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ORIGINAL ARTICLE

Functional human Th17 clones with WT1-specific helper activity

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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

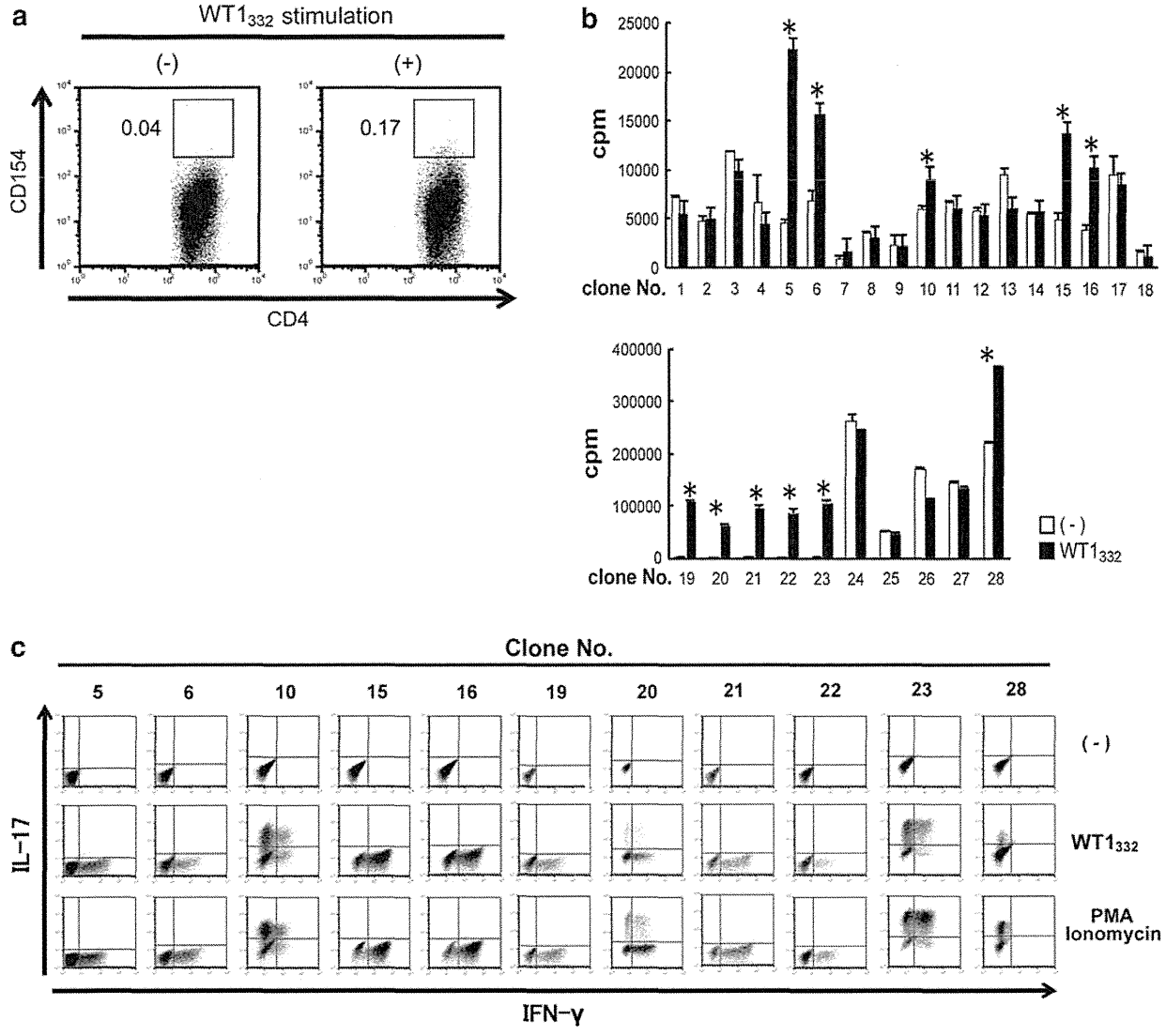

Fig. 1 Establishment of $WT1_{332}$-specific IL-17-producing clones. PBMCs from a healthy donor were cultured with $WT1_{332}$ peptide under Th17-polarizing conditions. **a** On the day 10, the cells were re-stimulated with the $WT1_{332}$ peptide, and $CD154^+CD4^+$ T cells were single-cell-sorted and 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.

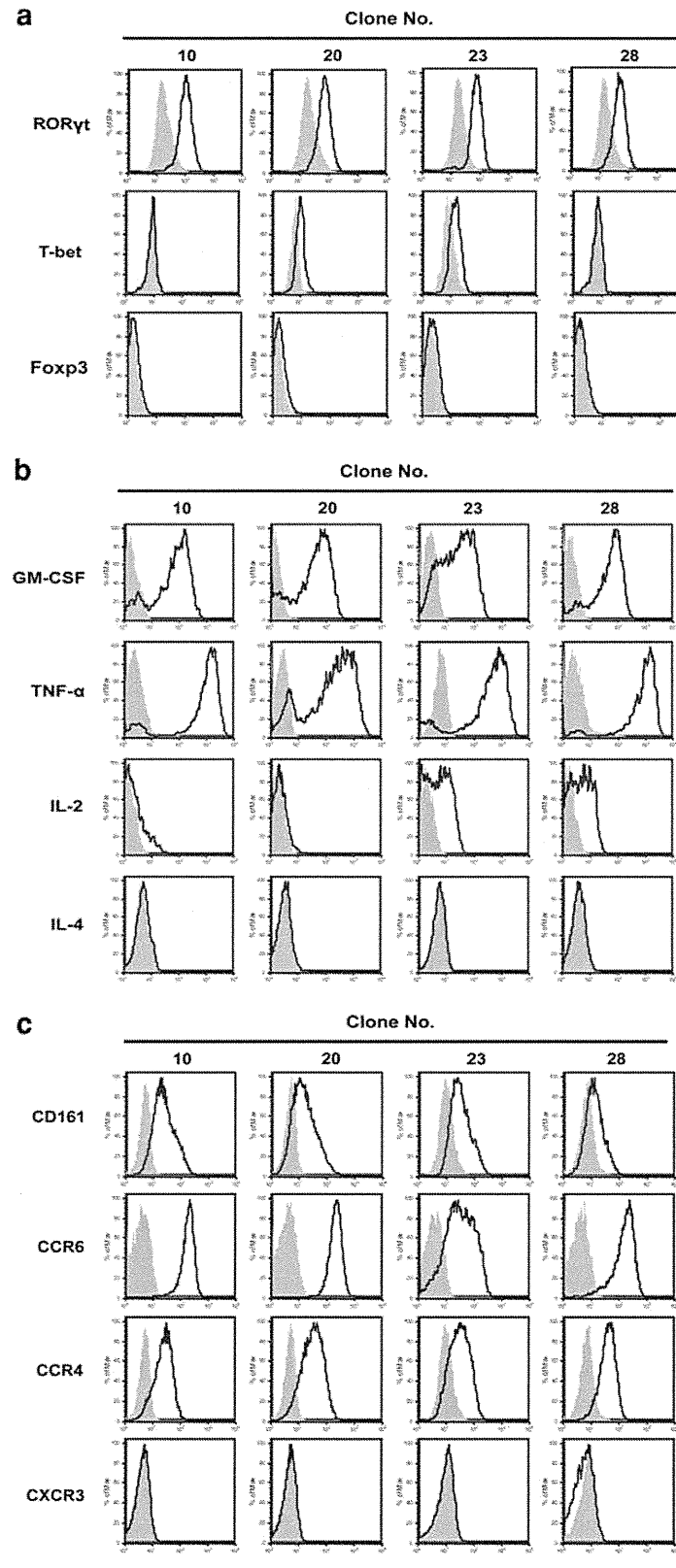

Fig. 2 Characterization of IL-17-producing clones. **a** Flow cytometric analysis of expression of RORγt, T-bet, and Foxp3 in IL-17-producing clones in resting state. **b** Flow cytometric analysis of expression of GM-CSF, TNF-α, IL-2, and IL-4 by stimulation with $WT1_{332}$ peptide. **c** Flow cytometric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

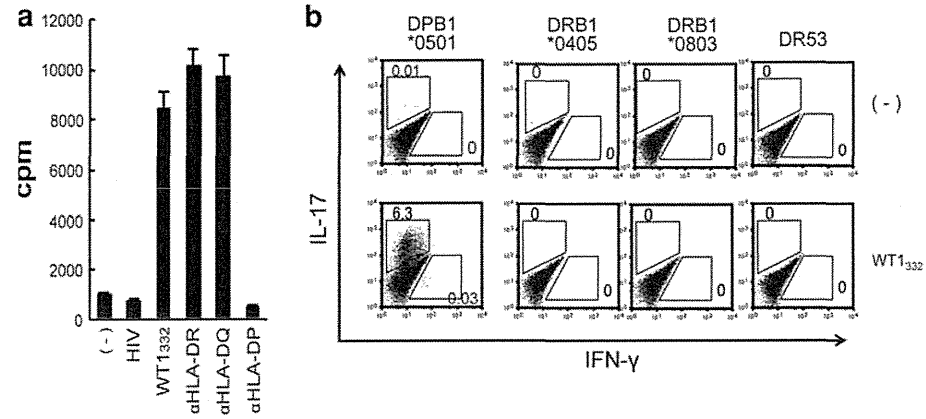

Fig. 3 $WT1_{332}$-specific response of Th17 clone10 is HLA-DPB1*0501-restricted. **a** Clone10 was stimulated by autologous PBMCs pulsed with $WT1_{332}$ peptide in the presence of HLA-DR, HLA-DQ, or HLA-DP blocking antibodies. ^{3}H-thymidine uptake (mean + S.D. of triplicate) is shown on the day 4.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

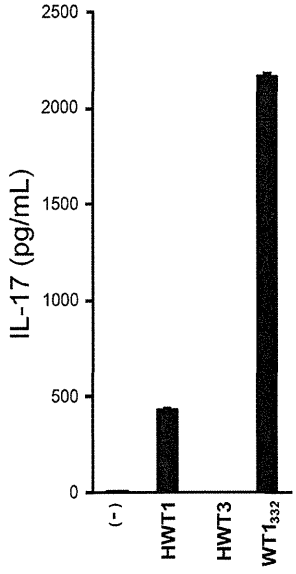

Fig. 4 Th17 clone10 is responsive to the product processed naturally from WT1 protein. Clone10 was stimulated by autologous PBMCs pulsed with the full-length WT1 protein (HWT1) or $WT1_{332}$-deficient WT1 protein (HWT3), and IL-17 concentrations in the culture supernatant were examined by ELISA. Clone10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

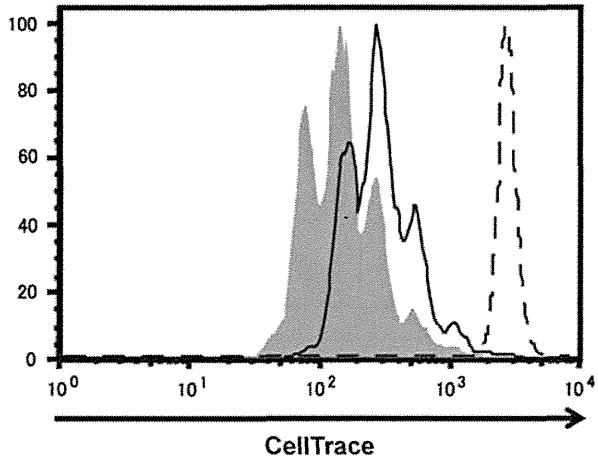

Fig. 5 Th17 clone10 helps the proliferation of conventional $CD4^+$ T cells in a $WT1_{332}$-specific manner CellTrace™-Violet-labeled $CD25^-CD4^+$ conventional T cells were co-cultured with clone10 with TCR Vβ18 and autologous DCs pulsed with TSST-1 in the presence (*gray shadow*) or absence (*solid 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.

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

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BASIC STUDY

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

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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.[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

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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 1×10^8 tumor cells resuspended in 1 mL of PBS. Lysates were used at 5×10^5 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 1×10^5 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