mL), PHA-induced lymphoblast lysate, TF-1 leukemia cell lysate, or K562 leukemia cell lysate. Proliferative responses (E) and IFN-γ production (F) of the CD4+ T cells were evaluated by [^{3}H]-thymidine incorporation and enzyme-linked immunosorbent assay, respectively. Columns represent mean values ± SEM from triplicated wells. Asterisks (*) indicate significant ($P<0.05$) proliferative response compared to control sample. These experiments were repeated several times and similar results were obtained. Representative data are shown. NS indicates not significant.

positive TF-1 cells that were pretreated with 100 μM granzyme B inhibitor, ac-IETD-Cho were used as target cells. The cytotoxicity of the $WT1_{332}$ TCR-transduced CD4+ T cells against ac-IETD-Cho-pretreated TF-1 cells remarkably decrease compared to that against DMSO-pretreated TF-1 cells (Fig. 5F).

Taken together, the $WT1_{332}$ TCR-transduced CD4+ T cells established here could directly recognize WT1-

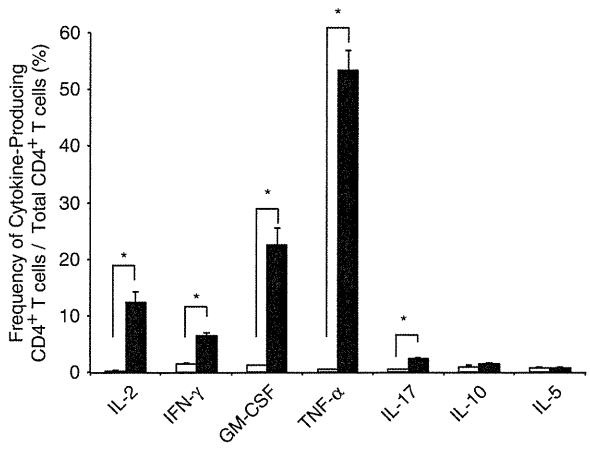

FIGURE 4. Wilms tumor gene 1 $(WT1)_{332}$ T-cell receptor (TCR)-transduced CD4+ T cells have a Th1 type-cytokine profile. $WT1_{332}$ TCR-transduced CD4+ T-cell lines were established from 3 different healthy donors as described in Figure 3. The CD4+ T cells were incubated with or without $WT1_{332}$ peptide in the presence of CD28/CD49d Costimulatory Reagent and Brefeldin A for 4 hours and then intracellular cytokine assay was performed. Columns represent mean values ± SEM of results from 3 different healthy donors. Asterisks (*) indicate significant difference ($P < 0.05$).

expressing, HLA-DP5-positive leukemia cells and kill them through a granzyme B/perforin pathway.

$WT1_{332}$ TCR-transduced CD4+ T Cells Enhance the Induction of WT1-specific CD8+ Cytotoxic Lymphocytes

We previously demonstrated that $WT1_{332}$-specific $CD4^+$ T-cell clones established previously were Th1-type and could enhance the induction of WT1-derived CTL epitope-specific $CD8^+$ CTLs. It is interesting to note that, all $WT1_{332}$ TCR-transduced $CD4^+$ T cells established from 3 healthy donors also showed a Th1-type cytokine profile as shown in Figure 4. It was therefore expected that the $WT1_{332}$ TCR-transduced $CD4^+$ T cells could enhance the induction of WT1-specific $CD8^+$ CTLs.

To confirm the helper activity of $WT1_{332}$ TCR-transduced $CD4^+$ T cells, the $CD4^+$ T cells were cocultured with HLA-A*24:02-positive autologous PBMCs in the presence of $WT1_{332}$ helper peptide and modified $WT1_{235}$ peptides ($WT1_{235m}$, HLA-A*24:02-restricted CTL-epitope). After 1 week of coculture, the cells were restimulated with $WT1_{235m}$-pulsed, irradiated autologous PBMCs, and further cultured for 1 week. After the serial culture, the frequencies of $CD8^+$ T cells and $WT1_{235m}$-specific $CD8^+$ CTLs were evaluated by flow cytometry. Expectedly, when $WT1_{332}$ TCR-transduced $CD4^+$ T cells were added to autologous PBMCs, the frequencies of $CD8^+$ T cells and $WT1_{235m}$-specific $CD8^+$ CTLs significantly increased, compared to the addition of mock-transduced $CD4^+$ T cells to the autologous PBMCs (Figs. 6A, B). Cell numbers of $WT1_{235m}$-specific $CD8^+$ CTLs increased 10.8- or 27.6-fold by the addition of $WT1_{332}$ TCR-transduced $CD4^+$ T cell at ratio of auto-PBMCs:$CD4^+$ T cells, 10:1 or 10:2, respectively (Fig. 6C). To rule out that the increased frequency of $WT1_{235m}$-specific $CD8^+$ CTLs was due to nonspecific tetramer binding, IFNγ expression of these cells was assessed in response to $WT1_{235m}$. Consistent with the results of tetramer assay, $CD8^+$ T cells that were cultured with $WT1_{332}$ TCR-transduced $CD4^+$ T cells could express IFNγ in response to $WT1_{235m}$ (Figs. 6D, 6E). However, no significant IFNγ expression was observed in the $CD8^+$ T cells that were cultured with the mock-transduced $CD4^+$ T cells. In addition, the average frequencies of IFNγ-producing cells in $CD8^+$ T cells were 3.7% or 6.8% when $WT1_{332}$ TCR-transduced $CD4^+$ T cells were added at ratio of auto-PBMCs:$CD4^+$ T cells, 10:1 or 10:2, respectively (Fig. 6E).

Thus, these results clearly demonstrated that $WT1_{332}$ TCR-transduced $CD4^+$ T cells could enhance the induction of WT1-specific $CD8^+$ CTLs dependently on cell number of the $CD4^+$ T cells.

DISCUSSION

Although a number of studies of TAA-specific TCR gene therapy were reported in last decade,[14–16,32–34] there seemed to be few studies focusing on HLA class II-restricted, TAA-specific TCR gene therapy.[35–37] It is likely that the following 3 steps is necessary for induction an optimal antitumor immune response[38]: first, antigen-presenting cells such as dendritic cell and macrophage phagocytose necrotic/apoptotic tumor cells and present TAAs to $CD4^+$ T cells in context with MHC class II; second, the TAA-specific $CD4^+$ T cells recognize TAAs and activate the antigen-presenting cells; and third, the TAA-specific $CD8^+$ CTLs are induced by the activated antigen-presenting cells through cross-priming and kill the TAA-expressing tumor cells. Accordingly, $CD4^+$ T cells bearing HLA class II-restricted TCR specific for TAAs (ie, TAA-specific $CD4^+$ T cells) facilitate a link between antigen-presenting cells and $CD8^+$ CTLs and play a crucial and central role in induction of an optimal antitumor immune response. It is therefore expected that TCR gene therapy using HLA class II-restricted TCR with combination of HLA class I-directed therapies such as HLA class I-restricted peptide vaccine or TCR therapy specific for TAAs can accelerate antitumor immune response. Thus, it was strongly indicated that $WT1_{332}$-specific TCR gene cloned here should be useful for an HLA class II-restricted TCR gene therapy.

In the present study, it was clearly demonstrated that $WT1_{332}$ TCR-transduced $CD4^+$ T cells had a potent cytotoxicity against WT1-expressing hematological malignant cells through granzyme B/perforin pathway. The granzyme B/perforin-dependent cytotoxicity of $CD4^+$ CTLs had been demonstrated in previous investigations.[39–41] In general, expression of HLA class II, unlike that of HLA class I, is not ubiquitous and is usually observed only on antigen-presenting cells. However, hematological malignant cells such as leukemia and lymphoma often express not only HLA class II but also costimulatory molecules (CD80, CD86, and CD54) on the cell surface. Furthermore, it has been shown that many solid tumors, including melanoma, gastric carcinoma, colorectal carcinoma, breast cancer, head and neck squamous cell carcinoma, osteosarcoma, lung cancer, and ovarian cancer also express HLA class II molecules.[42,43] Consequently, HLA class II-expressing tumor cells will be recognized and killed by TAA-specific $CD4^+$ T cells. In fact, it has been demonstrated that loss of HLA class II expression in lymphoma is related to decreased tumor immunosurveillance and poor patient survival.[44] In addition, most recent report has demonstrated that human leukemic cells acquire the

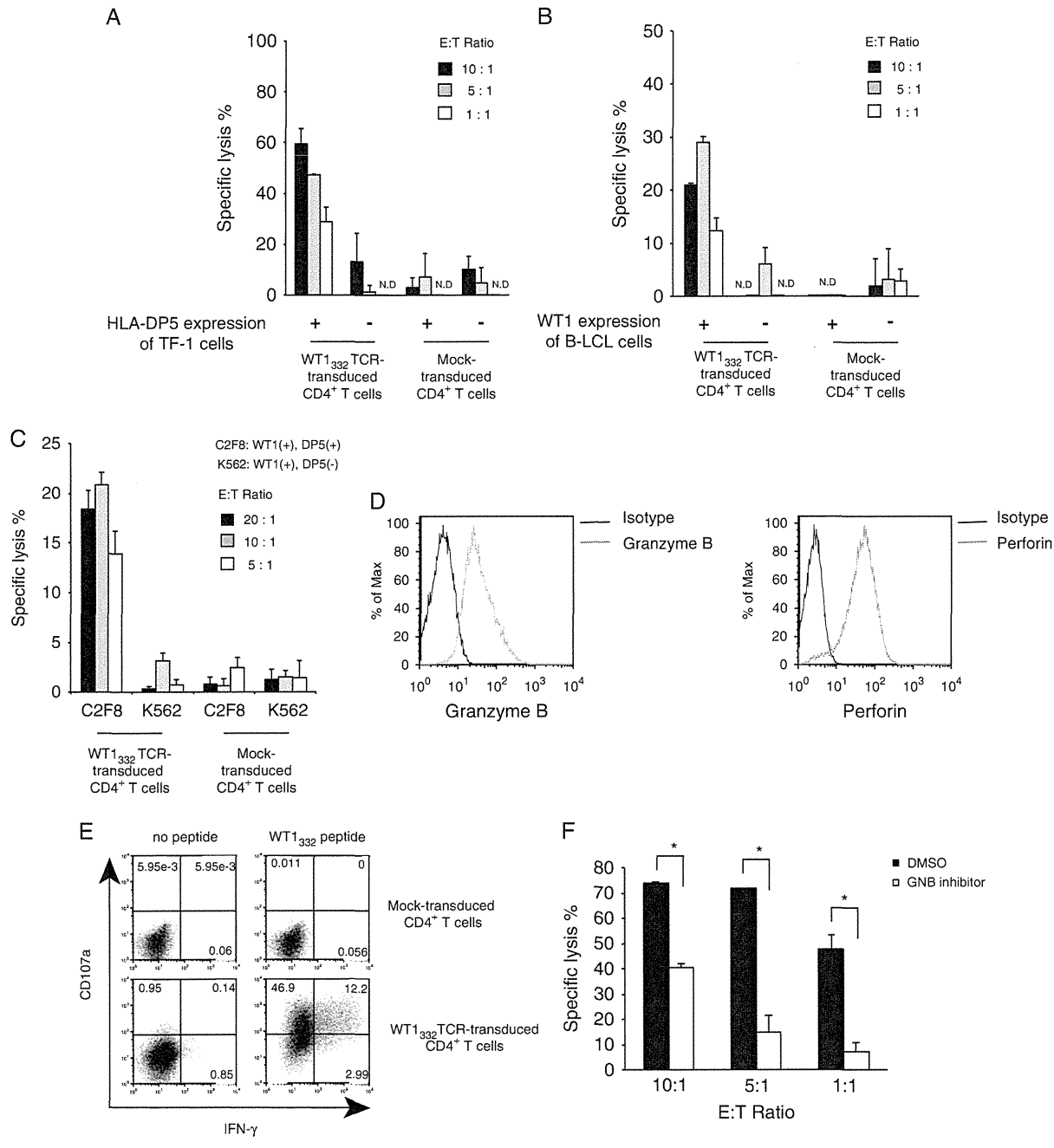

FIGURE 5. Wilms tumor gene 1 $(WT1)_{332}$ T-cell receptor (TCR)-transduced CD4+ T cells can directly kill leukemia cells through granzyme B/perforin pathway in an HLA-DPB1*05:01-restricted, $WT1_{332}$-specific manner. A–C, $WT1_{332}$ TCR-transduced CD4+ T cells were tested for cytotoxic activity against HLA-DPB1*05:01 positive or HLA-DPB1*05:01 negative, WT1-expressing TF-1 leukemia cell lines (A), HLA-DPB1*05:01 positive, WT1-expressing or WT1-unexpressing B-LCL cells (B) and HLA-DPB1*05:01 positive, WT1-expressing (C2F8) and HLA-DPB1*05:01-negative, WT1-expressing (K562) cell lines (C). $WT1_{332}$ TCR-transduced CD4+ T cells were incubated with ^{51}Cr-labeled target cells at the indicated E/T ratio for 18 hours. Columns represent mean values ± SEM from triplicated wells. These experiments were repeated several times and similar results were obtained. E/T ratio indicates ratio of effector:target cells; N.D., not detected. (D, Expression of perforin and granzyme B in $WT1_{332}$ TCR-transduced CD4+ T cells was detected by flow cytometry. Representative histograms are shown. E, $WT1_{332}$ TCR-transduced and empty vector (mock)-transduced CD4+ T cells were cocultured with $WT1_{332}$ peptide-pulsed or peptide-unpulsed HLA-DP5-positive TF-1 in the presence of anti-CD107a-APC mAb for 5 hours and then intracellular interferon (IFN)-γ staining was performed. The plots are gated on Venus+ CD4+ T cells, and the percentage on each quadrant is shown on the plot. F, HLA-DP*05:01-positive TF-1 cells were pretreated with 100 μM of granzyme B inhibitor (Ac-IETD-Cho) or DMSO as a control for 2 hours and then labeled with ^{51}Cr, and incubated with $WT1_{332}$ TCR-transduced CD4+ T cells and ^{51}Cr release assay was performed. Columns represent mean values ± SEM from triplicated wells. Asterisks (*) indicate significant difference ($P < 0.05$). These experiments were repeated several times and similar results were obtained.

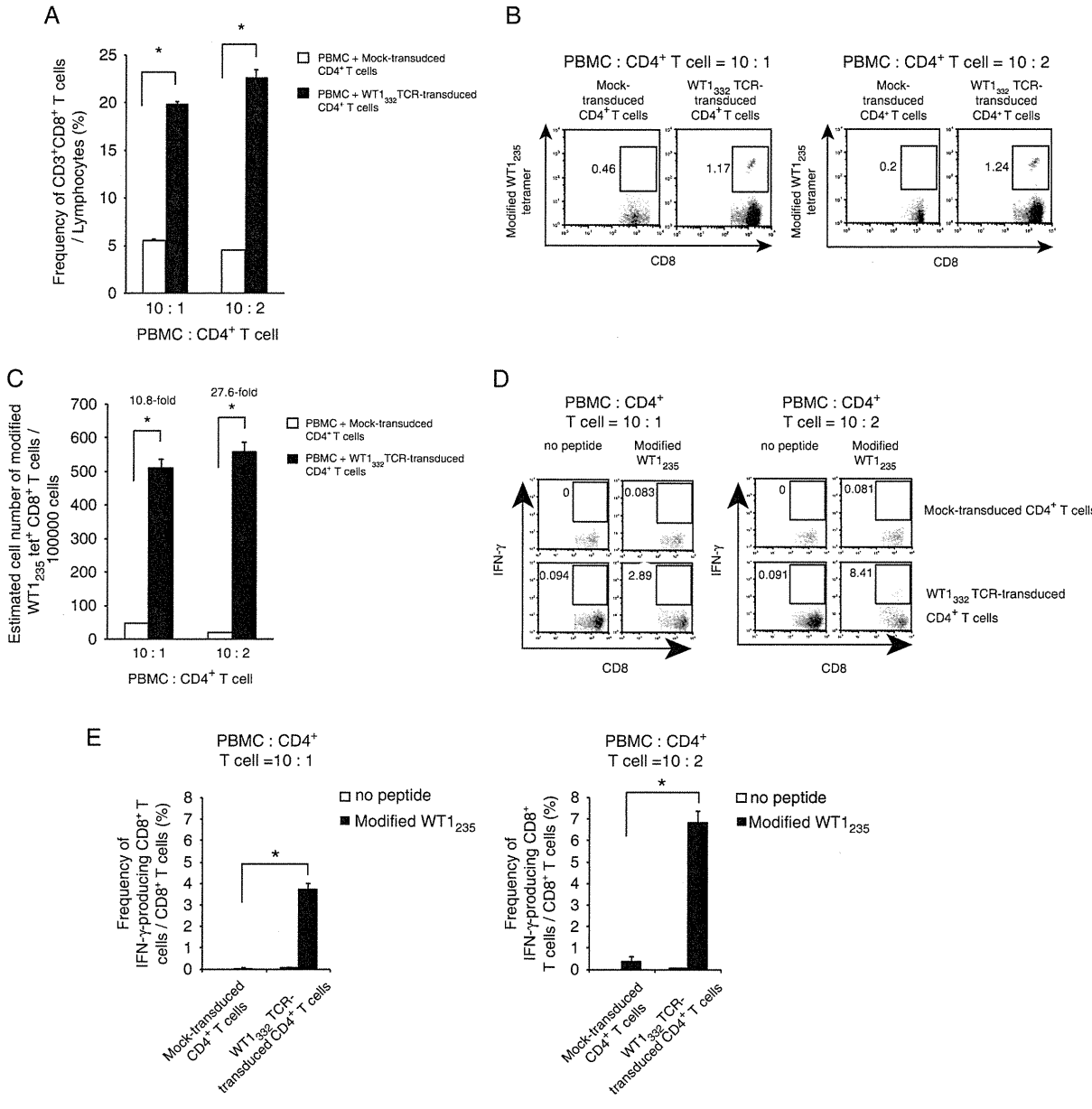

FIGURE 6. Enhancement of the induction of Wilms tumor gene 1 (WT1)-specific CD8+ CTLs by $WT1_{332}$ T-cell receptor (TCR)-transduced CD4+ T cells. $WT1_{332}$ TCR-transduced or empty vector (mock)-transduced CD4+ T cells were added to 3×10^6 autologous PBMCs with HLA-A*24:02/HLA-DPB1*05:01 at the indicated ratios. The mixed cells were cultured in the presence of $WT1_{332}$ peptide and WT1-derived CTL epitope, modified $WT1_{235}$ peptide, $WT1_{235m}$. On day 7, the cells were restimulated with irradiated, $WT1_{235m}$-pulsed autologous PBMCs, and further cultured for a week. These cultures were performed in exogenous recombinant IL-2-free medium. Seven days after the restimulation, frequencies of CD8+ T cells (A), $WT1_{235m}$ tetramer+ CD8+ T cells (B and C), and interferon (IFN)γ-producing CD8+ T cells in response to $WT1_{235m}$ (D and E) were investigated using flow cytometry. A, Frequencies of CD8+ T cells are shown. CD8+ T cells were determined as $Venus^-CD3^+$ CD8+ cells in a 7-AAD− lymphocyte population. B, Representative dot plots of $WT1_{235m}$/HLA-A*24:02-tetramer and CD8 are shown. The plots are gated on 7-AAD−$Venus^-CD3^+CD8^+$ lymphocytes. C, Cell numbers of $WT1_{235m}$-specific CTLs per 1×105 lymphocytes were estimated from the frequency of $WT1_{235m}$ tetramer+ CD8+ T cells in CD8+ T cells. D and E, The cells cultured as described earlier were restimulated with $WT1_{235m}$ peptide in the presence of CD28/CD49d Costimulatory Reagent and Brefeldin A for 4 hours and intracellular IFN-γ-staining assay was performed. Representative dot plots (D) and summarized data (E) are shown. All data shown in columns (A, C, and E) represent mean values ± SEM from duplicated assays. Asterisks (*) indicate significant difference ($P < 0.05$). These experiments were repeated 2 times and similar results were obtained.

phenotypes like an antigen-presenting cell by the contact with CD4+ T cells, resulting in good targets of CD4+ T-cell–mediating cytotoxicity.[45] Other studies showed that in solid tumors, HLA class II expression correlated with good clinical outcome.[42,46,47] Friedman et al[48] reported that HLA class II-restricted, melanoma-specific CD4+ T cells were contained in tumor-infiltrating lymphocytes (TILs) and that adoptive cell transfer of the TILs into the patient let metastatic melanoma regress dramatically. These findings strongly indicate that both hematological malignancy

and solid tumor are good targets for cytotoxic $CD4^+$ T cells.

The present study clearly demonstrated that $WT1_{332}$ TCR-transduced $CD4^+$ T cells displayed helper activity for WT1-specific CTL induction and cytotoxicity against leukemia cells. This observation that $WT1_{332}$ TCR-transduced $CD4^+$ T cells had 2 function (helper and cytotoxicity) raised a hypothesis that function of $CD4^+$ T cells was divided into 2 phases: helper and cytotoxicity phases. It is generally known that IL-2 derived from $CD4^+$ T cells is a crucial factor for exhibition of helper activity to enhance CTL function and that undifferentiated, proliferative $CD4^+$ T cells can more produce IL-2 compared to differentiated, nonproliferative $CD4^+$ T cells. $WT1_{332}$ TCR-transduced $CD4^+$ T cells rapidly proliferated and produced a large amount of IL-2 at early phase within 1 month from the beginning of the culture, but stopped producing IL-2 with less proliferation at late phase after repeated antigen-stimulation (data not shown). It was previously reported that cytotoxic $CD4^+$ T cells appeared after repeated antigen-stimulation and possessed a phenotype-like terminally differentiated effector cells such as $CD27^-$, $CD28^-$, and $CD57^+$.[41] These findings supported our hypothesis that $CD4^+$ T cell might transiently exert a helper activity (helper $CD4^+$ T cells) at early phase and then a cytotoxic activity (cytotoxic $CD4^+$ T cells) at late phase.

As WT1 was selected from 75 defined tumor antigens to rank as the most promising cancer vaccine target in a prioritization study carried out by National Cancer Institute,[13] WT1-targeted cancer immunotherapy is thought to be also the most promising strategy for cure of cancer. TCR gene therapy using $WT1_{332}$-specific TCR should be a useful tool for cancer immunotherapy because the TCR-transduced $CD4^+$ T cells elicited both a helper activity for the induction of WT1-specific $CD8^+$ CTLs and a cytotoxic activity against tumor. Furthermore, combination TCR gene therapy of HLA class I-restricted, WT1-specific TCR and HLA class II-restricted, $WT1_{332}$-specific TCR is expected to be more efficient. In addition, $WT1_{332}$-specific TCR gene therapy should be also effective in combination with HLA class I-restricted WT1 peptide vaccine.

CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

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ORIGINAL PAPER

WT1 peptide immunotherapy for gynecologic malignancies resistant to conventional therapies: a phase II trial

Takashi Miyatake · Yutaka Ueda · Akiko Morimoto · Takayuki Enomoto · Sumiyuki Nishida · Toshiaki Shirakata · Yoshihiro Oka · Akihiro Tsuboi · Yusuke Oji · Naoki Hosen · Shin-ichi Nakatsuka · Satoshi Morita · Junichi Sakamoto · Haruo Sugiyama · Tadashi Kimura

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Abstract
Objective The aim of the present study was to analyze the long-term survival effects of WT1 peptide vaccine, in addition to its anti-tumor effects and toxicity.
Methods A phase II clinical trial was conducted during the period of 2004–2010 at Osaka University Hospital, Osaka, Japan. The patients who had gynecologic malignancies progressing against previous treatments received WT1 peptide vaccine intradermally at 1-week intervals for 12 weeks. The vaccination was allowed to further continue, unless the patient's condition became significantly worse due to the disease progression.
Results Forty out of 42 patients, who met all the inclusion criteria, underwent WT1 peptide vaccine. Among these 40 patients, stable disease was observed in 16 cases (40 %). Skin toxicity of a grade 1, 2 and 3 occurred in 25 cases (63 %), 9 cases (23 %) and a single case (3 %), respectively, and liver toxicity of grade 1 in a single case (3 %). The overall survival period was significantly longer in cases positive for the WT1 peptide-specific delayed-type hypersensitivity (DTH) reaction after the vaccination, compared to those negative for the DTH reaction ($p = 0.023$). Multivariate Cox proportional hazards analysis demonstrated that the adjusted hazard ratio for the

T. Miyatake · Y. Ueda (✉) · A. Morimoto · T. Enomoto · T. Kimura
Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan
e-mail: ZVF03563@nifty.ne.jp

S. Nishida · A. Tsuboi · Y. Oji
Department of Cancer Immunotherapy, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan

T. Shirakata
Department of Biomedical Informatics, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan

Y. Oka
Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan

Y. Oka
Department of Immunopathology WPI Immunology Frontier Research Center, Osaka University, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan

N. Hosen
Department of Cancer Stem Cell Biology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan

S. Nakatsuka
Department of Pathology, Kansai Rosai Hospital, 69-1-3 Inabasou, Amagasaki, Hyogo 660-8511, Japan

S. Morita
Department of Clinical Statistics, Yokohama City University Medical Center, 4-57, Minami-ku Urabune-cho, Yokohama, Kanagawa 232-0024, Japan

J. Sakamoto
Department of Health and Community Medicine, Nagoya University Graduate School of Medicine, 65, Showa-ku Tsurumai-cho, Nagoya, Aichi 466-8550, Japan

H. Sugiyama
Department of Functional Diagnostic Science, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan

negative DTH reaction was 2.73 (95 % CI 1.04–7.19, $p = 0.043$).

Conclusion WT1 peptide vaccine may be a potential treatment, with limited toxicity, for gynecologic malignancies that have become resistant to conventional therapies. Larger scale of clinical studies is required to establish the efficacy of the WT1 peptide vaccine for gynecologic malignancies.

Keywords WT1 peptide immunotherapy · Gynecologic malignancy · Anti-tumor effect · Survival · Stable disease · Toxicity

Abbreviations

CR	Complete response
CT	Computed tomography
HLA	Human leukocyte antigen
HPV	Human papillomavirus
OS	Overall survival
PD	Progressive disease
PFS	Progression-free survival
PR	Partial response
PS	Performance status
RECIST	Response evaluation criteria in solid tumor
RR	Responsive rate
SD	Stable disease
TC	Paclitaxel and carboplatin

Introduction

The Wilms tumor gene, *WT1*, was first identified as a tumor suppressor gene responsible for Wilms tumors of the kidney. However, a series of investigations demonstrated that *WT1* possesses an oncogenic, rather than a tumor-suppressive, function, and WT1 protein is expressed in various kinds of hematological and solid malignancies, indicating that immunotherapy targeting WT1 could potentially be used for treatment of a variety of such malignancies (Oka and Sugiyama 2010). In fact, WT1 has been regarded as one of the most promising target antigens for cancer immunotherapy by an American National Cancer Institute pilot project (Cheever et al. 2009). It has already been demonstrated that WT1 vaccination is safe, and encouraging reports that showed its efficacy for several kinds of tumors have been accumulated (Oka and Sugiyama 2010; Hashii et al. 2010; Oji et al. 2010; Izumoto et al. 2008). A previous phase I study empirically determined a safe dose of the WT1 peptide, which was intradermally injected with Montanide ISA 51 adjuvant for patients with solid tumors, as 3 mg per injection, and this dose was shown to have little toxicity except skin reaction of the vaccination sites (Morita et al. 2006).

Ovarian carcinoma accounts for 5 % of all cancers among women and will eventually develop in 1 of every 58 women. It has an extremely high mortality rate; consequently, aggressive cytoreductive surgery followed by chemotherapy with taxane and platinum is the gold standard for its therapy. Endometrial carcinoma is an even more common malignant neoplasm of the female pelvis and is the fourth most common cancer of women today. Endometrial carcinoma is usually confined to the uterus or pelvis, has a lower mortality rate than ovarian cancer and is commonly treated by resection of the uterus and adnexae, with or without co-resection of the regional lymph nodes. Another common gynecological tumor, uterine cervical carcinoma, is mostly associated with a human papilloma virus (HPV) infection, and its incidence appears to vary from one locality to another. It is important to note that in some Asian and South American countries, cervical carcinoma accounts for the largest percentage of cancer deaths in women. Cervical carcinoma is usually treated by radical surgery and/or radiation therapy. And lastly, yet another kind of uterine tumor, the leiomyosarcoma, although rare, has an extremely poor prognosis (DiSaia and Creasman 2002).

Tumors in the early stage of all these diseases are usually treated relatively successfully, while the advanced and recurrent forms of these diseases are often very difficult to treat. Salvage, second-line and third-line chemotherapies are effective in only a fraction of the cases, and the best available supportive care is usually proposed to the patients whose tumors have become resistant to prior therapies.

An immunotherapeutic approach that is less toxic than available chemotherapies might be a more promising option for those whose gynecologic malignancies continue to progress despite conventional chemotherapy and radiation treatments. A previous small study showed that disease stabilization was achieved in 3 (25 %) of 12 gynecologic malignancies by vaccination with an antigenic WT peptide (Ohno et al. 2009). There is only one case report on the effect of WT1 peptide for the survival elongation in a ovarian cancer case (Dohi et al. 2011). In the present phase II trial, we have analyzed for the first time the long-term survival effect of the WT1 peptide vaccine, as well as its anti-tumor effects, evaluated by the usual response evaluation criteria in solid tumor (RECIST) and toxicity.

Materials and methods

Eligibility

This phase II trial was conducted at Osaka University Hospital, Osaka, Japan, during the period of 2004–2010. Major inclusion criteria were as follows: having a gynecologic malignancy progressing despite previous treatments;

WT1 protein expression in the primary or metastatic tumor tissue using anti-WT1 rabbit polyclonal antibody C-19 (Santa Cruz Biotechnology) or anti-WT1 mouse monoclonal antibody 6F-H2 (Dako Cytometry); positive status for human leukocyte antigen (HLA)-A*2402; performance status (PS) of 0–2; and life expectancy >3 months.

Vaccination schedule

The HLA-A*2402-restricted, 9-mer modified WT1 peptide (amino acids 235–243: CYTWNQMNL) emulsified with Montanide ISA 51 adjuvant, was used for the vaccination, as previously described (Hashii et al. 2010). The dose of WT1 peptide injected was 3 mg per body. The WT1 vaccination was scheduled to be performed intradermally every week for 12 weeks but was allowed to continue even after 12 weeks, unless the patient's condition became significantly worse due to the disease progression.

Evaluation of the WT1 vaccine effects

The primary endpoints of the WT1 vaccine study were its anti-tumor effect and its toxicity. Computed tomography (CT) was performed every 4 weeks to evaluate tumor size. The anti-tumor effect was evaluated by the RECIST (version 1.1) (Eisenhauer et al. 2009) after the vaccination during 12 weeks. Adverse effects were graded based on the National Cancer Institute's Common Toxicity Criteria (version 2.0). A test for delayed-type hypersensitivity (DTH) reaction specific to the WT1 peptide used for vaccination was performed at week 4 and 8. We regarded the patient as DTH positive, if the DTH reaction of the patient was positive either at week 4 or at week 8.

Secondary endpoints were progression-free survival (PFS) and overall survival (OS). PFS was defined as the period from the date of the start of WT1 vaccination to the date of the radiologic or pathologic relapse, or to the date of the last follow-up. OS was defined as the period from the start of the vaccination to the patient's death or to the date of the last follow-up. OS was analyzed for its association with DTH.

Cancellation or termination of WT1 vaccination

If grade 3 toxicity was observed, the next injection of the WT1 vaccine was postponed until the toxicity returned to grade 2 or less. The vaccination was permanently terminated if grade 4 toxicity was detected or if a performance status of 3 or worse was observed.

Statistical analysis

MedCalc (MedCalc Software, Mariakerke, Belgium) was used for statistical analysis. The association between DTH induction and anti-tumor effect, including RECIST evaluation, PFS and OS, was analyzed by Fisher's exact test. OS curves were constructed using the Kaplan–Meier method and evaluated for statistical significance by the log-rank test. Multivariate Cox proportional hazards model (stepwise method) for the factors including age, origin of the disease, histology, evaluation of the previous therapy and number of recurrence was calculated to evaluate whether DTH was a significantly important factor on OS. Results were considered to be significant when the p value was <0.05.

Statements of ethics

This study was approved by the Institutional Review Board and the Ethics Committee of the Osaka University Hospital. All patients provided written informed consent. (Approval of this analysis: #10302, approved on March 11, 2011).

Results

Clinical characteristics of the patients and completion rate of the study schedule

During the study period, 42 patients entered the study. Among these, 2 patients were excluded from the present analysis due to protocol violation. The clinicopathological characteristics of these patients are shown in Table 1. The median age was 56 (35–75). The histological diagnosis was obtained as ovarian carcinoma in 24 cases, cervical carcinoma in 11 cases, uterine sarcoma (leiomyosarcoma and carcinosarcoma) in 5 cases. These patients had already received 1–11 (median: 3) kinds of treatments prior to the WT1 vaccination and were considered to have disease

Table 1 Clinical characteristics of patients enrolled in the phase II study

Characteristics	
Number (cases)	40
Median age (years) (range)	56 (35–75)
Type of malignancy	
Ovarian carcinoma	24 (60 %)
Cervical carcinoma	11 (28 %)
Uterine leiomyosarcoma/carcinosarcoma	5 (13 %)
Performance status	
0	35 (88 %)
1	4 (10 %)
2	1 (3 %)
Median number of previous treatment regimens (range)	3 (1–11)

resistant to conventional therapies such as chemotherapy and radiotherapy.

Injection of the WT1 vaccine was performed weekly for 1–50 (median: 14.5) times. The 12 injections prescheduled upon entry to this trial were completed in 32 of the 40 cases (80 %). Vaccination was terminated prior to the 12th injection due to progression of the disease including worsening of PS in 8 cases (20 %).

Anti-tumor effect of the WT1 peptide vaccine evaluated by RECIST

Among the 40 patients who received the WT1 vaccination, neither complete response (CR) nor partial response (PR) was obtained. Encouragingly, however, stable disease (SD) of 3 months or more was observed in 16 cases (40 %), including 10 cases of ovarian carcinoma, 5 cases of cervical carcinoma and a single case of uterine leiomyosarcoma, respectively.

The WT1 peptide-specific DTH reaction appeared after the vaccination in 27 cases (68 %); however, the vaccine's anti-tumor effect evaluated by RECIST was not correlated to the appearance of DTH (data not shown).

Toxicity of the WT1 vaccination

An adverse effect was observed in 36 cases (90 %): grade 1, 2 and 3 of skin reaction in 25 cases (63 %), 9 cases (23 %) and a single case (3 %), respectively, and grade 1 liver toxicity in a single case (3 %). The skin reactions had definite relationship with WT1 injection because the reactions were observed only in WT1 injected area. The liver toxicity occurred after first injection of WT1, and the relationship between WT1 vaccine and liver toxicity was probable. Postponement of the next injection due to adverse effects occurred in one case with grade 3 of skin reaction. However, termination of the WT1 vaccine injection due to adverse effects was never required.

Prognosis of the patients treated with WT1 peptide vaccine: the vaccines' survival effect

The PFS was 84 days (11–497). Surprisingly, among these WT1-vaccinated cases, which had been already resistant to conventional therapies and the disease had exhibited continuous progression against various other treatments for 40–1,198 days (median: 185 days), progression-free survival for a range of 67–427 days (median: longer than 160 days) was achieved in 16 SD cases (Table 2). The median OS of all the patients was 193 days (29–941).

Although an association between an anti-tumor effect evaluated RECIST and an appearance of DTH reaction was not observed, the PFS tended to be longer in DTH-positive cases than DTH-negative ones ($p = 0.23$ by the log-rank test), and the OS was significantly longer in DTH-positive cases than DTH-negative ones ($p = 0.023$ by the log-rank test) (Fig. 1).

Table 2 Duration of disease progression before WT1 vaccination was begun and progression-free period afterward in stable disease (SD) cases

Case number	Duration of disease progression before WT1 vaccine (days)	Progression-free survival after WT1 vaccine (days)
1	40	105[a]
2	55	67
3	61	427[a]
4	81	320
5	97	126
6	142	145
7	155	92
8	178	273
9	192	140[a]
10	324	84
11	405	175
12	434	196
13	439	84[a]
14	655	196
15	737	219
16	1,198	180[a]
Median	185	160[a]

The duration of disease progression before the WT1 vaccine, and the progression-free period after the start of WT1 vaccination in 16 SD cases, is demonstrated

[a] The cases in which the disease was stable after WT1 vaccination without progression

Multivariate Cox proportional hazards analysis

We utilized the multivariate Cox proportional hazards model in order to find evidence to further support our belief that the DTH reaction was significantly associated with the survival. The DTH reaction was demonstrated to be an independent factor for overall survival of the patients (Table 3). The adjusted hazard ratio (HR) for the DTH reaction (− vs. +) was 2.73 (95 % CI 1.04–7.19, $p = 0.043$).

Discussion

A National Cancer Institute pilot project recently suggested that WT1 was one of the most promising targets for cancer immunotherapy (Cheever et al. 2009), and it has been demonstrated that WT1 vaccination is safe and has therapeutic potential for at least several kinds of tumors (Oka and Sugiyama 2010; Hashii et al. 2010; Oji et al. 2010;

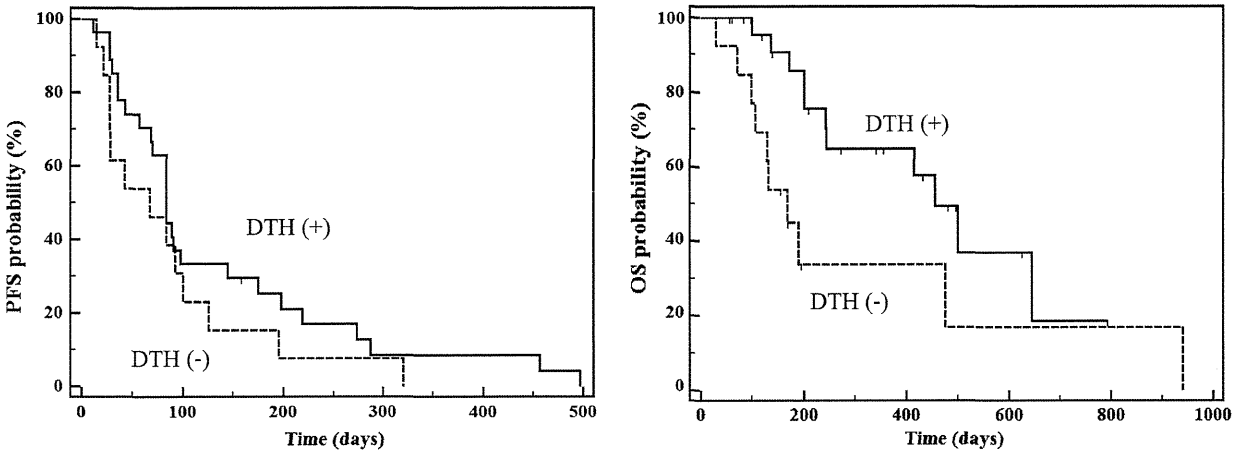

Fig. 1 PFS and OS association with DTH after WT1 vaccination. **a** The PFS tended to be longer in positive DTH cases than in DTH-negative cases ($p = 0.23$ by the log-rank test). **b** The OS was significantly longer in positive DTH cases than in DTH-negative cases ($p = 0.023$ by the log-rank test). *Solid line*: DTH (*plus*), *broken line*: DTH (*minus*)

Izumoto et al. 2008; Ohno et al. 2009). In this current phase II trial, we have tested the efficacy and safety of WT1 immunotherapy for gynecologic malignancies that were progressing, that is, resistant against conventional therapies.

In general, gynecologic tumors, including ovarian, endometrial and cervical carcinomas and uterine sarcomas, are very difficult to further treat, once the disease become resistant to conventional therapies such as chemotherapy or radiotherapy. For example, when ovarian carcinoma is first treated with cytoreductive surgery, the surgery is immediately followed by combination chemotherapy with paclitaxel and carboplatin (TC). If there is a failure of this first-line treatment, a single drug or combination chemotherapy for the recurrent disease, chosen based on the patient's treatment-free interval, can still be performed effectively in some cases (Koensgen et al. 2008; Markman et al. 2003; Harries and Gore 2002; Dizon et al. 2003). However, even though some third-line regimens have been reported to be occasionally effective for second relapses of some of these advanced stage diseases (Vergote et al. 2009; Chiyoda et al. 2010); the efficacy of each attempt becomes progressively lower as the number of previous treatment failures increases.

In the present study, the median number of the previous treatment regimens was 3 (range 1–11 treatments). Since all of the patients in the present trial had exhibited resistance to previous therapies, normally supportive care would have been considered as the only remaining option for them; however, the experimental WT1 vaccination immunotherapy was offered to them as an alternative.

A previous small study showed that stable disease was achieved by WT vaccination in 3 (25 %) of 12 gynecologic malignancies (Ohno et al. 2009). However, that study was so small that a survival effect was not analyzed. The response rate (CR + PR/all) in our study was 0 % (0 of 40 cases). However, the disease control rate (CR + PR + SD/all), which corresponds to disease stabilization lasting at least 3 months from the start of the vaccination, was 40 % (16 of 40 cases). The median PFS was 84 days (11–497), and the median OS of all the patients was 193 days (29–941). Considering that these cases were resistant to various kinds of therapies, and the diseases were progressing prior to the vaccination, these results of disease control rate and PFS time may be favorable, and were consistent with results of the previous smaller study that suggested the therapeutic potential of WT1 vaccine for gynecologic malignancies. Furthermore, surprisingly, in these SD cases, whose tumors had continuously progressed against previous therapies during the median of 185 days of treatments (range 40–1,198 days), the disease was durably controlled, without significant progression of the disease, for the median of longer than 160 days (range 67–427 days) after starting the WT1 immunotherapy (Table 2), implying an improved survival effect of the WT1 peptide vaccine. The adverse effect by the WT1 peptide-based immunotherapy with the dosage and schedule adopted here was limited and largely tolerable.

We next investigated the association of DTH and the efficacy of the WT1 immunotherapy. The OS of the patients with a positive DTH reaction was significantly better than that of those with a negative DTH reaction ($p = 0.023$ by the log-rank test) (Fig. 1). Moreover, the DTH reaction was demonstrated to be an independent factor for overall survival of the patients by multivariate Cox proportional hazards analysis (Table 3). These findings suggested that the induction of WT1-specific immune response, that is, the peptide-specific DTH, is a potential

Table 3 Multivariate Cox proportional hazards analysis on overall survival

Variable	Number of cases	Adjusted HR	95 % CI	*p* value
Age (years)				0.44
<60	24	1		
≥60	16	0.64	0.21–1.96	
Origin of the disease				0.75
Uterus	16	1		
Ovary	24	1.17	0.44–3.14	
Histology				0.98
Carcinoma	35	1		
Sarcoma, carcinosarcoma	5	0.99	0.28–3.42	
Evaluation of the previous therapy				0.39
SD	4	1		
PD	36	1.88	0.46–7.71	
Number of previous therapy regimens				0.034
<3	12	1		
≥3	28	4.28	1.12–16.37	
DTH				0.043
+	27	1		
−	13	2.73	1.04–7.19	

Multivariate Cox proportional hazards analysis (stepwise method) for the factors including age, origin of the disease, histology, evaluation of the previous therapy, number of previous therapy regimens and DTH was performed to evaluate whether DTH was an independently significant factor on OS

SD stable disease, *PD* progressive disease

predictor for the induction of clinical response, leading to a better prognosis.

The number of previous treatment regimens was also demonstrated to be an independent factor for survival prognosis after WT1 immunotherapy. The response rate of the first-line chemotherapy was quite high for ovarian carcinoma, however, that of second-line and the third-line chemotherapy was 34.5 and 27.5 %, respectively (Nishio et al. 2006). Effectiveness of WT1 was demonstrated to be associated with the number of previous treatment regimens, which was similar to that of the cell toxic chemotherapy. As the number of chemotherapy regimen increases, the tumor cells are considered to become resistant to the next line therapy. Furthermore, immunological potentials of the patients treated by chemotherapy with many courses might be dampened, leading to the poor response to the administered cancer vaccine. WT1 peptide vaccination soon after the first-line therapy, including the vaccination to prevent relapse after the operation, chemotherapy or radiation therapy, may be a favorable setting for the next clinical trial.

In the present phase II prospective study with a single arm, we have, for the first time, analyzed the survival effect of the WT1 vaccine for gynecologic malignancies, in addition to its anti-tumor effect conventionally evaluated by RECIST and toxicity, which had previously been reported in a smaller pilot study (Ohno et al. 2009). It was strongly suggested that WT1 peptide vaccination could induce the peptide-specific immune response in patients whose gynecological tumors have become resistant to conventional therapies, leading to a better survival. Larger two-arm randomized studies will be required to confirm the efficacy and clinical usefulness of the WT1 peptide vaccine for gynecologic malignancies.

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Conflict of interest The authors have no conflict of interest.

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Biased usage of T cell receptor β-chain variable region genes of Wilms' tumor gene (WT1)-specific CD8$^+$ T cells in patients with solid tumors and healthy donors

Soyoko Morimoto,[1] Yoshihiro Oka,[1] Akihiro Tsuboi,[2] Yukie Tanaka,[3] Fumihiro Fujiki,[4] Hiroko Nakajima,[4] Naoki Hosen,[5] Sumiyuki Nishida,[2] Jun Nakata,[1] Yoshiki Nakae,[1] Motohiko Maruno,[6] Akira Myoui,[7] Takayuki Enomoto,[8] Shuichi Izumoto,[9] Mitsugu Sekimoto,[10] Naoki Kagawa,[11] Naoya Hashimoto,[11] Toshiki Yoshimine,[11] Yusuke Oji,[12] Atsushi Kumanogoh[1] and Haruo Sugiyama[5,13]

Departments of [1]Respiratory Medicine, Allergy and Rheumatic Diseases, [2]Cancer Immunotherapy, Osaka University Graduate School of Medicine, Osaka; [3]Division of Hematology, Saitama Medical Center, Jichi Medical University, Saitama; Departments of [4]Cancer Immunology, [5]Functional Diagnostic Science, Osaka University Graduate School of Medicine, Osaka; [6]Department of Neurosurgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka; [7]Medical Center for Translational Research, Osaka University Hospital, Osaka; [8]Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Osaka; [9]Department of Neurosurgery, Hyogo College of Medicine, Hyogo; Departments of [10]Gastroenterological Surgery, [11]Neurosurgery, [12]Cancer Stem Cell Biology, Osaka University Graduate School of Medicine, Osaka, Japan

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Wilms' tumor gene 1 (WT1) protein is a promising tumor-associated antigen. In patients with WT1-expressing malignancies, WT1-specific CTLs are spontaneously induced as a result of an immune response to the WT1 protein. In the present study, we performed single cell-level comparative analysis of T cell receptor β-chain variable region (TCR-BV) gene families of a total of 750 spontaneously induced $WT1_{126}$ peptide (amino acids 126–134, $WT1_{126}$)-specific CTLs in both HLA-A*0201$^+$ patients with solid tumors and healthy donors (HDs). This is the first report of direct usage analysis of 24 kinds of TCR-BV gene families of $WT1_{126}$-specific CTLs at the single cell level. Usage analysis with single-cell RT-PCR of TCR-BV gene families of individual FACS-sorted $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells showed, for the first time, that: (i) BVs 3, 6, 7, 20, 27, and 28 were commonly biased in patients and HDs; (ii) BVs 2, 11, and 15 were biased only in patients; and (iii) BVs 4, 5, 9, and 19 were biased only in HDs. However, statistical analysis of similarity of individual usage frequencies of 24 kinds of TCR-BV gene families between patients and HDs indicated that the usage frequencies of TCR-BV gene families in patients reflected those in HDs. These results should provide us with a novel insight for a better understanding of WT1-specific immune responses. (*Cancer Sci* 2012; 103: 408–414)

Wilms' tumor gene (*WT1*) encodes a zinc-finger transcription factor and plays important roles in the regulation of cell proliferation, differentiation, and apoptosis.[1–3] The *WT1* gene was originally isolated as the gene responsible for a childhood renal neoplasm, namely Wilms' tumor, and was first categorized as a tumor-suppressor gene.[4,5] However, based on the result of a series of studies,[6–8] we proposed that the wild-type *WT1* gene had an oncogenic rather than a tumor-suppressor function in various kinds of hematological malignancies and solid tumors. Indeed, the *WT1* gene is expressed at high levels in acute myeloid leukemia (AML), acute lymphocytic leukemia, chronic myelogenous leukemia, and myelodysplastic syndromes (MDS), as well as in various types of solid tumors.[9–14] Because a correlation has been shown between *WT1* mRNA transcript levels and the amount of minimal residual disease (MRD) in the peripheral blood (PB) or bone marrow of leukemia patients,[15–17] measurement of *WT1* mRNA transcripts is now being used to monitor MRD in leukemia patients.

Previous studies have reported that WT1-specific CTLs can be generated from human PBMC in a human leukocyte antigen (HLA) Class I-restricted manner and can lyse WT1-expressing tumor cells as well as WT1 peptide-pulsed target cells.[18,19] Mice immunized with WT1 peptide or WT1 plasmid DNA elicit WT1-specific CTLs and reject challenges by WT1-expressing tumor cells.[20,21] Furthermore, WT1-specific CTLs and antibodies are induced spontaneously in WT1-expressing tumor-bearing patients.[22–24] These results indicate that the WT1 protein is highly immunogenic and a promising target antigen for cancer immunotherapy. In fact, WT1 has been rated as the most promising cancer antigen of 75 tumor-associated antigens.[25]

On the basis of the results of these preclinical studies, clinical studies of WT1 peptide vaccination were undertaken,[26–29] with promising clinical effects, including a reduction in leukemic blast cells and tumor size, as well as long-term stable disease, being seen in association with an increase in the frequency of WT1-specific CD8$^+$ T cells in PB.[26,27] In this context, analysis of the clonality of the WT1-specific CTLs is important to gain a better understanding of the WT1-specific CTL response in WT1-expressing tumor-bearing patients and, further, to obtain clues as to how to enhance WT1-specific CTL responses in WT1 immunotherapy.

Recently, using single-cell RT-PCR analysis of the T cell receptor β-chain variable region (TCR-BV) genes of individual FACS-sorted WT1 tetramer$^+$ CD8$^+$ T cells, we demonstrated biased usage of TCR-BV gene families of $WT1_{235}$ peptide (amino acids 235–243)-specific CTLs in HLA-A*2402$^+$ patients with AML or MDS, which reflected the biased usage in healthy donors (HDs).[30]

In the present study, we examined usage frequencies of TCR-BV gene families of CTLs specific for $WT1_{126}$, an HLA-A*0201-restricted CTL epitope, in both patients with solid tumors and HDs and found biased usage for these TCR-BV gene families in both the patients and HDs and that the patterns of biased usage were very similar between the two groups.

[13]To whom correspondence should be addressed.
E-mail: sugiyama@sahs.med.osaka-u.ac.jp

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Table 1. Characteristics of the patients and healthy donors

	Gender	Age (years)	Disease	Clinical stage	Prior therapy
Patients					
PT-1	M	33	GBM	N/A	Ope/RT
PT-2	F	56	GBM	N/A	Ope/RT
PT-3	M	28	GBM	N/A	Ope/RT/Chemo
PT-4	M	18	PNET	IV	Ope/RT/Chemo/ auto-PBSCT
PT-5	F	53	Ovarian cancer	IIIc	Ope/Chemo
PT-6	F	73	Cecal cancer	IV	Ope/Chemo
Healthy donors					
HD-1	F	23			
HD-2	M	45			
HD-3	F	24			
HD-4	F	25			
HD-5	M	37			

auto-PBSCT, autologous peripheral blood stem cell transplantation; Chemo, chemotherapy; GBM, glioblastoma multiforme; N/A, not available; Ope, operation; PNET, primitive neuroectodermal tumor; RT, radiation therapy.

Materials and Methods

Samples of PB from patients with solid tumors and HDs. Analysis of $WT1_{126}$-specific CTLs in PBMC was approved by the Institutional Review Board for Clinical Research, Osaka University Hospital. After written informed consent had been obtained, PB samples were obtained from six HLA-A*0201$^+$ patients with a solid tumor (patient (PT)-1, -2, -3, -4, -5, and -6) and five HLA-A*0201$^+$ HDs. Expression of WT1 protein in tumor cells was determined by immunohistochemical analysis, as described elsewhere.[31] The PBMC were separated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use. Table 1 summarizes the characteristics of both the patients and HDs.

Flow cytometric analysis and single-cell sorting of WT1 tetramer$^+$ CD8$^+$ T cells. Thawed PBMC were rested at 37°C for 1.5 h in RPMI 1640 containing 10% FBS before being stained with phycoerythrin (PE)-labeled HLA-A*0201/$WT1_{126}$ tetramer ($WT1_{126}$ tetramer; MBL, Tokyo, Japan) in FACS buffer composed of PBS containing 5% FBS at 37°C for 30 min. The PBMC were then stained with a panel of mAbs at 4°C for 25 min in the dark, washed three times with FACS buffer, and finally resuspended in appropriate quantities of FACS buffer. The following mAbs were used: anti-CD4-FITC, anti-CD16-FITC, anti-CD45RA-allophycocyanin (APC) (BioLegend, San

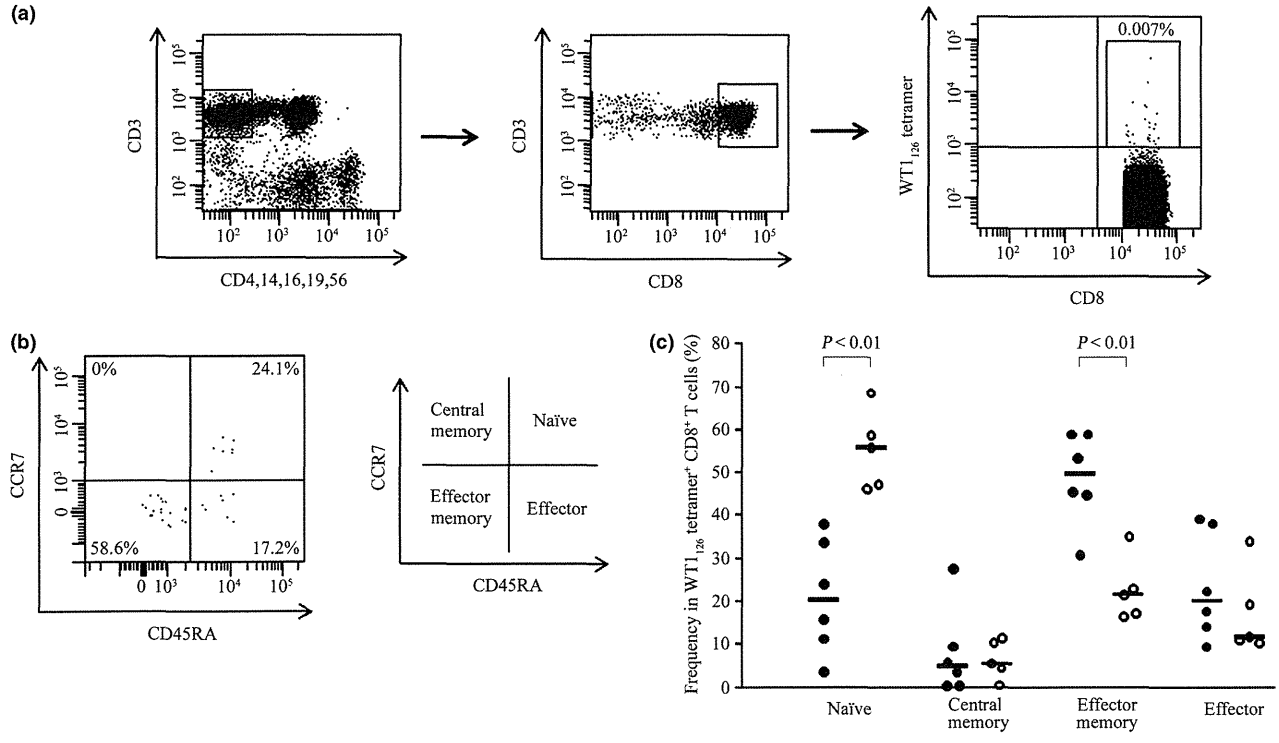

Fig. 1. Frequencies of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells in peripheral blood of patients with a solid tumor and healthy donors and phenotypic analysis of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. (a) Representative data of flow cytometric analysis using $WT1_{126}$ tetramer. CD4$^-$, CD14$^-$, CD16$^-$, CD19$^-$, CD56$^-$, and $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were defined as $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. The percentages shown represent the frequencies of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells among total CD3$^+$ CD8$^+$ T cells. (b) $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were classified into four distinct differentiation stages according to the cell surface expression of CCR7 and CD45RA as follows: (i) CCR7$^+$ CD45RA$^+$ (naïve) cells; (ii) CCR7$^+$ CD45RA$^-$ (central memory) cells; (iii) CCR7$^-$ CD45RA$^-$ (effector memory) cells; and (iv) CCR7$^-$ CD45RA$^+$ (effector) cells. Representative data from Patient 3 are shown. (c) Frequencies of each subset of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. Closed and open circles represent patients and healthy donors, respectively. Bars indicate the median values of the frequencies.

Diego, CA, USA); anti-CD19-FITC, anti-CCR7-PE-Cy7 (BD Pharmingen, San Diego, CA, USA); anti-CD3-peridinin chlorophyII protein (PerCP), anti-CD8-APC-Cy7, anti-CD14-FITC (BD Biosciences, San Jose, CA, USA); and anti-CD56-FITC (eBioscience, San Diego, CA, USA). In the present study, lineage antigen (CD4, CD14, CD16, CD19, and CD56)-negative, CD3-, CD8-, and $WT1_{126}$ tetramer-positive lymphocytes were defined as $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. The $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were single-cell sorted using a FACSAria instrument (BD Biosciences), and data were analyzed using FACSDiva software (BD Biosciences).

Synthesis of cDNA from a single cell-sorted $WT1_{126}$ tetramer$^+$ CD8$^+$ T cell and determination of TCR-BV gene families. The $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were directly single-cell sorted into PCR tubes containing cDNA reaction mix, and cDNA synthesis was performed as described previously.[30] The cDNA was amplified using 24 kinds of TCR-BV gene family-specific forward primers and a constant region-specific reverse primer.[30] Next, the PCR products were amplified by semi-nested PCR for the screening of the BV gene family as follows: the first PCR product was put into eight separate tubes, each of which was filled with a reaction mix containing the reagents, one of eight kinds of screening sets of BV gene family-specific forward primers and the reverse primer. The eight kinds of screening sets used in the present study were the same as those used in a previous study.[30] Each screening PCR product was run on a 2% agarose gel to identify the positive reaction among the eight kinds of screening sets. Finally, the TCR-BV gene family was identified by the second round of PCR using an individual TCR-BV gene family-specific forward primer, which was contained in the positive screening set, and the reverse primer. As a negative control, three PCR tubes without sorted cells were prepared in each experiment and were subjected to the same RT-PCR procedures.

A total of 750 $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were analyzed in six patients (i.e. 59, 66, 46, 67, 88, and 73 cells from PT-1, -2, -3, -4, -5, and -6, respectively) and five HDs (i.e. 53, 57, 77, 79, and 85 cells from HD-1, -2, -3, -4, and -5, respectively). The International Immunogenetics Information System (IMGT) database site (http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=humanTcR, accessed 15 Nov 2011) was used to identify the human TCR-BV gene family.

Statistical analysis. The Mann–Whitney *U*-test was used to evaluate differences in frequencies and subset compositions of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells and CD3$^+$ CD8$^+$ T cells between patients and HDs. The significance of differences in usage frequencies of the 24 kinds of BV gene families between patients and HDs was also assessed using the Mann–Whitney *U*-test. Analyses were performed with the Stat Flex statistical software package (Artech, Osaka, Japan).

Results

Increase in $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells with effector memory phenotype in HLA-A*0201$^+$ patients with solid tumors. The CTL responses to an HLA-A*0201-restricted epitope $WT1_{126}$ of the WT1 protein were examined in HLA-A*0201$^+$ patients with solid tumors. The PBMC were FACS analyzed by using $WT1_{126}$ tetramer (Fig. 1), with Figure 1(a) showing representative profiles of the FACS analysis of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. The frequencies of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells in patients and HDs were 0.007–0.122% (median 0.039%) and 0.009–0.079% (median 0.016%), respectively. Although there was a tendency for higher frequencies in patients than in HDs, the differences failed to reach statistical significance (data not shown).

Human CD3$^+$ CD8$^+$ T cells can be divided into four distinct differentiation stages according to the cell surface expression of CCR7 and CD45RA as follows: (i) CCR7$^+$ CD45RA$^+$ (naïve) cells; (ii) CCR7$^+$ CD45RA$^-$ (central memory) cells; (iii) CCR7$^-$ CD45RA$^-$ (effector memory) cells; and (iv) CCR7$^-$ CD45RA$^+$ (effector) cells.[32,33] These cell surface markers were used to classify the differentiation stages of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells and a representative pattern from PT-3 is shown in Figure 1(b). The frequency of the naïve phenotype of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells was significantly higher in HDs than in patients (45.8–68.4% [median 55.6%] *vs* 3.4–37.9% [median 19.9%], respectively; $P < 0.01$), while the frequency of the effector memory phenotype of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells was significantly higher in patients than in HDs (30.3–58.6% [median 49.0%] *vs* 15.8–34.4% [median 20.7%], respectively; $P < 0.01$; Fig. 1c). In contrast, there were no significant differences in frequencies of the four subsets of the whole CD3$^+$ CD8$^+$ T cells between patients and HDs (data not shown),

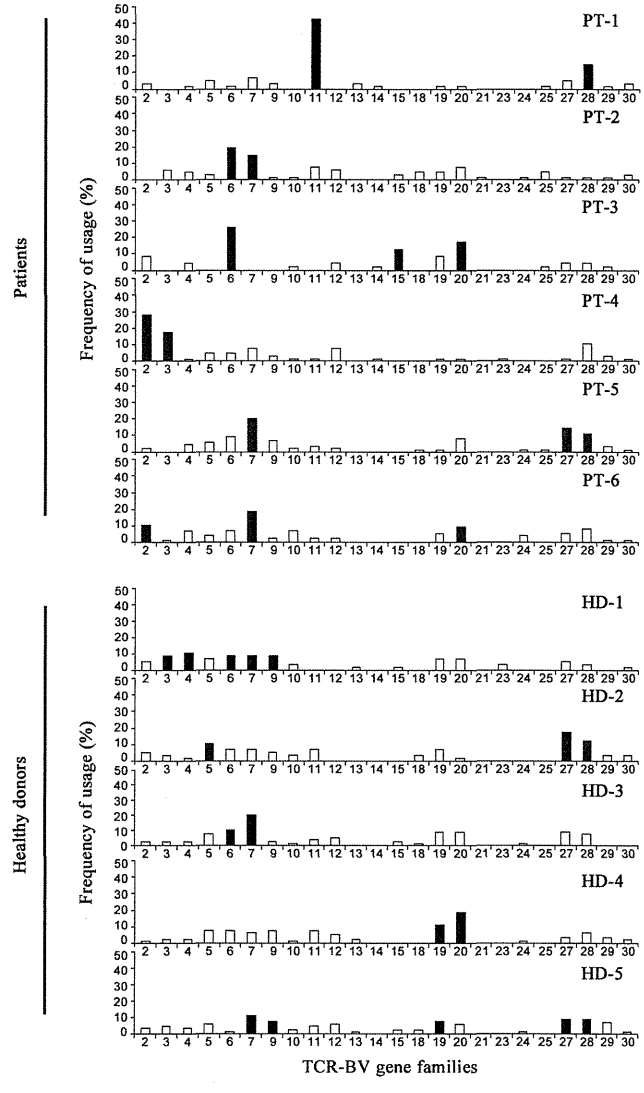

Fig. 2. Frequencies of T cell receptor β-chain variable region (TCR-BV) gene families used by T cell receptors (TCRs) in $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. The usage frequencies were defined as the ratio of (the number of a given TCR-BV gene family used)/(the total number of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells analyzed). Closed columns indicate that the usage frequency is higher than the mean value + 1SD.

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