Cancer Immunol Immunother (2013) 62:801–810
DOI 10.1007/s00262-012-1385-3

ORIGINAL ARTICLE

# Functional human Th17 clones with WT1-specific helper activity

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Received: 3 September 2012 / Accepted: 7 December 2012 / Published online: 29 December 2012


**Abstract** Th17 plays important roles in the pathogenesis of various inflammatory and autoimmune diseases. Although the importance of Th17 in tumor immunity has also been suggested, precise roles of tumor-associated antigen-specific Th17 still remain poorly understood, especially in humans. We previously identified $WT1_{332}$, a 16-mer helper epitope derived from tumor-associated antigen Wilms' tumor gene 1 (WT1) product, and $WT1_{332}$-specific Th1 clones were established. In the present study, WT1-specific Th17 clones were established by the stimulation of peripheral blood mononuclear cells with the $WT1_{332}$ helper peptide under human Th17-polarizing conditions. The WT1-specific Th17 clone exhibited the helper function for proliferation of conventional $CD4^+$ T cells in the antigenic stimulation-specific manner. This is the first report of establishment of functional Th17 clones with both antigen ($WT1_{332}$) specificity and antigen-specific helper activity. Th17 clones established here and the method to establish antigen-specific Th17 clones should be a useful tool to further analyze the roles of human Th17 in tumor immunity.

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

## Introduction

Interleukin (IL)-17-producing T helper cells (Th17) have been identified as a distinct subset of $CD4^+$ T cells characterized by production of pro-inflammatory cytokine IL-17 [1–3] and simply defined by production of IL-17 and expression of RORγt [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 $CD4^+$ 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 $WT1_{332}$ and established $WT1_{332}$-specific $CD4^+$ T-cell clones by the stimulation of peripheral blood mononuclear cells (PBMCs) with the $WT1_{332}$ peptide [26, 27]. However, all the $WT1_{332}$-specific $CD4^+$ T cell clones were T helper type 1 (Th1) [26]. These results encouraged us to try to establish $WT1_{332}$-specific Th17 clones under Th17-polarizing conditions.

In the present study, we describe the establishment of $WT1_{332}$-specific Th17 clones and demonstrate that Th17 clones have antigen-specific helper activity for the proliferation of conventional $CD4^+$ T cells. This is the first report of establishment of functional Th17 clones with $WT1_{332}$-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-γ-FITC, anti-GM-CSF-PE, anti-TNF-α-APC, anti-CCR6-biotin; anti-Foxp3-FITC monoclonal antibodies (mAbs); streptavidin-APC-Cy7 (BD Pharmingen, San Diego, CA, USA), anti-IL-17-PE, anti-RORγt-APC, anti-T-bet-PE (eBiosciences, San Diego, CA, USA), anti-TCR Vβ18-PE, anti-TCR Vβ2-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-1β, IL-6, IL-23, GM-CSF, IL-4, and TNF-α 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 $CD14^+$ 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-α (100 IU/ml). Floating cells were harvested and used as antigen-presenting cells (APCs).

### In vitro induction of $WT1_{332}$-specific IL-17-producing $CD4^+$ T cells from PBMCs

PBMCs from the healthy donor were stimulated with $WT1_{332}$ peptide under the human Th17-polarizing conditions containing IL-1β (10 ng/ml), IL-6 (50 ng/ml), and neutralizing antibodies for IFN-γ and IL-4 (10 μg/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 $WT1_{332}$-specific Th17 cells, were single-cell-sorted, as previously described [30]. In brief, the cells were re-stimulated with $WT1_{332}$ 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 $CD4^+CD154^+$ cells were single-cell-sorted by means of FACSAria (BD Biosciences). The sorted cells were expanded by PHA HA16 (2 μg/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 $WT1_{332}$ peptide in the presence of IL-2.

### Proliferation assay

Cell proliferation was assessed with a standard $^3$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 μg/mL of $WT1_{332}$ peptide. HIV peptide was used as an irrelevant control peptide. $^3$H-thymidine (Amersham Biosciences Corp, NJ) was added after culturing for 80 h, and the cells were cultured in the presence of $^3$H-thymidine for additional 18 h. The cells were then harvested onto glass-fiber filters, and their radioactivity was measured on a β-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 $WT1_{332}$ peptide, or with phorbol-12-myristate-13-acetate (PMA; 25 ng/ml) and ionomycin (1 μg/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 $WT1_{332}$ 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-RORγt, anti-T-bet, and anti-FoxP3 mAbs.

### TCR-Vβ sequence analysis

T-cell receptor (TCR)-Vβ CDR3 amino acid sequences of Th17 clones were obtained from the DNA sequences of RT-PCR products of the TCR-Vβ genes, as described previously [31]. In brief, complementary DNAs (cDNAs) of TCR-β were synthesized by the reverse transcription of mRNA of resting clones with a TCR-β gene constant region-specific primer and used for two-step semi-nested PCR using 24 kinds of TCR-Vβ gene family-specific primers and two kinds of TCR-Vβ constant region-specific primers to identify the Vβ gene family used in each clone. After identification of the Vβ gene family used in each clone, direct sequencing of the V–D–J CDR3 region with Vβ 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 $WT1_{332}$ 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

$CD4^+CD25^-$ T cells were isolated from PBMC of the healthy donor from which the Th17 clones had been generated. The $CD4^+CD25^-$ T cells were labeled with CellTrace™-Violet (eBiosciences), and co-cultured with the Th17 clones and either $WT1_{332}$ peptide-pulsed or -unpulsed DCs in the presence of 1 μg/ml of TSST-1. Since TSST-1 is a Vβ2 family-specific superantigen, it stimulated a Vβ2 family TCR-bearing population of $CD4^+CD25^-$ T cells via a TCR signaling, but did not stimulate the Th17

clones that did not have TCR Vβ2. After 5 days of incubation, the proliferation of TCR Vβ2-expressing $CD4^+CD25^-$ T cells was assessed by the evaluation of the dilution of CellTrace™-Violet by means of flow cytometric analysis.

## Results

### Induction and establishment of $WT1_{332}$-specific IL-17-producing $CD4^+$ T cell clones from human PBMCs

$WT1_{332}$ peptide, a human WT1 protein-derived helper epitope, was previously identified, and $WT1_{332}$-specific Th1-type $CD4^+$ T cell clones were established. Here, it was examined whether $WT1_{332}$-specific Th17 clones could be generated from PBMCs by stimulation with $WT1_{332}$ 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 $WT1_{332}$ peptide under Th17-polarizing conditions for 10 days. The cells were re-stimulated with the $WT1_{332}$ 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 $WT1_{332}$-specific. Each clone was cultured with irradiated autologous PBMCs in the presence or absence of the $WT1_{332}$ peptide for 3 days, and the uptake of $^3$H-thymidine by proliferating cells was measured. Consequently, 11 of the 28 clones showed the $WT1_{332}$-specific proliferation (Fig. 1b), and thus, 11 $WT1_{332}$-specific $CD4^+$ T cell clones were established.

To investigate whether the established $WT1_{332}$-specific clones produced IL-17 in response to stimulation with the $WT1_{332}$ peptide, each of clones was stimulated with the $WT1_{332}$ peptide for 4 h, and the cytoplasmic cytokines were stained with anti-IL-17 and anti-IFN-γ 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 $WT1_{332}$ peptide stimulation. All of the four IL-17-producing clones contained IFN-γ-producing cells, although the frequencies of IFN-γ-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-γ in response to the $WT1_{332}$ peptide stimulation, they were considered to be Th1 clones.

Since IL-17 single-positive, IFN-γ 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 expectedly, 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 Vβ18 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 $WT1_{332}$ peptide stimulation under the Th17-polarizing conditions.

### $WT1_{332}$-specific IL17-producing clones are Th17

Recent studies have demonstrated that transcriptional factor RORγt 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, RORγt, 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 RORγt, but not Foxp3, while T-bet was expressed in two clones (clones 20 and 23). Since RORγt 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-α in response to the $WT1_{332}$ 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 $WT1_{332}$-specific Th17 clones could be generated from PBMCs, other rounds of the same experiments were performed. Expectedly, other $WT1_{332}$-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 $WT1_{332}$ peptide in an HLA-DPB1*05:01-restricted manner

$WT1_{332}$ peptide was originally identified as a helper peptide with the restriction of HLA-DRB1*04:05, and

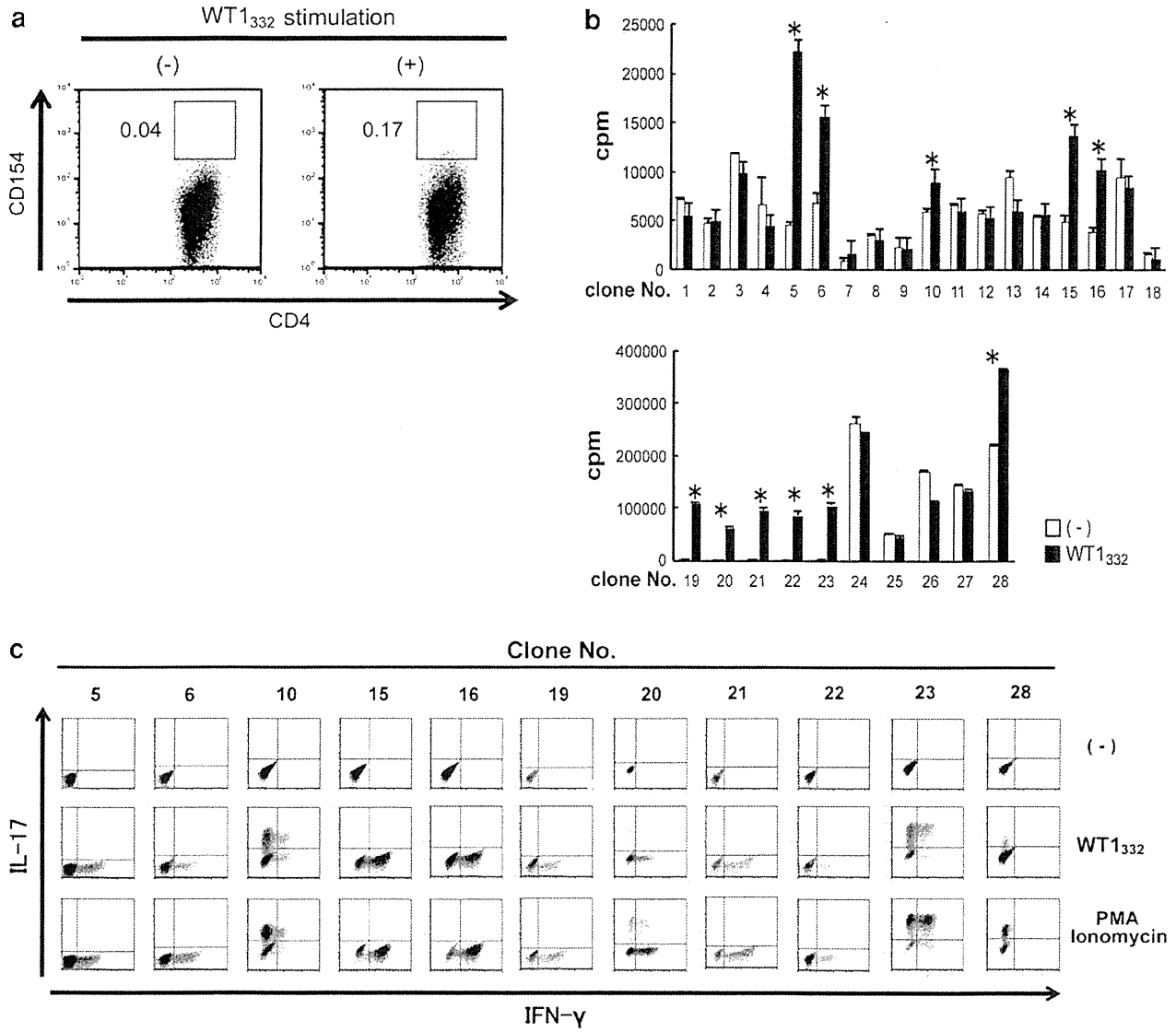


**Fig. 1** Establishment of $WT1_{332}$-specific IL-17-producing clones. PBMCs from a healthy donor were cultured with $WT1_{332}$ peptide under Th17-polarizing conditions. **a** On the day 10, the cells were re-stimulated with the $WT1_{332}$ peptide, and $CD154^{+}CD4^{+}$ T cells were single-cell-sorted and expanded in the presence of PHA and IL-2. **b** Result of $WT1_{332}$-specific proliferation of each clone assessed by proliferation assay is shown. $^{3}$H-thymidine uptake (mean + S.D. of triplicate) by the $WT1_{332}$-stimulated (*black columns*) or unstimulated (*white columns*) cells is shown. *Asterisks* show a $WT1_{332}$-specific proliferation. **c** Result of flow cytometric analysis of IFN-γ and IL-17 production in each clone stimulated by the $WT1_{332}$ 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 $WT1_{332}$-specific response was examined in Th17 clone10 as a representative of the four Th17 clones.

$WT1_{332}$-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 $WT1_{332}$-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 $WT1_{332}$ peptide, while Th17 clone10 did not produce IL-17 in response to the HLA-DPB1*05:01-expressing L cells unpulsed with $WT1_{332}$ peptide, or the HLA-DRB1*04:05-, *08:03- or -DR53-expressing L cells pulsed with $WT1_{332}$ peptide (Fig. 3b). These results, taken together, indicated that Th17 clone10 recognized $WT1_{332}$ peptide in a restriction to HLA-DPB1*05:01.

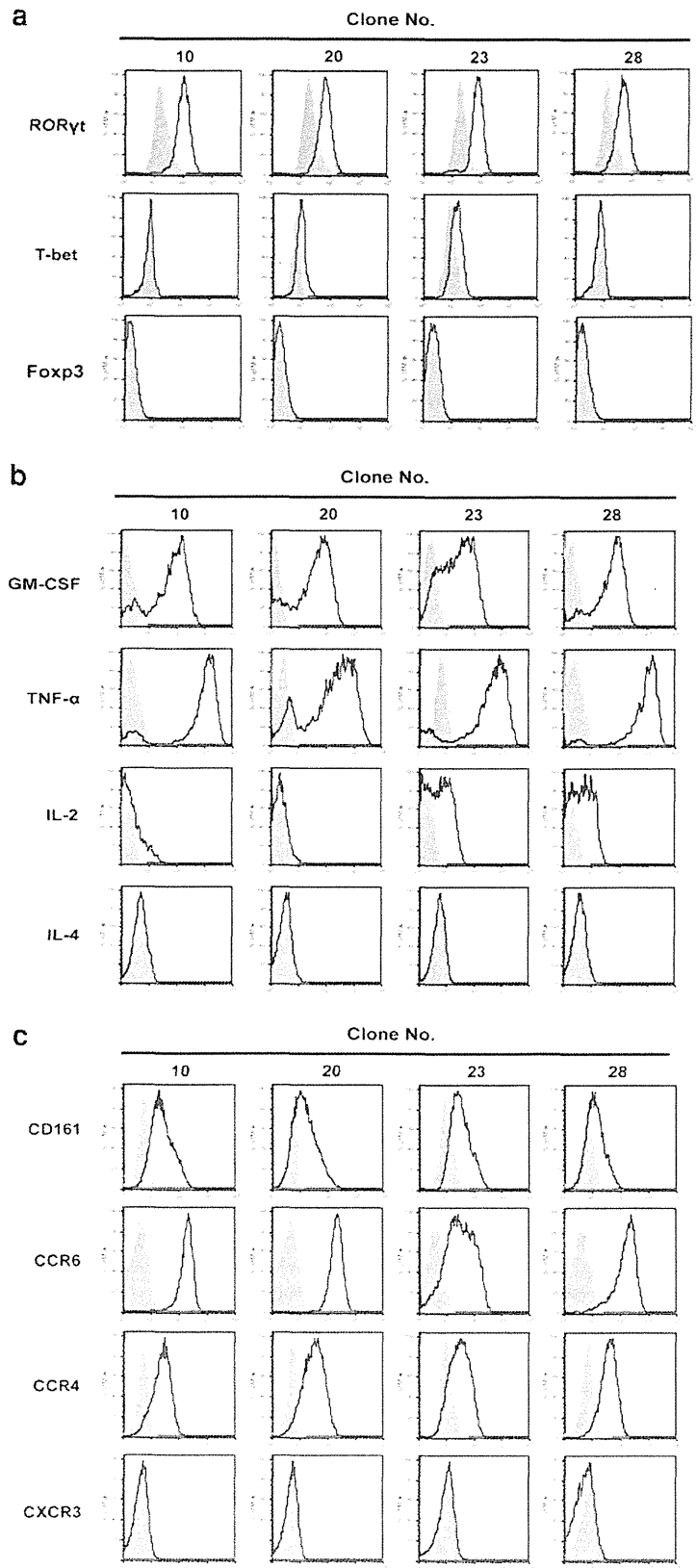


**Fig. 2** Characterization of IL-17-producing clones. **a** Flow cytometric analysis of expression of RORγt, T-bet, and Foxp3 in IL-17-producing clones in resting state. **b** Flow cytometric analysis of expression of GM-CSF, TNF-α, IL-2, and IL-4 by stimulation with $WT1_{332}$ peptide. **c** Flow cytometric analysis of expression of cell surface molecules, CD161, CCR6, CCR4, and CXCR3. *Solid lines* and *shadows* represent $WT1_{332}$-stimulated or -unstimulated cells, respectively

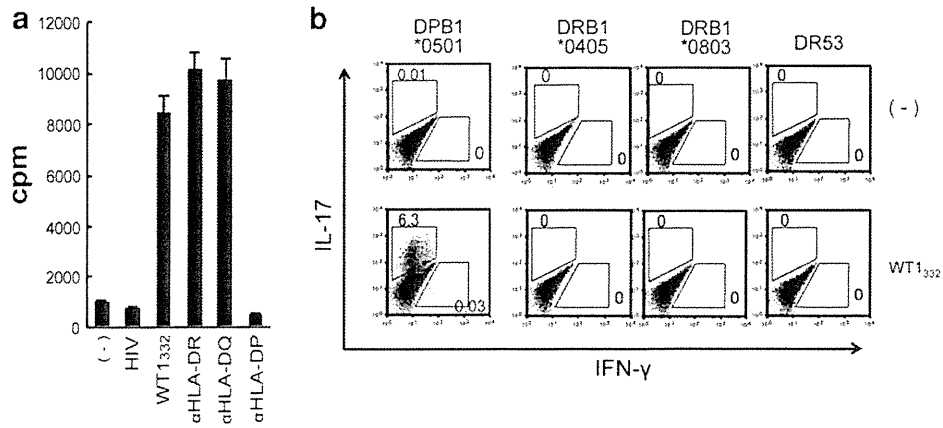


**Fig. 3** $WT1_{332}$-specific response of Th17 clone10 is HLA-DPB1*0501-restricted. **a** Clone10 was stimulated by autologous PBMCs pulsed with $WT1_{332}$ peptide in the presence of HLA-DR, HLA-DQ, or HLA-DP blocking antibodies. $^{3}$H-thymidine uptake (mean + S.D. of triplicate) is shown on the day 4. HIV peptide ($RT_{171-190}$) was used as an irrelevant antigen. **b** Clone10 was stimulated by $WT1_{332}$-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

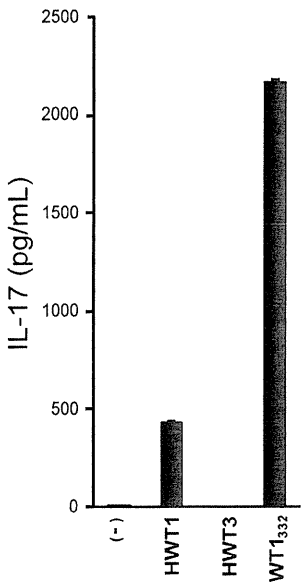


**Fig. 4** Th17 clone10 is responsive to the product processed naturally from WT1 protein. Clone10 was stimulated by autologous PBMCs pulsed with the full-length WT1 protein (HWT1) or $WT1_{332}$-deficient WT1 protein (HWT3), and IL-17 concentrations in the culture supernatant were examined by ELISA. Clone10 that was stimulated by autologous PBMCs pulsed with $WT1_{332}$ 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 $WT1_{332}$ epitope processed naturally from WT1 protein.

## Th17 clone functions as a helper for the proliferation of conventional $CD4^+$ T cells in a $WT1_{332}$-specific manner

To investigate whether Th17 clone10 had a helper or suppressive activity, the effect of the $WT1_{332}$-stimulated Th17 clone10 on the proliferation of conventional $CD4^+CD25^-$ T cells was examined.

In order to stimulate the conventional $CD4^+CD25^-$ 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 $WT1_{332}$-stimulated Th17 clone10 on the TSST-1-stimulated responder T cell proliferation was examined (Fig. 5).

CellTrace$^{TM}$-Violet-labeled conventional $CD4^+CD25^-$ T cells were co-cultured with Th17 clone10 and mature DCs pulsed or unpulsed with $WT1_{332}$ peptide in the presence of TSST-1. On the day 5, the proliferation of the $CD4^+CD25^-$ T cells (responder T cells) was examined by the evaluation of the dilutions of CellTrace$^{TM}$-Violet. As shown in Fig. 5, the responder T-cell proliferation was

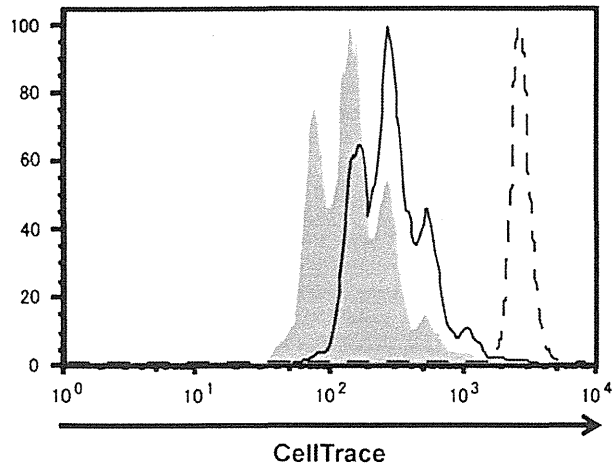


**Fig. 5** Th17 clone10 helps the proliferation of conventional $CD4^+$ T cells in a $WT1_{332}$-specific manner CellTrace™-Violet-labeled $CD25^-CD4^+$ conventional T cells were co-cultured with clone10 with TCR Vβ18 and autologous DCs pulsed with TSST-1 in the presence (*gray shadow*) or absence (*solid line*) of $WT1_{332}$ peptide. After 5 days of culture, the proliferation of the $V\beta2^+CD25^-CD4^+$ T cells was evaluated of the dilutions of CellTrace™-Violet by flow cytometric analysis. A *dashed line* shows $V\beta2^-CD25^-CD4^+$ T cells that did not respond to TSST-1

enhanced by co-culture with the $WT1_{332}$ peptide-stimulated Th17 clone10, compared to co-culture with unstimulated Th17 clone10. When the $WT1_{332}$ 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 $WT1_{332}$ peptide-stimulated Th17 clone10 induced the responder T-cell proliferation. Taken together, these results clearly demonstrated that $WT1_{332}$ peptide-stimulated Th17 clone10 promoted the proliferation of the responder T cell, indicating that Th17 clone10 had helper activity in a $WT1_{332}$-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, co-stimulatory cell surface molecule as an activation marker and expanded, was useful for establishing TAA-specific Th clones, such as $WT1_{332}$-specific Th17 clones. Hamaï 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 $WT1_{332}$ peptide stimulation. In addition, Th17 clone10 produced GM-CSF and TNF-α 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.

$WT1_{332}$ peptide is a promiscuous peptide and has the ability to induce $WT1_{332}$-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 $WT1_{235\text{-}243}$) vaccine and $WT1_{332}$-specific Th1 and Th2 responses, which indicates that the possibility of prediction of clinical effect of the peptide vaccine by assessing the $WT1_{332}$-specific Th responses [40]. Thus, analysis of the correlation between clinical effect of HLA-A*24:02-restricted WT1 peptide vaccine and $WT1_{332}$-specific Th17 response should be interesting.

**Acknowledgments** This study was supported, in part, by Grant-in-Aid from the Ministry of Education, Science, and Technology and Ministry of Health, Labor and Welfare of Japan.

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

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# HLA-DPB1*05:01-restricted $WT1_{332}$-specific TCR-transduced $CD4^+$ 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 $WT1_{332}$ that could elicit Th1-type $CD4^+$ T-cell response and bind to multiple HLA class II molecules. In this study, we examined the feasibility of adoptive therapy using $CD4^+$ T cells that were transduced an HLA-DPB1*05:01-restricted, $WT1_{332}$-specific T-cell receptor (TCR). HLA-DPB1*05:01-restricted, $WT1_{332}$-specific TCR-transduced $CD4^+$ T cells were successfully generated using lentiviral vector and exhibited strong proliferative response and Th1-type cytokine production in response to $WT1_{332}$ peptide, WT1 protein, or WT1-expressing tumor cell lysate. Furthermore, the $WT1_{332}$-specific TCR-transduced $CD4^+$ 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 $WT1_{235}$ peptide, $WT1_{235m}$) and $WT1_{332}$ helper peptide in the presence of $WT1_{332}$-specific TCR-transduced $CD4^+$ T cells strikingly enhanced the induction of $WT1_{235m}$-specific CTLs. Thus, these results demonstrated the feasibility of immunotherapy based on adoptive transfer of $WT1_{332}$-specific TCR-transduced $CD4^+$ T cells for the treatment of leukemia.

**Key Words:** WT1, $CD4^+$ helper T cell, HLA class II, helper peptide, TCR gene therapy

(*J Immunother* 2013;00:000–000)

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.[1] On the basis of the results of a series of studies,[2–4] 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.[5,6] 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.[7]

Some WT1-derived, HLA class I-restricted CTL epitopes were identified[8,9] and WT1 peptide vaccination using these CTL epitopes[10–12] has been performing for patients with leukemia and solid cancers with good clinical responses such as a reduction in leukemic blast cells[10] and tumor size, and prolonged survival.[11] These good clinical responses were associated with an increase in the frequency of WT1-specific $CD8^+$ T cells in peripheral blood.[7,10] 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.[13]

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[14,15] or solid tumor.[16,17] 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.[14,15] 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 $CD4^+$ T cell in both elimination of infectious disease[18] and antitumor immunity. $CD4^+$ T cells have been demonstrated to be critical for, maintenance of cell numbers, recruitment to the tumor sites, and memory response, of $CD8^+$ T cells.[19] Recent investigations showed more direct evidence to support the benefit of the use of tumor-reactive $CD4^+$ T cells for cancer immunotherapy.[20,21] We previously identified a WT1-derived HLA class II-restricted, 16-mer helper peptide, $WT1_{332}$, and showed that $WT1_{332}$ helper peptide had promiscuous characteristics that could bind to many types of HLA class II.[22,23] $WT1_{332}$-specific $CD4^+$ T cells that were induced by in vitro stimulation of PBMCs by $WT1_{332}$ peptide could enhance in vitro induction of WT1-specific CTLs[22] and kill WT1-expressing leukemia cells from patients.[24] Furthermore, importantly, $WT1_{332}$-specific $CD4^+$ T-cell responses correlated with good clinical responses of HLA-A*24:02-restricted, WT1

Received for publication September 10, 2012; accepted October 8, 2012.
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peptide vaccine.[25] These findings suggested that gene therapy using $WT1_{332}$-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, $WT1_{332}$-specific *TCR* gene and the usefulness of the $WT1_{332}$-specific TCR-transduced $CD4^+$ T cells for cancer immunotherapy. The $WT1_{332}$-specific TCR-transduced $CD4^+$ 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 $WT1_{332}$-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,[26] 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).[27] Endogenously WT1-expressing TF-1 (human erythroleukemia cell line)[8] was cultured in RPMI1640 (Nacalai TesqueI 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.[22] 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

$WT1_{332}$ peptide (KRYFKLSHLQMHSRKH) and 14 truncated $WT1_{332}$ 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.[28,29] Tumor cell lysates were prepared by 5 freeze-thaw cycles from $1 \times 10^8$ tumor cells resuspended in 1 mL of PBS. Lysates were used at $5 \times 10^5$ 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-IFNγ-PE-Cy7, anti-CD154-APC (BD Bioscience, San Jose, CA), anti-IFNγ-PE, anti-TNFα-APC, anti-GM-CSF-PE, anti-mouse CD3ε-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.[30]

### Generation of $WT1_{332}$-specific $CD4^+$ T-Cell Clones

Three million PBMCs from an HLA-DPB1*05:01 $^+$ donor were cultured in the presence of 20 μg/mL of $WT1_{332}$ peptide in 24-well plates. After a week, CD154 expression assay[31] was performed to sort $WT1_{332}$-specific $CD4^+$ T cells. Briefly, the PBMCs were restimulated with 20 μg/mL $WT1_{332}$ peptide in the presence of anti-CD154-APC mAb, 2 μL BD GolgiStopTM [BD Bioscience and 2 μg/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 $CD154^+$ $CD4^+$ 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 $1 \times 10^5$ irradiated allogeneic PBMCs, 100 IU/mL IL-2 and 3 μg/mL HA16 phytohaemagglutinin (PHA, Remel Inc., Lenexa, KS). Established single-cell–derived $CD4^+$ T-cell clones were screened for $WT1_{332}$-specificity by proliferation assay as described later and used for further experiments.

### Cloning of Full-Length TCR α and TCR β genes From a $WT1_{332}$-specific $CD4^+$ T-Cell Clone

HLA-DPB1*05:01-restricted, $WT1_{332}$-specific TCR α and β genes were cloned from a $WT1_{332}$-specific $CD4^+$ 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: Cα3′UTR-primer; 5′-CACAGGCTGTCT TACAATCTTGCAGATC-3′, Cβ1-3′UTR-primer; 5′-CTC CACTTCCAGGGCTGCCTTCA-3′, and Cβ2-3′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) for the identification of human TCR α and β chains.

### Construction of Lentiviral Vector Expressing $WT1_{332}$-specific TCRs and Preparation of Recombinant Lentiviruses

To construct a functional $WT1_{332}$-specific TCRs, the isolated TCR α and TCR β chain were linked with picornaviral 2A-like sequence (p2A) by using Vα primer 1; 5′-CGCTCTGCGGCCGCGCCACCATGGCAGGCATTCG AGCT-3′, Vβ primer 2; 5′-GCCACGAACTTCTCTCTGT TAAAGCAAGCAGGAGACGTGGAAGAAAACCCCG GTCCCATGAGCATCGGGCTCCTG-3′, Vα primer 3; 5′-GGGACCGGGGTTTTCTTCCACGTCTCCTGCTTG CTTTAACAGAGAGAAGTTCGTGGCTCCGGAACCG TCTTACAATCTTGCAGATC-3′, Vβ primer 4; 5′-CGCT CTGGATCCTCCACTTCCAGGGCTGCCTTCA-3′, and the TCR α-p2A-TCR β 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

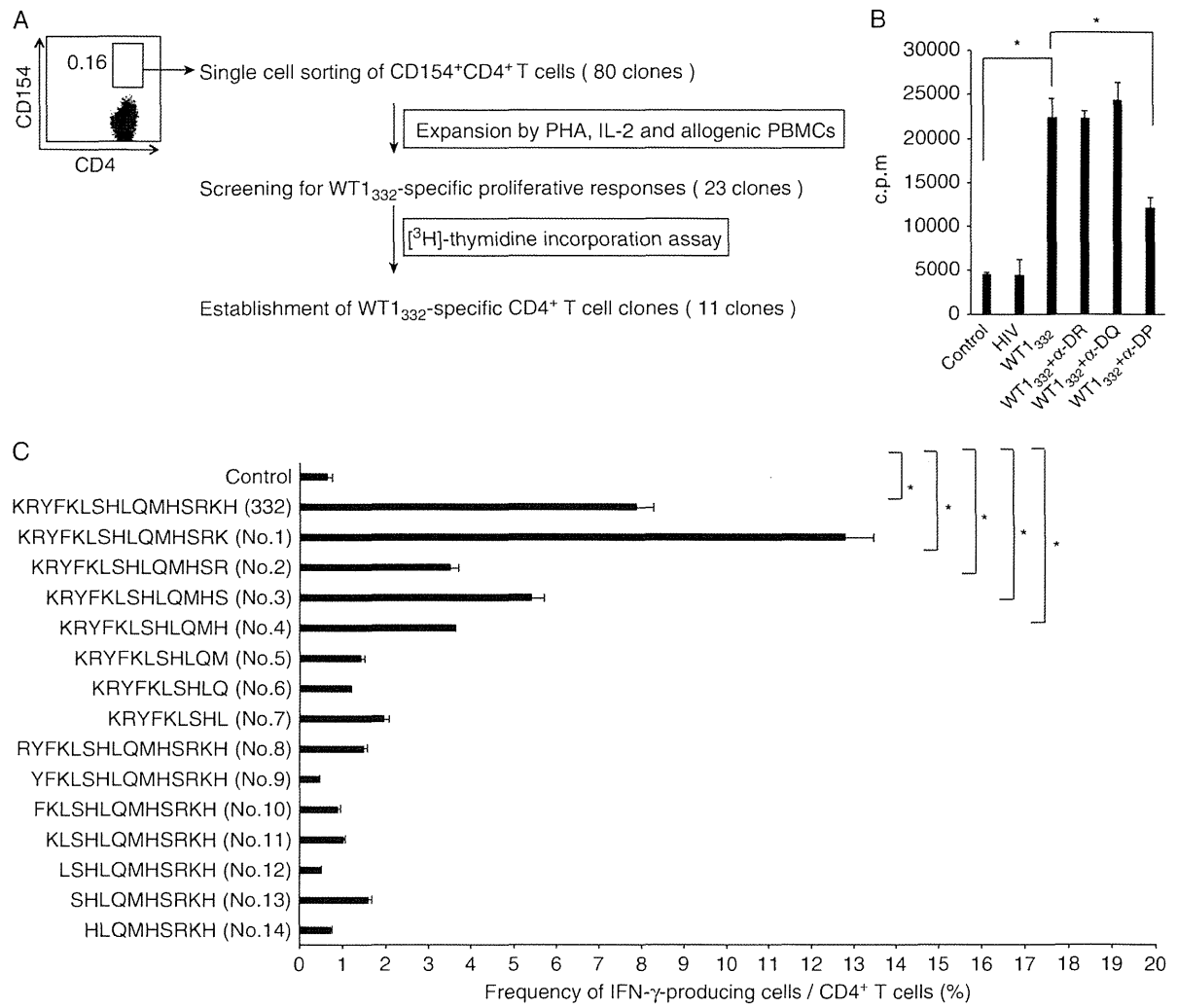


**FIGURE 1.** Establishment of HLA-DPB1*05:01-restricted, Wilms tumor gene 1 ($WT1)_{332}$-specific CD4+ T cell clone. A, Establishment of HLA-DPB1*05:01-restricted, $WT1_{332}$-specific CD4+ T-cell clones. $WT1_{332}$ peptide-primed PBMCs were restimulated with $WT1_{332}$ peptide in the presence of anti-CD154-APC mAb for 6 hours, and CD154+ CD4+ T cells were single-cell sorted and expanded in the presence of irradiated allogeneic PBMCs and IL-2 for 1–2 weeks. The expanded clones were screened for $WT1_{332}$-specific proliferative response by [$^3$H]-thymidine incorporation as described in the Materials and methods section. B, A $WT1_{332}$-specific CD4 T-cell clone, clone 10, was cocultured with irradiated homozygous DPB1*05:01-expressing autologous PBMCs pulsed or unpulsed with $WT1_{332}$ peptide in the presence of HLA-DR-blocking, HLA-DQ-blocking, or HLA-DP-blocking mAb and tested for proliferative responses by [$^3$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 $WT1_{332}$-specific TCR gene or empty plasmid, pCAG-HIVgp, and pCMV-VSUG-RSV-Rev (kindly provided by Dr H Miyoshi) using Polyethyleneimine "Max" (Polyscience Inc., Warrington, PA). After 12 hours of transfection, the medium was changed and the cells were further cultured for 48 hours. The supernatant containing the recombinant lentiviruses were collected, filtered through 0.45-μm filters and concentrated by using PEG-it Virus Concentration Solution (System Biosciences, Mountain View, CA) according to the manufacture's procedures. The concentrated viruses were dissolved in Hanks' balanced salt solution and stored at −80°C.

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

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

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

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

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

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

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

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

### Intracellular Cytokine Staining Assay and CD107a Mobilization Assay

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

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

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

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

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

### $^{51}Cr$ release assay

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

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

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

## Statistical Analysis

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

## RESULTS

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

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

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

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

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

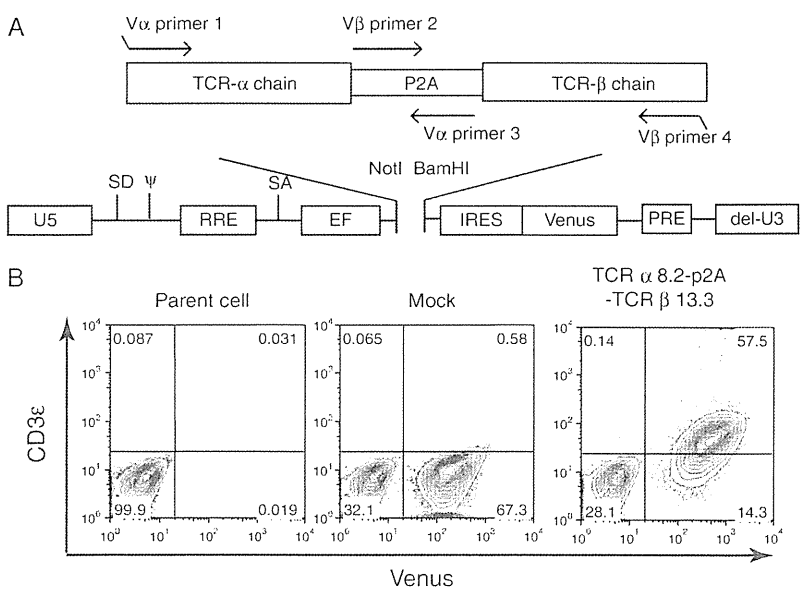


**FIGURE 2.** Cloning and expression of *TCR α 8.2/TCR β 13.3* gene using lentiviral vector. A, Construction of a lentiviral vector encoding full-length *TCR α 8.2* and β *13.3* genes derived from clone 10, and primer positions for cloning of *TCR*. Lentiviral vector constructions are as follows: SD,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

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

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

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

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

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

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

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

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

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

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

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

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

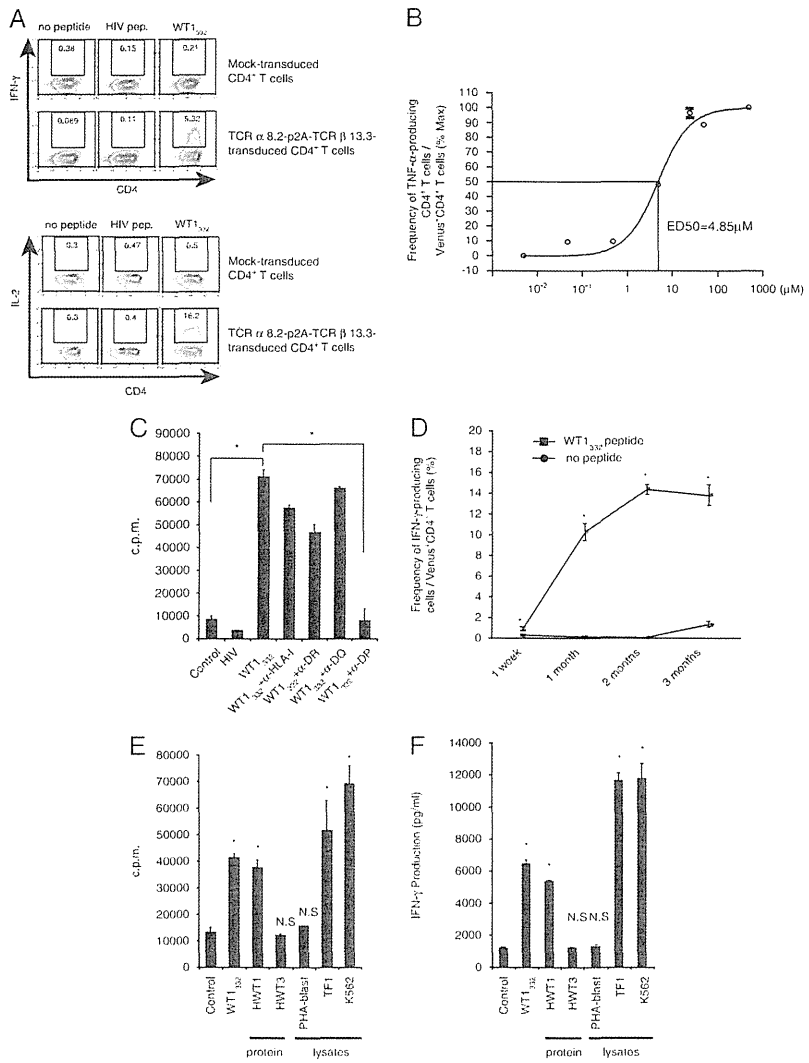


**FIGURE 3.** T-cell receptor (TCR) α 8.2-p2A-TCR β 13.3-transduced CD4$^+$ T cells displays antigen-specific T-cell responses. PBMCs from a healthy donor were transduced with TCR α 8.2-p2A-TCR β 13.3 cassette-encoding lentiviral vector (TCR α 8.2-p2A-TCR β 13.3) or empty lentiviral vector (Mock) and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

positive TF-1 cells that were pretreated with 100 μM granzyme B inhibitor, ac-IETD-Cho were used as target cells. The cytotoxicity of the WT1$_{332}$ TCR-transduced CD4$^+$ T cells against ac-IETD-Cho-pretreated TF-1 cells remarkably decrease compared to that against DMSO-pretreated TF-1 cells (Fig. 5F).

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

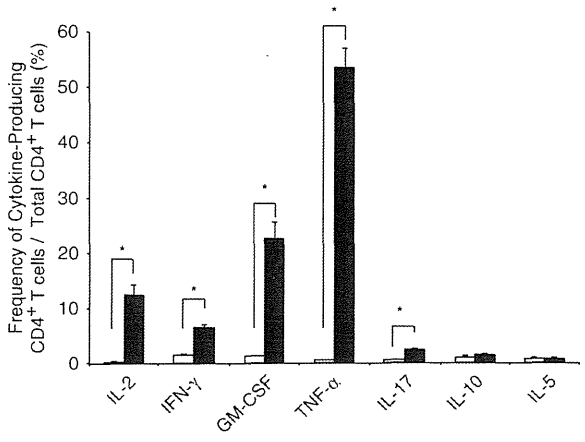


**FIGURE 4.** Wilms tumor gene 1 $(WT1)_{332}$ T-cell receptor (TCR)-transduced CD4+ T cells have a Th1 type-cytokine profile. $WT1_{332}$ TCR-transduced CD4+ T-cell lines were established from 3 different healthy donors as described in Figure 3. The CD4+ T cells were incubated with or without $WT1_{332}$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

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

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

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

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

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

## DISCUSSION

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

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

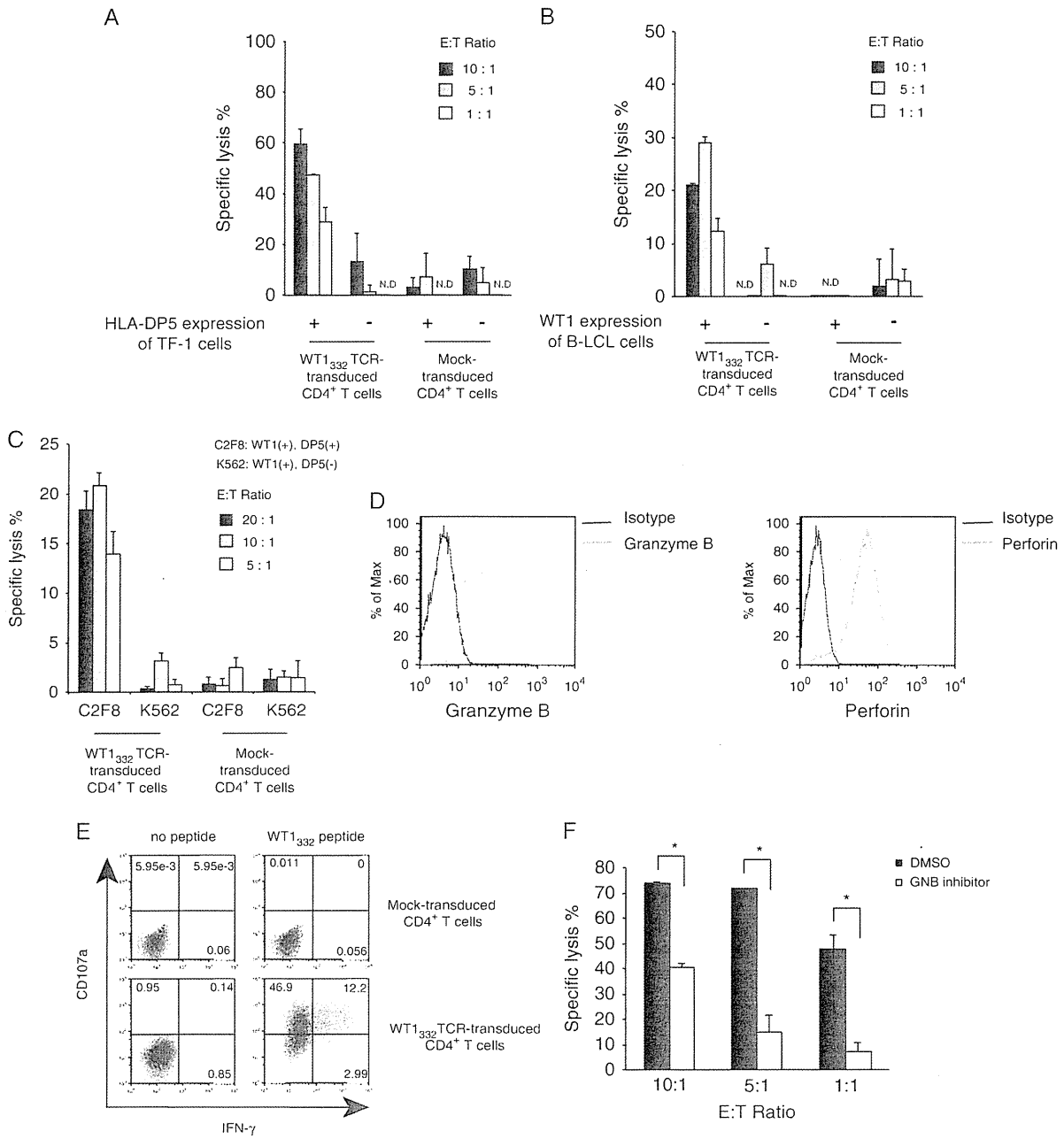


**FIGURE 5.** Wilms tumor gene 1 $(WT1)_{332}$ T-cell receptor (TCR)-transduced CD4+ T cells can directly kill leukemia cells through granzyme B/perforin pathway in an HLA-DPB1*05:01-restricted, $WT1_{332}$-specific manner. A–C, $WT1_{332}$ TCR-transduced CD4+ T cells were tested for cytotoxic activity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

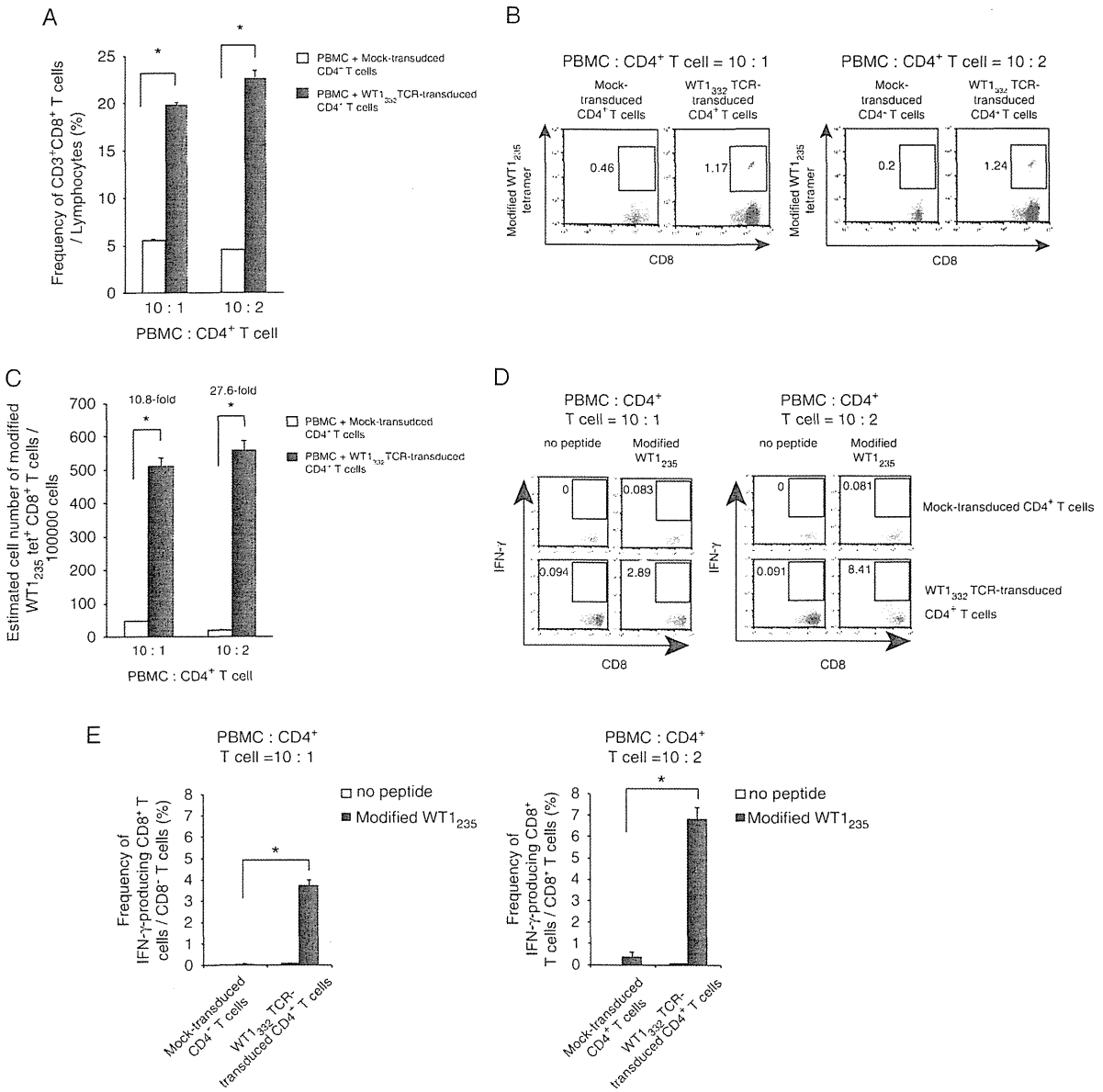


**FIGURE 6.** Enhancement of the induction of Wilms tumor gene 1 (WT1)-specific CD8+ CTLs by $WT1_{332}$ T-cell receptor (TCR)-transduced CD4+ T cells. $WT1_{332}$ TCR-transduced or empty vector (mock)-transduced CD4+ T cells were added to $3 \times 10^6$ autologous PBMCs with HLA-A*24:02/HLA-DPB1*05:01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

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