

**Keywords** Th17 · WT1 · Tumor immunity · Human immunology

## Introduction

Interleukin (IL)-17-producing T helper cells (Th17) have been identified as a distinct subset of CD4<sup>+</sup> T cells characterized by production of pro-inflammatory cytokine IL-17 [1–3] and simply defined by production of IL-17 and expression of ROR $\gamma$ t [4]. Th17 plays important roles in the pathogenesis of a wide array of auto-inflammatory diseases [5–7]. Recent studies have demonstrated that Th17 also plays important roles in tumor immunity. Several investigations have suggested a beneficial role of Th17 in tumor immunity [8–11]. Among ovarian cancer patients, for example, a group with high accumulation of Th17 into tumor sites showed better prognosis than a group with low accumulation. This positive association between Th17 accumulation and prognosis was thought to be caused by the production of chemokines that enhance recruitment of effector cells into tumor sites [8]. Consistent with this result, it was demonstrated that the transfer of tumor-specific CD4<sup>+</sup> T cells that had been polarized in vitro into Th17 induced drastic tumor regression in a mouse model [8]. Other studies, however, have suggested a negative effect of Th17 on tumor immunity [12–14]. For example, in colorectal cancer patients, a group with high accumulation of Th17 into tumor sites showed poor prognosis than a group with low accumulation. This negative association between Th17 accumulation and prognosis was thought to be caused by pro-angiogenic and pro-tumoral activity of the Th17 signature cytokine, IL-17 [12]. Thus, the influences of Th17 accumulation in tumor sites remain controversial. For further understanding of the roles of Th17 in tumor immunity, establishment of tumor-associated antigen (TAA)-specific Th17 clones has been awaited.

Wilms' tumor gene 1 (*WT1*) encodes a zinc-finger transcription factor and plays important roles in the regulation of cell proliferation, differentiation, and apoptosis [15–17]. *WT1* has an oncogenic function [18–20] and is expressed in various kinds of malignancies [21–24]. WT1 protein, as the product of this gene, is one of the most promising target antigens for cancer immunotherapy [25]. We previously identified WT1 protein-derived 16-mer helper epitope WT1<sub>332</sub> and established WT1<sub>332</sub>-specific CD4<sup>+</sup> T-cell clones by the stimulation of peripheral blood mononuclear cells (PBMCs) with the WT1<sub>332</sub> peptide [26, 27]. However, all the WT1<sub>332</sub>-specific CD4<sup>+</sup> T cell clones were T helper type 1 (Th1) [26]. These results encouraged us to try to establish WT1<sub>332</sub>-specific Th17 clones under Th17-polarizing conditions.

In the present study, we describe the establishment of WT1<sub>332</sub>-specific Th17 clones and demonstrate that Th17 clones have antigen-specific helper activity for the proliferation of conventional CD4<sup>+</sup> T cells. This is the first report of establishment of functional Th17 clones with WT1<sub>332</sub>-specific helper activity.

## Materials and methods

### Donor

Peripheral blood samples were obtained from a healthy donor with HLA-DRB1\*04:05/08:03, HLA-DR53, HLA-DQB1\*04:01/06:01, and HLA-DPB1\*05:01/-. Informed consent was given before the peripheral blood (PB) samples by the donor were obtained for the experiments.

### Antibodies and cytokines

Anti-CD3-PerCP, anti-CD4-PE, anti-CD4-APC-H7, anti-CD154-APC, anti-CD161-FITC, anti-CD25-PE-Cy7, anti-IFN- $\gamma$ -FITC, anti-GM-CSF-PE, anti-TNF- $\alpha$ -APC, anti-CCR6-biotin; anti-Foxp3-FITC monoclonal antibodies (mAbs); streptavidin-APC-Cy7 (BD Pharmingen, San Diego, CA, USA), anti-IL-17-PE, anti-ROR $\gamma$ t-APC, anti-T-bet-PE (eBiosciences, San Diego, CA, USA), anti-TCR V $\beta$ 18-PE, anti-TCR V $\beta$ 2-biotin mAbs (Beckman Coulter, Fullerton, CA, USA), anti-CCR4-FITC mAb (R&D systems, Minneapolis, MN, USA), and anti-HLA-DQ blocking antibody (Immunotech, Miami, FL, USA) were used. Anti-HLA-DR and anti-HLA-DP blocking antibodies were obtained from culture supernatant of hybridomas, L243 and B7/21 cells, respectively, and used at their optimal concentrations for all experiments. Hybridomas and L cells were provided by Prof. Nishimura (Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan). Recombinant human IL-1 $\beta$ , IL-6, IL-23, GM-CSF, IL-4, and TNF- $\alpha$  were purchased from PeproTech EC, London, UK.

### Generation of dendritic cells (DCs) in vitro

Peripheral blood mononuclear cells (PBMCs) were obtained from the healthy donor, and CD14<sup>+</sup> cells were enriched by using BD IMag anti-human CD14 magnetic particles-DM (BD Biosciences). In order to let the cells differentiate into DCs, the cells were cultured in X-VIVO15 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 1 % AB serum (Nabi Biopharmaceuticals, Miami, FL, USA) in the presence of GM-CSF and IL-4 (1,000 IU/ml each) for 5 days, as described elsewhere. Next, the maturation of the DCs was induced by

an additional 12-hour culture with TNF- $\alpha$  (100 IU/ml). Floating cells were harvested and used as antigen-presenting cells (APCs).

#### In vitro induction of WT1<sub>332</sub>-specific IL-17-producing CD4<sup>+</sup> T cells from PBMCs

PBMCs from the healthy donor were stimulated with WT1<sub>332</sub> peptide under the human Th17-polarizing conditions containing IL-1 $\beta$  (10 ng/ml), IL-6 (50 ng/ml), and neutralizing antibodies for IFN- $\gamma$  and IL-4 (10  $\mu$ g/ml each), as described elsewhere [28, 29]. IL-23 (20 ng/ml) and IL-2 (10U/ml) were added on the days 3 and 5, respectively. On the day 10, CD154-expressing cells, which were candidates for WT1<sub>332</sub>-specific Th17 cells, were single-cell-sorted, as previously described [30]. In brief, the cells were re-stimulated with WT1<sub>332</sub> peptide for 6 h in the presence of Golgi stop and anti-CD154-APC mAb, followed by staining with anti-CD4-PE mAb and streptavidin-APC-Cy7, and then CD4<sup>+</sup>CD154<sup>+</sup> cells were single-cell-sorted by means of FACSAria (BD Biosciences). The sorted cells were expanded by PHA HA16 (2  $\mu$ g/ml; Remel Europe Ltd., Dartford, UK) and IL-2 (100 IU/ml) in the presence of irradiated allogenic-PBMC (allo-PBMC) cocktail from three healthy donors. The cloned cells were maintained by periodic stimulation with autologous APCs pulsed with WT1<sub>332</sub> peptide in the presence of IL-2.

#### Proliferation assay

Cell proliferation was assessed with a standard <sup>3</sup>H-thymidine incorporation assay. Cells were plated in 96-well U-bottomed plate and cultured with irradiated autologous PBMC that were pulsed or unpulsed with 20  $\mu$ g/mL of WT1<sub>332</sub> peptide. HIV peptide was used as an irrelevant control peptide. <sup>3</sup>H-thymidine (Amersham Biosciences Corp, NJ) was added after culturing for 80 h, and the cells were cultured in the presence of <sup>3</sup>H-thymidine for additional 18 h. The cells were then harvested onto glass-fiber filters, and their radioactivity was measured on a  $\beta$ -scintillation counter in triplicate wells. The monoclonal antibodies L243, SPVL3, and B7/21 were used for blocking of HLA-DR, HLA-DQ, and HLA-DP, respectively.

#### Intracellular cytokine staining analysis

The cells were stimulated with WT1<sub>332</sub> peptide, or with phorbol-12-myristate-13-acetate (PMA; 25 ng/ml) and ionomycin (1  $\mu$ g/ml) in the presence of brefeldin A (Sigma, St Louis, MO, USA) for 6 h, and stained for cytoplasmic cytokines according to the protocol of BD intracellular staining kit (BD Biosciences). For the analysis of HLA-restriction, murine L cells that were transduced

with HLA-DRB1\*04:05, HLA-DRB1\*08:03, HLA-DR53, or HLA-DPB1\*05:01 were used as APCs. Established Th17 clones were stimulated by WT1<sub>332</sub> peptide-pulsed L cells, followed by the analysis of IL-17 production.

#### Analysis of the expression of transcriptional factors

Resting state-clone cells were fixed and permeabilized with Human FoxP3 Buffer Set (BD Bioscience), followed by intracellular staining with anti-ROR $\gamma$ t, anti-T-bet, and anti-FoxP3 mAbs.

#### TCR-V $\beta$ sequence analysis

T-cell receptor (TCR)-V $\beta$  CDR3 amino acid sequences of Th17 clones were obtained from the DNA sequences of RT-PCR products of the TCR-V $\beta$  genes, as described previously [31]. In brief, complementary DNAs (cDNAs) of TCR- $\beta$  were synthesized by the reverse transcription of mRNA of resting clones with a TCR- $\beta$  gene constant region-specific primer and used for two-step semi-nested PCR using 24 kinds of TCR-V $\beta$  gene family-specific primers and two kinds of TCR-V $\beta$  constant region-specific primers to identify the V $\beta$  gene family used in each clone. After identification of the V $\beta$  gene family used in each clone, direct sequencing of the V-D-J CDR3 region with V $\beta$  gene family-specific primers was performed to confirm the sequence of CDR3.

#### Recognition of WT1 protein processed APCs by the Th17 clone

Autologous PBMCs were pulsed with full-length WT1 protein (HWT1) [32] or truncated WT1 protein that is not containing WT1<sub>332</sub> amino acid sequence (HWT3) [33] for 7 h and used as APCs after irradiation. Th17 clones were co-cultured with the autologous PBMCs processed as described above for 3 days, and the concentration of IL-17 in the culture supernatant was measured by means of ELISA (R&D Systems).

#### Helper activity analysis

CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated from PBMC of the healthy donor from which the Th17 clones had been generated. The CD4<sup>+</sup>CD25<sup>-</sup> T cells were labeled with Cell-Trace<sup>TM</sup>-Violet (eBiosciences), and co-cultured with the Th17 clones and either WT1<sub>332</sub> peptide-pulsed or -unpulsed DCs in the presence of 1  $\mu$ g/ml of TSST-1. Since TSST-1 is a V $\beta$ 2 family-specific superantigen, it stimulated a V $\beta$ 2 family TCR-bearing population of CD4<sup>+</sup>CD25<sup>-</sup> T cells via a TCR signaling, but did not stimulate the Th17

clones that did not have TCR V $\beta$ 2. After 5 days of incubation, the proliferation of TCR V $\beta$ 2-expressing CD4<sup>+</sup>CD25<sup>-</sup> T cells was assessed by the evaluation of the dilution of CellTrace<sup>TM</sup>-Violet by means of flow cytometric analysis.

## Results

### Induction and establishment of WT1<sub>332</sub>-specific IL-17-producing CD4<sup>+</sup> T cell clones from human PBMCs

WT1<sub>332</sub> peptide, a human WT1 protein-derived helper epitope, was previously identified, and WT1<sub>332</sub>-specific Th1-type CD4<sup>+</sup> T cell clones were established. Here, it was examined whether WT1<sub>332</sub>-specific Th17 clones could be generated from PBMCs by stimulation with WT1<sub>332</sub> peptide under the Th17-polarizing conditions.

PBMCs from a healthy donor (HLA-DRB1\*04:05, \*08:03, HLA-DR53, DQB1\*04:01, \*06:01, and HLA-DPB1\*05:01) were cultured with WT1<sub>332</sub> peptide under Th17-polarizing conditions for 10 days. The cells were restimulated with the WT1<sub>332</sub> peptide, and CD154-positive cells were single-cell-sorted and expanded (Fig. 1a). As a result, 28 clones were established.

A proliferation assay was performed to determine which of the expanded 28 clones were WT1<sub>332</sub>-specific. Each clone was cultured with irradiated autologous PBMCs in the presence or absence of the WT1<sub>332</sub> peptide for 3 days, and the uptake of <sup>3</sup>H-thymidine by proliferating cells was measured. Consequently, 11 of the 28 clones showed the WT1<sub>332</sub>-specific proliferation (Fig. 1b), and thus, 11 WT1<sub>332</sub>-specific CD4<sup>+</sup> T cell clones were established.

To investigate whether the established WT1<sub>332</sub>-specific clones produced IL-17 in response to stimulation with the WT1<sub>332</sub> peptide, each of clones was stimulated with the WT1<sub>332</sub> peptide for 4 h, and the cytoplasmic cytokines were stained with anti-IL-17 and anti-IFN- $\gamma$  mAbs, followed by flow cytometric analysis. As shown in Fig. 1c, 4 of the 11 clones (clones 10, 20, 23, and 28) produced IL-17 in response to the WT1<sub>332</sub> peptide stimulation. All of the four IL-17-producing clones contained IFN- $\gamma$ -producing cells, although the frequencies of IFN- $\gamma$ -producing cell populations varied among the four clones with IL-17-production. Thus, these four IL-17-producing clones were considered to be Th17 clones. On the other hand, since the remaining seven IL-17-non-producing clones produced IFN- $\gamma$  in response to the WT1<sub>332</sub> peptide stimulation, they were considered to be Th1 clones.

Since IL-17 single-positive, IFN- $\gamma$  single-positive, or the double-positive cell populations were detected in the IL-17-producing clones, the possibility that different types

(Th1, Th17, and Th1/Th17) of plural cells were simultaneously sorted in a single well by experimental errors could be raised. To exclude this possibility, the sequences of TCR CDR3 regions of the four IL-17-producing clones established here were analyzed. As expected, each clone had only one TCR CDR3 sequence (data not shown), indicating that the cells are truly clones. Furthermore, it was revealed that all the four IL-17-producing clones had the same CDR3 sequence of TCR V $\beta$ 18 family (data not shown). These results indicated that the four Th17 clones were subclones derived from a single cell that had expanded before single-cell-sorting in the initial culture of PBMCs with the WT1<sub>332</sub> peptide stimulation under the Th17-polarizing conditions.

### WT1<sub>332</sub>-specific IL17-producing clones are Th17

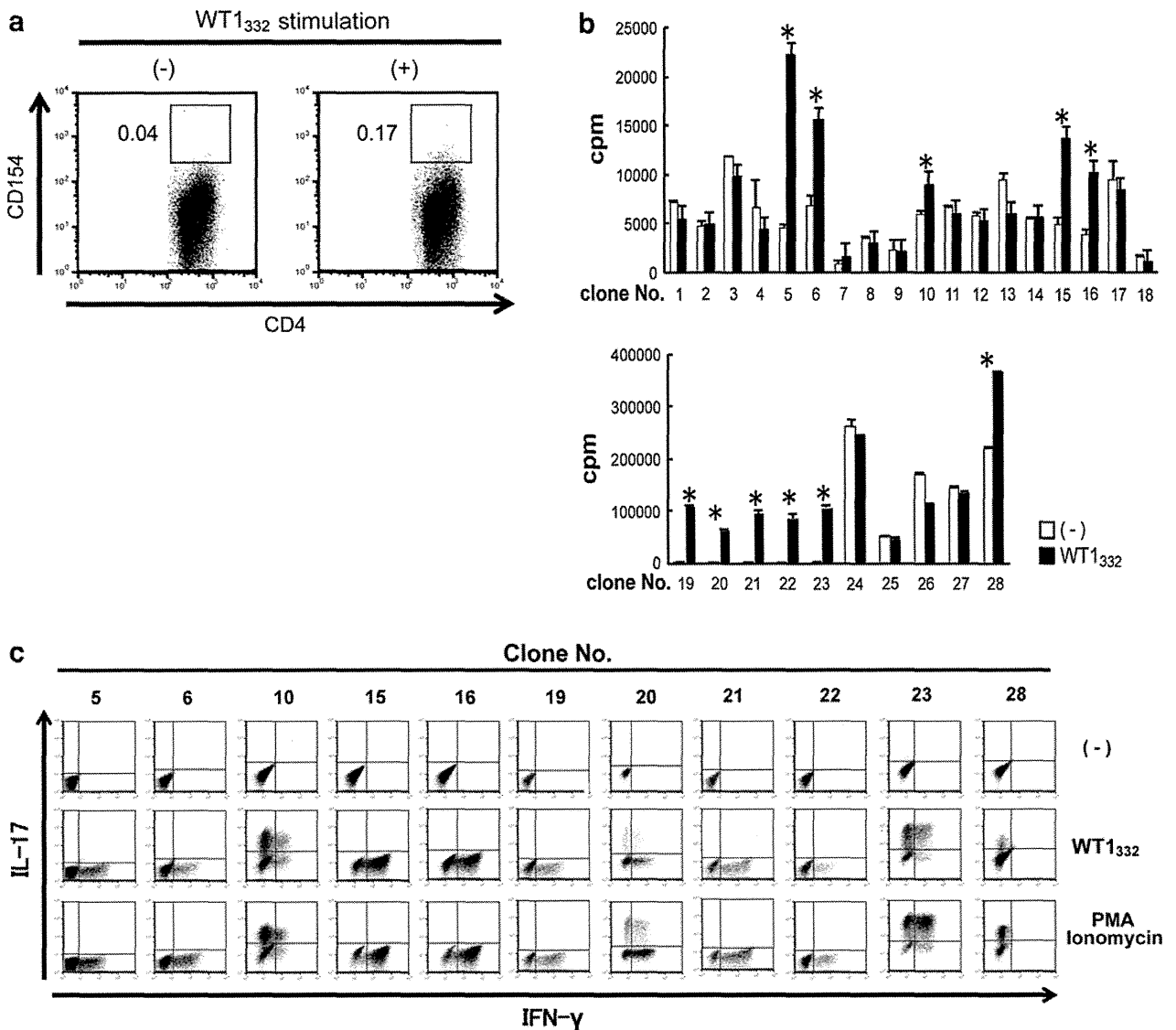
Recent studies have demonstrated that transcriptional factor ROR $\gamma$ t is specifically expressed in Th17 cells and functions as a master regulator of Th17-lineage. In order to molecularly confirm that the four IL-17-producing clones were Th17 clones, expression of three lineage-specific transcriptional factors, ROR $\gamma$ t, T-bet, and Foxp3, was examined in the four IL-17-producing clones in their resting state (Fig. 2a). Consequently, all of the four IL-17-producing clones expressed ROR $\gamma$ t, but not Foxp3, while T-bet was expressed in two clones (clones 20 and 23). Since ROR $\gamma$ t expression is a molecular Th17 signature, all of the four IL-17-producing clones were considered to be actual Th17 clones.

To further examine the lineage of the four IL-17-producing clones, cytokine profiles and cell surface phenotypes were examined in more detail (Fig. 2b, c). All of the four IL-17-producing clones produced GM-CSF and TNF- $\alpha$  in response to the WT1<sub>332</sub> peptide stimulation (Fig. 2b), which was consistent with the cytokine profiles of Th17. As shown in Fig. 2c, all of the four clones expressed CD161, CCR6, and CCR4, but not CXCR3, which was consistent with the surface phenotype of Th17.

Taken together, these results strongly indicated that these four clones were truly Th17 clones. To confirm the generality that WT1<sub>332</sub>-specific Th17 clones could be generated from PBMCs, other rounds of the same experiments were performed. Expectedly, other WT1<sub>332</sub>-specific Th17 clones with a TCR CDR3 sequence different from that of the four Th17 clones were established (data not shown), and, thus the generality was confirmed.

### Th17 clone specifically responds to WT1<sub>332</sub> peptide in an HLA-DPB1\*05:01-restricted manner

WT1<sub>332</sub> peptide was originally identified as a helper peptide with the restriction of HLA-DRB1\*04:05, and



**Fig. 1** Establishment of WT1<sub>332</sub>-specific IL-17-producing clones. PBMCs from a healthy donor were cultured with WT1<sub>332</sub> peptide under Th17-polarizing conditions. **a** On the day 10, the cells were re-stimulated with the WT1<sub>332</sub> peptide, and CD154<sup>+</sup>CD4<sup>+</sup> T cells were single-cell-sorted and expanded in the presence of PHA and IL-2. **b** Result of WT1<sub>332</sub>-specific proliferation of each clone assessed

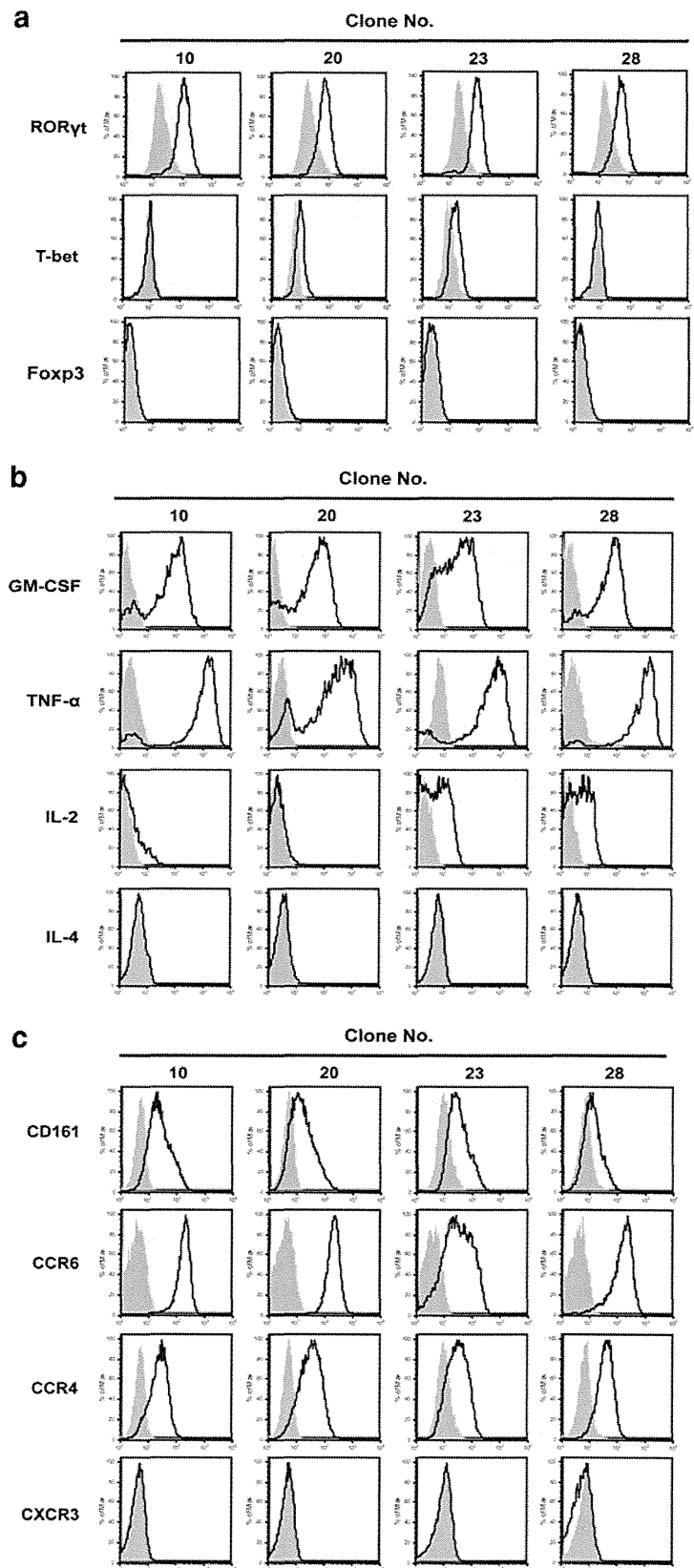
by proliferation assay is shown. <sup>3</sup>H-thymidine uptake (mean + S.D. of triplicate) by the WT1<sub>332</sub>-stimulated (*black columns*) or unstimulated (*white columns*) cells is shown. Asterisks show a WT1<sub>332</sub>-specific proliferation. **c** Result of flow cytometric analysis of IFN- $\gamma$  and IL-17 production in each clone stimulated by the WT1<sub>332</sub> peptide or PMA/ionomycin

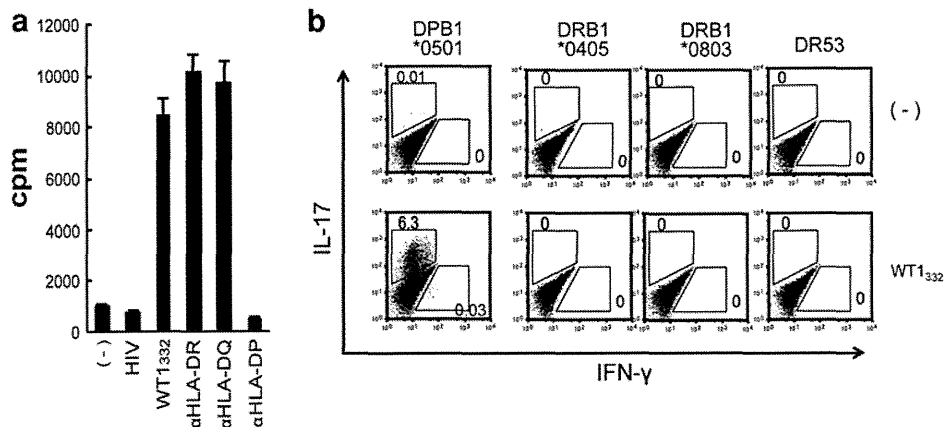
afterward defined as a promiscuous epitope that binds to at least HLA-DRB1\*15:01, \*15:02, and -DPB1\*09:01 molecules. Since all the four Th17 clones had the same TCR CDR3 sequences, HLA-restriction of WT1<sub>332</sub>-specific response was examined in Th17 clone10 as a representative of the four Th17 clones.

WT1<sub>332</sub>-specific proliferative responses of Th17 clone10 were significantly inhibited by anti-HLA-DP antibody, but not by anti-HLA-DR or anti-HLA-DQ antibody (Fig. 3a), indicating that the WT1<sub>332</sub>-specific proliferative responses

of Th17 clone10 were HLA-DP-restricted. Moreover, Th17 clone10 produced IL-17 when it was stimulated by the HLA-DPB1\*05:01-expressing L cells pulsed with WT1<sub>332</sub> peptide, while Th17 clone10 did not produce IL-17 in response to the HLA-DPB1\*05:01-expressing L cells unpulsed with WT1<sub>332</sub> peptide, or the HLA-DRB1\*04:05-, \*08:03- or -DR53-expressing L cells pulsed with WT1<sub>332</sub> peptide (Fig. 3b). These results, taken together, indicated that Th17 clone10 recognized WT1<sub>332</sub> peptide in a restriction to HLA-DPB1\*05:01.

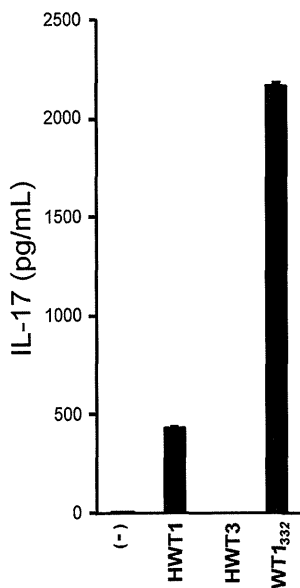
**Fig. 2** Characterization of IL-17-producing clones. **a** Flow cytometric analysis of expression of ROR $\gamma$ t, T-bet, and Foxp3 in IL-17-producing clones in resting state. **b** Flow cytometric analysis of expression of GM-CSF, TNF- $\alpha$ , IL-2, and IL-4 by stimulation with WT1<sub>332</sub> peptide. **c** Flow cytometric analysis of expression of cell surface molecules, CD161, CCR6, CCR4, and CXCR3. *Solid lines* and *shadows* represent WT1<sub>332</sub>-stimulated or -unstimulated cells, respectively





**Fig. 3** WT<sub>1332</sub>-specific response of Th17 clone10 is HLA-DPB1\*0501-restricted. **a** Clone10 was stimulated by autologous PBMCs pulsed with WT<sub>1332</sub> peptide in the presence of HLA-DR, HLA-DQ, or HLA-DP blocking antibodies. <sup>3</sup>H-thymidine uptake (mean + S.D. of triplicate) is shown on the day 4. HIV peptide (RT<sub>171-190</sub>) was used as an irrelevant antigen. **b** Clone10 was

stimulated by WT<sub>1332</sub>-pulsed or-unpulsed L cells that expressed one each of HLA-DPB1\*05:01, DRB1\*04:05, DRB1\*08:03, and DR53 molecules and examined for IL-17 production. Flow cytometric analysis of IL-17 production is shown as a representative of 3 experiments



**Fig. 4** Th17 clone10 is responsive to the product processed naturally from WT1 protein. Clone10 was stimulated by autologous PBMCs pulsed with the full-length WT1 protein (HWT1) or WT<sub>1332</sub>-deficient WT1 protein (HWT3), and IL-17 concentrations in the culture supernatant were examined by ELISA. Clone10 that was stimulated by autologous PBMCs pulsed with WT<sub>1332</sub> peptide was used as a positive control. A representative data of 3-independent experiments are shown

Th17 clone recognizes a naturally processed WT1 helper epitope

Whether or not Th17 clone10 recognized and responded to a naturally processed WT1 helper epitope was examined.

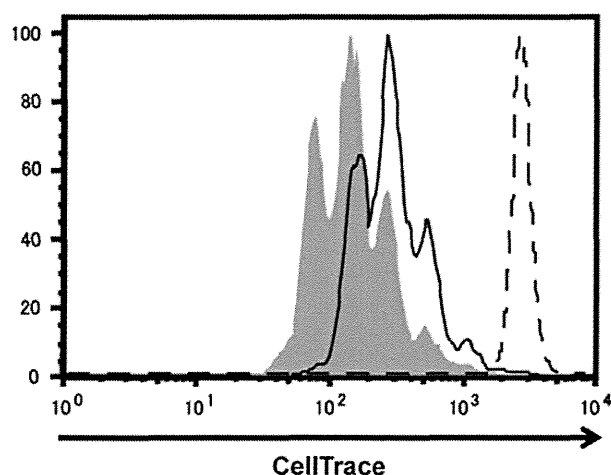
As shown in a Fig. 4, Th17 clone10 produced IL-17 in response to the full-length WT1 protein (HWT1)-pulsed autologous PBMCs with HLA-DPB1\*05:01, while it did not respond to the protein-unpulsed or truncated WT1 protein, which is not containing WT1332 amino acid sequence (HWT3)-pulsed autologous PBMCs. These results indicated that Th17 clone could recognize and respond to the WT<sub>1332</sub> epitope processed naturally from WT1 protein.

Th17 clone functions as a helper for the proliferation of conventional CD4<sup>+</sup> T cells in a WT<sub>1332</sub>-specific manner

To investigate whether Th17 clone10 had a helper or suppressive activity, the effect of the WT<sub>1332</sub>-stimulated Th17 clone10 on the proliferation of conventional CD4<sup>+</sup>CD25<sup>-</sup> T cells was examined.

In order to stimulate the conventional CD4<sup>+</sup>CD25<sup>-</sup> T cells (responder T cells), superantigen TSST-1 was used because it stimulates T cells with Vβ2 and Vβ4 families that accounted for a considerable proportion of the responder T cells have, while the TSST-1 did not stimulate Th17 clone10 with Vβ18 family. The effect of the WT<sub>1332</sub>-stimulated Th17 clone10 on the TSST-1-stimulated responder T cell proliferation was examined (Fig. 5).

CellTrace<sup>TM</sup>-Violet-labeled conventional CD4<sup>+</sup>CD25<sup>-</sup> T cells were co-cultured with Th17 clone10 and mature DCs pulsed or unpulsed with WT<sub>1332</sub> peptide in the presence of TSST-1. On the day 5, the proliferation of the CD4<sup>+</sup>CD25<sup>-</sup> T cells (responder T cells) was examined by the evaluation of the dilutions of CellTrace<sup>TM</sup>-Violet. As shown in Fig. 5, the responder T-cell proliferation was



**Fig. 5** Th17 clone10 helps the proliferation of conventional  $CD4^+$  T cells in a WT1<sub>332</sub>-specific manner CellTrace<sup>TM</sup>-Violet-labeled  $CD25^-CD4^+$  conventional T cells were co-cultured with clone10 with TCR  $V\beta 18$  and autologous DCs pulsed with TSST-1 in the presence (*gray shadow*) or absence (*solid line*) of WT1<sub>332</sub> peptide. After 5 days of culture, the proliferation of the  $V\beta 2^+CD25^-CD4^+$  T cells was evaluated of the dilutions of CellTrace<sup>TM</sup>-Violet by flow cytometric analysis. A *dashed line* shows  $V\beta 2^-CD25^-CD4^+$  T cells that did not respond to TSST-1

enhanced by co-culture with the WT1<sub>332</sub> peptide-stimulated Th17 clone10, compared to co-culture with unstimulated Th17 clone10. When the WT1<sub>332</sub> peptide alone was added to the culture of the TSST-1-stimulated  $CD4^+CD25^-$  T cell without addition of Th17 clone10, proliferation was not enhanced (data not shown), indicating that the WT1<sub>332</sub> peptide-stimulated Th17 clone10 induced the responder T-cell proliferation. Taken together, these results clearly demonstrated that WT1<sub>332</sub> peptide-stimulated Th17 clone10 promoted the proliferation of the responder T cell, indicating that Th17 clone10 had helper activity in a WT1<sub>332</sub>-specific manner.

## Discussion

Recently, a number of clinical investigations have demonstrated that Th17 is highly accumulated in tumor sites, and that the degree of Th17-accumulation is clearly associated with prognosis [8–14, 34–36]. Although Th17 is thought to play important roles in cancer immunity, detailed roles of tumor-associated antigen (TAA)-specific Th17 still remain unclear. One way to address this issue is to establish TAA-specific Th17 clones. In the present study, we succeeded to establish TAA WT1-specific human Th17 clones from the PBMCs of a healthy donor and precisely confirmed that established Th17 clones maintained the phenotypes of typical Th17 regardless of in vitro stimulation and expansion. Furthermore, our

findings suggest one aspect of TAA-specific Th17 that Th17 has a potential to help proliferation of conventional  $CD4^+$  T cell by using established Th17 clones. Therefore, TAA-specific Th17 clones are useful for the study of the roles of human Th17 in cancer immunity.

One of the difficulties to study the roles of human TAA-specific Th17 in tumor immunity is the low frequency of TAA-specific Th17 or its precursor in human PBMCs. To overcome this difficulty, establishment of Th17 clones was awaited. However, this was not easy to accomplish because of the low frequency of Th17 cells in PBMCs. The present study demonstrated that the method used here, in which antigen-activated  $CD4^+$  T cells that existed at low frequency in the samples were sorted by using a CD154, costimulatory cell surface molecule as an activation marker and expanded, was useful for establishing TAA-specific Th clones, such as WT1<sub>332</sub>-specific Th17 clones. Hamai et al. [37] also demonstrated, very recently, that MAGE-A3-specific Th17 clones could be generated from PBMCs of a lung cancer patient with the same method as ours. Taken together, these results showed that the use of CD154 expression as an activation marker should be useful for the establishment of TAA-specific Th17 clones. Furthermore, these studies made it evident that TAA-specific Th17 or their precursors existed in PB. These Th17 clones established by this method should be useful to further analyze the roles of Th17 in cancer immunity.

Clinical investigations indicated that Th17 was highly accumulated into tumor sites in various types of tumors. However, high accumulation of Th17 is associated, either positively or negatively, with prognosis [8–14, 34–36]. At present time, it is likely that whether Th17 functions positively or negatively in tumor immunity depend on the kinds and characteristics of tumors. In this study, the TAA-specific Th17 clone helped proliferation of conventional  $CD4^+$  T cells in an antigen-specific manner. This might demonstrate an aspect of anti-tumoral function of Th17. Further studies are needed to obtain a deeper understanding of biphasic function of Th17.

The present study demonstrated that Th17 clone10 had the helper activity for the proliferation of conventional T cells, while Th17 clone10 produced only small amount of IL-2 (Fig. 2b). Recent studies showed that, besides IL-2 production, helper T cells enhance immune responses through activation and maturation of APCs. One of the most important mechanisms of the activation of APCs by helper T cells is the interaction of CD40 and its ligand CD154 [38], as Th17 clone10 expressed CD154 in response to the WT1<sub>332</sub> peptide stimulation. In addition, Th17 clone10 produced GM-CSF and TNF- $\alpha$  that are essential for differentiation and survival, and maturation of DCs, respectively [39], as the Th17 clone10 did. Therefore, one of the mechanisms of helper function of Th17 clone10

may be activation of APCs. Further studies are needed to address this issue.

WT1<sub>332</sub> peptide is a promiscuous peptide and has the ability to induce WT1<sub>332</sub>-specific Th1 cells that actually helped the induction of WT1-specific CTLs [26]. We demonstrated that there is a clear correlation between clinical effect of HLA-A\*24:02-restricted WT1 peptide (modified WT1<sub>235-243</sub>) vaccine and WT1<sub>332</sub>-specific Th1 and Th2 responses, which indicates that the possibility of prediction of clinical effect of the peptide vaccine by assessing the WT1<sub>332</sub>-specific Th responses [40]. Thus, analysis of the correlation between clinical effect of HLA-A\*24:02-restricted WT1 peptide vaccine and WT1<sub>332</sub>-specific Th17 response should be interesting.

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**Conflict of interest** The authors declare that they have no conflict of interest.

## References

- Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT (2005) Interleukin 17-producing CD4<sup>+</sup> effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6(11):1123–1132
- Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201(2):233–240
- Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6(11):1133–1141
- Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR (2006) The orphan nuclear receptor ROR $\gamma$  directs the differentiation program of proinflammatory IL-17<sup>+</sup> T helper cells. *Cell* 126(6):1121–1133
- Leipe J, Grunke M, Dechant C, Reindl C, Kerzendorf U, Schulze-Koops H, Skapenko A (2010) Role of Th17 cells in human autoimmune arthritis. *Arthritis Rheum* 62(10):2876–2885
- Kebir H, Kreymborg K, Ifergan I, Dodelet-Devillers A, Cayrol R, Bernard M, Giuliani F, Arbour N, Becher B, Prat A (2007) Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med* 13(10):1173–1175
- Abraham C, Cho J (2009) Interleukin-23/Th17 pathways and inflammatory bowel disease. *Inflamm Bowel Dis* 15(7):1090–1100
- Kryczek I, Banerjee M, Cheng P, Vatan L, Szeliga W, Wei S, Huang E, Finlayson E, Simeone D, Welling TH, Chang A, Coukos G, Liu R, Zou W (2009) Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood* 114(6):1141–1149
- Li J, Mo HY, Xiong G, Zhang L, He J, Huang ZF, Liu ZW, Chen QY, Du ZM, Zheng LM, Qian CN, Zeng YX (2012) Tumor microenvironment MIF directs the accumulation of IL-17-producing tumor-infiltrating lymphocytes and predicts favorable survival in nasopharyngeal carcinoma patients. *J Biol Chem* 287(42):35484–35495
- Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A, Paulos CM, Palmer DC, Touloukian CE, Ptak K, Gattinoni L, Wrzesinski C, Hinrichs CS, Kerstann KW, Feigenbaum L, Chan CC, Restifo NP (2008) Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* 112(2):362–373
- Yang L, Qi Y, Hu J, Tang L, Zhao S, Shan B (2012) Expression of Th17 cells in breast cancer tissue and its association with clinical parameters. *Cell Biochem Biophys* 62(1):153–159
- Liu J, Duan Y, Cheng X, Chen X, Xie W, Long H, Lin Z, Zhu B (2011) IL-17 is associated with poor prognosis and promotes angiogenesis via stimulating VEGF production of cancer cells in colorectal carcinoma. *Biochem Biophys Res Commun* 407(2):348–354
- Zhang JP, Yan J, Xu J, Pang XH, Chen MS, Li L, Wu C, Li SP, Zheng L (2009) Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients. *J Hepatol* 50(5):980–989
- Iida T, Iwahashi M, Katsuda M, Ishida K, Nakamori M, Nakamura M, Naka T, Ojima T, Ueda K, Hayata K, Nakamura Y, Yamaue H (2011) Tumor-infiltrating CD4<sup>+</sup> Th17 cells produce IL-17 in tumor microenvironment and promote tumor progression in human gastric cancer. *Oncol Rep* 25(5):1271–1277
- Drummond IA, Madden SL, Rohwer-Nutter P, Bell GI, Sukhatme VP, Rauscher FJ 3rd (1992) Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. *Science* 257(5070):674–678
- Hewitt SM, Hamada S, McDonnell TJ, Rauscher FJ 3rd, Saunders GF (1995) Regulation of the proto-oncogenes bcl-2 and c-myc by the Wilms' tumor suppressor gene WT1. *Cancer Res* 55(22):5386–5389
- Kim J, Prawitt D, Bardeesy N, Torban E, Vicaner C, Goodyer P, Zabel B, Pelletier J (1999) The Wilms' tumor suppressor gene (wt1) product regulates Dax-1 gene expression during gonadal differentiation. *Mol Cell Biol* 19(3):2289–2299
- Yamagami T, Sugiyama H, Inoue K, Ogawa H, Tatekawa T, Hirata M, Kudoh T, Akiyama T, Murakami A, Maekawa T (1996) Growth inhibition of human leukemic cells by WT1 (Wilms tumor gene) antisense oligodeoxynucleotides: implications for the involvement of WT1 in leukemogenesis. *Blood* 87(7):2878–2884
- Inoue K, Tamaki H, Ogawa H, Oka Y, Soma T, Tatekawa T, Oji Y, Tsuboi A, Kim EH, Kawakami M, Akiyama T, Kishimoto T, Sugiyama H (1998) Wilms' tumor gene (WT1) competes with differentiation-inducing signal in hematopoietic progenitor cells. *Blood* 91(8):2969–2976
- Tsuboi A, Oka Y, Ogawa H, Elisseeva OA, Tamaki H, Oji Y, Kim EH, Soma T, Tatekawa T, Kawakami M, Kishimoto T, Sugiyama H (1999) Constitutive expression of the Wilms' tumor gene WT1 inhibits the differentiation of myeloid progenitor cells but promotes their proliferation in response to granulocyte-colony stimulating factor (G-CSF). *Leuk Res* 23(5):499–505
- Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, Kita K, Hiraoka A, Masaoka T, Nasu K et al (1994) WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. *Blood* 84(9):3071–3079
- Oji Y, Ogawa H, Tamaki H, Oka Y, Tsuboi A, Kim EH, Soma T, Tatekawa T, Kawakami M, Asada M, Kishimoto T, Sugiyama H (1999) Expression of the Wilms' tumor gene WT1 in solid tumors and its involvement in tumor cell growth. *Jpn J Cancer Res* 90(2):194–204
- Oji Y, Suzuki T, Nakano Y, Maruno M, Nakatsuka S, Jomgeow T, Abeno S, Tatsumi N, Yokota A, Aoyagi S, Nakazawa T, Ito K,



- Kanato K, Shirakata T, Nishida S, Hosen N, Kawakami M, Tsuboi A, Oka Y, Aozasa K, Yoshimine T, Sugiyama H (2004) Overexpression of the Wilms' tumor gene WT1 in primary astrocytic tumors. *Cancer Sci* 95(10):822–827
24. Oji Y, Yano M, Nakano Y, Abeno S, Nakatsuka S, Ikeba A, Yasuda T, Fujiwara Y, Takiguchi S, Yamamoto H, Fujita S, Kanato K, Ito K, Jomgeow T, Kawakami M, Tsuboi A, Shirakata T, Nishida S, Hosen N, Oka Y, Aozasa K, Monden M, Sugiyama H (2004) Overexpression of the Wilms' tumor gene WT1 in esophageal cancer. *Anticancer Res* 24(5B):3103–3108
  25. Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, Mellman I, Prindiville SA, Viner JL, Weiner LM, Matrisian LM (2009) The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin Cancer Res* 15(17):5323–5337
  26. Fujiki F, Oka Y, Tsuboi A, Kawakami M, Kawakatsu M, Nakajima H, Elisseeva OA, Harada Y, Ito K, Li Z, Tatsumi N, Sakaguchi N, Fujioka T, Masuda T, Yasukawa M, Udaka K, Kawase I, Oji Y, Sugiyama H (2007) Identification and characterization of a WT1 (Wilms Tumor Gene) protein-derived HLA-DRB1\*0405-restricted 16-mer helper peptide that promotes the induction and activation of WT1-specific cytotoxic T lymphocytes. *J Immunother* 30(3):282–293
  27. Fujiki F, Oka Y, Kawakatsu M, Tsuboi A, Nakajima H, Elisseeva OA, Harada Y, Li Z, Tatsumi N, Kamino E, Shirakata T, Nishida S, Taniguchi Y, Kawase I, Oji Y, Sugiyama H (2008) A WT1 protein-derived, naturally processed 16-mer peptide, WT1(332), is a promiscuous helper peptide for induction of WT1-specific Th1-type CD4(+) T cells. *Microbiol Immunol* 52(12):591–600
  28. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F (2007) Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 8(9):942–949
  29. Stritesky GL, Yeh N, Kaplan MH (2008) IL-23 promotes maintenance but not commitment to the Th17 lineage. *J Immunol* 181(9):5948–5955
  30. Chattopadhyay PK, Yu J, Roederer M (2005) A live-cell assay to detect antigen-specific CD4<sup>+</sup> T cells with diverse cytokine profiles. *Nat Med* 11(10):1113–1117
  31. Tanaka-Harada Y, Kawakami M, Oka Y, Tsuboi A, Katagiri T, Elisseeva OA, Nishida S, Shirakata T, Hosen N, Fujiki F, Murao A, Nakajima H, Oji Y, Kanda Y, Kawase I, Sugiyama H (2010) Biased usage of BV gene families of T-cell receptors of WT1 (Wilms' tumor gene)-specific CD8<sup>+</sup> T cells in patients with myeloid malignancies. *Cancer Sci* 101(3):594–600
  32. Oji Y, Kitamura Y, Kamino E, Kitano A, Sawabata N, Inoue M, Mori M, Nakatsuka S, Sakaguchi N, Miyazaki K, Nakamura M, Fukuda I, Nakamura J, Tatsumi N, Takakuwa T, Nishida S, Shirakata T, Hosen N, Tsuboi A, Nezu R, Maeda H, Oka Y, Kawase I, Aozasa K, Okumura M, Miyoshi S, Sugiyama H (2009) WT1 IgG antibody for early detection of nonsmall cell lung cancer and as its prognostic factor. *Int J Cancer* 125(2):381–387
  33. Elisseeva OA, Oka Y, Tsuboi A, Ogata K, Wu F, Kim EH, Soma T, Tamaki H, Kawakami M, Oji Y, Hosen N, Kubota T, Nakagawa M, Yamagami T, Hiraoka A, Tsukaguchi M, Udaka K, Ogawa H, Kishimoto T, Nomura T, Sugiyama H (2002) Humoral immune responses against Wilms tumor gene WT1 product in patients with hematopoietic malignancies. *Blood* 99(9):3272–3279
  34. Kryczek I, Wei S, Szeliga W, Vatan L, Zou W (2009) Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood* 114(2):357–359
  35. Kryczek I, Wei S, Zou L, Altuwajri S, Szeliga W, Kolls J, Chang A, Zou W (2007) Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. *J Immunol* 178(11):6730–6733
  36. Su X, Ye J, Hsueh EC, Zhang Y, Hoft DF, Peng G (2010) Tumor microenvironments direct the recruitment and expansion of human Th17 cells. *J Immunol* 184(3):1630–1641
  37. Hamai A, Pignon P, Raimbaud I, Duperrier-Amouriaux K, Senellart H, Huret S, Douillard JY, Bennouna J, Ayyoub M, Valmori D (2012) Human T(H)17 immune cells specific for the tumor antigen MAGE-A3 convert to IFN-gamma-secreting cells as they differentiate into effector T cells in vivo. *Cancer Res* 72(5):1059–1063
  38. Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G (1996) Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T–T help via APC activation. *J Exp Med* 184(2):747–752
  39. Markowicz S, Engleman EG (1990) Granulocyte-macrophage colony-stimulating factor promotes differentiation and survival of human peripheral blood dendritic cells in vitro. *J Clin Invest* 85(3):955–961
  40. Fujiki F, Oka Y, Kawakatsu M, Tsuboi A, Tanaka-Harada Y, Hosen N, Nishida S, Shirakata T, Nakajima H, Tatsumi N, Hashimoto N, Taguchi T, Ueda S, Nonomura N, Takeda Y, Ito T, Myoui A, Izumoto S, Maruno M, Yoshimine T, Noguchi S, Okuyama A, Kawase I, Oji Y, Sugiyama H (2009) A clear correlation between WT1-specific Th response and clinical response in WT1 CTL epitope vaccination. *Anticancer Res* 30(6):2247–2254



## BASIC STUDY

# HLA-DPB1\*05:01-restricted WT1<sub>332</sub>-specific TCR-transduced CD4<sup>+</sup> T Lymphocytes Display a Helper Activity for WT1-specific CTL Induction and a Cytotoxicity Against Leukemia Cells

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**Summary:** Wilms tumor gene 1 (WT1) is overexpressed in various malignant neoplasms, and has been demonstrated as an attractive target for cancer immunotherapy. We previously reported the identification of a WT1 protein-derived, 16-mer helper peptide WT1<sub>332</sub> that could elicit Th1-type CD4<sup>+</sup> T-cell response and bind to multiple HLA class II molecules. In this study, we examined the feasibility of adoptive therapy using CD4<sup>+</sup> T cells that were transduced an HLA-DPB1\*05:01-restricted, WT1<sub>332</sub>-specific T-cell receptor (TCR). HLA-DPB1\*05:01-restricted, WT1<sub>332</sub>-specific TCR-transduced CD4<sup>+</sup> T cells were successfully generated using lentiviral vector and exhibited strong proliferative response and Th1-type cytokine production in response to WT1<sub>332</sub> peptide, WT1 protein, or WT1-expressing tumor cell lysate. Furthermore, the WT1<sub>332</sub>-specific TCR-transduced CD4<sup>+</sup> T cells lysed HLA-DPB1\*05:01-positive, WT1-expressing human leukemia cells through granzyme B/perforin pathway. Furthermore, stimulation of peripheral blood mononuclear cells with both HLA-A\*24:02-restricted CTL-epitope peptide (modified 9-mer WT1<sub>235</sub> peptide, WT1<sub>235m</sub>) and WT1<sub>332</sub> helper peptide in the presence of WT1<sub>332</sub>-specific TCR-transduced CD4<sup>+</sup> T cells strikingly enhanced the induction of WT1<sub>235m</sub>-specific CTLs. Thus, these results demonstrated the feasibility of immunotherapy based on adoptive transfer of WT1<sub>332</sub>-specific TCR-transduced CD4<sup>+</sup> T cells for the treatment of leukemia.

**Key Words:** WT1, CD4<sup>+</sup> helper T cell, HLA class II, helper peptide, TCR gene therapy

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Wilms tumor gene 1 (WT1), a zinc finger transcription factor, has been proved to play an important role in the regulation of cell differentiation, proliferation, and apoptosis.<sup>1</sup> On the basis of the results of a series of studies,<sup>2–4</sup> we proposed that the wild-type *WT1* gene had an oncogenic function in various kinds of hematological malignancies and solid tumors although it was originally

defined as a tumor-suppressor gene.<sup>5,6</sup> Indeed, the *WT1* gene is overexpressed in acute myeloid leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, and myelodysplastic syndromes as well as various types of solid cancers.<sup>7</sup>

Some WT1-derived, HLA class I-restricted CTL epitopes were identified<sup>8,9</sup> and WT1 peptide vaccination using these CTL epitopes<sup>10–12</sup> has been performing for patients with leukemia and solid cancers with good clinical responses such as a reduction in leukemic blast cells<sup>10</sup> and tumor size, and prolonged survival.<sup>11</sup> These good clinical responses were associated with an increase in the frequency of WT1-specific CD8<sup>+</sup> T cells in peripheral blood.<sup>7,10</sup> These results indicated that WT1-targeted immunotherapy should be a promising strategy for cancer treatment. In fact, WT1 was selected as the most promising tumor-associated antigen (TAA) among identified 75 TAAs.<sup>13</sup>

Accumulating evidence showed that adoptive transfer of TAA-specific, HLA class I-restricted T-cell receptor (TCR) gene-transduced T cells was also a promising strategy to treat patients with leukemia<sup>14,15</sup> or solid tumor.<sup>16,17</sup> WT1-specific, HLA-A\*02:01-restricted or A\*2402-restricted *TCR* genes have been already cloned and adoptive transfer of T cells transduced with these *TCR* genes could elicit a potent antileukemia effect.<sup>14,15</sup> However, clinical results of *TCR* gene therapy were still limited and not yet sufficient. Thus, improvement of clinical effect of *TCR* gene-transduced T-cell therapy is awaited.

A number of studies have indicated the importance of CD4<sup>+</sup> T cell in both elimination of infectious disease<sup>18</sup> and antitumor immunity. CD4<sup>+</sup> T cells have been demonstrated to be critical for, maintenance of cell numbers, recruitment to the tumor sites, and memory response, of CD8<sup>+</sup> T cells.<sup>19</sup> Recent investigations showed more direct evidence to support the benefit of the use of tumor-reactive CD4<sup>+</sup> T cells for cancer immunotherapy.<sup>20,21</sup> We previously identified a WT1-derived HLA class II-restricted, 16-mer helper peptide, WT1<sub>332</sub>, and showed that WT1<sub>332</sub> helper peptide had promiscuous characteristics that could bind to many types of HLA class II.<sup>22,23</sup> WT1<sub>332</sub>-specific CD4<sup>+</sup> T cells that were induced by *in vitro* stimulation of PBMCs by WT1<sub>332</sub> peptide could enhance *in vitro* induction of WT1-specific CTLs<sup>22</sup> and kill WT1-expressing leukemia cells from patients.<sup>24</sup> Furthermore, importantly, WT1<sub>332</sub>-specific CD4<sup>+</sup> T-cell responses correlated with good clinical responses of HLA-A\*24:02-restricted, WT1

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peptide vaccine.<sup>25</sup> These findings suggested that gene therapy using WT1<sub>332</sub>-specific, HLA class II-restricted TCR should be promising as a cancer immunotherapy.

In this study, we describe the isolation of HLA-DPB1\*05:01-restricted, WT1<sub>332</sub>-specific TCR gene and the usefulness of the WT1<sub>332</sub>-specific TCR-transduced CD4<sup>+</sup> T cells for cancer immunotherapy. The WT1<sub>332</sub>-specific TCR-transduced CD4<sup>+</sup> T cells enhance the induction of WT1-specific CTLs and directly kill leukemic cells through granzyme B/perforin pathway. These results allow us to expect the clinical benefit of WT1<sub>332</sub>-specific TCR-transduced T cell therapy.

## MATERIALS AND METHODS

### Cell Lines

TG40, a cell surface TCR-negative and intracytoplasmic CD3-positive mutant of the mouse T-cell line,<sup>26</sup> was obtained from Dr Toshio Kitamura (Tokyo University, Tokyo, Japan). WT1-expressing and HLA-DPB1\*05:01-positive C2F8 (early erythroblastic leukemia cell line) was kindly provided from Dr Tatsuo Furukawa (Niigata University, Niigata, Japan).<sup>27</sup> Endogenously WT1-expressing TF-1 (human erythroleukemia cell line)<sup>8</sup> was cultured in RPMI1640 (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10% heat inactivated FBS (Euro-lone, Milano, Italia), 1% penicillin/streptomycin (Nacalai Tesque Inc., Kyoto, Japan) and 4 ng/mL GM-CSF (PeproTech, Rocky Hill, NJ). Epstein-Barr virus-transformed B-cell line, B-LCL(-), and WT1-overexpressed B-LCL(+) were previously established from an HLA-DPB1\*05:01-positive donor.<sup>22</sup> X-VIVOTM 15 (Lonza, MD) supplemented with 10% AB serum (Gemini, NC) and 20 IU/mL IL-2 (kindly donated by Shionogi & Co. Ltd., Osaka, Japan) was used for culture of PBMCs and T cells.

### Antibodies, Peptides, and Reagents

WT1<sub>332</sub> peptide (KRYFKLSHLQMSHRKH) and 14 truncated WT1<sub>332</sub> peptides (as described in Fig. 1) for epitope mapping were synthesized by Sigma-Aldrich (Hokkaido, Japan) or MBL (Nagoya, Japan), respectively. Recombinant full length of WT1 protein (HWT1) and a truncated WT1 protein (HWT3) including 1–294 amino acid sequence were obtained as previously described.<sup>28,29</sup> Tumor cell lysates were prepared by 5 freeze-thaw cycles from 1 × 10<sup>8</sup> tumor cells resuspended in 1 mL of PBS. Lysates were used at 5 × 10<sup>5</sup> tumor cells equivalents per mL. An anti-HLA class I mAb, W6/32 (obtained from ATCC), anti-HLA-DR mAb, L243 (obtained from ATCC), and anti-HLA-DP mAb, B7/21 (kindly provided by Dr Yasuharu Nishimura, Kumamoto University, Kumamoto, Japan) were prepared from their hybridoma supernatants. Anti-HLA-DQ mAb, SPVL3 was obtained from Immunotech (Miami, FL). For flow cytometric analysis, the following mAbs were used: anti-CD107a-APC, anti-CD3-Pacific Blue, anti-CD4-APC-H7, anti-granzyme B-PE, anti-IL-5-PE, anti-IL-10-PE, anti-IFN $\gamma$ -PE-Cy7, anti-CD154-APC (BD Bioscience, San Jose, CA), anti-IFN $\gamma$ -PE, anti-TNF $\alpha$ -APC, anti-GM-CSF-PE, anti-mouse CD3 $\epsilon$ -PE, anti-Perforin-APC, anti-IL-17A-PE, (eBioscience, San Diego, CA), and anti-IL-2-APC (BioLegend, San Diego, CA). Ac-IETD-Cho, the caspase 8 inhibitor (granzyme B inhibitor) was purchased from Merck (Darmstadt, Germany) and used as previously described.<sup>30</sup>

### Generation of WT1<sub>332</sub>-specific CD4<sup>+</sup> T-Cell Clones

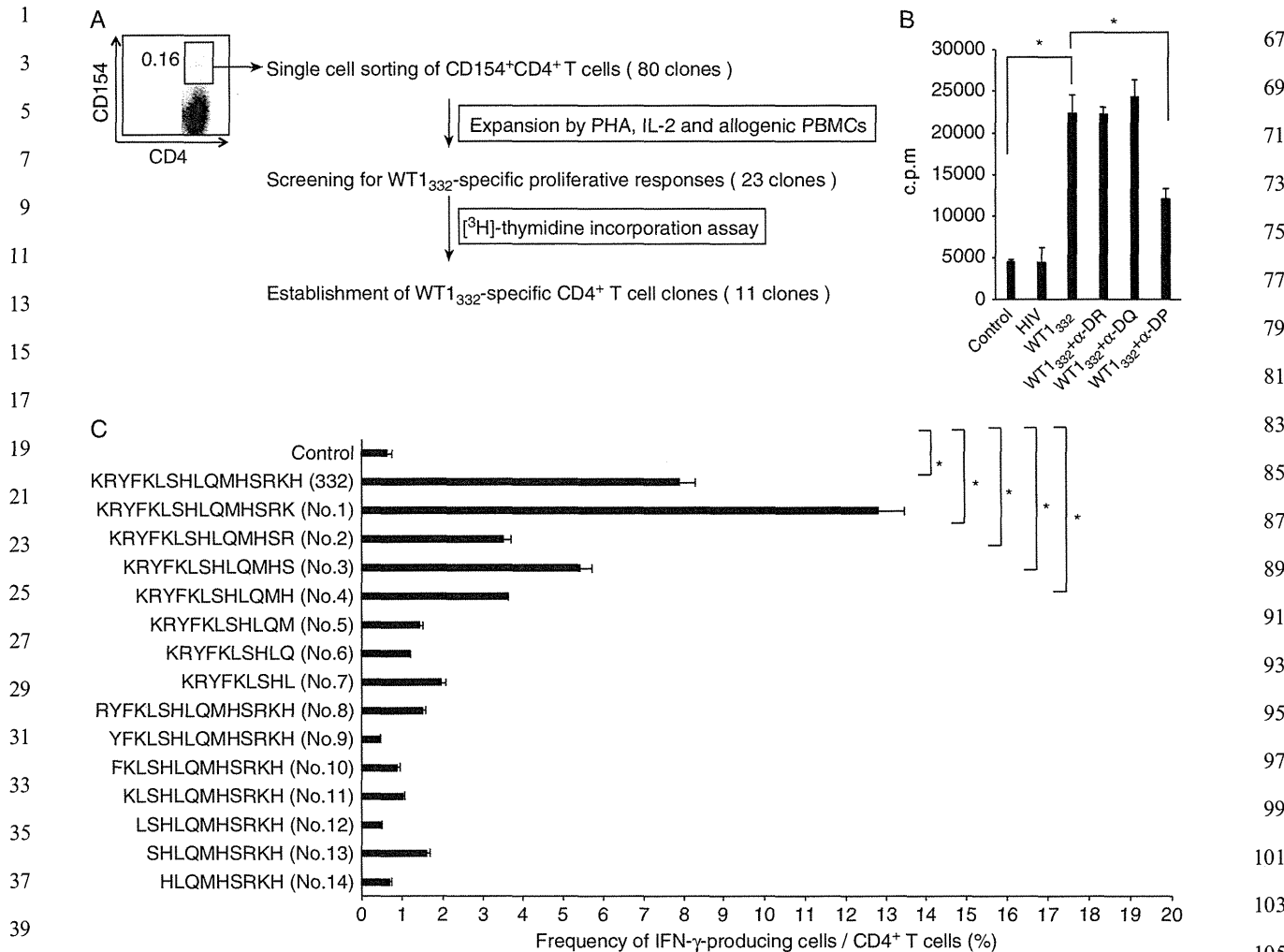
Three million PBMCs from an HLA-DPB1\*05:01<sup>+</sup> donor were cultured in the presence of 20  $\mu$ g/mL of WT1<sub>332</sub> peptide in 24-well plates. After a week, CD154 expression assay<sup>31</sup> was performed to sort WT1<sub>332</sub>-specific CD4<sup>+</sup> T cells. Briefly, the PBMCs were restimulated with 20  $\mu$ g/mL WT1<sub>332</sub> peptide in the presence of anti-CD154-APC mAb, 2  $\mu$ L BD GolgiStopTM [BD Bioscience and 2  $\mu$ g/mL CD28/CD49d Costimulatory Reagent (BD Bioscience)]. After 6 hours of incubation, the cells were stained with anti-CD4-APC-H7 and anti-CD3-Pacific Blue mAbs, and then CD154<sup>+</sup> CD4<sup>+</sup> T cells were single-cell sorted into 96-well U-bottomed plate by FACSaria (BD Bioscience). The sorted cells were expanded in the presence of 1 × 10<sup>5</sup> irradiated allogeneic PBMCs, 100 IU/mL IL-2 and 3  $\mu$ g/mL HA16 phytohaemagglutinin (PHA, Remel Inc., Lenexa, KS). Established single-cell-derived CD4<sup>+</sup> T-cell clones were screened for WT1<sub>332</sub>-specificity by proliferation assay as described later and used for further experiments.

### Cloning of Full-Length TCR $\alpha$ and TCR $\beta$ genes From a WT1<sub>332</sub>-specific CD4<sup>+</sup> T-Cell Clone

HLA-DPB1\*05:01-restricted, WT1<sub>332</sub>-specific TCR  $\alpha$  and  $\beta$  genes were cloned from a WT1<sub>332</sub>-specific CD4<sup>+</sup> T-cell clone (clone 10) using SMARTerTM RACE cDNA Amplification Kit (Clontech/TaKaRa Bio Co., Shiga, Japan) and Advantage 2 PCR kit (Clontech/TaKaRa). For the amplification of TCRs, the following gene-specific primers were made: C $\alpha$ 3'UTR-primer; 5'-CACAGGCTGTCTACAATCTTGCGAGATC-3', C $\beta$ 1-3'UTR-primer; 5'-CTC CACTTCCAGGGGCTGCCTTCA-3', and C $\beta$ 2-3'UTR-primer; 5'-TGACCTGGGATGGTTTTGGAGCTA-3'. Polymerase chain reaction (PCR) was conducted as follows: 95°C for 1 minute, 5 cycles (94°C for 30 s, 72°C for 2 min), 5 cycles (94°C for 30 s, 70°C for 30 s, and 72°C for 2 min), and then 25 cycles (94°C for 30 s, 68°C for 30 s, and 72°C for 2 min). The PCR products were cloned into pCR2.1 cloning vector (Invitrogen Life Technologies, Carlsbad, CA) and the inserted DNA fragments were sequenced by using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Forster City, CA). The DNA sequence data were analyzed using The International Immunogenetics Information System ([http://www.imgt.org/IMGT\\_vquest/vquest?livret=0&Option=humanTcR](http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=humanTcR)) for the identification of human TCR  $\alpha$  and  $\beta$  chains.

### Construction of Lentiviral Vector Expressing WT1<sub>332</sub>-specific TCRs and Preparation of Recombinant Lentiviruses

To construct a functional WT1<sub>332</sub>-specific TCRs, the isolated TCR  $\alpha$  and TCR  $\beta$  chain were linked with picornaviral 2A-like sequence (p2A) by using V $\alpha$  primer 1; 5'-CGCTCTGCGCCGCGCCACCATGGCAGGCATTCGAGCT-3', V $\beta$  primer 2; 5'-GCCACGAATCTCTCTGT TAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGTCCCATGAGCATCGGGCTCCTG-3', V $\alpha$  primer 3; 5'-GGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTAAACAGAGAGAAGTTCGTGGCTCCGGAACCGTCTTACAATCTTGAGATC-3', V $\beta$  primer 4; 5'-CGCTCTGGATCCTCCACTTCCAGGGCTGCCTTCA-3', and the TCR  $\alpha$ -p2A-TCR  $\beta$  cassette was cloned into the Not I and BamH I site of CSII-EF-MCS-IRES2-Venus lentiviral vector (kindly provided from Drs Hiroyuki Miyoshi and Atsushi Miyawaki, RIKEN). For the generation of recombinant lentiviruses, 293 T cells were cotransfected



**FIGURE 1.** Establishment of HLA-DPB1\*05:01-restricted, Wilms tumor gene 1 (WT1)<sub>332</sub>-specific CD4<sup>+</sup> T cell clone. A, Establishment of HLA-DPB1\*05:01-restricted, WT1<sub>332</sub>-specific CD4<sup>+</sup> T-cell clones. WT1<sub>332</sub> peptide-primed PBMCs were restimulated with WT1<sub>332</sub> peptide in the presence of anti-CD154-APC mAb for 6 hours, and CD154<sup>+</sup> CD4<sup>+</sup> 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<sub>332</sub>-specific proliferative response by [<sup>3</sup>H]-thymidine incorporation as described in the Materials and methods section. B, A WT1<sub>332</sub>-specific CD4 T-cell clone, clone 10, was cocultured with irradiated homozygous DPB1\*05:01-expressing autologous PBMCs pulsed or unpulsed with WT1<sub>332</sub> peptide in the presence of HLA-DR-blocking, HLA-DQ-blocking, or HLA-DP-blocking mAb and tested for proliferative responses by [<sup>3</sup>H]-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<sub>332</sub>-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'-CAGGGTCCCCTGGCCCCGGGGGTC-3', DPA1 primer Reverse; 5'-GGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTACAGAGAGAAGTTCGTGGCTCCGGAACCCAGGTTCCCTGGGCCCGGGGTC-3', DPB1 primer Forward;

1 5'-GCCACGAACTTCTCTCTGTTAAAGCAAGCAGGA  
 2 GACGTGGAAGAAAACCCCGTCCCATGATGGTTC  
 3 TGCAGGTTTCTGCG-3', DPB1 primer Reverse; 5'-ATG  
 4 ATGGTTCTGCAGGTTTCTGCG-3'. PCR conditions  
 5 were as follows: 94°C for 2 minutes and 25 cycles (98°C for  
 6 10 s, 60°C for 30 s, and 68°C for 90 s). Amplified HLA-  
 7 DPA1-p2A-HLA-DPB1 cassette was cloned into the *EcoRI*  
 8 and *XhoI* site of pcDNA3.1 (+) cloning vector (Invitrogen)  
 9 then sequenced by BigDye Terminator v3.1 cycle sequenc-  
 10 ing kit.

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

### 17 Transduction of WT1<sub>332</sub>-specific TCR Gene into 18 Mouse TG40 Cells

19 Thirty thousand TG40 cells were added to a 48-well  
 20 plate and incubated with WT1<sub>332</sub>-specific TCR genes-  
 21 encoding (WT1<sub>332</sub>-TCR) or control lentiviruses (Mock)  
 22 in the presence of 8 µg/mL polybrene (Sigma, St Louis, MO).  
 23 After 16 hours of incubation, the medium was changed and  
 24 the transduced cells were further cultured and analyzed for  
 25 the expression of CD3 molecules on their cell surface.

### 27 Generation of WT1<sub>332</sub>-specific TCR Gene-transduced 28 CD4<sup>+</sup> T Cells

29 PBMCs were stimulated with plate-bound anti-CD3  
 30 (5 µg/mL) and anti-CD28 (1 µg/mL) mAbs in the presence  
 31 of 40 IU/mL IL-2 for 2 days. Thirty thousand activated  
 32 cells were incubated in the presence of recombinant lenti-  
 33 viruses and 8 µg/mL polybrene on a RetroNectin-coated  
 34 (TaKaRa Bio Co., Shiga, Japan) 48-well plate. The plate  
 35 containing the cells was centrifuged at 2000 rpm at 33°C for  
 36 1 hour. After 12 hours of incubation, medium change was  
 37 carried out and the cells were further incubated for 48–72  
 38 hour. Then the transduced cells, Venus<sup>+</sup> CD4<sup>+</sup> T cells,  
 39 were sorted by FACSARIA and restimulated with irradiated,  
 40 WT1<sub>332</sub> peptide-pulsed autologous PBMCs. Mock-trans-  
 41 duced CD4<sup>+</sup> T cells were stimulated with 3 µg/mL PHA in  
 42 the presence of irradiated, autologous PBMCs. One week  
 43 later, the established CD4<sup>+</sup> T cells were used for various  
 44 experiments as described later or stored. To investigate the  
 45 stability of the established CD4<sup>+</sup> T cells, they were weekly  
 46 restimulated with irradiated, WT1<sub>332</sub>-pulsed autologous  
 47 PBMCs.

### 49 Intracellular Cytokine Staining Assay and CD107a 50 Mobilization Assay

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

61 For CD107a mobilization assay, 1 × 10<sup>5</sup> CD4<sup>+</sup> T cells  
 62 were incubated with 1 × 10<sup>5</sup> WT1<sub>332</sub> peptide-pulsed or  
 63 peptide-nonpulsed HLA-DP5-positive TF-1 in the presence  
 64 of 2 µg BD GolgiStopTM and anti-CD107a-APC mAb for 5  
 65

hours. Then, the cells were harvested and intracellular  
 cytokine staining was performed as described earlier.

### 67 Proliferation Assay and Enzyme-Linked 68 Immunosorbent Assay (ELISA)

69 Proliferative capacity was assessed using a standard  
 70 [<sup>3</sup>H]-thymidine incorporation assay. In brief, CD4<sup>+</sup> T cells  
 71 were plated at a concentration of 1 × 10<sup>4</sup>/well (U-bottomed  
 72 96-well plate), and cultured with 1 × 10<sup>5</sup> irradiated (30 Gy)  
 73 PBMCs pulsed or unpulsed with tumor lysate, WT1 protein  
 74 (100 µg/mL), or WT1 peptides (20 µg/mL). [<sup>3</sup>H]-thymidine  
 75 (Amersham Biosciences, NJ) was added after culture for 2  
 76 days, and the cells were harvested onto glass-fiber filters 18  
 77 hours after the addition of [<sup>3</sup>H]-thymidine. Radioactivity  
 78 was then measured on a β-scintillation counter in triplicate  
 79 wells. For the blocking assays, W6/32, L243, SPVL3, and  
 80 B7/21 mAbs were added to the proliferation assays at their  
 81 optimal concentrations for blocking of HLA class I, HLA-  
 82 DR, HLA-DQ, and HLA-DP, respectively, and cell pro-  
 83 liferation was measured as described earlier.

84 For evaluation of interferon (IFN)γ-release from  
 85 CD4<sup>+</sup> T cells, culture supernatants were collected before  
 86 the addition of [<sup>3</sup>H]-thymidine and frozen at -80°C until  
 87 use. IFNγ in the supernatants was measured by double  
 88 sandwich ELISA using the Quantikine provided by R&D  
 89 Systems (Minneapolis, MN).

### 91 <sup>51</sup>Cr release assay

92 <sup>51</sup>Cr release assays were performed as described pre-  
 93 viously with minor modification. Briefly, target cells (1 ×  
 94 10<sup>4</sup> cells) labeled with <sup>51</sup>Cr were added to wells containing  
 95 varying numbers of effector cells in 96-well plates. After 18  
 96 hours of incubation at 37°C, the supernatant was collected  
 97 and measured for radioactivity. The percentage of specific  
 98 lysis (% specific lysis) was calculated as follows: percentage  
 99 of specific lysis = (cpm of experimental release - cpm of  
 100 spontaneous release)/(cpm of maximal release - cpm of  
 101 spontaneous release) × 100. Maximal release and sponta-  
 102 neous release were determined from supernatants of target  
 103 cells incubated with 1% Triton X-100 and those incubated  
 104 without effector cells, respectively. For granzyme B inhib-  
 105 ition, target cells were pretreated with 100 µM Ac-IETD-  
 106 Cho or DMSO as a control at 37°C for 2 hours, washed  
 107 extensively, and used for the <sup>51</sup>Cr release assays.

### 113 Enhancement of the Induction of WT1-specific CTL by 114 WT1<sub>332</sub>-TCR-transduced CD4<sup>+</sup> T Cells

115 Three million of PBMCs from an HLA-DPB1\*05:01-  
 116 positive and HLA-DPB1-A\*24:02-positive healthy donor  
 117 were freshly isolated and cocultured with WT1<sub>332</sub>-TCR-  
 118 transduced or mock-transduced CD4<sup>+</sup> T cells at the indi-  
 119 cated ratios in the presence of 20 µg/mL WT1<sub>332</sub> peptide  
 120 and 10 µg/mL modified WT1<sub>235</sub> peptide (WT1<sub>235m</sub>;  
 121 CYTWNQMNL), which is an HLA-A\*24:02-restricted  
 122 CTL epitope. The X-VIVO 15 medium supplemented with  
 123 10% AB serum but not with exogenous IL-2 was used to  
 124 evaluate a helper activity of CD4<sup>+</sup> T cells. One week later,  
 125 the cells were restimulated with 2 × 10<sup>6</sup> irradiated, WT1<sub>235m</sub>  
 126 peptide-pulsed autologous PBMCs, and cultured for fur-  
 127 ther 1 week. Then, the frequencies of WT1-specific CD8<sup>+</sup>  
 128 T cells were determined using WT1<sub>235m</sub> tetramer-staining  
 129 and WT1<sub>235m</sub>-specific IFN-γ expression by flow cytometry.

**1 Statistical Analysis**

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

**7 RESULTS**

Identification of a novel HLA-DPB1\*05:01-restricted CD4<sup>+</sup> T-cell epitope in WT1<sub>332</sub> helper peptide.

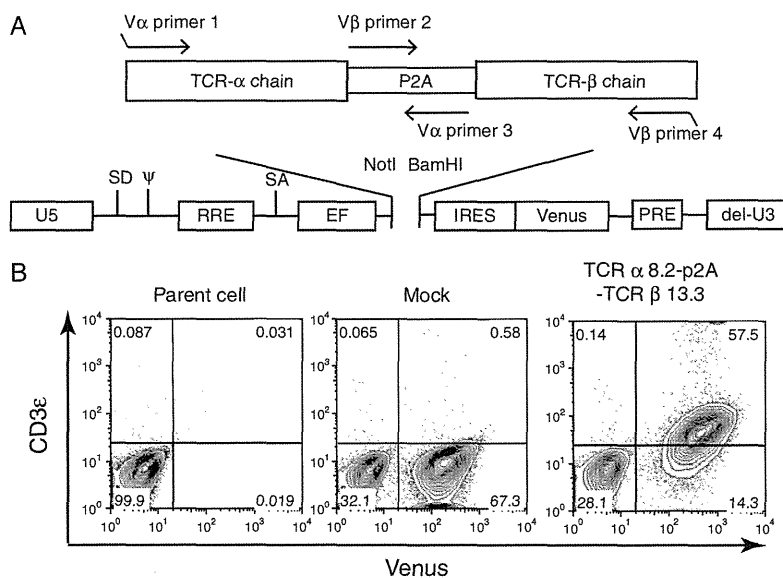
We previously identified WT1<sub>332</sub> helper peptide (332-347: KRYFKLSHLQMHSRKH) that could promiscuously bind to multiple HLA class II molecules including HLA-DRB1\*04:05, 15:02, 15:01, and HLA-DPB1\*09:01 and induce the peptide-specific CD4<sup>+</sup> T cells.<sup>22,23</sup> First, whether or not WT1<sub>332</sub> helper peptide could bind to HLA-DPB1\*05:01 (DP5), which was most popular in Japanese, and induce WT1<sub>332</sub>-specific CD4<sup>+</sup> T cells was examined. PBMCs obtained from an HLA-DP5<sup>+</sup> donor were stimulated with WT1<sub>332</sub> for a week, and then CD154-expressing cells, which were CD4<sup>+</sup> T cells specifically activated by WT1<sub>332</sub>, were sorted for cloning. As shown in Figure 1A, the CD154-expressing CD4<sup>+</sup> T cells that were detected at the frequency of 0.16% after the stimulation with WT1<sub>332</sub> 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<sub>332</sub>-specific proliferation (data not shown). To screen HLA-DP5-restricted CD4<sup>+</sup> T-cell clones, blocking assays against their proliferative responses to WT1<sub>332</sub> were performed (Fig. 1B). As the proliferative responses of clone 10 to WT1<sub>332</sub> 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<sub>332</sub> in an HLA-DP5-restriction manner. Thus, clone 10 had been established as a WT1<sub>332</sub>-specific, HLA-DP5-restricted CD4<sup>+</sup> T-cell clone.

To confirm the WT1<sub>332</sub>-specific response of clone 10, it was stimulated with various deletion peptides and the frequencies of IFN $\gamma$ -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<sub>332</sub> peptide. These results confirmed that clone 10 specifically responded to WT1<sub>332</sub> peptide whose core amino acid sequence was KRYFKLSHLQMHSRK.

**Cloning of HLA-DP5-restricted, WT1<sub>332</sub>-specific TCR Genes**

Full-length TCR  $\alpha$ -chain and  $\beta$ -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  $\alpha$  and  $\beta$  chains, and the resultant TCR  $\alpha$  8.2-p2A-TCR  $\beta$  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  $\alpha$  8.2-p2A-TCR  $\beta$  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  $\alpha/\beta$  expression.<sup>26</sup> Both TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-transduced and mock-transduced TG40 cells showed high Venus fluorescence protein expression compared to parental TG40 cells (Fig. 2B), whereas only TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-transduced TG40 cells showed CD3 $\epsilon$  expression in



**FIGURE 2.** Cloning and expression of TCR  $\alpha$  8.2/TCR  $\beta$  13.3 gene using lentiviral vector. **A**, Construction of a lentiviral vector encoding full-length TCR  $\alpha$  8.2 and  $\beta$  13.3 genes derived from clone 10, and primer positions for cloning of TCR. Lentiviral vector constructions are as follows: SD, splicing donor site;  $\Psi$ , packaging signal; RRE, Rev responsive element; SA, splicing acceptor site; EF, human elongation factor 1- $\alpha$  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)  $\alpha$  8.2-p2A-TCR  $\beta$  13.3 cassette-encoding lentiviral vector (TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3) or empty lentiviral vector (Mock). After 3 days of transduction, parental TG40 and lentiviral vector-transduced cells were stained with anti-mouse CD3 $\epsilon$ -PE mAb and analyzed with flow cytometry.

1 the Venus<sup>+</sup> cell population. Thus, these results clearly  
2 indicated that the TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3 could be  
3 correctly expressed on the surface of the TG40 cells.

#### 7 **Functional Expression of TCR $\alpha$ 8.2-p2A-TCR $\beta$ 8 **13.3 in human CD4<sup>+</sup> T Cells****

9 Next, whether or not TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3 was  
10 functional in human CD4<sup>+</sup> T cells was investigated. To  
11 establish TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-transduced CD4<sup>+</sup> T  
12 cells, PBMCs with homozygous HLA-DP5 were transfected  
13 with TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-encoding lentiviral vec-  
14 tor. After 72 hours of transfection, the Venus<sup>+</sup> CD4<sup>+</sup> T  
15 cells were sorted and cocultured with irradiated, and  
16 WT1<sub>332</sub>-pulsed autologous PBMCs. After 1 week of cul-  
17 ture, intracellular cytokine assay was performed to inves-  
18 tigate WT1<sub>332</sub> specificity of the expanded CD4<sup>+</sup> T cells. As  
19 expected, the expanded TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-  
20 transduced CD4<sup>+</sup> T cells expressed IFN- $\gamma$  and IL-2 only  
21 by the stimulation with WT1<sub>332</sub> (Fig. 3A), whereas mock-  
22 transduced CD4<sup>+</sup> T cells did not show any cytokine  
23 expression in response to WT1<sub>332</sub>. In addition, the cytokine  
24 expression of the TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-transduced  
25 CD4<sup>+</sup> T cells was dependent on the concentration  
26 of WT1<sub>332</sub> peptide (Fig. 3B). Furthermore, proliferative  
27 response to WT1<sub>332</sub> of the TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-  
28 transduced CD4<sup>+</sup> T cells was remarkably inhibited by  
29 addition of an anti-HLA-DP antibody, but not by addition  
30 of anti-HLA class I, anti-HLA-DR, or anti-HLA-DQ  
31 antibody (Fig. 3C). As PBMCs with homozygous HLA-  
32 DP5 were used as a stimulator, it was concluded that the  
33 TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-transduced CD4<sup>+</sup> T cells  
34 recognized WT1<sub>332</sub> in an HLA-DP5-restriction manner.  
35 Importantly, WT1<sub>332</sub>-specific cytokine expression in the  
36 TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-transduced CD4<sup>+</sup> T cells was  
37 observed even after 3 months of culture (Fig. 3D), indi-  
38 cating that function of TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-trans-  
39 duced CD4<sup>+</sup> T cells were stable for long-term culture.

40 In our previous studies, it was demonstrated that  
41 WT1<sub>332</sub> (WT1<sub>332-347</sub>, KRYFKLSHLQMHSRKH) was a  
42 natural epitope for CD4<sup>+</sup> T cells with the restriction of  
43 HLA-DRB1\*04:05, 15:01, 15:02, and HLA-DPB1\*09:01.  
44 To examine whether or not TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-  
45 transduced CD4<sup>+</sup> T cells could recognize the natural  
46 epitope of WT1 protein in an HLA-DP5-restriction manner,  
47 the CD4<sup>+</sup> T cells were cocultured with WT1 peptide-  
48 pulsed, WT1 protein-pulsed, or WT1-expressing tumor  
49 lysate-pulsed autologous PBMCs that were used as a stim-  
50 ulator and the proliferative responses of the CD4<sup>+</sup> T cells  
51 were measured. Consequently, the TCR  $\alpha$  8.2-p2A-TCR  $\beta$   
52 13.3-transduced CD4<sup>+</sup> T cells showed proliferative  
53 responses to WT1<sub>332</sub> peptide-pulsed or full-length WT1  
54 protein (HWT1)-pulsed autologous PBMCs, but not to  
55 those pulsed with the truncated WT1 protein not containing  
56 WT1<sub>332</sub> peptide sequences (HWT3). Furthermore, the  
57 CD4<sup>+</sup> T cells could strongly proliferate (Fig. 3E) and  
58 produce IFN- $\gamma$  (Fig. 3F) in response to the PBMCs pulsed  
59 with the lysate of WT1-expressing leukemia cell line (TF-1  
60 and K562).

61 Taken together, these results clearly demonstrated that  
62 cloned TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3 really encoded WT1<sub>332</sub>-  
63 specific, HLA-DP5-restricted TCR of clone 10. Thus, in the  
64 following chapters, "WT1<sub>332</sub> TCR-transduced" was used in  
65 exchange for "TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-transduced."

#### 67 **WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T Cells are Th1 68 Type-Cytokine Profile**

69 It was previously reported that WT1<sub>332</sub> could domi-  
70 nantly induce Th1-type CD4<sup>+</sup> T cells. Therefore, whether  
71 or not WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells displayed  
72 Th1 dominant cytokine profile was examined. WT1<sub>332</sub>  
73 TCR-transduced CD4<sup>+</sup> T cells from 3 healthy donors with  
74 HLA-DP5 were established as shown in Figure 3 and  
75 examined for cytokine production by intracellular cytokine  
76 staining assay (Fig. 4). As expected, all the 3 established  
77 CD4<sup>+</sup> T-cell lines expressed at high frequencies the Th1-  
78 type cytokines such as IL-2, IFN- $\gamma$ , TNF- $\alpha$ , and GM-CSF  
79 in response to WT1<sub>332</sub> stimulation. However, the expres-  
80 sions of Th2-type cytokines (IL-5 and IL-10) or Th17-type  
81 cytokine (IL-17) were at low frequencies or undetectable in  
82 all the 2 CD4<sup>+</sup> T-cell lines.

83 Thus, the results that the cloned WT1<sub>332</sub> TCR let  
84 CD4<sup>+</sup> T cells endow Th1-type were consistent with those  
85 that WT1<sub>332</sub>-specific CD4<sup>+</sup> T-cell clones established from  
86 PBMCs from healthy donors were dominantly Th1-type.

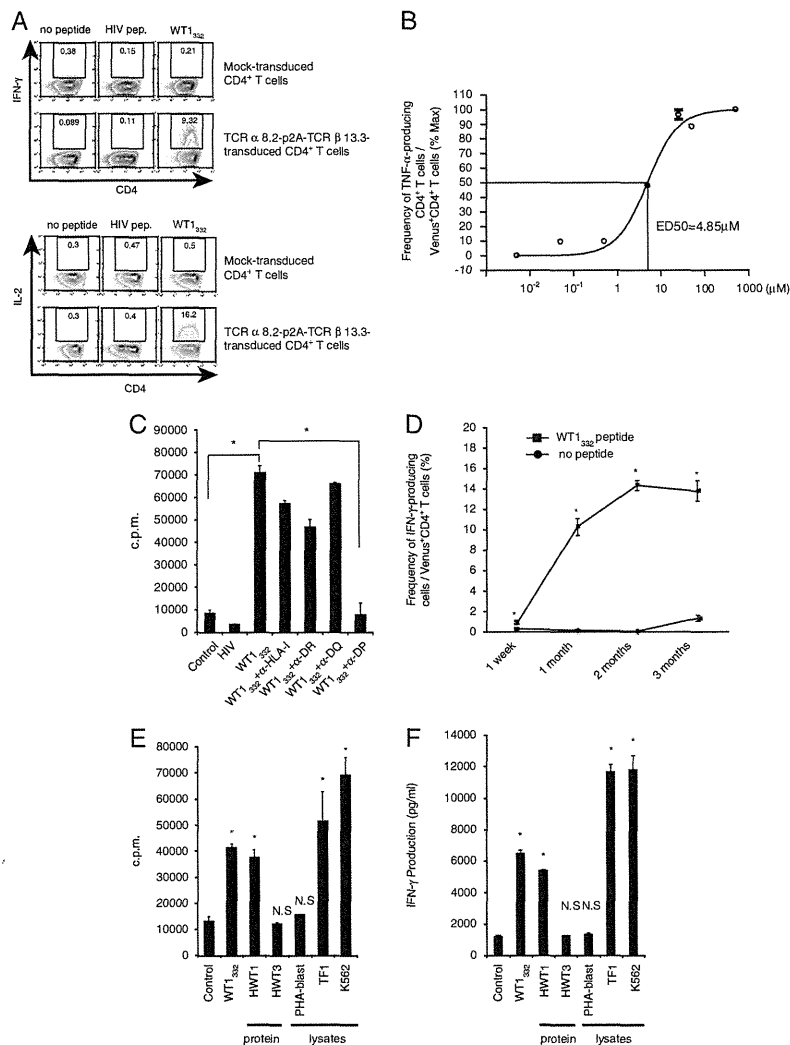
#### 89 **WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T Cells can 90 Directly Recognize and Kill WT1-expressing 91 Leukemia Cell Lines through Perforin/Granzyme 92 B Pathway**

93 Next, whether WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells  
94 could recognize and kill WT1-expressing leukemia cell line  
95 in HLA-DP5-restriction manner was examined.

96 HLA-DPA1\*01:DPB1\*05:01-expression vector was  
97 transduced into TF-1 cells and HLA-DP5-positive TF-1  
98 cells were established. The HLA-DP5-positive TF-1 cells  
99 established could present WT1<sub>332</sub> peptide to the WT1<sub>332</sub>  
100 TCR-transduced CD4<sup>+</sup> T cells and were useful as target  
101 cells in killing assay (data not shown). WT1<sub>332</sub>-TCR-  
102 transduced CD4<sup>+</sup> T cells showed strong cytotoxic activity  
103 against HLA-DP5-positive TF-1 cells, but not against  
104 parental TF-1 cells (Fig. 5A). In contrast, empty lentiviral  
105 vector-transduced CD4<sup>+</sup> T cells did not show cytotoxicity  
106 against both target cells. To confirm WT1-specific cyto-  
107 toxicity of the WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells,  
108 WT1-overexpressing autologous B-LCL (B-LCL(+)) and  
109 its parental B-LCL [B-LCL(-)] were used as target cells  
110 (Fig. 5B). As expected, the WT1<sub>332</sub> TCR-transduced  
111 CD4<sup>+</sup> T cells effectively lysed B-LCL(+) cells compared to  
112 B-LCL(-) cells. Furthermore, the WT1<sub>332</sub> TCR-transduced  
113 CD4<sup>+</sup> T cells could lyse endogenously WT1-expressing  
114 and HLA-DP5-positive C2F8 leukemia cells (Fig. 5C).  
115 Thus, these results clearly demonstrated that the WT1<sub>332</sub>  
116 TCR-transduced CD4<sup>+</sup> T cells had a potent cytotoxic  
117 activity against WT1-expressing, HLA-DP5-positive  
118 malignant cells such as leukemia cells.

119 Next, whether or not the WT1<sub>332</sub> TCR-transduced  
120 CD4<sup>+</sup> T cells exerted the cytotoxic activity through a  
121 granzyme B and perforin pathway was investigated. High  
122 expression of granzyme B and perforin was observed in the  
123 WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells (Fig. 5D). Fur-  
124 thermore, the simultaneous expression of IFN- $\gamma$  and  
125 CD107a, which reflected degranulation, was observed only  
126 when the WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells were  
127 incubated with WT1<sub>332</sub>-pulsed HLA-DP5-positive TF-1  
128 cells (Fig. 5E). Finally, in order to confirm that the cyto-  
129 toxicity of the WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells was  
130 dependent on granzyme B/perforin pathway, HLA-DP5-





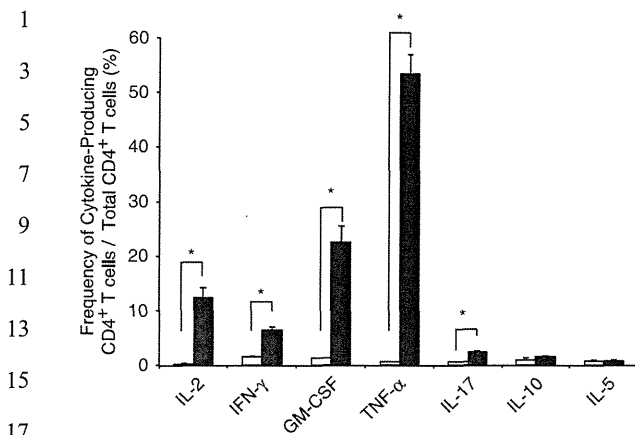
**FIGURE 3.** T-cell receptor (TCR)  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-transduced CD4<sup>+</sup> T cells displays antigen-specific T-cell responses. PBMCs from a healthy donor were transduced with TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3 cassette-encoding lentiviral vector (TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3) or empty lentiviral vector (Mock) and then transduced CD4<sup>+</sup> T cells were sorted and expanded for a week as described in the Materials and Methods section. **A**, The transduced CD4<sup>+</sup> T cells were stimulated with or without the indicated peptides for 4 hours and then intracellular interferon (IFN) $\gamma$  (upper) and IL-2 (lower) were analyzed by flow cytometry. Representative data from 3 independent experiments are shown. **B**, The TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-transduced CD4<sup>+</sup> T cells were stimulated with various concentrations of Wilms tumor gene 1 (WT1)<sub>332</sub> for 4 hours and intracellular cytokine staining assay was performed. Each plot represents mean values  $\pm$  SEM from duplicated wells. A half maximum effective dose (ED50) calculated is shown. Data are representative of several independent experiments. **C**, The TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-transduced CD4<sup>+</sup> T cells were cocultured with irradiated homozygous DPB1\*05:01-expressing autologous PBMCs pulsed or unpulsed with Wilms tumor gene 1 (WT1)<sub>332</sub> 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 [<sup>3</sup>H]-thymidine incorporation. Columns represent mean values  $\pm$  SEM from triplicated wells. Asterisks (\*) indicate significant difference ( $P < 0.05$ ). Representative data from 3 independent experiments are shown. **D**, The TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-transduced CD4<sup>+</sup> T cells that were cultured with weekly WT1<sub>332</sub> peptide stimulation were tested for the capacity of WT1<sub>332</sub>-specific IFN $\gamma$  production by intracellular cytokine staining in response to WT1<sub>332</sub> peptide stimulation at the indicated time points. Data represent mean values  $\pm$  SEM from triplicated assays. The TCR  $\alpha$  8.2-p2A-TCR  $\beta$  13.3-transduced CD4<sup>+</sup> T cells were cocultured with autologous irradiated PBMCs pulsed or unpulsed with WT1<sub>332</sub> peptide (20  $\mu$ g/mL), HWT1 (full-length WT1 protein, 100  $\mu$ g/mL), HWT3 (truncated WT1 protein, 1–294 amino acids, 100  $\mu$ g/mL), PHA-induced lymphoblast lysate, TF-1 leukemia cell lysate, or K562 leukemia cell lysate. Proliferative responses (E) and IFN- $\gamma$  production (F) of the CD4<sup>+</sup> T cells were evaluated by [<sup>3</sup>H]-thymidine incorporation and enzyme-linked immunosorbent assay, respectively. Columns represent mean values  $\pm$  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  $\mu$ M granzyme B inhibitor, ac-IETD-Cho were used as target cells. The cytotoxicity of the WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> 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<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells established here could directly recognize WT1-





**FIGURE 4.** Wilms tumor gene 1 (WT1)<sub>332</sub> T-cell receptor (TCR)-transduced CD4<sup>+</sup> T cells have a Th1 type-cytokine profile. WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T-cell lines were established from 3 different healthy donors as described in Figure 3. The CD4<sup>+</sup> T cells were incubated with or without WT1<sub>332</sub> 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<sub>332</sub> TCR-transduced CD4<sup>+</sup> T Cells Enhance the Induction of WT1-specific CD8<sup>+</sup> Cytotoxic Lymphocytes

We previously demonstrated that WT1<sub>332</sub>-specific CD4<sup>+</sup> T-cell clones established previously were Th1-type and could enhance the induction of WT1-derived CTL epitope-specific CD8<sup>+</sup> CTLs. It is interesting to note that, all WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> 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<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells could enhance the induction of WT1-specific CD8<sup>+</sup> CTLs.

To confirm the helper activity of WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells, the CD4<sup>+</sup> T cells were cocultured with HLA-A\*24:02-positive autologous PBMCs in the presence of WT1<sub>332</sub> helper peptide and modified WT1<sub>235m</sub> peptides (WT1<sub>235m</sub>, HLA-A\*24:02-restricted CTL-epitope). After 1 week of coculture, the cells were restimulated with WT1<sub>235m</sub>-pulsed, irradiated autologous PBMCs, and further cultured for 1 week. After the serial culture, the frequencies of CD8<sup>+</sup> T cells and WT1<sub>235m</sub>-specific CD8<sup>+</sup> CTLs were evaluated by flow cytometry. Expectedly, when WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells were added to autologous PBMCs, the frequencies of CD8<sup>+</sup> T cells and WT1<sub>235m</sub>-specific CD8<sup>+</sup> CTLs significantly increased, compared to the addition of mock-transduced CD4<sup>+</sup> T cells to the autologous PBMCs (Figs. 6A, B). Cell numbers of WT1<sub>235m</sub>-specific CD8<sup>+</sup> CTLs increased 10.8- or 27.6-fold by the addition of WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cell at ratio of auto-PBMCs:CD4<sup>+</sup> T cells, 10:1 or 10:2, respectively (Fig. 6C). To rule out that the increased frequency of WT1<sub>235m</sub>-specific CD8<sup>+</sup> CTLs was due to nonspecific tetramer binding, IFN $\gamma$  expression of these cells was assessed in response to WT1<sub>235m</sub>. Consistent with the results of

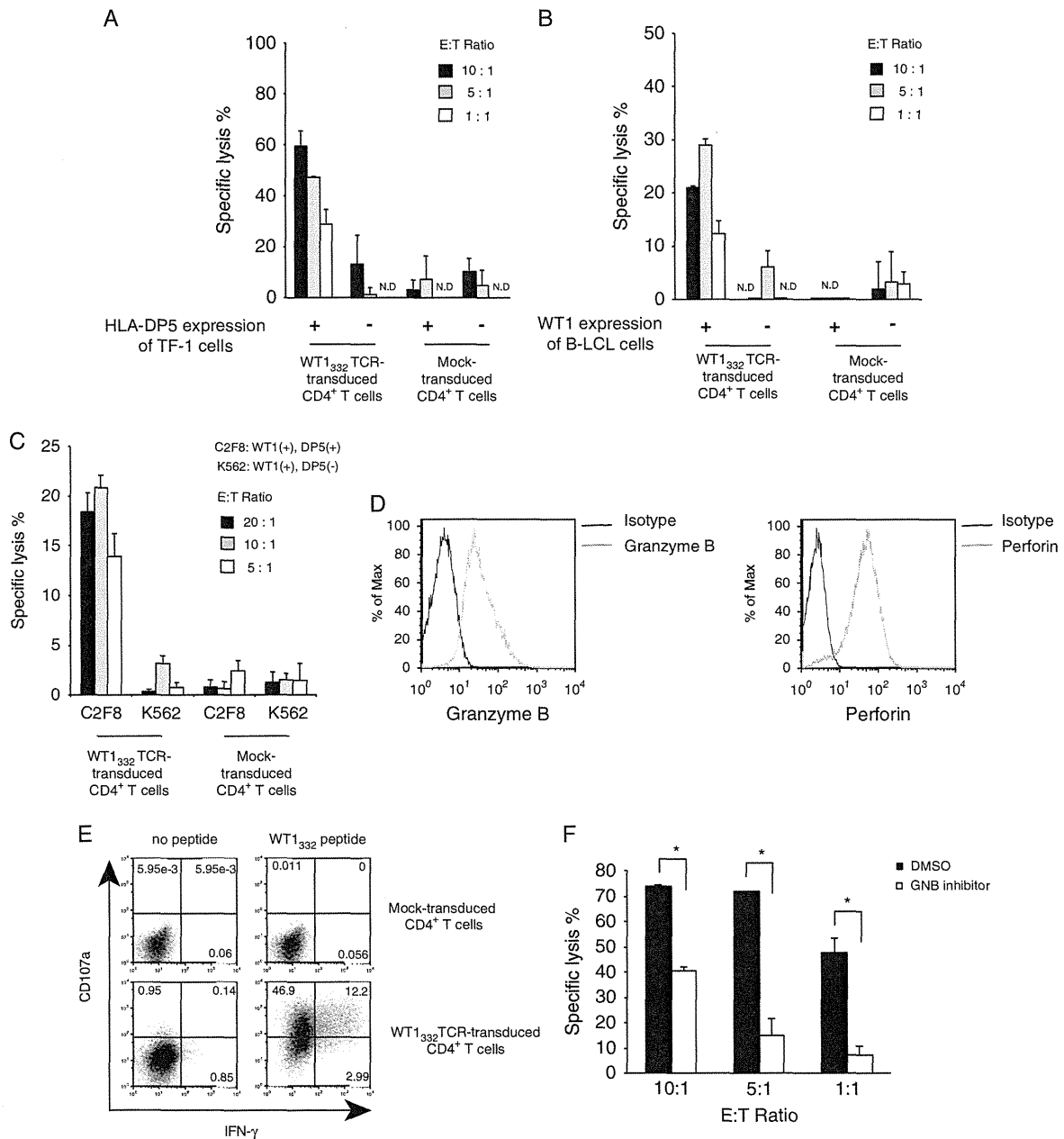
tetramer assay, CD8<sup>+</sup> T cells that were cultured with WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells could express IFN $\gamma$  in response to WT1<sub>235m</sub> (Figs. 6D, 6E). However, no significant IFN $\gamma$  expression was observed in the CD8<sup>+</sup> T cells that were cultured with the mock-transduced CD4<sup>+</sup> T cells. In addition, the average frequencies of IFN $\gamma$ -producing cells in CD8<sup>+</sup> T cells were 3.7% or 6.8% when WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells were added at ratio of auto-PBMCs:CD4<sup>+</sup> T cells, 10:1 or 10:2, respectively (Fig. 6E).

Thus, these results clearly demonstrated that WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells could enhance the induction of WT1-specific CD8<sup>+</sup> CTLs dependently on cell number of the CD4<sup>+</sup> T cells.

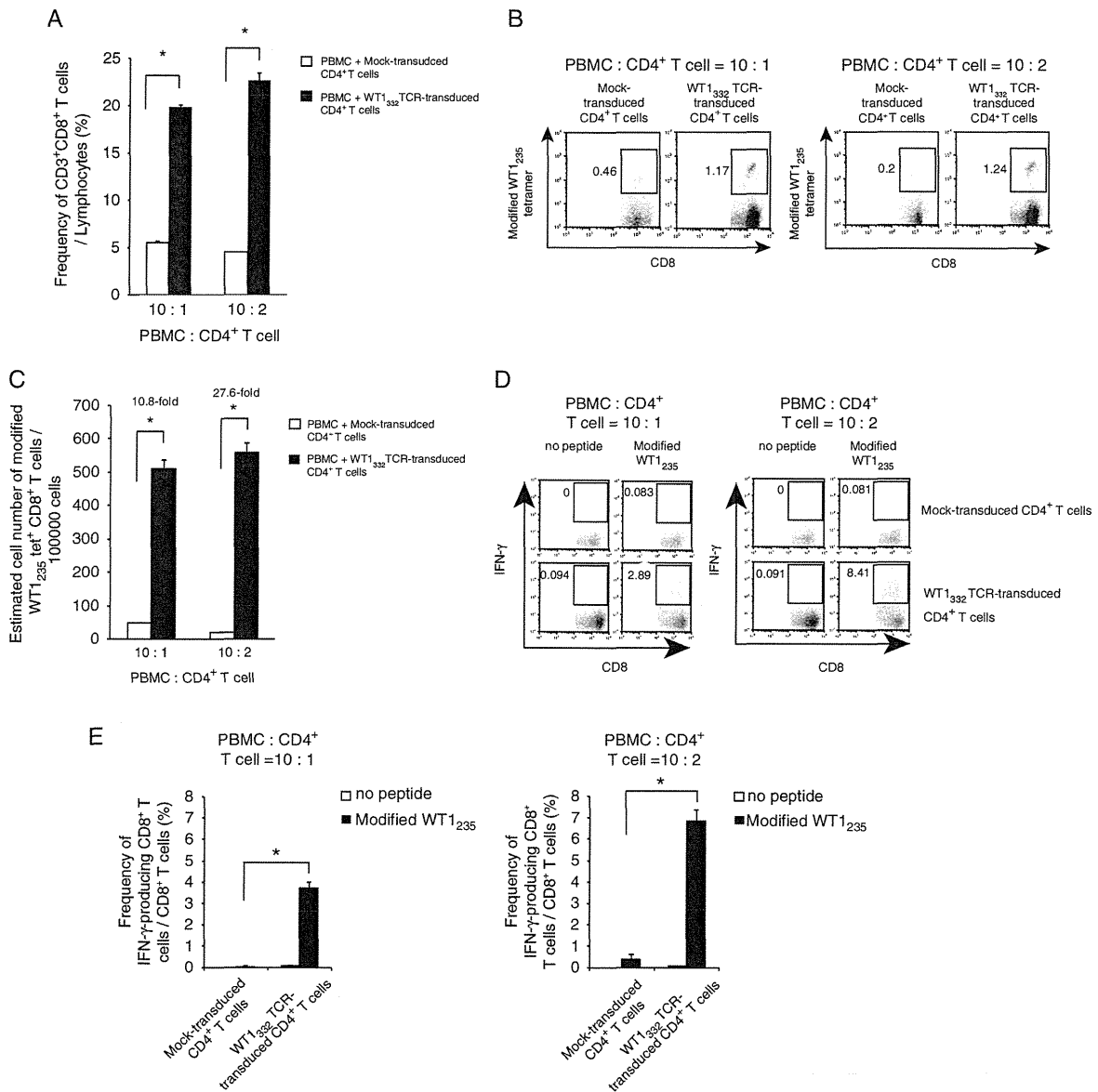
## DISCUSSION

Although a number of studies of TAA-specific TCR gene therapy were reported in last decade,<sup>14-16,32-34</sup> there seemed to be few studies focusing on HLA class II-restricted, TAA-specific TCR gene therapy.<sup>35-37</sup> It is likely that the following 3 steps is necessary for induction an optimal antitumor immune response<sup>38</sup>: first, antigen-presenting cells such as dendritic cell and macrophage phagocytose necrotic/apoptotic tumor cells and present TAAs to CD4<sup>+</sup> T cells in context with MHC class II; second, the TAA-specific CD4<sup>+</sup> T cells recognize TAAs and activate the antigen-presenting cells; and third, the TAA-specific CD8<sup>+</sup> CTLs are induced by the activated antigen-presenting cells through cross-priming and kill the TAA-expressing tumor cells. Accordingly, CD4<sup>+</sup> T cells bearing HLA class II-restricted TCR specific for TAAs (ie, TAA-specific CD4<sup>+</sup> T cells) facilitate a link between antigen-presenting cells and CD8<sup>+</sup> 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<sub>332</sub>-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<sub>332</sub> TCR-transduced CD4<sup>+</sup> 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<sup>+</sup> CTLs had been demonstrated in previous investigations.<sup>39-41</sup> 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.<sup>42,43</sup> Consequently, HLA class II-expressing tumor cells will be recognized and killed by TAA-specific CD4<sup>+</sup> 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.<sup>44</sup> In addition, most recent report has demonstrated that human leukemic cells acquire the



**FIGURE 5.** Wilms tumor gene 1 (WT1)<sub>332</sub> T-cell receptor (TCR)-transduced CD4<sup>+</sup> T cells can directly kill leukemia cells through granzyme B/perforin pathway in an HLA-DPB1\*05:01-restricted, WT1<sub>332</sub>-specific manner. A–C, WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> 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<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells were incubated with <sup>51</sup>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<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells was detected by flow cytometry. Representative histograms are shown. E, WT1<sub>332</sub> TCR-transduced and empty vector (mock)-transduced CD4<sup>+</sup> T cells were cocultured with WT1<sub>332</sub> 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<sup>+</sup> CD4<sup>+</sup> 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 <sup>51</sup>Cr, and incubated with WT1<sub>332</sub> TCR-transduced CD4<sup>+</sup> T cells and <sup>51</sup>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.



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**FIGURE 6.** Enhancement of the induction of Wilms tumor gene 1 (WT1)-specific CD8<sup>+</sup> CTLs by WT1<sub>332</sub> T-cell receptor (TCR)-transduced CD4<sup>+</sup> T cells. WT1<sub>332</sub> TCR-transduced or empty vector (mock)-transduced CD4<sup>+</sup> T cells were added to 3 × 10<sup>6</sup> 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<sub>332</sub> peptide and WT1-derived CTL epitope, modified WT1<sub>235</sub> peptide, WT1<sub>235m</sub>. On day 7, the cells were restimulated with irradiated, WT1<sub>235m</sub>-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<sup>+</sup> T cells (A), WT1<sub>235m</sub> tetramer<sup>+</sup> CD8<sup>+</sup> T cells (B and C), and interferon (IFN)γ-producing CD8<sup>+</sup> T cells in response to WT1<sub>235m</sub> (D and E) were investigated using flow cytometry. A, Frequencies of CD8<sup>+</sup> T cells are shown. CD8<sup>+</sup> T cells were determined as Venus<sup>-</sup>CD3<sup>+</sup> CD8<sup>+</sup> cells in a 7-AAD<sup>-</sup> lymphocyte population. B, Representative dot plots of WT1<sub>235m</sub>/HLA-A\*24:02-tetramer and CD8 are shown. The plots are gated on 7-AAD<sup>-</sup>Venus<sup>-</sup>CD3<sup>+</sup>CD8<sup>+</sup> lymphocytes. C, Cell numbers of WT1<sub>235m</sub>-specific CTLs per 1 × 10<sup>5</sup> lymphocytes were estimated from the frequency of WT1<sub>235m</sub> tetramer<sup>+</sup> CD8<sup>+</sup> T cells in CD8<sup>+</sup> T cells. D and E, The cells cultured as described earlier were restimulated with WT1<sub>235m</sub> 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<sup>+</sup> T cells, resulting in good targets of CD4<sup>+</sup> T-cell-mediated cytotoxicity.<sup>45</sup> Other studies showed that in solid tumors, HLA class II expression correlated with good clinical outcome.<sup>42,46,47</sup> Friedman et al<sup>48</sup> reported that

HLA class II-restricted, melanoma-specific CD4<sup>+</sup> 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

1 and solid tumor are good targets for cytotoxic CD4<sup>+</sup> T  
2 cells.

3 The present study clearly demonstrated that WT1<sub>332</sub>  
4 TCR-transduced CD4<sup>+</sup> T cells displayed helper activity  
5 for WT1-specific CTL induction and cytotoxicity against  
6 leukemia cells. This observation that WT1<sub>332</sub> TCR-trans-  
7 duced CD4<sup>+</sup> T cells had 2 function (helper and cytotoxic-  
8 ity) raised a hypothesis that function of CD4<sup>+</sup> T cells was  
9 divided into 2 phases: helper and cytotoxicity phases. It is  
10 generally known that IL-2 derived from CD4<sup>+</sup> T cells is a  
11 crucial factor for exhibition of helper activity to enhance  
12 CTL function and that undifferentiated, proliferative  
13 CD4<sup>+</sup> T cells can more produce IL-2 compared to differentiat-  
14 ed, nonproliferative CD4<sup>+</sup> T cells. WT1<sub>332</sub> TCR-trans-  
15 duced CD4<sup>+</sup> T cells rapidly proliferated and produced a  
16 large amount of IL-2 at early phase within 1 month from the  
17 beginning of the culture, but stopped producing IL-2 with  
18 less proliferation at late phase after repeated antigen-stim-  
19 ulation (data not shown). It was previously reported that  
20 cytotoxic CD4<sup>+</sup> T cells appeared after repeated antigen-  
21 stimulation and possessed a phenotype-like terminally dif-  
22 ferentiated effector cells such as CD27<sup>-</sup>, CD28<sup>-</sup>, and  
23 CD57<sup>+</sup>.<sup>41</sup> These findings supported our hypothesis that  
24 CD4<sup>+</sup> T cell might transiently exert a helper activity (helper  
25 CD4<sup>+</sup> T cells) at early phase and then a cytotoxic activity  
26 (cytotoxic CD4<sup>+</sup> T cells) at late phase.

27 As WT1 was selected from 75 defined tumor antigens  
28 to rank as the most promising cancer vaccine target in a  
29 prioritization study carried out by National Cancer Insti-  
30 tute,<sup>13</sup> WT1-targeted cancer immunotherapy is thought to  
31 be also the most promising strategy for cure of cancer. TCR  
32 gene therapy using WT1<sub>332</sub>-specific TCR should be a useful  
33 tool for cancer immunotherapy because the TCR-trans-  
34 duced CD4<sup>+</sup> T cells elicited both a helper activity for the  
35 induction of WT1-specific CD8<sup>+</sup> CTLs and a cytotoxic  
36 activity against tumor. Furthermore, combination TCR  
37 gene therapy of HLA class I-restricted, WT1-specific TCR  
38 and HLA class II-restricted, WT1<sub>332</sub>-specific TCR is  
39 expected to be more efficient. In addition, WT1<sub>332</sub>-specific  
40 TCR gene therapy should be also effective in combination  
41 with HLA class I-restricted WT1 peptide vaccine.

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44 **CONFLICTS OF INTEREST/  
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54 **REFERENCES**

55 1. Hohenstein P, Hastie ND. The many facets of the Wilms'  
56 tumour gene, WT1. *Hum Mol Genet.* 2006;15:R196-R201.  
57 2. Yamagami T, Sugiyama H, Inoue K, et al. Growth inhibition  
58 of human leukemic cells by WT1 (Wilms tumor gene) antisense  
59 oligodeoxynucleotides: implications for the involvement of  
60 WT1 in leukemogenesis. *Blood.* 1996;87:2878-2884.  
61 3. Inoue K, Tamaki H, Ogawa H, et al. Wilms' tumor gene  
62 (WT1) competes with differentiation-inducing signal in hem-  
63 atopoietic progenitor cells. *Blood.* 1998;91:2969-2976.  
64 4. Tsuboi A, Oka Y, Ogawa H, et al. Constitutive expression of  
65 the Wilms' tumor gene WT1 inhibits the differentiation of

myeloid progenitor cells but promotes their proliferation in  
response to granulocyte-colony stimulating factor (G-CSF).  
*Leuk Res.* 1999;23:499-505. 67  
5. Call KM, Glaser T, Ito CY, et al. Isolation and character-  
ization of a zinc finger polypeptide gene at the human  
chromosome 11 Wilms' tumor locus. *Cell.* 1990;60:509-520. 69  
6. Gessler M, Poustka A, Cavenee W, et al. Homozygous deletion  
in Wilms tumours of a zinc-finger gene identified by  
chromosome jumping. *Nature.* 1990;343:774-778. 73  
7. Sugiyama H. WT1 (Wilms' tumor gene 1): biology and cancer  
immunotherapy. *Jpn J Clin Oncol.* 2010;40:377-387. 75  
8. Oka Y, Elisseeva OA, Tsuboi A, et al. Human cytotoxic T-  
lymphocyte responses specific for peptides of the wild-type  
Wilms' tumor gene (WT1) product. *Immunogenetics.* 2000;51:  
99-107. 77  
9. Ohnishi H, Yasukawa M, Fujita S. HLA class I-restricted  
lysis of leukemia cells by a CD8(+) cytotoxic T-lymphocyte  
clone specific for WT1 peptide. *Blood.* 2000;95:286-293. 79  
10. Oka Y, Tsuboi A, Taguchi T, et al. Induction of WT1 (Wilms'  
tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide  
vaccine and the resultant cancer regression. *Proc Natl Acad Sci  
USA.* 2004;101:13885-13890. 81  
11. Tsuboi A, Oka Y, Kyo T, et al. Long-term WT1 peptide  
vaccination for patients with acute myeloid leukemia with  
minimal residual disease. *Leukemia.* 2011;26:1410-1413. 83  
12. Morita S, Oka Y, Tsuboi A, et al. A phase I/II trial of a WT1  
(Wilms' tumor gene) peptide vaccine in patients with solid  
malignancy: safety assessment based on the phase I data. *Jpn J  
Clin Oncol.* 2006;36:231-236. 85  
13. Cheever MA, Allison JP, Ferris AS, et al. The prioritization of  
cancer antigens: a national cancer institute pilot project for the  
acceleration of translational research. *Clin Cancer Res.*  
2009;15:5323-5337. 87  
14. Ochi T, Fujiwara H, Okamoto S, et al. Novel adoptive T-cell  
immunotherapy using a WT1-specific TCR vector encoding  
silencers for endogenous TCRs shows marked antileukemia  
reactivity and safety. *Blood.* 2011;118:1495-1503. 89  
15. Xue SA, Gao L, Hart D, et al. Elimination of human leukemia  
cells in NOD/SCID mice by WT1-TCR gene-transduced  
human T cells. *Blood.* 2005;106:3062-3067. 91  
16. Parkhurst MR, Yang JC, Langan RC, et al. T cells targeting  
carcinoembryonic antigen can mediate regression of metastatic  
colorectal cancer but induce severe transient colitis. *Mol Ther.*  
2011;19:620-626. 93  
17. Davis JL, Theoret MR, Zheng Z, et al. Development of human  
anti-murine T-cell receptor antibodies in both responding and  
nonresponding patients enrolled in TCR gene therapy trials.  
*Clin Cancer Res.* 2010;16:5852-5861. 95  
18. Marshall NB, Swain SL. Cytotoxic CD4 T cells in antiviral  
immunity. *J Biomed Biotechnol.* 2011;2011:954602. 97  
19. Janssen EM, Lemmens EE, Wolfe T, et al. CD4 + T cells are  
required for secondary expansion and memory in CD8 + T  
lymphocytes. *Nature.* 2003;421:852-856. 99  
20. Hunder NN, Wallen H, Cao J, et al. Treatment of metastatic  
melanoma with autologous CD4 + T cells against NY-ESO-1.  
*N Engl J Med.* 2008;358:2698-2703. 101  
21. Quezada SA, Simpson TR, Peggs KS, et al. Tumor-reactive  
CD4(+) T cells develop cytotoxic activity and eradicate large  
established melanoma after transfer into lymphopenic hosts.  
*J Exp Med.* 2010;207:637-650. 103  
22. Fujiki F, Oka Y, Tsuboi A, et al. Identification and char-  
acterization of a WT1 (Wilms Tumor Gene) protein-derived  
HLA-DRB1\*0405-restricted 16-mer helper peptide that pro-  
motes the induction and activation of WT1-specific cytotoxic T  
lymphocytes. *J Immunother.* 2007;30:282-293. 105  
23. Fujiki F, Oka Y, Kawakatsu M, et al. A WT1 protein-derived,  
naturally processed 16-mer peptide, WT1(332), is a promiscu-  
ous helper peptide for induction of WT1-specific Th1-type  
CD4(+) T cells. *Microbiol Immunol.* 2008;52:591-600. 107  
24. Guo Y, Niiya H, Azuma T, et al. Direct recognition and lysis  
of leukemia cells by WT1-specific CD4 + T lymphocytes in an  
HLA class II-restricted manner. *Blood.* 2005;106:1415-1418. 109  
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