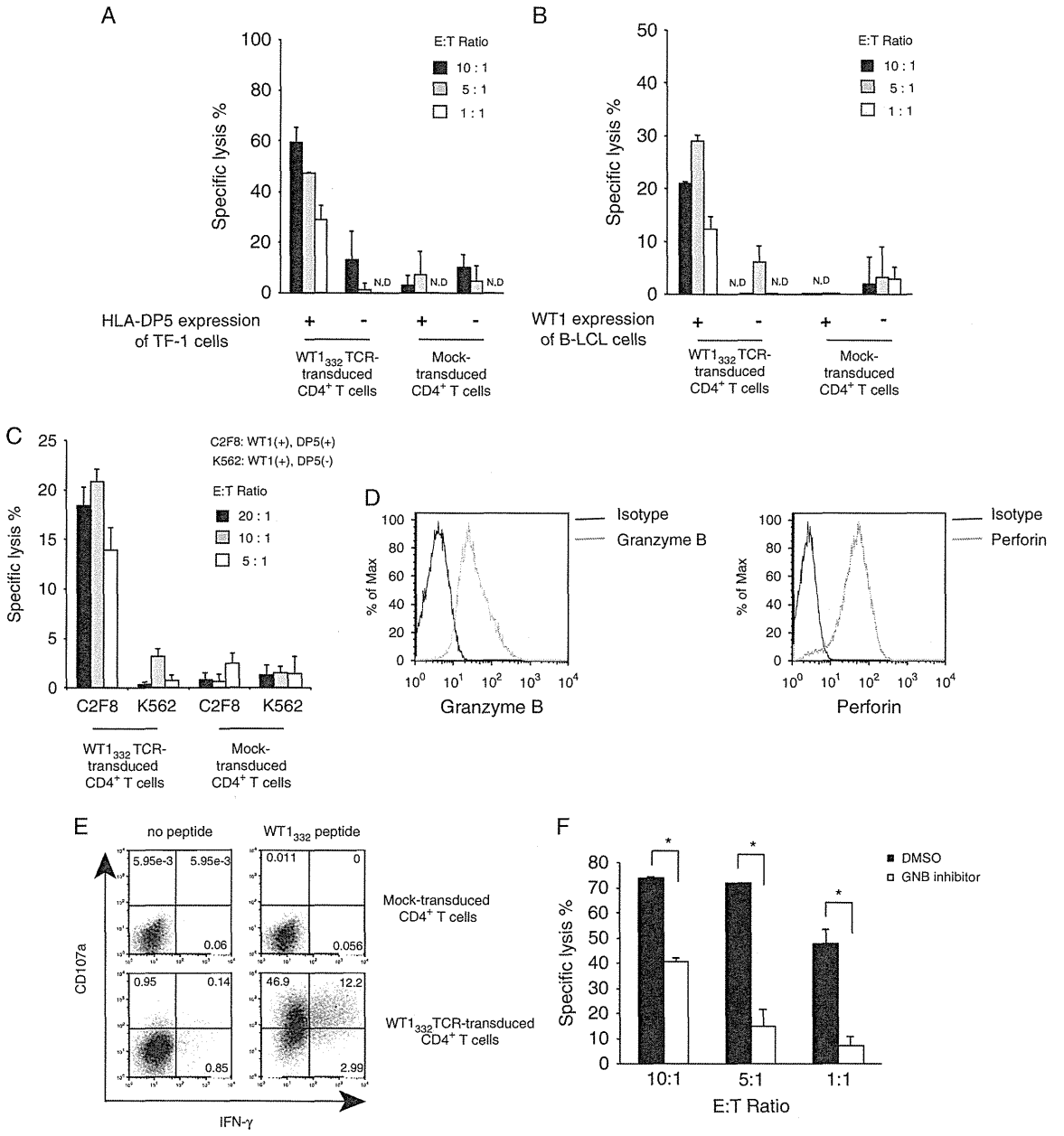

FIGURE 5. Wilms tumor gene 1 $(WT1)_{332}$ T-cell receptor (TCR)-transduced $CD4^+$ T cells can directly kill leukemia cells through granzyme B/perforin pathway in an HLA-DPB1*05:01-restricted, $WT1_{332}$-specific manner. A–C, $WT1_{332}$ TCR-transduced $CD4^+$ T cells were tested for cytotoxic activity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

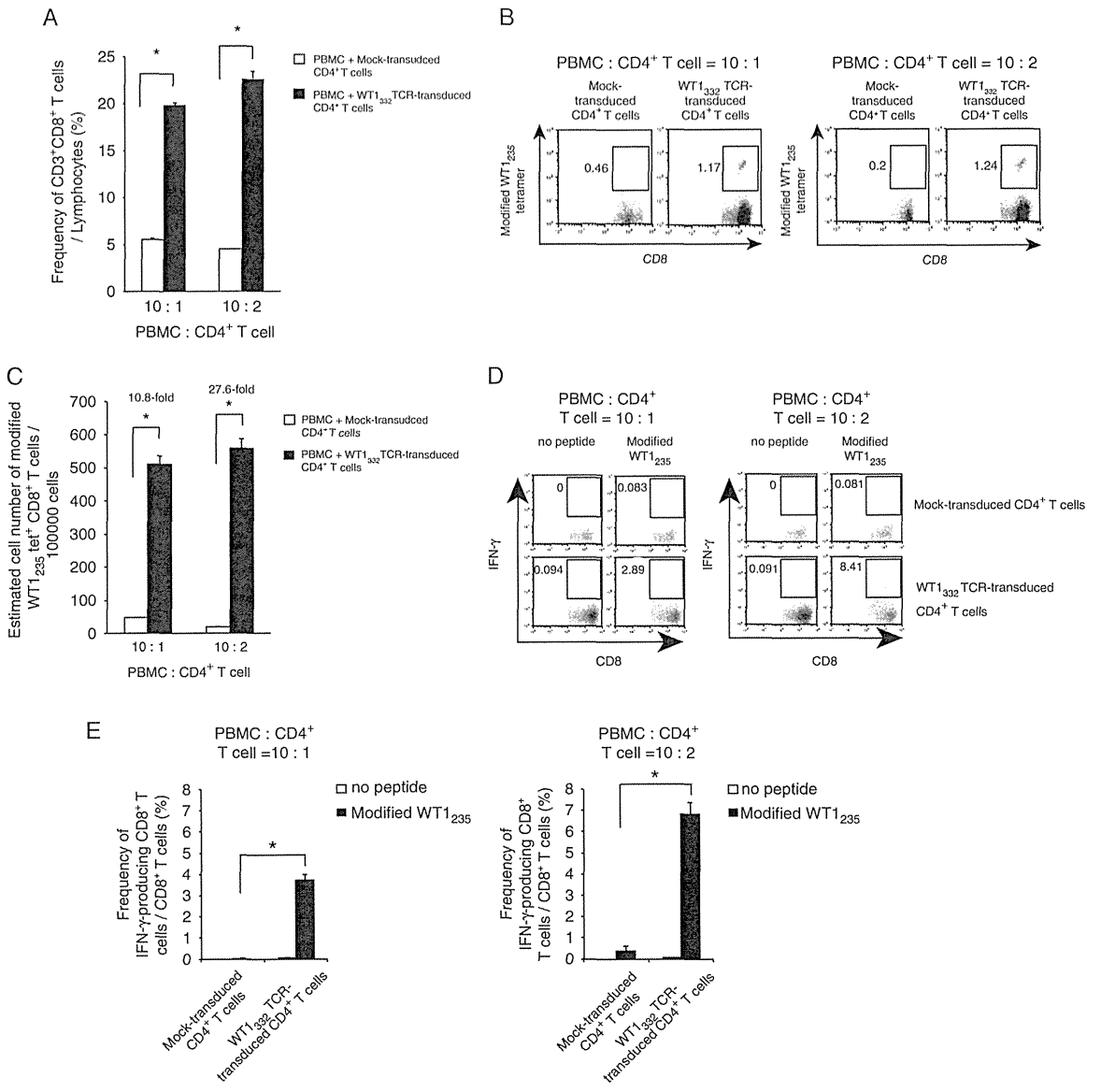

FIGURE 6. Enhancement of the induction of Wilms tumor gene 1 (WT1)-specific CD8+ CTLs by $WT1_{332}$ T-cell receptor (TCR)-transduced CD4+ T cells. $WT1_{332}$ TCR-transduced or empty vector (mock)-transduced CD4+ T cells were added to 3×10^6 autologous PBMCs with HLA-A*24:02/HLA-DPB1*05:01 at the indicated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

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

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

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

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

CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

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ORIGINAL 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

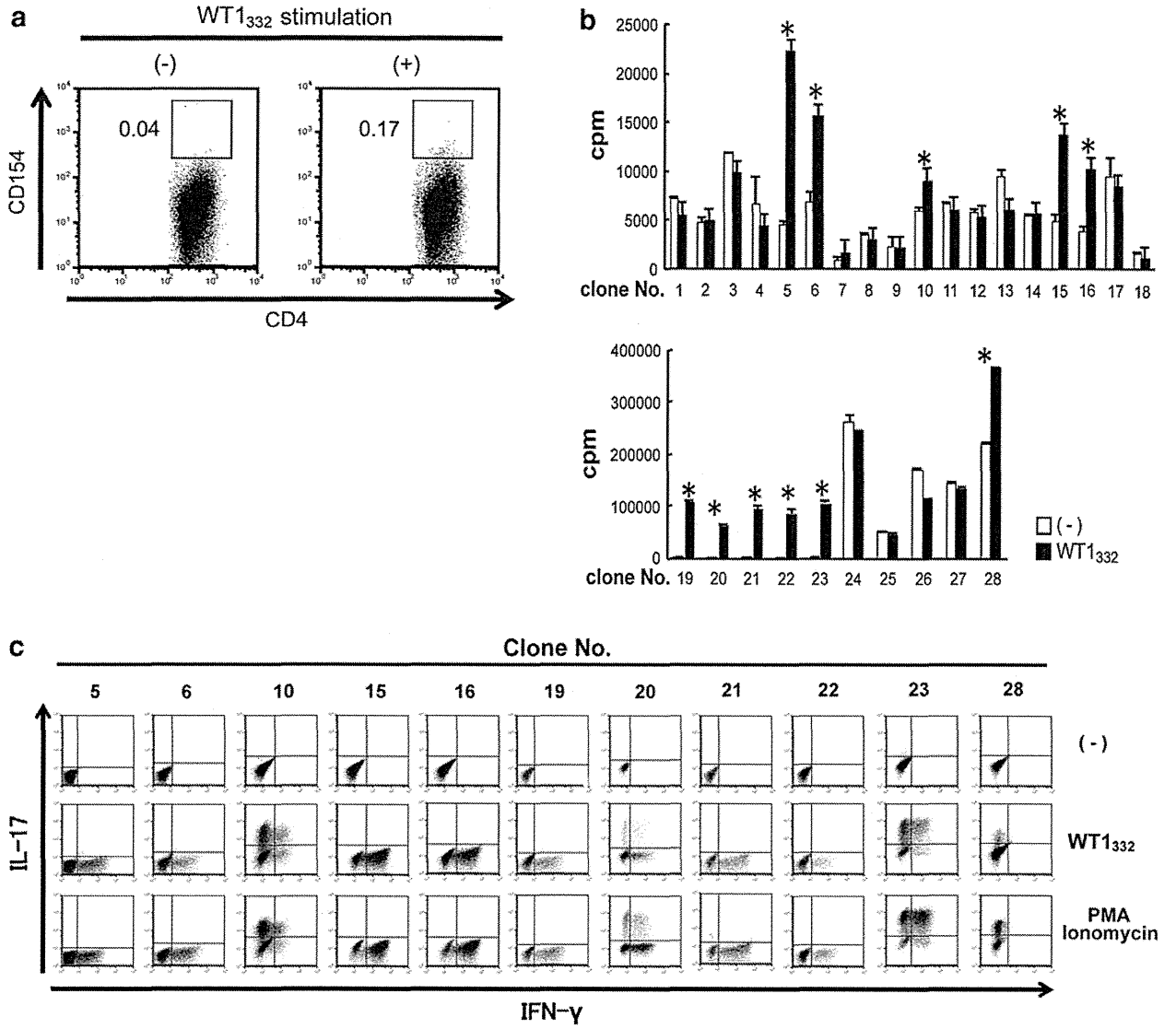

Fig. 1 Establishment of $WT1_{332}$-specific IL-17-producing clones. PBMCs from a healthy donor were cultured with $WT1_{332}$ peptide under Th17-polarizing conditions. **a** On the day 10, the cells were re-stimulated with the $WT1_{332}$ peptide, and $CD154^{+}CD4^{+}$ T cells were single-cell-sorted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

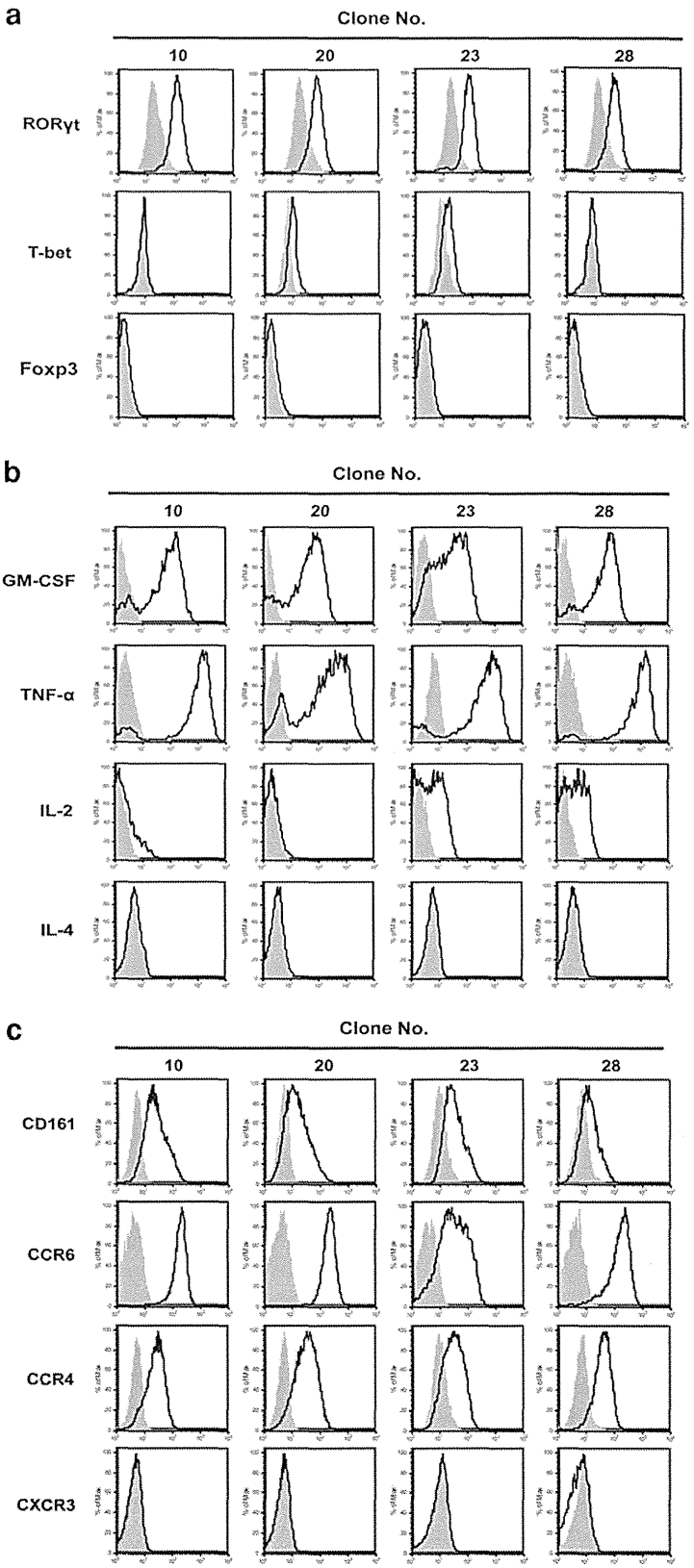

Fig. 2 Characterization of IL-17-producing clones. **a** Flow cytometric analysis of expression of RORγt, T-bet, and Foxp3 in IL-17-producing clones in resting state. **b** Flow cytometric analysis of expression of GM-CSF, TNF-α, IL-2, and IL-4 by stimulation with $WT1_{332}$ peptide. **c** Flow cytometric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

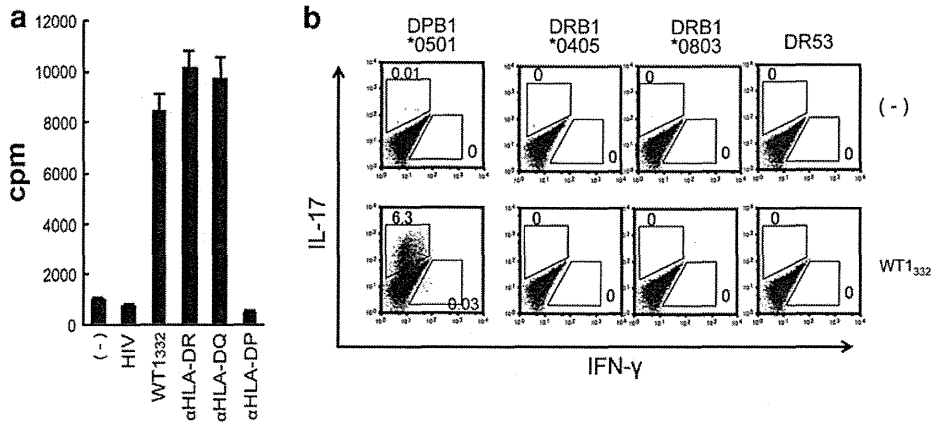

Fig. 3 $WT1_{332}$-specific response of Th17 clone10 is HLA-DPB1*0501-restricted. **a** Clone10 was stimulated by autologous PBMCs pulsed with $WT1_{332}$ peptide in the presence of HLA-DR, HLA-DQ, or HLA-DP blocking antibodies. ^{3}H-thymidine uptake (mean + S.D. of triplicate) is shown on the day 4.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

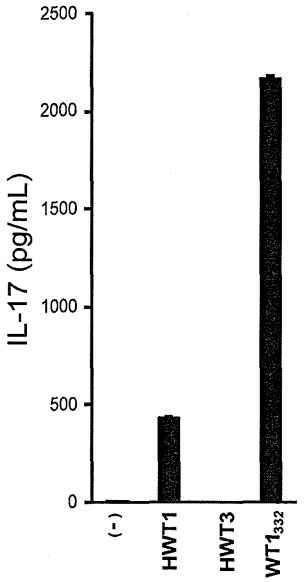

Fig. 4 Th17 clone10 is responsive to the product processed naturally from WT1 protein. Clone10 was stimulated by autologous PBMCs pulsed with the full-length WT1 protein (HWT1) or $WT1_{332}$-deficient WT1 protein (HWT3), and IL-17 concentrations in the culture supernatant were examined by ELISA. Clone10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

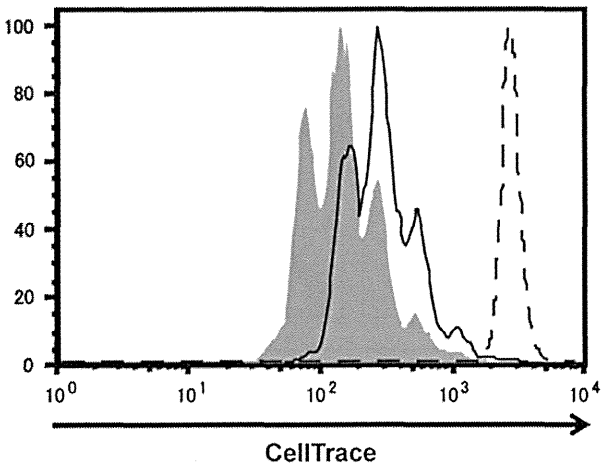

Fig. 5 Th17 clone10 helps the proliferation of conventional $CD4^+$ T cells in a $WT1_{332}$-specific manner CellTrace™-Violet-labeled $CD25^-CD4^+$ conventional T cells were co-cultured with clone10 with TCR Vβ18 and autologous DCs pulsed with TSST-1 in the presence (*gray shadow*) or absence (*solid 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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Clinical evaluation of WT1 mRNA expression levels in peripheral blood and bone marrow in patients with myelodysplastic syndromes

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Abstract

A study to evaluate WT1 mRNA expression levels in peripheral blood (PB) and bone marrow aspirate (BM) was conducted in 172 patients, including 115 with myelodysplastic syndromes (MDS), in Japan. The level of WT1 mRNA expression was evaluated according to the French–American–British (FAB) and World Health Organization (WHO) classifications (2001, 2008) and using the International Prognostic Scoring System and the WHO Prognostic Scoring System scales. WT1 mRNA expression levels in PB and BM were well correlated ($r = 0.85$), and they tended to increase with disease stage progression and in those at higher risk of leukemic transformation. WT1 mRNA expression can be a useful marker for the diagnosis and risk evaluation of MDS.

Keywords: Myelodysplastic syndromes, WT1 mRNA expression, classification system, peripheral blood, bone marrow

Introduction

Myelodysplastic syndrome (MDS), a clonal disorder of pluripotent hematopoietic stem cells, is a blood disease characterized by dysplasia and ineffective hemopoiesis. Approximately 20–30% of cases of MDS undergo transformation to acute myeloid leukemia (AML) [1].

The expression of Wilms' tumor gene (WT1) has been found to be a new prognostic factor and marker for the detection of minimal residual disease (MRD) in acute leukemia, including AML and acute lymphocytic leukemia (ALL) [2]. A recent study has revealed the clinical relevance of measuring WT1 mRNA for monitoring MRD in AML, primarily due to its high rate of expression (93.9%) in the peripheral blood (PB) of incipient untreated patients with AML, secondarily due to its ability to predict relapse after complete remission (CR), and finally because its levels after consolidation therapy

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show a significant correlation between disease-free survival, overall survival and early relapse [3]. WT1 mRNA expression occurs not only in AML but also in the PB and bone marrow (BM) of patients with MDS [4–9].

Tamaki *et al.* [4] examined the level of WT1 mRNA expression in PB and BM from 57 patients with MDS grouped by the French-American-British (FAB) classification, and 12 patients experienced AML-MDS progression. The results revealed that WT1 mRNA expression in both PB and BM progressively increased with disease stage progression, from refractory anemia (RA), refractory anemia with excess of blasts (RAEB), refractory anemia with excess of blasts in transformation (RAEB-t), and to AML, suggesting the possibility that the WT1 mRNA expression level reflects the disease stage progression of MDS. Particularly, the patient group who developed leukemia from RAEB or RAEB-t within 6 months showed significantly higher WT1 mRNA expression in PB compared with the group who did not [4].

In accordance with that study, Cilloni *et al.* [6] measured WT1 mRNA expression levels in PB and BM from 131 patients with MDS, and found that: (1) WT1 mRNA expression in PB and BM was confirmed in 78% and 65% of patients with RA, respectively; (2) WT1 mRNA expression in PB and BM was confirmed in all patients with RAEB and secondary AML; (3) the level of WT1 mRNA expression increased with disease stage progression; and (4) the WT1 mRNA expression level was well correlated with the International Prognostic Scoring System (IPSS) scores established by Greenberg *et al.* [10].

In addition to the IPSS, the World Health Organization (WHO) Classification-Based Prognostic Scoring System (WPSS) has been proposed as a prognostic scoring system for MDS [11]. The WPSS consists of three characteristics: WHO subtype classification, considered to be important as a prognostic factor; IPSS-based karyotype abnormalities; and transfusion dependency.

Both the IPSS and WPSS require a chromosomal test as a primary parameter. However, because there are cases in which chromosomal abnormalities cannot be determined [12–14], it is necessary to establish molecular- and genetic-based methods to diagnose and determine the prognosis of MDS. The relatively rapid quantitation of WT1 mRNA is considered to be a useful test to determine the prognosis of MDS and has potential for clinical application, to become a novel marker to complement the current IPSS and WPSS criteria. We performed a clinical study in patients with MDS to demonstrate the usefulness of measuring the WT1 mRNA expression level in PB and BM in the diagnosis and treatment of MDS.

Patients and methods

This study was conducted in accordance with the Declaration of Helsinki, and preliminary approval was obtained from the Institutional Review Board or equivalent organization of each participating institution. Explanations of the study protocol were provided to all patients, and written informed consent was obtained from them before study enrollment.

Patients

From December 2008 to September 2009, 175 patients with MDS, suspected MDS and AML-MDS examined at 17 Japanese medical institutions were enrolled in the study. The subjects were 20 years of age or older and entered in the study regardless of gender, inpatient/outpatient status, or presence or absence of treatment. The 175 patients comprised 106 men (age range 27–88 years, average 65.5 years) and 69 women (age range 22–85 years, average 64.5 years). PB and BM samples from each patient were collected on the same day and used for WT1 mRNA measurement. Three of the 175 enrolled patients were excluded because BM could not be collected due to a dry tap or because the subtype could not be diagnosed. A total of 172 patients were therefore included in the final analysis set.

Diagnosis

Diagnosis of MDS was carried out using a central review format based on the FAB classification [15], the 2001 WHO classification [16] and the 2008 WHO classification [17]. Central review of the bone marrow smear-stained specimens, blood smear-stained specimens, iron-stained specimens, and clot hematoxylin and eosin-stained specimens was carried out by two individuals, one each in the Department of Hemato-Oncology, Saitama International Medical Center, Saitama Medical University, and the Department of Laboratory Medicine, Kawasaki Medical School.

WT1 mRNA measurement method

mRNA was extracted from PB leukocytes and BM nucleated cells at SRL, Inc., Tokyo, Japan using the RNeasy Mini-Kit (Qiagen, Valencia, CA), and the amount containing WT1 mRNA was measured at the Research Laboratory, Diagnostic Division, Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan using a WT1 mRNA Assay Kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). cDNA was synthesized from 1 μg of extracted RNA in a reverse-transcription reaction using random hexamer primers. The amounts of WT1 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA were quantitated using real-time polymerase chain reaction (PCR) with a COBAS TaqMan48 analyzer (Roche Diagnostics, Pleasanton, CA), and the respective amounts of WT1 and GAPDH RNA in the sample were calculated by simultaneous reaction with standards of known concentrations.

Method for calculating WT1 mRNA expression

mRNA of the universally expressed housekeeping gene GAPDH was used for correction of variations in the efficiencies of RNA extraction and reverse transcription. As shown in the following formula, the level of WT1 mRNA expression was calculated by dividing the measured amount of WT1 mRNA by the measured amount of GAPDH mRNA and multiplying that value by the average number of copies of GAPDH mRNA found in 1 μg of RNA from PB leukocytes of healthy adults (GAPDH mRNA expression). The average GAPDH mRNA expression in PB leukocytes of healthy adults was reported to be 2.7×10^7 copies/μg RNA based on independent tests in healthy adults [3].

WT1 mRNA expression (copies/μg RNA) = (measured WT1 mRNA [copies/mL]/measured GAPDH mRNA [copies/mL]) × 2.7 × 10^7 (copies/μg RNA)

PB cut-off value

The lower limit of the WT1 mRNA measurement range in the WT1 assay kit is 2500 copies/mL, or 50 copies/μg RNA when converted to copies per microgram of RNA. In this study, a value of 50 copies/μg RNA was set as the cut-off value for WT1 mRNA expression, and a value of 50 or more copies/μg RNA was judged as positive according to the instruction manual of the WT1 mRNA assay kit.

Statistical analysis

The mean ± SD for the log-transformed values of WT1 mRNA expression (copies/μg RNA) was calculated, and then converted back to base 10 and used as the geometric mean. All data below the detection limit were shown as 49 copies/μg RNA. For intergroup comparison of WT1 mRNA expression, a Tukey-Kramer honestly significant difference (HSD) test was performed at the level of significance of $p < 0.05$ using log-transformed values of WT1 mRNA expression (copies/μg RNA). For comparison of WT1 mRNA expression between the aplastic anemia (AA) and RA groups, a Wilcoxon rank-sum test and Steel test were performed at the level of significance of $p < 0.05$ using log-transformed values of WT1 mRNA expression (copies/μg RNA). The Pearson correlation coefficient was used for analysis of each correlation.

Results

As a result of the central review conducted on all 172 patients, 115 were classified as patients with MDS in the FAB classification, excluding chronic myelomonocytic leukemia (CMML). Similarly, 98 patients in the 2001 WHO classification and 97 in the 2008 WHO classification were classified as patients with MDS (Figure 1).

Analytical results based on FAB classification

WT1 mRNA expression in PB and BM

The 172 patients eligible for analysis were categorized by disease type, and their WT1 mRNA expression levels in PB and BM are shown in Table I. The mean WT1 mRNA expression level in the 115 patients with MDS (excluding CMML) was 360 copies/μg RNA in PB and 2240 copies/μg RNA in BM, and these values were the second highest after the values obtained in patients with AML-MDS (PB: 12 600 copies/μg RNA; BM: 33 100 copies/μg RNA). On the other hand, the WT1 mRNA expression level was less than 50 copies/μg RNA in PB and 90–630 copies/μg RNA in BM in patients with AA, idiopathic cytopenia of unknown significance (ICUS), idiopathic thrombocytopenic purpura (ITP), paroxysmal nocturnal hemoglobinuria (PNH), pure red-cell aplasia (PRCA) and erythroid hypoplasia, which were all lower compared with the level in MDS.

The relationship between WT1 mRNA expression in PB and BM was evaluated in all patients. The regression line formula $y = 0.7329x + 1.4407$ was obtained, indicating a strong correlation ($r = 0.85$) (Figure 2).

WT1 mRNA expression in PB and BM for each MDS disease stage

When the WT1 mRNA expression levels in PB and BM were compared for each MDS subtype based on the FAB classification [Figure 3(a)], the level in both increased proportionally with each MDS classification as the disease

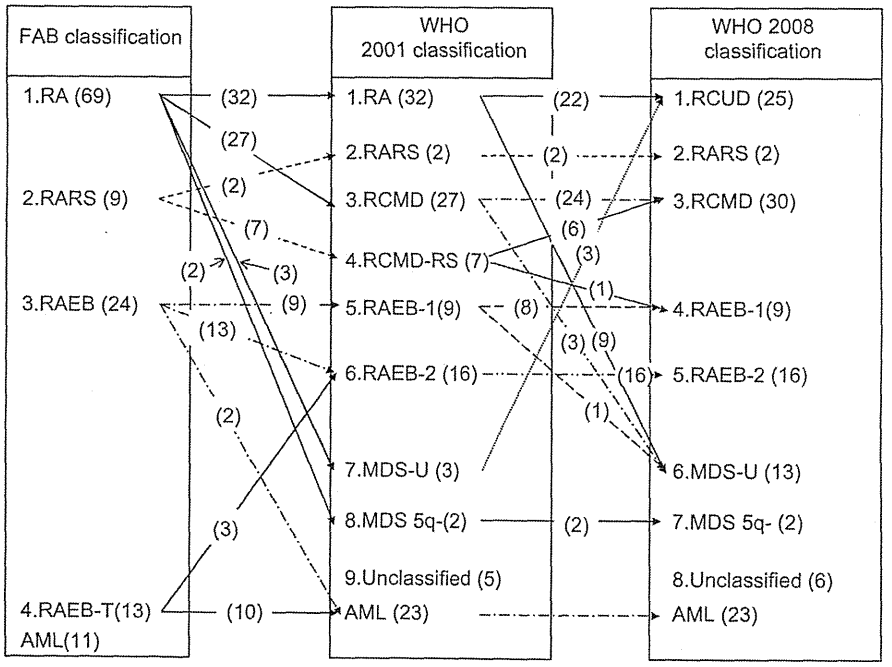

Figure 1. FAB and WHO classification of myelodysplastic syndromes in this study. FAB classification-based MDS subtypes (four subtypes: RA, RARS, RAEB and RAEB-t), 2001 WHO-based MDS subtypes (eight subtypes: RA, RARS, RCMD, RCMD-RS, RAEB-1, RAEB-2, MDS-U and MDS 5q−), 2008 WHO-based MDS subtypes (seven subtypes: RCUD, RARS, RCMD, RAEB-1, RAEB-2, MDS-U and MDS 5q−). Numbers in parentheses represent numbers of patients.

Table I. WT1 mRNA expression levels in PB and BM from patients with different MDS subtypes and AML-MDS according to FAB classification.

		WT1 mRNA expression level			
		Peripheral blood		Bone marrow	
Disease	No. of patients	Log (mean ± SD)	Geometric mean (copies/μg RNA)	Log (mean ± SD)	Geometric mean (copies/μg RNA)
MDS	115	2.56 ± 1.05	360	3.35 ± 0.87	2240
AML-MDS	11	4.10 ± 0.96	12 600	4.52 ± 0.77	33 100
AML-MDS (CR)	2	1.89 ± 0.20	80	2.98 ± 0.39	1000
CMML	3	2.17 ± 0.54	150	3.04 ± 0.54	1100
CLL	1	1.92	80	3.33	2140
Atypical CML	1	—	<50	1.95	90
AA	8	—	<50	2.64 ± 0.37	440
ICUS	3	—	<50	2.16 ± 0.36	140
ITP	1	—	<50	2.13	130
PNH	1	—	<50	2.8	630
PRCA	2	—	<50	2.17 ± 0.12	150
Erythroid hypoplasia	1	—	<50	1.94	90
Unclassified	23	2.14 ± 0.56	140	2.96 ± 0.61	910
Total	172	2.50 ± 1.05	320	3.27 ± 0.90	1860

PB, peripheral blood; BM, bone marrow; MDS, myelodysplastic syndromes; AML-MDS, acute myeloid leukemia-evolved MDS; FAB, French-American-British; CR, complete remission; CMML, chronic myelomonocytic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; AA, aplastic anemia; ICUS, idiopathic cytopenia of unknown significance; ITP, idiopathic thrombocytopenic purpura; PNH, paroxysmal nocturnal hemoglobinuria; PRCA, pure red-cell aplasia.

stage progressed. Significant differences in both PB and BM expression were seen between RA and RAEB, RA and RAEB-t, refractory anemia with ringed sideroblasts (RARS) and RAEB, and RARS and RAEB-t ($p < 0.05$).

WT1 mRNA expression in PB and BM for each IPSS risk group

WT1 mRNA expression levels in PB and BM for each IPSS risk group were compared in the 115 patients with MDS. A tendency for WT1 mRNA expression to increase in both PB and BM was observed in each IPSS risk group as the risk of transformation to AML increased from low to high. Significant differences ($p < 0.05$) in WT1 mRNA expression were observed in risk groups between low and intermediate-2, low and high, intermediate-1 and intermediate-2, and intermediate-1 and high in PB samples; and between low and intermediate-1, low and intermediate-2, low and high, intermediate-1 and intermediate-2, and intermediate-1 and higher in BM samples [Figure 3(b)]. The correlation between IPSS score and WT1 mRNA expression was evaluated, and a correlation of $r = 0.57$ was found for both PB and BM samples.

Next, the WT1 mRNA expression levels in PB and BM between IPSS risk groups were compared in the 69 patients with RA [Figure 3(c)]. As the risk increased from low to intermediate-2, the level of WT1 mRNA expression in both PB and BM increased. Moreover, when the distribution of WT1 mRNA expression between each risk group was evaluated, a significant difference ($p < 0.05$) was found in PB between low and intermediate-2; in BM, significant differences were found between low and intermediate-1, and low and intermediate-2.

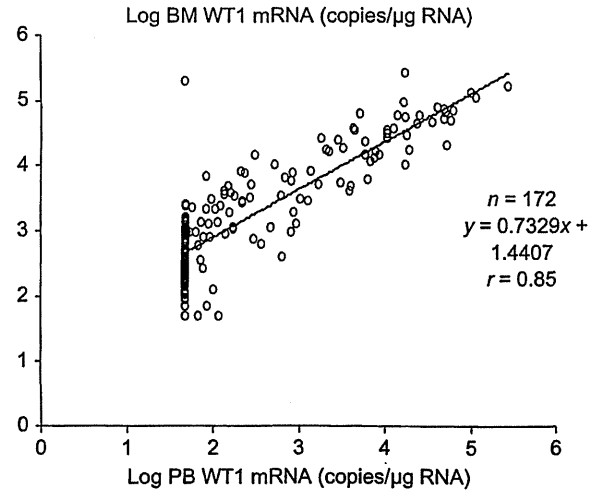

Figure 2. Correlation of WT1 mRNA expression in PB and WT1 mRNA expression in BM.

Correlation between IPSS karyotype and WT1 mRNA expression

A total of 114 patients with MDS were categorized into the three prognostic groups of good, intermediate and poor in accordance with their IPSS karyotype, and the levels of WT1 mRNA expression in their PB and BM samples were compared. One patient with MDS was excluded from this analysis because chromosome testing was not performed. The WT1 mRNA expression level increased in both PB and BM samples as the karyotype indicated a poorer prognosis. Among karyotypes, significant differences ($p < 0.05$) in WT1 mRNA expression were found between the good and intermediate and between the good and poor groups [Figure 3(d)].

Correlation between WT1 mRNA expression and percentage of blasts in BM

The correlation between blast ratio and WT1 mRNA expression in PB and BM was investigated in 114 patients with MDS (excluding one patient in whom the blast ratio could not be measured). The correlation between blast ratio and PB WT1 mRNA expression was $r = 0.51$, and the correlation between blast ratio and BM WT1 mRNA expression was $r = 0.48$.

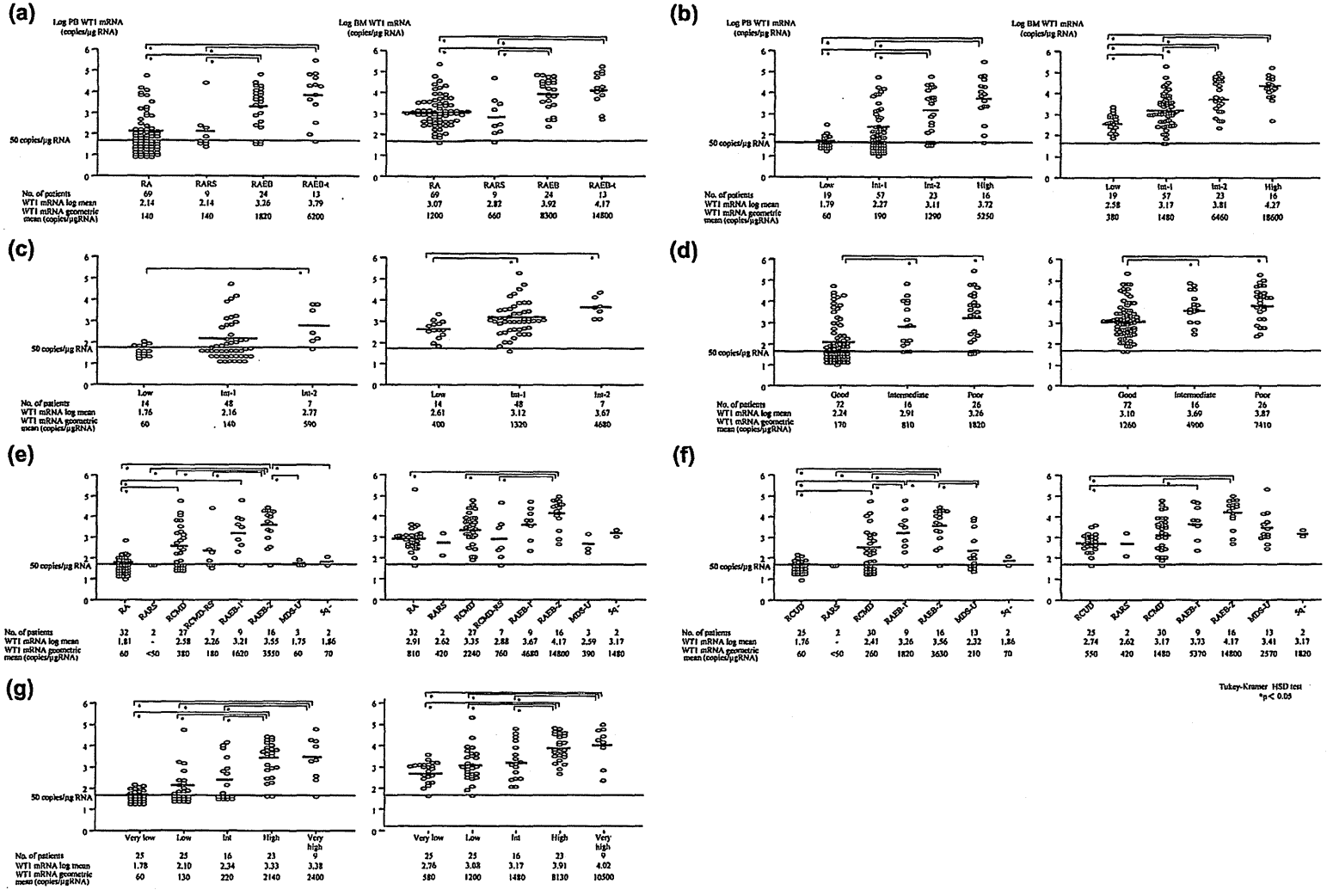

Figure 3. WT1 mRNA expression levels in PB and BM from patients with MDS (a) according to FAB classification, (b) according to IPSS category, (c) patients with RA according to IPSS category, (d) according to chromosomal karyotype, (e) according to WHO 2001 classification, (f) according to WHO 2008 classification, (g) according to WPSS category. In intergroup comparison of WT1 mRNA expression, the Tukey–Kramer HSD test was performed using log-transformed values of WT1 mRNA expression with a level of significance of $p < 0.05$. Bold lines represent mean WT1 mRNA expression after log transformation. Fine lines represent lower limit of detection of WT1 mRNA (50 copies/μg RNA).

Analytical results based on 2001 WHO classification

WT1 mRNA expression in PB and BM for each MDS disease stage based on 2001 WHO classification

Figure 3(e) shows the assay results for WT1 mRNA expression in PB and BM in 98 patients in various MDS disease stages categorized on the basis of the 2001 WHO classification. The WT1 mRNA expression levels in both PB and BM tended to increase with the progression to each MDS subtype. When the levels of WT1 mRNA expression in each disease stage were investigated, significant differences ($p < 0.05$) were found in PB between RA and refractory cytopenia with multilineage dysplasia (RCMD), RA and RAEB-1, RA and RAEB-2, RARS and RAEB-2, RCMD and RAEB-2, RCMD with ringed sideroblasts (RCMD-RS) and RAEB-2, RAEB-2 and unclassified MDS (MDS-U), and RAEB-2 and 5q− syndrome; in BM, significant differences were found between RA and RAEB-2, RCMD and RAEB-2, and RCMD-RS and RAEB-2.

Correlation between WT1 mRNA expression and percentage of blasts in BM based on 2001 WHO Classification

The correlation between the blast ratio and WT1 mRNA expression in PB and BM was investigated in 97 patients with MDS (excluding one patient in whom the blast ratio could not be measured). The correlations between the blast ratio and WT1 mRNA expression were $r = 0.50$ in PB and $r = 0.46$ in BM.

Analytical results based on 2008 WHO classification

WT1 mRNA expression in PB and BM for each MDS disease stage based on 2008 WHO classification

Figure 3(f) shows the assay results for WT1 mRNA expression in PB and BM in a total of 97 patients in various MDS disease stages categorized on the basis of the 2008 WHO classification. WT1 mRNA expression in both PB and BM tended to increase with the progression to each MDS subtype.

When the distribution of WT1 mRNA expression for each disease stage was examined, significant differences ($p < 0.05$) were found in PB between refractory cytopenia with unilineage dysplasia (RCUD) and RCMD, RCUD and RAEB-1, RCUD and RAEB-2, RARS and RAEB-2, RCMD and RAEB-1, RCMD and RAEB-2, RAEB-1 and MDS-U, and RAEB-2 and MDS-U; in BM, significant differences were found between RCUD and RAEB-1, RCUD and RAEB-2, and RCMD and RAEB-2.

Correlation between WT1 mRNA expression and percentage of blasts in BM based on 2008 WHO classification

The correlations between blast ratio and WT1 mRNA expression in 96 patients (excluding one patient with MDS whose blast ratio could not be measured) were $r = 0.50$ in PB and $r = 0.46$ in BM.

WT1 mRNA expression in PB and BM for each WPSS risk group

WT1 mRNA expression in PB and BM was compared in 98 patients with MDS classified according to WPSS risk group [Figure 3(g)]. As the risk increased from very low to very high, WT1 mRNA expression in both PB and BM also tended to rise. When the distribution of WT1 mRNA for each risk group was evaluated, significant differences ($p < 0.05$) were found in both PB and BM between very low and high, very low and very high, low and high, low and very high, intermediate and high, and intermediate and very high. Moreover, when the correlation between the WPSS score and WT1 mRNA expression was investigated, the values were $r = 0.61$ in PB and $r = 0.55$ in BM.

Differential diagnosis between RA and AA

Differential diagnosis based on WT1 mRNA expression in PB samples

The WT1 mRNA expression level in PB was less than 50 copies/μg RNA in all eight patients with AA, whereas it was less than 50 copies/μg RNA in 34 patients with RA and 50–52 100 copies/μg RNA in 35 of 69 patients with RA. The statistical analysis by Wilcoxon rank-sum test revealed a statistical difference between eight patients with AA and 65 patients with RA ($p = 0.01$). Sixty-nine patients with RA were further categorized into three groups by bone marrow findings: hypoplastic RA ($n = 20$), hyperplastic RA ($n = 15$) and normoplastic RA ($n = 30$), excluding the non-categorized RA ($n = 4$). Significant differences were observed between AA and each of hypoplastic ($p = 0.04$) or normoplastic RA ($p = 0.02$), whereas no difference was shown between the AA and hyperplastic RA group ($p = 0.10$) by Steel test (Figure 4). From these findings, a differential diagnostic cut-off value between RA and AA of 50 copies/μg RNA for WT1 mRNA expression in PB is considered appropriate, for which the sensitivity was 50.7% (35/69) and the specificity was 100% (8/8).

Differential diagnosis based on WT1 mRNA expression in BM samples

The WT1 mRNA expression level in BM was 251–2600 copies/μg RNA in eight patients with AA, whereas it was less than 50 copies/μg RNA in one of 69 patients with RA and 69–196 000 copies/μg RNA in the others. The statistical analysis by Wilcoxon rank-sum test revealed no statistical difference between eight patients with AA and 65 patients with RA. Sixty-nine patients with RA were similarly categorized into three groups: hypoplastic, hyperplastic and normoplastic RA, excluding the non-categorized RA. Statistical analysis by Steel test revealed a significant difference between AA and normoplastic RA groups ($p = 0.04$), whereas there were no significant differences between the AA and each of hypoplastic RA and hyperplastic RA groups (Figure 4).

When receiver operating characteristic (ROC) analysis was performed to evaluate the performance of BM WT1 mRNA expression as an indicator to differentiate between RA and AA, the area under the curve was 0.713, and the Youden index [18] showed 432 copies/μg RNA. Moreover, the sensitivity was 69.6% (48/69), and the specificity was 75.0% (6/8) (Supplementary Figure to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.745074).

When the PB cut-off value of 50 copies/μg RNA was inserted into the regression line formula obtained