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Prognostic significance of WT1 mRNA and anti-WT1 antibody levels in peripheral blood in patients with myelodysplastic syndromes

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ABSTRACT

Wilms tumor gene (WT1) mRNA expression in peripheral blood cells was examined in 80 patients with myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) transformed from MDS. Serum anti-WT1 antibody titers were also determined in 45 patients. Their long-term follow-up showed that the survival rate became worse as the WT1 mRNA level increased. In particular, a high WT1 mRNA level was a strong predictor of a short time to AML transformation even if adjusted by the International Prognostic Scoring System category. Moreover, high values of anti-WT1 antibody were an independent predictor of longer survival. These data may justify therapeutic strategies targeting WT1 molecules in MDS.

1. Introduction

The Wilms tumor gene WT1, located on chromosome 11p13, was originally identified as a tumor suppressor gene responsible for Wilms tumor, a pediatric kidney cancer. WT1 mRNA is overexpressed in tumor cells from various solid cancers as well as hematologic malignancies including acute myeloid leukemia (AML), acute lymphocytic leukemia, chronic myeloid leukemia (CML), and myelodysplastic syndromes (MDS) [1–3]. It was reported that WT1 mRNA monitoring in peripheral blood (PB) and bone marrow (BM) is useful for estimating minimal residual disease and predicting relapse in leukemias [4–6]. Furthermore, recent data have suggested that the anti-WT1 immune response elicited by WT1 peptide vaccine may induce tumor regression in some patients [7].

MDS are clonal hematologic disorders characterized by cytopenias and a risk of progression to AML. The prognosis of MDS is generally poor but shows significant heterogeneity among patients. The International Prognostic Scoring System (IPSS) has been the most widely applied system for prognostication [8], and the World Health Organization (WHO) Classification-Based Prognostic Scoring System and other systems have been proposed for further improvement of prognostication [9,10]. Meanwhile, the recent introduction of new therapeutic agents has shown promise in treating MDS. One of these agents, lenalidomide, has a variety of immunomodulatory functions and suppresses or eradicates MDS clones in a substantial proportion of patients [11,12]. Therefore, it is speculated that an appropriate immune response may modulate prognosis in MDS.

We reported previously that WT1 mRNA expression in PB cells as well as BM cells increased in MDS patients compared with normal individuals and that a humoral immune response, IgG-type or IgM-type anti-WT1 antibody (Ab), was detected in sera from most MDS patients [13,14]. In this study, we investigated whether WT1 mRNA expression and anti-WT1 Ab titers in PB, as a marker of MDS disease stage and a marker of anti-MDS immune response, respectively, are associated with prognosis in MDS patients by examining their long-term follow-up data.

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Table 1
Patient characteristics.

Sex (M/F)	53/27
Age, median (range)	70 (28–91)
FAB subtype	
RA	35
RARS	5
RAEB	24
RAEB-t	5
AML-MDS	11
IPSS[a]	
Low	13
Intermediate-1	34
Intermediate-2	8
High	14

Data are number of patients except for years for age.
[a] AML-MDS patients were excluded.

2. Patients and methods

2.1. Patients

PB samples were obtained from 80 patients with MDS or AML transformed from MDS (AML-MDS) after obtaining written informed consent. Their diagnoses were 35 refractory anemia (RA), 5 RA with ringed sideroblasts (RARS), 24 RA with excess blasts (RAEB), 5 RAEB in transformation (RAEB-t), and 11 AML-MDS in the French-American-British (FAB) classification [15]. The WHO classification and IPSS were applied according to previous reports [8,16]. The clinical characteristics of the patients are summarized in Table 1. PB mononuclear cells (PBMCs) were isolated and used immediately for the WT1 mRNA assay, while sera were stored at −20 °C until use. During the follow-up period, 13 MDS patients transformed to AML.

2.2. Real-time quantitative polymerase chain reaction

RNA was isolated from PBMCs and converted into cDNA. The levels of WT1 mRNA expression were assessed using the real-time quantitative polymerase chain reaction (PCR), as described previously [2,17]. The lowest detection limit of our WT1 mRNA assay was 50 copies/μg RNA [18].

2.3. Detection of WT1 Abs in PB

The dot-blot assay was used to detect IgG and IgM Abs against WT1, as reported previously [14]. Briefly, truncated WT1 protein was bound on a nitrocellulose membrane (Optitran, Schleicher & Schuell, Dassel, Germany), and the membrane was loaded onto a dot-blot apparatus (Schleicher & Schuell). The sera for measuring anti-WT1 antibodies, which had been diluted with phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.1% Tween 20, were applied to wells and incubated for 1 h. After washing with PBS, the membrane was reacted with horseradish peroxidase-conjugated goat anti-human IgM Ab (ICN Pharmaceuticals, Cleveland, OH, USA) or rabbit anti-human IgG Ab (ICN Pharmaceuticals). After intensive washing with PBS, the membrane was incubated with the substrate solution Renaissance (NEN Life Science Products, Boston, MA, USA) for 1 min and exposed to Hyperfilm MP (Amersham Pharmacia Biotech, Buckinghamshire, UK). Densities of dot-blots were measured as densitometric units with a computerized scanning analyzer system (Molecular Dynamics, Sunnyvale, CA, USA).

2.4. Statistical analysis

We analyzed whether the levels of WT1 mRNA expression in PBMCs or serum WT1 Abs were associated with clinical characteristics (hemoglobin value, white blood cell count, neutrophils, lymphocytes, blast percentages in PB, IPSS score, and MDS subtype in the FAB and WHO classifications), survival, and time of freedom from AML transformation. The median survival of the present cohort was 55.9 months. Differences between two groups of data of continuous variables were analyzed using Student's *t*-test. Differences in categorical variables were evaluated using the chi-square test. Correlations between two parameters were analyzed with Spearman's test. The patients were divided into two or more groups using various cut-off points for WT1 mRNA levels or anti-WT1 Ab levels to examine whether these levels were associated with prognosis. The times of survival and freedom from AML transformation were estimated using the Kaplan–Meier method and compared in the log-rank test. Multivariate Cox proportional hazard regression models were used to determine significant predictors of survival and AML transformation. A *P*-value of less than 0.05 was regarded as representing a statistically significant difference.

Table 2
Prognostic value of WT1 mRNA expression and IPSS in 69 MDS patients.

Variables	Survival		Freedom from AML	
	Univariate analysis	Multivariate analysis	Univariate analysis	Multivariate analysis
IPSS	<0.0001	0.0009	<0.0001	0.0037
WT1 mRNA[a]	0.0186	NS[b]	<0.0001	0.0005

Data are *P*-values.
[a] Data divided into three groups as shown in Fig. 2 were analyzed.
[b] Not significant.

3. Results

3.1. Expression of WT1 mRNA in PB in MDS

As observed in samples from patients with acute leukemias [5], WT1 mRNA expression was detectable in PB samples from MDS patients even if they did not contain blasts in 200 leukocytes examined microscopically (Supplementary Figure). The WT1 mRNA level was not correlated with hemoglobin value or leukocyte, neutrophil, lymphocyte, and platelet counts. WT1 mRNA levels increased in accordance with the aggressiveness of disease subtype in MDS, particularly when the patients were diagnosed using the FAB classification (Fig. 1a). When a high level of WT1 mRNA expression was defined as more than 50 copies/μg mRNA, which most normal samples did not exceed [18], a high level was observed in 15 of 35 (42.9%), 2 of 5 (40%), 20 of 24 (83.3%), 4 of 5 (80.0%), and 11 of 11 (100%) patients in the RA, RARS, RAEB, RAEB-t, and AML-MDS categories in the FAB classification, respectively. Furthermore, WT1 mRNA levels increased in accordance with the aggressiveness of the IPSS category (Fig. 1c).

In four patients, we were able to analyze WT1 mRNA levels sequentially at diagnosis and at disease progression to AML. WT1 levels increased with the AML transformation in two of the four patients, one with an initial diagnosis of RARS (the WT1 mRNA level increased from 570 to 67,000 copies/μg) and the other with an initial diagnosis of RAEB (from 320 to 1900 copies/μg). The data of the remaining two patients did not change significantly after AML transformation.

3.2. Prognostic significance of WT1 expression

When the patients were divided into three groups based on the WT1 mRNA level (less than 10^2 copies/μg, 10^2 to 10^4 copies/μg, and more than 10^4 copies/μg), their survival rates differed significantly: survival worsened in accordance with the increase in the WT1 mRNA level (Fig. 2a). In addition to the WT1 mRNA level, the IPSS category had predictive power for survival in univariate analysis, as expected (Table 2). However, when these two prognostic parameters were analyzed using multivariate analysis, only the IPSS category had independent prognostic power (Table 2). When examining the time of freedom from AML transformation, both the WT1 mRNA level and IPSS category were prognostic factors in both univariate and multivariate analyses (Fig. 2b and Table 2). A high WT1 mRNA level was a strong predictor of a short time to AML transformation.

3.3. Clinical significance of WT1 Abs

We analyzed whether the level of WT1 Abs in sera was associated with patient characteristics and survival. The IgM and IgG Abs to WT1 could be analyzed in 45 of the 80 patients: 15 RA, 3 RARS, 18 RAEB, 3 RAEB-t, and 6 AML-MDS patients (Fig. 3). In our previous study using the same assay, 16% and 5% of 43 healthy volunteers had detectable IgM and IgG WT1 Abs in their sera, respectively [14].

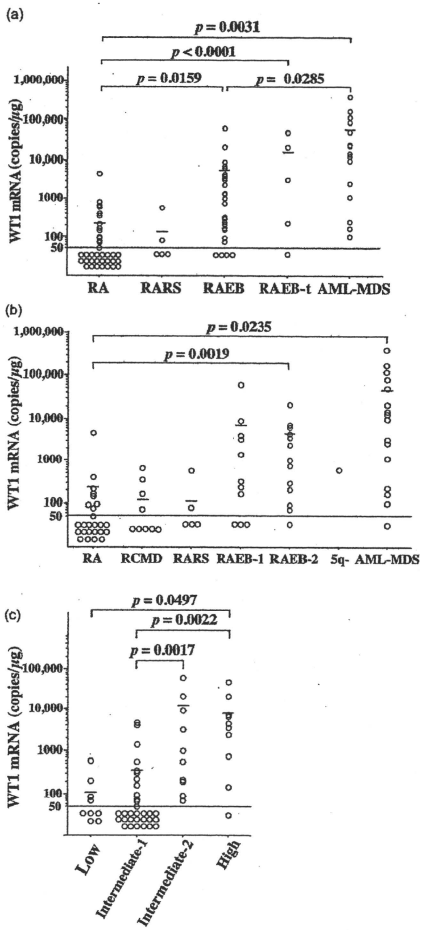

Fig. 1. WT1 mRNA expression levels in PB from patients with different MDS subtypes and AML-MDS according to the FAB classification (a) and WHO classification (b), and IPSS category (c). The horizontal bars accompanying each group of data indicate mean values. RCMD, refractory cytopenia with multilineage dysplasia; 5q-, MDS associated with isolated del(5q).

In this study, IgM and IgG WT1 Abs were detected in 31 (79.5%) and 34 (87.2%) MDS patients, and 5 (83.3%) and 6 (100%) AML-MDS patients, respectively. The WT1 Abs levels were not related to the FAB subtype, IPSS category, or WT1 mRNA expression in PBMCs, with the exception that AML-MDS patients had higher IgG WT1 Ab levels compared with RA patients ($P = 0.0292$) and with RAEB patients ($P = 0.0149$). Since the number of AML-MDS patients was small, this finding should be examined in a larger study. IgM and IgG WT1 Abs were positively correlated with the platelet count with

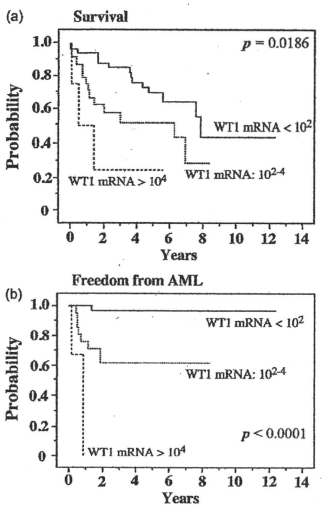

Fig. 2. Survival (a) and time of freedom from AML transformation (b) according to the WT1 mRNA expression level in PB in 69 MDS patients. Data from AML-MDS patients were excluded. The patients were divided into three groups according to the WT1 mRNA expression level: less than 10^2 copies/μg ($n = 37$), 10^2 to 10^4 copies/μg ($n = 28$), and more than 10^4 copies/μg ($n = 4$). The median survival times of these groups were 62.7, 29.9, and 11.6 months, respectively.

and without statistical significance ($P = 0.0043$ and 0.0698, respectively) but not with hemoglobin value or leukocyte, neutrophil, and lymphocyte counts.

Patients with high values of IgM WT1 Ab, defined as more than 1000 densitometric units, had significantly better survival compared with other patients ($P = 0.0207$, Fig. 4a). Similarly, patients with high values of IgG WT1 Ab, defined as more than 1200 densitometric units, had significantly better survival compared with other patients ($P = 0.0352$, Fig. 4b). When we combined these data, it was found that patients with high values of either IgM or IgG WT1 Ab had significantly better survival than patients whose IgM and IgG WT1 Abs values were both low ($P = 0.0007$, Fig. 4c). The prognostic power of high IgM or IgG WT1 Ab value was independent even if adjusted by the IPSS category ($P = 0.0019$, Table 3).

4. Discussion

In normal hematopoiesis, WT1 mRNA expression is observed in $CD34^+$ cells, decreases during differentiation, and becomes undetectable in mature cells [19]. However, in MDS, in which hematopoiesis is composed of predominantly clonal MDS cells with various degrees of differentiation and residual normal cells, higher WT1 mRNA expression is observed in $CD34^+$ cells as well as in more

Table 3
Prognostic value of anti-WT1 antibody titers and IPSS in 39 MDS patients.

Variables	Univariate analysis	Multivariate analysis
IPSS	0.0286	0.0564
WT1 antibody[a]	0.0007	0.0019

Data are *P*-values.
[a] Data divided into two groups as shown in Fig. 4c were analyzed.

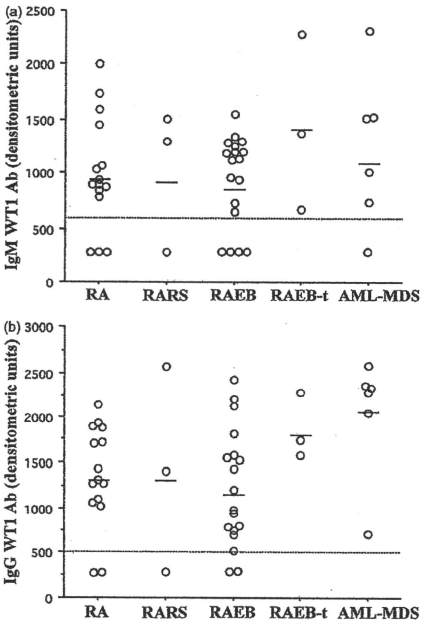

Fig. 3. Anti-WT1 Ab levels in sera from patients with different MDS subtypes and AML-MDS according to the FAB classification: (a) IgM-type Ab and (b) IgG-type Ab. The horizontal bars accompanying each group of data indicate mean values. The horizontal lines indicate the lowest detection limits of Abs.

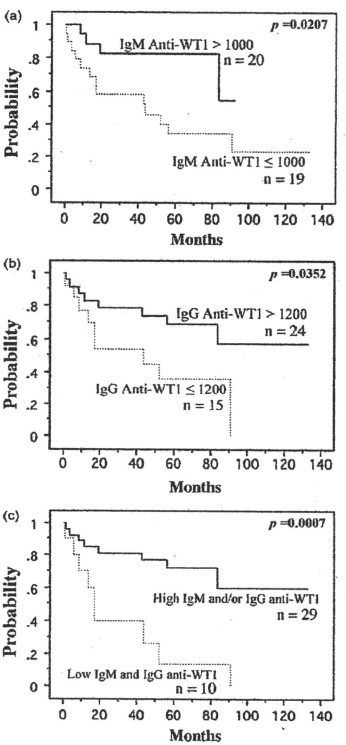

Fig. 4. Survival according to the level of WT1 Abs in 39 MDS patients. Data from AML-MDS patients were excluded. (a) The patients were divided into two groups according to the IgM-type WT1 Ab value: 1000 units or less and more than 1000 units. (b) The patients were divided into two groups according to the IgG-type WT1 Ab value: 1200 units or less and more than 1200 units. (c) The patients were divided into two groups according to the values of both antibodies: the IgM-type Ab more than 1000 units and/or IgG-type Ab more than 1200 units and other.

mature cells compared with healthy individuals [20]. Furthermore, it was confirmed that the high WT1 mRNA expression in MDS is due to MDS clonal cells rather than residual normal cells [21]. Because high WT1 expression blocks cell differentiation in hematopoietic progenitor cells [22,23], this may be implicated in dysregulated hematopoiesis in MDS. Our study demonstrated that the expression of WT1 mRNA in PBMCs was detected in approximately 60% of patients with MDS and in approximately 40% even in patients with low-grade MDS, RA, and RARS. Cilloni et al. reported that WT1 mRNA levels in BM were correlated with the IPSS category in MDS [24]. Here, we showed that WT1 mRNA levels in a more convenient source, PB, were correlated with the IPSS category as well as the FAB subtype and that this was a strong predictor of AML transformation in MDS.

In our previous studies, the humoral immune response as evidenced by IgG-type and IgM-type WT1 Abs were detected in sera in various hematologic malignancies in which tumor cells express high levels of WT1 mRNA, i.e., AML, CML, and MDS [14]. Furthermore, data on the IgG subclass of anti-WT1 IgG Ab in these diseases indicated that the anti-WT1 humoral immune response was biased toward the Th1 type [25]. Meanwhile, quite recently, we have reported that the elevation of WT1 Ab titers was significantly associated with long disease-free survival in non-small cell lung cancer, a disease that shows high WT1 mRNA expression [26]. Here, we showed that patients with high WT1 Ab titers were associated with longer survival compared with other MDS patients.

Our data showed for the first time that high WT1 mRNA expression and high WT1 Ab titers in PB affect the prognosis of MDS patients negatively and positively, respectively. Although these interesting findings should be confirmed in a larger study, the data suggest that an optimal immune response against WT1 may be beneficial for MDS patients. Recently, clinical trials of WT1 peptide-based immunotherapy have been conducted in various malignancies including MDS [7,27–29]. We treated 1 MDS and 1 AML-MDS patient in such a trial and they showed a WT1-specific cytotoxic T-cell response (CTL), decrease in MDS blasts, and severe leukopenia requiring intensive supportive care [7,30]. In two other vaccine trials with WT1 such hematotoxic side effects in MDS patients have not been observed [27,28]. Interestingly, in one of these studies, the emergence of WT1-specific CTL was associated with a 2-log or more reduction in WT1 mRNA expression in both MDS patients treated [27]. These data from clinical trials suggest that WT1-specific immunity can attack MDS clonal cells.

Our data presented here may provide a rationale for anti-WT-1 immunotherapy in MDS.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2009.11.029.

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Biased usage of BV gene families of T-cell receptors of WT1 (Wilms' tumor gene)-specific CD8$^+$ T cells in patients with myeloid malignancies

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WT1 (Wilms' tumor gene 1) protein is a potent pan-tumor-associated antigen (TAA) and WT1-specific cytotoxic T lymphocytes (WT1 tetramer$^+$ CD8$^+$ T cells) are spontaneously induced in patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). We conducted a single-cell level comparative analysis of T-cell receptor β-chain variable region (TCR-BV) gene families of a total of 1242 spontaneously induced WT1 tetramer$^+$ CD8$^+$ T cells in HLA-A*2402$^+$ patients with AML or MDS and those in healthy donors (HDs). This is the first report of direct usage analysis of TCR-BV gene families of individual TAA-specific CD8$^+$ T cells at single-cell level. Usage analysis using single-cell RT-PCR of TCR-BV gene families of individual FACS-sorted WT1 tetramer$^+$ CD8$^+$ T cells showed for the first time (i) that BVs 5, 6, 20, and 27 were commonly biased in both HDs and patients; (ii) that BV4 was commonly biased in HDs and MDS patients; (iii) that BV19 was commonly biased in the patients; and (iv) that BVs 7 and 28, BVs 9 and 15, and BVs 12 and 29 were specifically biased in HDs, AML, and MDS patients, respectively. However, statistical analysis of similarity among HD, AML, and MDS of individual usage frequencies of 24 kinds of TCR-BV gene families indicated that the usage frequencies of TCR-BV gene families in AML and MDS patients reflect those in HDs. These findings represent a novel insight for a better understanding of WT1-specific immune response. (*Cancer Sci* 2010; 101: 594–600)

Wilms' tumor gene *WT1*, which was originally isolated as a gene responsible for a pediatric neoplasm, Wilms' tumor, encodes a zinc finger transcription factor that is essentially involved in the regulation of cell proliferation and differentiation.[1–4] Although the *WT1* gene was first categorized as a tumor-suppressor gene, we proposed, based on accumulated experimental evidence, that the wild-type *WT1* gene played an oncogenic rather than a tumor-suppressor function in leukemogenesis and tumorigenesis. In fact, overexpression of the wild-type *WT1* gene was observed in acute myeloid leukemia (AML), acute lymphoblastic leukemia, chronic myelogenous leukemia, myelodysplastic syndrome (MDS), and various kinds of solid tumors.[5–7]

Mice immunized with WT1 peptide or *WT1* cDNA rejected challenges by *WT1*-expressing tumor cells and survived with no signs of auto-aggression against normal organs that physiologically expressed *WT1*.[8,9] In humans, WT1-specific cytotoxic T lymphocytes (CTLs) that could specifically lyse *WT1*-expressing tumor cells with an HLA class I restriction were generated *in vitro*[10–12] and detected in patients with hematopoietic malignancies. Furthermore, WT1 antibodies were detected at higher frequencies and titers in patients with hematopoietic malignancies than in healthy donors.[13,14] These findings indicated that the WT1 protein was highly immunogenic and could be a promising target antigen for cancer immunotherapy, that is a tumor-associated antigen (TAA). In fact, we and others performed clinical studies of WT1 peptide vaccination for cancer patients and reported induction of WT1 peptide-specific immunological responses such as an increase in frequencies of WT1 tetramer$^+$ CD8$^+$ T cells in peripheral blood (PB) and resultant clinical responses, including a decrease in leukemic blast cells and reduction in solid tumor size.[15,16] These results prompted us to analyze WT1-specific CTL responses in detail in the hope that this would be very useful for further elucidation of TAA-specific immune responses in cancer patients.

In order to analyze TAA-specific CTL responses, it is important to examine both their quantitative aspects, such as frequencies of TAA tetramer$^+$ CD8$^+$ T cells, and their qualitative aspects, such as clonality. One of the qualitative analyses involves defining the clonality of TAA tetramer$^+$ CD8$^+$ T cells. So far, several studies about T-cell receptor (TCR) usage of TAA-specific T cells have used TCR β-chain variable region (TCR-BV) gene family-specific monoclonal antibodies (mAbs) or PCR with primers directed for the BV gene families.[17–27] As for TCR usage in HLA-A2$^+$ melanoma patients, some investigations reported that melanoma-associated antigen (Melan-A)-specific CD8$^+$ T cells in PB or tumor sites showed selected usage of TCR-BV gene families, such as BV14,[17,20,23,24] while others reported no such selected usage.[25–27] Overall, knowledge concerning the TCR usage of TAA-specific T cells remains limited, although the occurrence of very selected TCR usage of viral antigen-specific T cells is well known.[28,29]

In the study reported here, we conducted a single-cell level comparative analysis of the usage of TCR-BV gene families of individual FACS-sorted WT1 tetramer$^+$ CD8$^+$ T cells in HLA-A*2402$^+$ patients with AML or MDS and in healthy donors (HDs), and describe biased usage of the TCR-BV gene families in the patients, which probably reflects biased usage in HDs.

Materials and Methods

PB samples from patients and healthy donors. After informed consent was obtained, PB samples were obtained from four AML patients (AML-1, -2, -3, and -4), four MDS patients (MDS-1, -2, -3, and -4), and five HDs. A summary of patients' clinical data and healthy donors' information is presented in Table 1. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Ficoll-Hypaque

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Table 1. Patients' and healthy donors' characteristics

	ID	Gender	Age	Disease	Prior therapy	Disease status	Residual disease detected by RT-PCR
Patients	AML-1	F	55	AML without maturation	Chemotherapy, WT1 vaccination	2nd CR	(High levels of WT1)
	AML-2	F	66	AML with maturation	Chemotherapy	1st CR	(High levels of WT1)
	AML-3	M	64	AML with *t*(8; 21)(q22; q22)	Chemotherapy	2nd CR	(Positivity of AML1/ETO)
	AML-4	F	45	AML with *t*(15; 17)(q22; q12)	Chemotherapy	2nd CR	(Positivity of PML/RARα)
	MDS-1	M	62	Secondary MDS, chronic myelomonocytic leukemia	Chemotherapy for prior AML	–	(High levels of WT1)
	MDS-2	M	66	MDS, refractory anemia with excess blasts-2	Best supportive care	–	(High levels of WT1)
	MDS-3	M	69	AML transformed from MDS	Chemotherapy	1st CR	(High levels of WT1)
	MDS-4	M	58	MDS, refractory anemia with excess blasts-2	Best supportive care	–	(High levels of WT1)
Healthy donors	HD-1	F	23				
	HD-2	M	24				
	HD-3	M	30				
	HD-4	F	25				
	HD-5	F	26				

AML, acute myeloid leukemia; CR, complete remission; ETO, eight twenty one; HD, healthy donor; MDS, myelodysplastic syndrome; PML, promyelocytic leukemia; RARα, retinoic acid receptor-α; WT1, Wilms' tumor gene 1.

(Pharmacia, Uppsala, Sweden) and then frozen in liquid nitrogen until use.

Flow cytometric analysis and single-cell sorting of WT1 tetramer$^+$ CD8$^+$ T cells. PBMCs were stained with PE-labeled HLA-A*2402-WT1$_{235}$ tetramer (MBL, Tokyo, Japan) at 37°C for 30 min and then with a panel of fluorescence-labeled monoclonal antibodies (mAbs) for 25 min on ice in the dark, and were finally FACS analyzed after washing with FACS buffer (phosphate-buffered saline containing 2% FBS). For this staining procedure, the following mAbs were used: anti-CD4-, CD14-, CD34-FITC, anti-CD3-PerCP (BD Bioscience, San Jose, CA, USA), anti-CD56-FITC (eBioscience, San Diego, CA, USA), anti-CD16-, CD19-, CD33-FITC, anti-CD8-APC-Cy7, anti-CD45RA-APC, and anti-CCR7-PE-Cy7 (BD Pharmingen, San Diego, CA). In this study, lineage antigens (CD4, CD14, CD16, CD19, CD33, CD34, and CD56)-negative, CD3-, CD8-, and

Table 2. Sequences of primers for PCR amplification

	2nd Mix	TRBV gene names†	Forward primer sequence (5′–3′)
PCR forward primers (TCR-BV gene family -specific primers)	S1	TRBV9	ACAGCAAGTGAC<TAG>CTGAGATGCTC
		TRBV5	
		TRBV25	GATCACTCTGGAATGTTCTCAAACC
		TRBV10	CCAAGACACAAGGTCACAGAGACA
	S2	TRBV20	GAGTGCCGTTCCCTGGACTTTCAG
		TRBV28	GTAACCCAGAGCTCGAGATATCTA
		TRBV2	GGTCACACAGATGGGACAGGAAGT
	S3	TRBV29	TCCAGTGTCAAGTCGATAGCCAAGTC
		TRBV7	ATGTAACT<CT>TCAGGTGTGATCCAA
		TRBV27	GTGACCCAGAACCCAAGATACCTC
	S4	TRBV7	GTGTGATCCAATTTCAGGTCATAC
		TRBV12	GGTGACAGAGATGGGACAAGAAGT
		TRBV11	CAGTCTCCCAGATATAAGATTATAGAG
	S5	TRBV19	CACTCAGTCCCCAAAGTACCTGTT
		TRBV30	GTCAGATCTCAGACTATTCATCAATGG
		TRBV4	TACGCAGACACCAA<GA>ACACCTGGTCA
		TRBV3	CCCAGACTCCAAAATACCTGGTCA
		TRBV18	TGCAGAACCCAAGACACCTGGTCA
	S6	TRBV21	AAGGTCACCCAGAGACCTAGACTT
		TRBV14	ATAGAAGCTGGAGTTACTCAGTTC
		TRBV23	ACAAAGATGGATTGTACCCCCGAA
	S7	TRBV6	GTGTCACTCAGACCCCAAAATTCC
		TRBV24	GTTACCCAGACCCCAAGGAATAGG
	S8	TRBV13	CTGATCAAAGAAAAGAGGGAAACAGCC
		TRBV15	CAAGATACCAGGTTACCCAGTTTG
Reverse primer sequence (5′–3′)	1st-PCR (3′BC)		GCTTCTGATGGCTCAAACACAGC
	Screening and specifying-PCR (5′BC)		GGAACACGTTTTTCAGGTCCT

†TCR-BV gene names are in accordance with IMGT unique gene nomenclatures. < >, mixture of nucleotides at this position.

HLA-A*2402-WT1$_{235}$ tetramer (WT1 tetramer)-positive lymphocytes were defined as WT1 tetramer$^+$ CD8$^+$ T cells. WT1 tetramer$^+$ CD8$^+$ T cells were single-cell sorted by FACSAria (BD Biosciences) and data analysis was performed with FACS Diva software (BD Biosciences).

cDNA synthesis from a single WT1 tetramer$^+$ CD8$^+$ T cell. WT1 tetramer$^+$ CD8$^+$ T cells were directly single cell-sorted into PCR tubes with 15 μL of a cDNA reaction mix containing lysis buffer (1 × cDNA buffer with 0.5% Triton X-100) with reverse transcriptase (SuperScript III; Invitrogen, Carlsbad, CA, USA), 0.5 mM dNTPs (Invitrogen), 20 U RNase inhibitor (Invitrogen), 100 μg/mL gelatin (Roche, Indianapolis, IN, USA), 100 μg/mL tRNA (Roche), and 200 nM TCR-β chain constant region (BC)-specific primer (5′-CACCAGTGTGGCCTTTTG-3′). The sorted samples were incubated in the cDNA reaction mix at 50°C for 90 min for cDNA synthesis, followed by further incubation at 95°C for 5 min to stop the reaction.

Semi-nested multiple PCR reaction of a single WT1 tetramer$^+$ CD8$^+$ T cell for identification of BV gene family. For the first PCR, 10 μL of synthesized cDNA products was added to 40 μL of a reaction mix containing 1 × PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 1.25 U DNA polymerase (Platinum Taq DNA Polymerase; Invitrogen), 5 nM of 24 kinds of BV gene family-specific forward primers (IMGT human TCR gene database site: http://imgt.cines.fr), and 5 nM 3′BC reverse primer (Table 2). The forward primers were synthesized as previously reported.[30] The PCR procedure for this step comprised a pre-PCR heating step at 95°C for 2 min and 40 cycles at 94°C for 45 s (denaturation), at 57°C for 45 s (annealing), and at 72°C for 50 s (extension). For the screening of the BV gene family, the first PCR product was subjected to second-round screening PCR. One μL of the first PCR products was added to eight separate tubes, each of which was filled with 24 μL of a reaction mix containing 1 × PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 U Taq polymerase, 150 nM each of eight kinds of screening sets of BV gene family-specific forward primers (S1–S8, Table 2), and 150 nM 5′BC reverse primer (Table 2). The PCR procedure for this step consisted of a pre-PCR heating step at 95°C for 2 min and 35 cycles at 94°C for 45 s, at 57°C for 45 s, and at 72°C for 40 s. Five μL of each screening-PCR product was run on 2% agarose gel to identify the positive reaction among the eight kinds of screening sets. Next, to identify the BV gene family, the second-round specifying PCR was performed by using individual 150 nM BV gene family-specific forward primers that were contained in the positive screening set. Finally, BV gene families were directly identified by their positive bands (approximately 300–350 bp) with electrophoresis of the samples on 2% agarose gel. For negative control, three tubes without sorted cells were prepared and subjected to the same RT-PCR procedures as those described above.

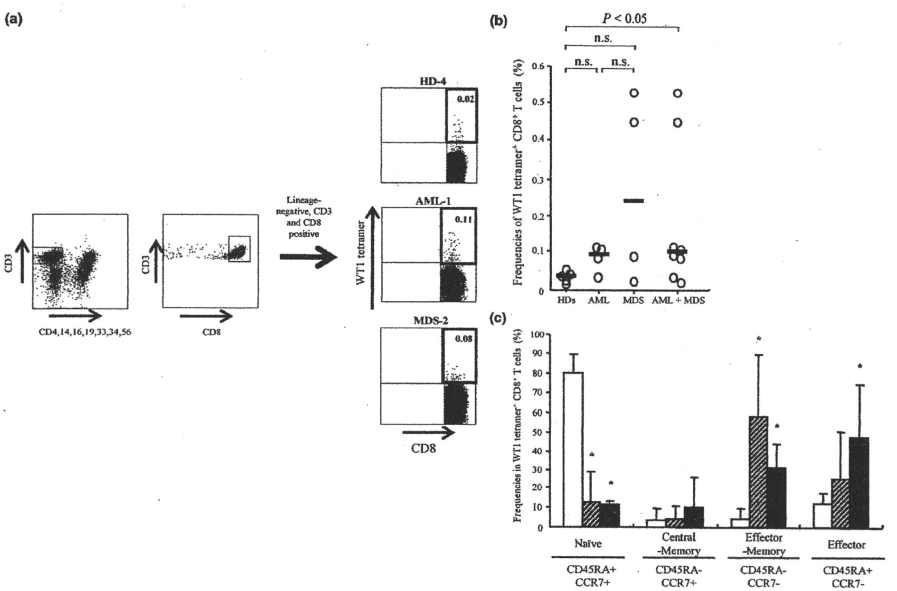

Fig. 1. Increase in WT1 (Wilms' tumor gene 1) tetramer$^+$ CD8$^+$ T cells with more maturated phenotypes in patients. (a) Representative profiles of flow cytometric analysis using WT1 tetramer. The numbers at the upper-right corners in dot plots of lineage antigens CD4, 14, 16, 19, 33, 34 and 56-negative and CD3-, CD8-positive gate represent the ratio of WT1 tetramer$^+$ CD8$^+$ T cells to total CD8$^+$ T cells. (b) Ratios of WT1 tetramer$^+$ CD8$^+$ T cells to total CD8$^+$ T cells in peripheral blood mononuclear cells (PBMC)s of healthy donors (HDs) (n = 5) and acute myeloid leukemia (AML) (n = 4), and myelodysplastic syndrome (MDS) (n = 4) patients. Bars represent medians; n.s., not significant. (c) Frequencies of four differentiation subsets of WT1 tetramer$^+$ CD8$^+$ T cells. White, hatched and black columns represent HDs, AML, and MDS patients, respectively. Statistical significance between HDs and AML, and HDs and MDS is indicated by asterisks (*P < 0.05).

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To distinguish BV5 and BV9 gene families, PCR products identified by 5′-ACAGCAAGTGAC<TAG>CTGAGATGCTC-3′ forward primer were sequenced as described elsewhere,[30] because this forward primer detects both the BV5 and BV9 gene families (Table 2). The two gene families were differentiated by identification of the BV gene family-specific sequence.

In this experiment, we analyzed BV gene family usages of a total of 1242 WT1 tetramer$^+$ CD8$^+$ T cells: 405 cells from five HDs (75, 71, 96, 76, and 87 from HD-1, -2, -3, -4, and -5, respectively), 403 cells from four AML patients (99, 105, 98, and 101 from AML-1, -2, -3, and -4, respectively), and 434 cells from four MDS patients (103, 126, 109, and 96 from MDS-1, -2, -3, and -4, respectively).

Statistical analysis. The nonparametric Mann–Whitney's two-tailed *U*-test was used for the evaluation of the statistical significance of differences in WT1 tetramer$^+$ CD8$^+$ T-cell frequencies or CTL differentiation subsets between HDs and either AML or MDS patients.

Statistical significance of the difference in usage frequencies (%) of the 24 kinds of BV gene families among HDs, AML, and MDS was assessed by one-factor ANOVA or the Kruskal–Wallis test. Usage frequencies of BVs 3, 5, 7, 11, 14, 20, 23, 25, and 27 were compared among HDs, AML, and MDS by one-factor ANOVA, and those of the other BV family usages were compared by using the Kruskal–Wallis test. Furthermore, the Tukey–Kramer *post-hoc* test was used to assess the difference in BV20 gene family usage among HDs, AML, and MDS.

Results

Increase in WT1 tetramer$^+$ CD8$^+$ T cells with effector phenotypes in patients with AML and MDS. For an examination of CTL responses to the WT1 CTL epitope peptide, PBMCs were FACS-analyzed by using the WT1 tetramer (Fig. 1). Figure 1a shows the representative profiles of WT1 tetramer$^+$ CD8$^+$ T cells in PBMCs of a healthy donor and two patients. Frequencies of WT1 tetramer$^+$ CD8$^+$ T cells were 0.03–0.11% for AML patients, 0.02–0.52% for MDS patients, and 0.01–0.05% for HDs, and thus significantly ($P < 0.05$) higher for the patients. However, the difference in frequency between the HDs and either AML or MDS patients was not significant because of the small number of patients (Fig. 1b).

CD45RA and CCR7 expression is used as the basis for the phenotypical subdivision of human CD8$^+$ T cells into naïve (CD45RA$^+$ CCR7$^+$), central-memory (CD45RA$^-$ CCR7$^+$), effector-memory (CD45RA$^-$CCR7$^-$), and terminal effector (CD45RA$^+$ CCR7$^-$) cells.[31] These surface markers were used in our study to analyze the phenotypes of WT1 tetramer$^+$ CD8$^+$ T cells. Frequencies of naïve phenotype-bearing WT1 tetramer$^+$ CD8$^+$ T cells were significantly higher in HDs (80.0 ± 8.4%) than in AML patients (12.5 ± 16.3%, $P < 0.05$) and MDS patients (11.2 ± 1.7%, $P < 0.05$) (Fig. 1c). In contrast, frequencies of effector-memory phenotype-bearing WT1 tetramer$^+$ CD8$^+$ T cells were significantly higher in AML patients (58.4 ± 30.7%, $P < 0.05$) and MDS patients (30.7 ± 13.2%, $P < 0.05$) than in HDs (4.5 ± 4.2%). In addition, frequencies of terminal effector phenotype-bearing WT1 tetramer$^+$ CD8$^+$ T cells were significantly higher in MDS patients (47.9 ± 26.5%, $P < 0.05$) than in HDs (11.9 ± 5.1%).

These results thus demonstrate that WT1 peptide-specific CD8$^+$ T-cell responses were higher in patients than in HDs, and that the WT1 tetramer$^+$ CD8$^+$ T cells featured more differentiated/activated phenotypes in patients than in HDs.

Biased usage of TCR-BV gene families in WT1 tetramer$^+$ CD8$^+$ T cells. TCR-BV gene families were investigated for single-cell-sorted WT1 tetramer$^+$ CD8$^+$ T cells from five HDs, four AML, and four MDS patients. Usage frequencies for a given BV gene family were defined as the ratios of the number of WT1 tetramer$^+$ CD8$^+$ T cells with the usage of the BV gene family to the total number of WT1 tetramer$^+$ CD8$^+$ T cells examined. When the usage frequencies of a given BV gene family were more than the mean values + 1SD for the usage of 24 different kinds of BV gene families, the usage was defined as biased. BV gene family usage is graphically shown in Figure 2.

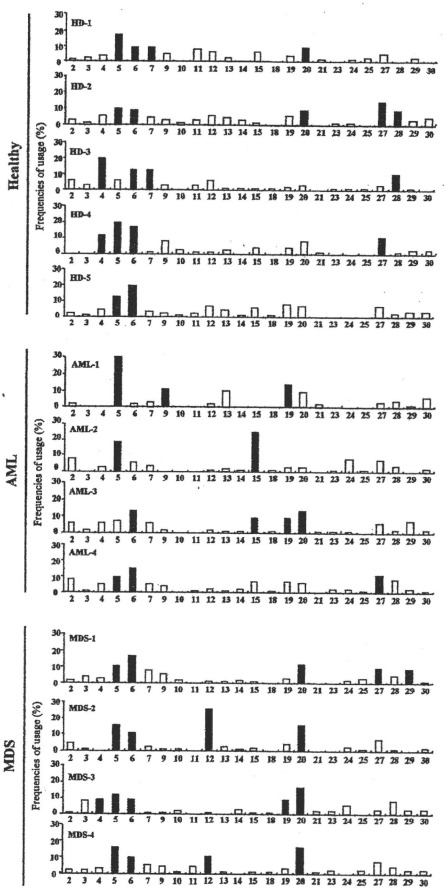

Fig. 2. Usage frequencies of T-cell receptor β-chain variable region (TCR-BV) gene families in WT1 (Wilms' tumor gene 1) tetramer$^+$ CD8$^+$ T cells. Usage frequencies (%) of each member of the BV gene family in WT1 tetramer$^+$ CD8$^+$ T cells of five healthy donors (HDs) and four acute myeloid leukemia (AML) and four myelodysplastic syndrome (MDS) patients. Black and white columns represent the TCR-BV gene families with usage frequencies above and below mean values + 1SD, respectively.

The following biased usages of TCR-BV gene families were observed: BV4, 2/5 in HDs and 1/4 in MDS; BV5, 4/5 in HDs, 3/4 in AML, and 4/4 in MDS; BV6, 5/5 in HDs, 2/4 in AML, and 4/4 in MDS; BV7, 2/5 in HDs; BV9, 1/4 in AML; BV12, 2/4 in MDS; BV15, 2/4 in AML; BV19, 2/4 in AML and 1/4 in MDS; BV20, 2/5 in HDs, 1/4 in AML and 4/4 in MDS; BV27, 2/5 in HDs, 1/4 in AML, and 1/4 in MDS; BV28, 2/5 in HDs; and BV29, 1/5 in MDS. The ratios of the number of HDs and patients with biased usage of TCR-BV gene families in the WT1 tetramer$^+$ CD8$^+$ T cells to the number of HDs and patients examined are graphically represented in Figure 3. These results showed that (i) BVs 5, 6, 20, and 27 were commonly biased in HDs and patients (AML and MDS); (ii) BV4 was commonly biased in both HDs and MDS; (iii) BV19 was commonly biased in the patients regardless of disease types (AML or MDS); and (iv) BVs 7 and 28, BVs 9 and 15, and BVs 12 and 29 were biased in HDs, AML, and MDS, respectively.

Usage frequencies of TCR-BV gene families in AML and MDS reflect those of TCR-BV gene families in HDs. As shown in Figure 4, the statistical significance of usage frequencies of 24 BV gene families was analyzed. In all the BV gene families except BV20, the usage frequencies were not statistically significantly different among HDs and AML and MDS patients, although there were more WT1 tetramer$^+$ CD8$^+$ T cells in AML and MDS than in HDs, and they were more maturated in AML and MDS than in HDs. Thus, usage frequency patterns of BV gene families were similar for HDs, AML, and MDS. BV20 usage alone was significantly ($P < 0.05$) more frequent in MDS than in HDs and AML. These results strongly indicate that the usage frequencies of TCR-BV gene families in AML and MDS reflect those in HDs.

Discussion

Many studies have been published about TCR-BV gene family usage of human TAA-reactive T cells.[17–27] In these studies, bulky lymphocyte populations were analyzed with conventional FACS-based methods using a panel of mAbs directed against one of the TCR-BV gene family products or with PCR-based methods using a panel of TCR-BV gene family-specific primers. The former methods cannot cover all of the BV gene segments distributed in each BV gene family, while the latter do not guarantee that all the TCR-BV gene families are amplified with equal efficiency because of variations in PCR amplification of cDNA from bulky lymphocyte populations.[32] For example, TCR-BV gene families of T cells that exist at very low frequencies are easily missed by these PCR methods.[27] However, since our study was performed at the single-cell level using WT1 tetramer$^+$ CD8$^+$ T cells instead of bulky lymphocyte populations, the shortcomings of the conventional methods described above could be overcome. Furthermore, since amplification efficiency of TCR-BV cDNA from a single WT1 tetramer$^+$ CD8$^+$ T cell was more than 80% (data not shown), it appears that our results directly reflect TCR-BV gene family usage in WT1 tetramer$^+$ CD8$^+$ T cells in PB.

Frequencies of WT1 tetramer$^+$ CD8$^+$ T cells were higher for patients with AML or MDS than for HDs, and the phenotypes of the T cells were more activated and maturated in patients than in HDs. These results strongly suggest that WT1-specific CTL responses were robustly induced and activated in the patients, probably due to continuous stimulation of the patients' immune system by the WT1 antigen of WT1-expressing leukemia cells. Surprisingly, regardless of such a striking difference in WT1-specific immune responses between patients and HDs, usage patterns of TCR-BV gene families in patients were similar to those in HDs, and patients and HDs shared the biased usage of TCR-BVs 4, 5, 6, 20, and 27. On the other hand, TCR-BVs 9, 15, and 19, and TCR-BVs 12, 19, and 29 were specifically biased in AML and MDS, respectively. Interestingly, five TCR-BVs (4, 5, 6, 20, and 27) out of seven (4, 5, 6, 7, 20, 27, and 28) with biased usage in the HDs also showed biased usage in the patients. Taken together, these results lead us to speculate that dominant WT1-specific CTL populations that existed prior to the onset of AML and MDS expanded and differentiated to maintain their dominancy in the WT1-expressing tumor-bearing patients, while a few WT1-specific CTL populations expanded and differentiated in an AML- or MDS-specific fashion. Furthermore, our findings seem to suggest that dominant WT1-specific CTL populations in HDs may play an important role in immune surveillance against tumors, and that the dominant populations may continue to expand as a result of stimulation of the tumor-derived WT1 protein after the initial occurrence of WT1-expressing tumors.

TCR usage of antigen-specific T cells is thought to be influenced by the affinity of the TCR to the antigen peptide/HLA class I complex on immune cells such as dendritic cells, while a few studies have reported that TCR usage was often influenced by genetic factors such as HLA allele types.[33–35] For example,

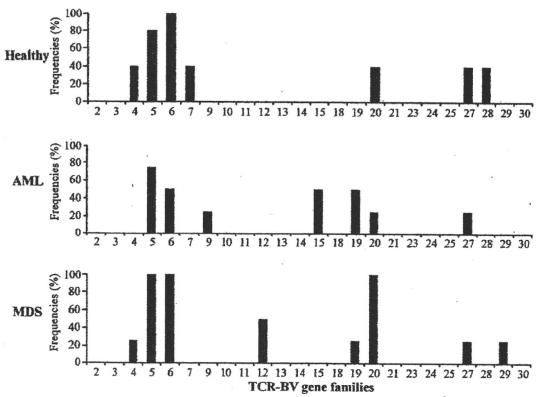

Fig. 3. Usage frequencies of T-cell receptor β-chain variable region (TCR-BV) gene families with the biased usage. Ratios of healthy donors (HDs) and patients with biased usage of the indicated TCR-BV gene families to the total number of HDs and patients examined, respectively.

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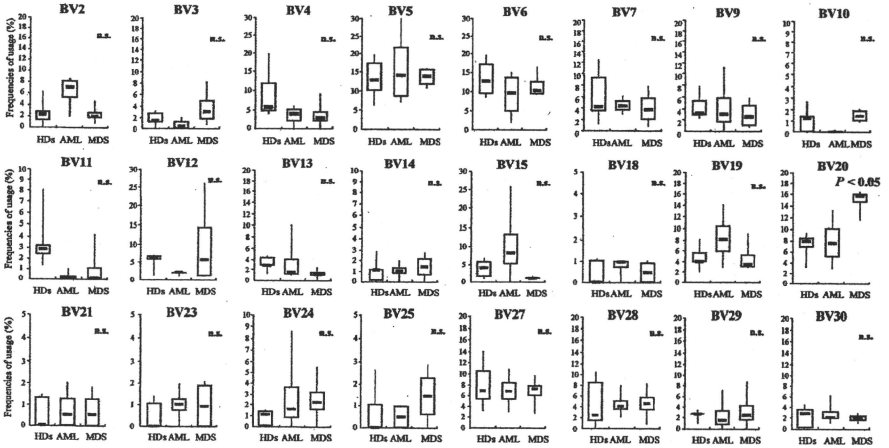

Fig. 4. Statistical comparison between healthy donors (HDs) and patients of usage frequencies of individual T-cell receptor β-chain variable region (TCR-BV) gene families in WT1 (Wilms' tumor gene 1) tetramer$^+$ CD8$^+$T cells. Statistical significance was assessed by one-factor ANOVA or Kruskal–Wallis test. n.s., not significant.

CTLs in HLA-A*2402$^+$ patients with acute parvovirus B19 infection preferentially used BV5,[36] which was one of the dominant TCR-BV gene families in the WT1 tetramer$^+$ CD8$^+$ T cells used in our study. As for tumor-associated antigens, SART-1 peptide-specific CTLs preferentially used BV7[37] in HLA-A*2402$^+$ patients with oral squamous cell carcinoma and BV18[38] in HDs. CTL sublines specific to cyclophilin B established from an HLA-A*2402$^+$ patient with lung adenocarcinoma preferentially used BVs 1, 15, and 16.[39] Our results and those of other studies suggest that TCR-BV gene family usage of CTLs mainly depends on the affinity of the interaction of the TCR with the antigen-derived peptide/HLA class I complex, rather than on HLA class I allele types.

Although a pre-existing higher number of WT1-specific CTLs with effector phenotypes were activated, no damage to the organs that physiologically expressed WT1 was observed. A plausible explanation for this phenomenon might be low expression of HLA class I molecules or low production of HLA class I-restricted WT1 peptide from WT1 protein, which resulted in low expression of HLA class I molecule/WT1 peptide complex in normal cells. These issues should be further addressed.

A question could be raised as to whether activation of pre-existing WT1-specific CTLs by WT1 cancer vaccine may not provide clinical benefits because tumors have already escaped immune surveillance by pre-existing WT1-specific CTLs. However, clonal analysis of T-cell receptors of the WT1-specific CTLs before and after WT1 vaccination showed that the pre-existing WT1-specific CTLs increased after the WT1 peptide vaccination (Y. Tanaka-Harada *et al.*, unpublished data, 2009). Therefore, these results indicated that at least a part of the clinical results of WT1 peptide vaccination could be ascribed to the activation of the pre-existing WT1-specific CTLs by WT1 peptide.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest.

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High frequencies of less differentiated and more proliferative WT1-specific CD8$^+$ T cells in bone marrow in tumor-bearing patients: An important role of bone marrow as a secondary lymphoid organ

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In tumor-bearing patients, tumor-associated antigen (TAA)-specific CTLs are spontaneously induced as a result of immune response to TAAs and play an important role in anti-tumor immunity. Wilms' tumor gene 1 (*WT1*) is overexpressed in various types of tumor and WT1 protein is a promising pan-TAA because of its high immunogenicity. In this study, to clarify the immune response to the WT1 antigen, WT1-specific CD8$^+$ T cells that were spontaneously induced in patients with solid tumor were comparatively analyzed in both bone marrow (BM) and peripheral blood (PB). WT1-specific CD8$^+$ T cells more frequently existed in BM than in PB, whereas frequencies of naïve (CCR7$^+$ CD45RA$^+$), central memory (CCR7$^+$ CD45RA$^-$), effector-memory (CCR7$^-$ CD45RA$^-$), and effector (CCR7$^-$ CD45RA$^+$) subsets were not significantly different between BM and PB. However, analysis of these subsets for the expression of CD57 and CD28, which were associated with differentiation, revealed that effector-memory and effector subsets of the WT1-specific CD8$^+$ T cells in BM had less differentiated phenotypes and more proliferative potential than those in PB. Furthermore, CD107a/b functional assay for WT1 peptide-specific cytotoxic potential and carboxyfluorescein diacetate succinimidyl ester dilution assay for WT1 peptide-specific proliferation also showed that WT1-specific CD8$^+$ T cells in BM were less cytotoxic and more proliferative in response to WT1 peptide than those in PB. These results implied that BM played an important role as a secondary lymphoid organ in tumor-bearing patients. Preferential residence of WT1-specific CD8$^+$ T cells in BM could be, at least in part, explained by higher expression of chemokine receptor CCR5, whose ligand was expressed on BM fibroblasts on the WT1-specific CD8$^+$ T cells in BM, compared to those in PB. These results should provide us with an insight into WT1-specific immune response in tumor-bearing patients and give us an idea of enhancement of clinical response in WT1 protein-targeted immunotherapy. (*Cancer Sci* 2010; 101: 848–854)

There is accumulating evidence that the immune system has the ability to recognize tumor-associated antigens (TAAs) and to eradicate the TAA-expressing malignant cells, also called 'tumor immunosurveillance'.[1,2] In tumor immunosurveillance, it is generally thought that CD8$^+$ CTLs are the main effector cells because they can effectively expand and kill malignant cells. Therefore, the most common approaches to combat tumors have centered on the induction of TAA-specific CTLs. Recent studies showed that CTLs with memory phenotypes also had potent anti-tumor activity.[3] Thus, not only the induction of effector CTLs but also maintenance of memory CTLs are required for ideal anti-tumor immune response in tumor-bearing patients. Regarding the maintenance of CTLs, infectious models using pathogens were well established. Interestingly, in chronic infection, in which antigens constitutively existed, it was reported that CTLs were continually activated by the antigens, finally resulting in exhaustion of the CTLs.[4] However, in tumor-bearing patients, TAAs constitutively exist for a long time, like the chronic infection. In contrast to patients with chronic infection, it appears that spontaneously induced TAA-specific CTLs in tumor-bearing patients are not exhausted but rather can be activated and expanded when the patients are treated with TAA peptide vaccines, because a considerable number of investigations showed the generation of TAA-specific CTLs from peripheral blood (PB) of tumor-bearing patients and an increase in CTL frequencies after treatment with TAA-specific vaccines.[5–8] This discrepancy in responsibility of CTLs between chronic infection and tumor bearing indicates the existence of a unique mechanism of maintenance of functional TAA-specific CTLs in tumor-bearing patients. Thus, to elucidate the unique mechanism, comprehensive analysis of the spontaneously induced TAA-specific CTLs is important.

Bone marrow (BM) has recently been shown to be an important site for T cell priming and reactivation, generation of T cell memory and recruitment of large amounts of circulating memory T cells and antigen-loaded dendritic cells (DCs).[9–13] Memory CD8$^+$ T cells in BM are more activated than those in the lymphoid periphery, and it was proposed that memory CD8$^+$ T cells in BM might receive stimulation from BM-resident cells through cell–cell contact or cytokines such as interleukin (IL)-7 or -15, resulting in their long-term maintenance in BM.[12,14–18] These findings indicated that BM was a crucial organ for migration of mature T cells and greatly contributed to long-term T cell memory. However, these findings mainly resulted from the analysis of immune responses to foreign pathogens such as virus, and the role of BM in immune response to self-antigens such as TAAs has not been investigated in detail.

Wilms' tumor gene (*WT1*), which has an oncogenic function, is highly expressed in various kinds of hematological malignancies and solid tumors.[19–23] Previous studies indicated that

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Table 1. Profile of patients who participated in this study

Pt. No.	Age, years/sex	Disease	Clinical stage	Prior treatments
1	56/F	Ovarian cancer (Serous papillary adenocarcinoma)	IIIc	Chemo
2	72/F	Ovarian cancer (Carcinosarcoma)	II	Chemo
3	16/F	Osteosarcoma	II	Chemo/TAE
4	51/M	Soft-tissue sarcoma(Clear cell sarcoma)	IV	Chemo/TAE
5	63/M	Lung cancer(Squamous cell carcinoma)	IIIb	Chemo/RT
6	74/M	Lung cancer(Adenocarcinoma)	IV	Chemo/RT
7	74/M	Lung cancer (Squamous cell carcinoma)	IV	Ope/RT

Chemo, chemotherapy; F, female; M, male; Ope, operation; Pt., patient; RT, radiation therapy; TAE, transcatheter arterial embolization.

stimulation of PBMCs with MHC class I-restricted WT1 protein-derived peptides induced WT1-specific $CD8^+$ T cells in an MHC class I-restricted manner, and the induced WT1-specific $CD8^+$ T cells specifically killed WT1-expressing tumor cells without affecting normal cells that physiologically expressed WT1, indicating that WT1 could be a promising target antigen for cancer immunotherapy.[22–27] WT1-specific $CD8^+$ T cells and WT1 IgM and IgG antibodies were spontaneously induced in patients with WT1-expressing tumors.[28] Clinical trials of WT1 peptide vaccination are now being carried out for patients with various types of malignancies, and WT1-specific $CD8^+$ T cell responses and the resultant clinical responses have been reported.[5,21,29–34] However, detailed analysis of spontaneously induced WT1-specific $CD8^+$ T cells in tumor-bearing patients has not yet been done. Furthermore, the majority of the findings resulted from the analysis of PB, and there is little data about WT1-specific $CD8^+$ T cells in BM, an important site for immune response to TAAs. Therefore, detailed comparative analysis of WT1-specific $CD8^+$ T cells in both PB and BM is interesting and necessary to understand further the WT1-directed immune responses, which should lead to enhancement of the clinical response of WT1 protein-targeted immunotherapy.

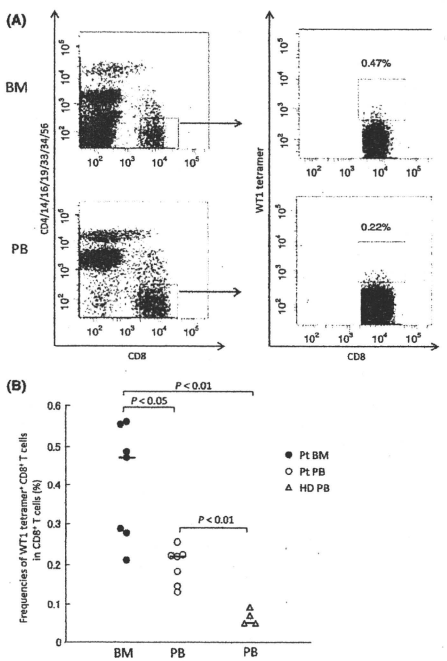

Fig. 1. Frequencies of WT1 tetramer$^+$ $CD8^+$ T cells in bone marrow (BM) and peripheral blood (PB) in patients with solid tumors. (A) Representative flow cytometric analysis of WT1 tetramer$^+$ $CD8^+$ T cells. Mononuclear cells from BM and PB were gated on $CD8^+$, $CD4^-$, $CD14^-$, $CD16^-$, $CD19^-$, $CD33^-$, $CD34^-$, and $CD56^-$ cells, and WT1 tetramer$^+$ $CD8^+$ T cells were defined as WT1-specific $CD8^+$ T cells. (B) Frequencies of WT1 tetramer$^+$ $CD8^+$ T cells in $CD8^+$ T cells in BM (closed circles) and PB (open circles) from patients (Pt), and PB from healthy donors (HD; open triangles). The horizontal bars indicate median values of the frequencies.

Materials and Methods

Patients and healthy donors. Three patients with lung cancer, two patients with ovarian cancer, one patient with osteosarcoma, and one patient with soft-tissue sarcoma were analyzed (Table 1). *WT1* expression in tumor cells was determined by immunohistochemistry. No bone marrow metastasis was detected in any patient. After written informed consent was given, PB and BM samples were obtained from seven HLA-A*2402$^+$ patients. PB samples were also obtained from four HLA-A*2402$^+$ healthy donors. PBMCs and BM mononuclear cells (BMMCs) were isolated by density gradient centrifugation using Ficoll–Hypaque and cryopreserved until analysis.

Flow cytometric analysis. PBMCs and BMMCs were incubated with phycoerythrin (PE)-conjugated HLA-A*2402/$WT1_{235-243}$ tetramer (MBL, Tokyo, Japan) in FACS buffer composed of PBS and 5% FBS at 37°C for 30 min. Subsequently, these cells were stained with a mixture of mAbs: (a) anti-CD8-APC-Cy7 (BD Biosciences, San Diego, CA, USA), anti-CD45RA-ECD (Beckman Coulter, Marseille, France), anti-CCR7-PE-Cy7 (BD Biosciences), FITC-labeled-anti-CD4 (Biolegend, San Diego, CA, USA), -CD14 (BD Biosciences), -CD16 (eBioscience, San Diego, CA, USA), -CD19, -CD33, -CD34 (all BD Biosciences), and -CD56 (eBioscience); or (b) anti-CD8-APC-Cy7, anti-CD45RA-ECD, anti-CCR7-PE-Cy7, anti-CD57-APC (Biolegend), and anti-CD28-FITC (eBioscience), at

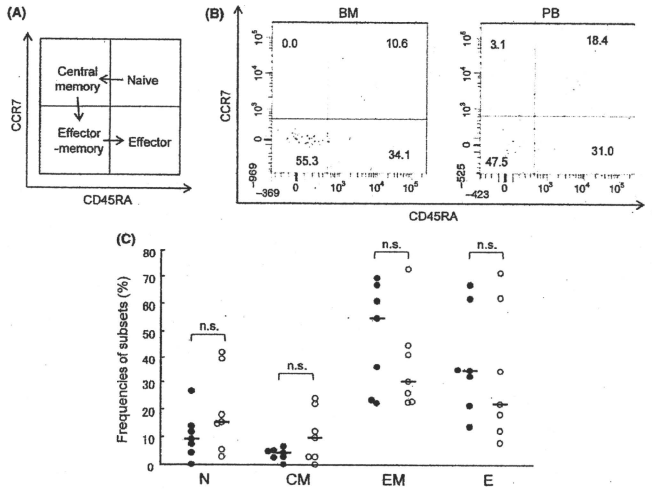

Fig. 2. Subset composition of WT1 tetramer$^+$ CD8$^+$ T cells in bone marrow (BM) and peripheral blood (PB). (A) WT1 tetramer$^+$ CD8$^+$ T cells are divided into four subsets according to the expression of CCR7 and CD45RA and differentiate as follows: naïve (N), CCR7$^+$ CD45RA$^+$ → central memory (CM), CCR7$^+$ CD45RA$^-$ → effector- memory (EM), CCR7$^-$ CD45RA$^-$ → effector (E), CCR7$^-$ CD45RA$^+$. (B) Representative subset analysis of WT1 tetramer$^+$ CD8$^+$ T cells in BM and PB. Frequencies of N, CM, EM, and E subsets are shown. (C), Frequencies of each subset of WT1 tetramer$^+$ CD8$^+$ T cells in BM (closed circles) and PB (open circles) are shown. The horizontal bars indicate median values of the frequencies.

4°C for 20 min, washed three times, and resuspended in FACS buffer. In staining with (a), CD8$^+$ and CD4$^-$, CD14$^-$, CD16$^-$ CD19$^-$, CD33$^-$, CD34$^-$, and CD56$^-$ cells were defined as CD8$^+$ T cells. CD8^{low+} cells, in which natural killer (NK) cells were contaminated, were gated out from the gating for CD8$^+$ T cells, because cells tended to give rise to non-specific binding to WT1 tetramer. Data acquisition was carried out on a FACSAria instrument (BD Biosciences), and the data were analyzed using FACSDiva software (BD Biosciences).

CD107a/b assay. PBMCs and BMMCs were stimulated *in vitro* with 10 μg/mL natural 9-mer $WT1_{235}$ peptide or mERK (QYIHSANVL) peptide (irrelevant peptide) in the presence of BD GolgiStop (BD Biosciences) and FITC-conjugated mAbs for CD107a and CD107b (BD Biosciences) for 3 h. The cells were then harvested, washed, stained with mAbs for anti-CD8-APC-Cy7, anti-CD45RA-ECD, and anti-CCR7-PE-Cy7, and gated on lymphocytes. Frequencies of CD107a/b$^+$ cells induced specifically by WT1 peptide stimulation were calculated by subtracting the frequencies of CD107a/b$^+$ cells induced by irrelevant mERK peptide stimulation from those of CD107a/b$^+$ cells induced by WT1 peptide stimulation.

Proliferation assay. Proliferative potential of WT1 peptide-specific CTLs was examined according to previous reports.[35] Briefly, PBMC and BMMC were labeled with 2.5 μM CFSE (Molecular Probes, Eugene, OR, USA), and 2×10^5 cells were plated in 96-well round plates in 100 μL X-VIVO 15 with 5% AB serum. The cells were stimulated with natural 9-mer $WT1_{235-243}$ peptide at a concentration of 10 μg/mL. After 2 days of culture, 100 μL X-VIVO 15 medium with 5% AB serum containing IL-2 (100 IU/mL) was added. After 10 days of peptide stimulation, the cells were re-stimulated for 6 h with or without the WT1 peptide (10 μg/mL), with the addition of 10 μg/mL Brefeldin A (SIGMA) for the last 5 h to block cytokine secretion. After 6 h of WT1 peptide stimulation, cells were washed, stained with anti-CD8-APC-Cy-7 and anti-CD3-PerCP, fixed, permeabilized, and stained with anti-interferon (IFN)-γ-APC. CD3$^+$ CD8$^+$ IFN-γ$^+$ cells were analyzed for CFSE dilution.

Statistical analysis. Differences between test groups were analyzed using the Mann–Whitney *U*-test.

Results

Bone marrow contains WT1-specific CD8$^+$ T cells at higher frequencies than PB in patients with solid tumor. Frequencies of WT1 tetramer$^+$ CD8$^+$ T cells in total CD8$^+$ T cells were measured in BM and PB by staining the mononuclear cells with HLA-A*2402/WT1 tetramer. BMMCs and PBMCs from seven HLA-A*2402$^+$ patients with solid tumor were examined (Table 1 and Fig. 1). The frequencies of WT1 tetramer$^+$ CD8$^+$ T cells in CD8$^+$ T cells were significantly higher in BM than in PB (median, 0.47% *vs* 0.22%; $P < 0.05$) (Fig. 1B). For reference, PB from four HLA-A*2402$^+$ healthy donors were similarly examined. Frequencies of WT1 tetramer$^+$ CD8$^+$ T cells in PB were significantly lower than those in BM and PB of seven patients (median, 0.05% [PB in healthy donors] *vs* 0.47% [BM in patients], 0.22% [PB in patients]; $P < 0.01$) (Fig. 1B), which was consistent with previous reports.[5]

Effector-memory and effector subsets of WT1-specific CD8$^+$ T cells in BM had less differentiated and more proliferative phenotypes than those in PB. To elucidate whether the WT1-specific CD8$^+$ T cells in BM phenotypically differed from those in PB, WT1-specific CD8$^+$ T cells are phenotypically classified into four distinct differentiation stages based on surface expression of CCR7 and CD45RA: naïve (N), CCR7$^+$ CD45RA$^+$; central memory (CM), CCR7$^+$ CD45RA$^-$; effector-memory (EM),

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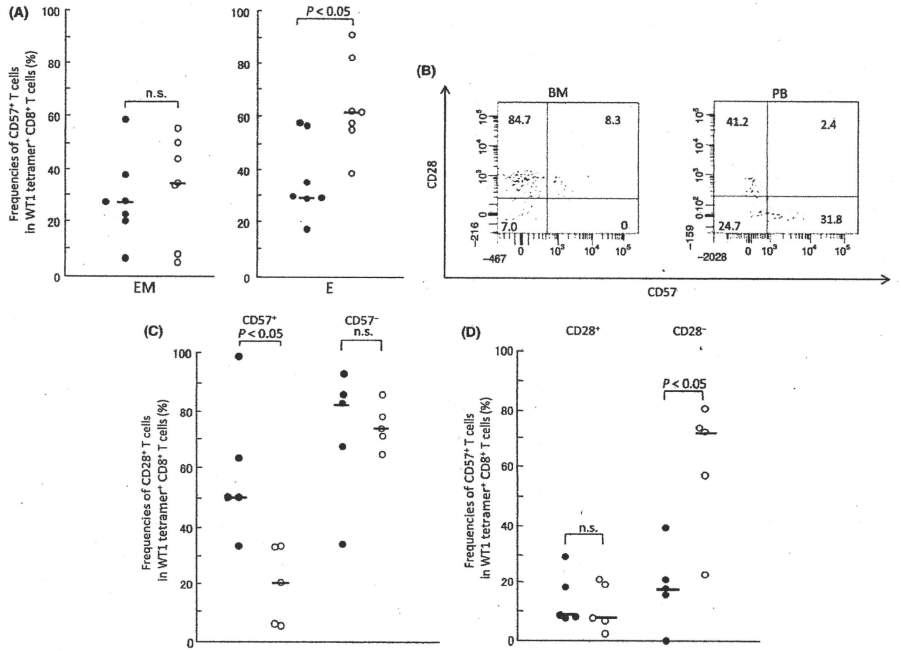

Fig. 3. Effector-memory (EM) and effector (E) subsets of WT1 tetramer$^+$ CD8$^+$ T cells in bone marrow (BM) had less differentiated and more proliferative phenotypes than those in peripheral blood (PB). (A) Frequencies of CD57$^+$ cells in EM and E subsets of WT1 tetramer$^+$ CD8$^+$ T cells in BM (closed circles) and PB (open circles). (B) Representative dot-plots of FACS analysis of CD28 and CD57 expression in the EM subset. Numbers represent frequencies (%) of cells in each fraction. (C) Frequencies of CD28$^+$ T cells in the CD57$^+$ or CD57$^-$ EM subset in WT1 tetramer$^+$ CD8$^+$ T cells in BM (closed circles) and PB (open circles). (D) Frequencies of CD57$^+$ T cells in the CD28$^+$ or CD28$^-$ EM subset in WT1 tetramer$^+$ CD8$^+$ T cells in BM (closed circles) and PB (open circles). Two patients were not evaluated because of the small number of cells. Horizontal bars indicate median values of the frequencies. n.s., not significant.

CCR7$^-$ CD45RA$^-$; and effector (E), CCR7$^-$ CD45RA$^+$.[36,37] It is well known that CD8$^+$ T cells differentiate as follows: N → CM → EM → E (Fig. 2A). As shown in Figure 2B, the majority of the WT1 tetramer$^+$ CD8$^+$ T cells belonged to EM and E subsets, and there was no significant difference in the frequencies of N, CM, EM, and E subsets of WT1 tetramer$^+$ CD8$^+$ T cells between BM and PB (Fig. 2C).

Subsets EM and E of WT1 tetramer$^+$ CD8$^+$ T cells, which accounted for the majority of the T cells, were further examined for the expression of CD57. Low CD57 expression means that cells are less differentiated and have sufficient proliferative potential. As shown in Figure 3A, frequencies of CD57$^+$ cells in E subset of WT1 tetramer$^+$ CD8$^+$ T cells were lower in BM than in PB (median, 28.5% *vs* 61.0%; $P < 0.05$). However, frequencies of CD57$^+$ cells in EM subset of WT1 tetramer$^+$ CD8$^+$ T cells were not significantly different between BM and PB. These results indicated that E subset of WT1 tetramer$^+$ CD8$^+$ T cells in BM had less differentiated and more proliferative phenotypes than that in PB.

Next, EM subset of WT1 tetramer$^+$ CD8$^+$ T cells, in which no significant difference in CD57 expression was found between BM and PB, was further examined for the expression of CD28, whose high expression means less differentiated state and sufficient proliferative potential (Fig. 3).[38,39] In CD57$^+$ cells in EM subset, CD28$^+$ cells (less differentiated) were more in BM than in PB (median, 50.0% *vs* 20.0%; $P < 0.05$), whereas in CD28$^-$ cells in EM subset, CD57$^+$ cells (more differentiated) were less in BM than in PB (median, 16.6% *vs* 71.4%; $P < 0.05$) (Fig. 3C,D). These results showed that WT1 tetramer$^+$ CD8$^+$ T cells in EM subset also had less differentiated phenotype in BM than in PB. Taken together, these results indicated that both EM and E subsets of WT1 tetramer$^+$ CD8$^+$ T cells in BM had less differentiated and more proliferative phenotypes than those of WT1 tetramer$^+$ CD8$^+$ T cells in PB.

WT1-specific CD8$^+$ T cells in BM have higher proliferative potential than those in PB. As we demonstrated that EM and E subsets of WT1-specific CD8$^+$ T cells in BM had less differentiated and more proliferative phenotypes than those in PB, proliferative activity of WT1-specific CD8$^+$ T cells was examined. CFSE-labeled BMMCs and PBMCs were stimulated with WT1 peptide. After 10 days of the peptide stimulation, expanded BMMCs and PBMCs were restimulated with WT1 peptide for

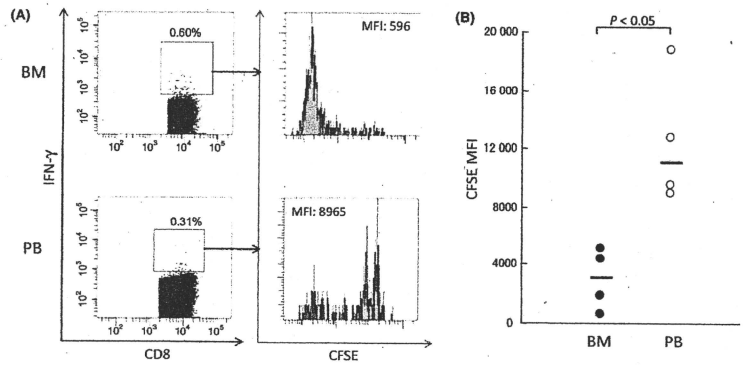

Fig. 4. Proliferative potential of WT1-reactive $CD8^+$ T cells. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled bone marrow (BM) and peripheral blood (PB) mononuclear cells were stimulated with WT1 peptides, and CFSE dilution was analyzed by FACS. (A) Representative flow cytometric analysis of CFSE dilution in interferon (IFN)-γ^+ $CD8^+$ $CD3^+$ T cells. (B) CFSE mean fluorescence intensity (MFI) of IFN-γ^+ $CD8^+$ $CD3^+$ T cells in BM (closed circles) and PB (open circles). Horizontal bars indicate median values of MFI.

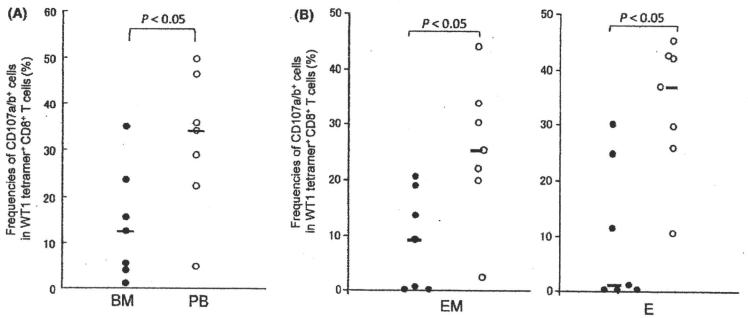

Fig. 5. Cytotoxic potential of WT1 $tetramer^+$ $CD8^+$ T cells. Mononuclear cells from bone marrow (BM) and peripheral blood (PB) were stimulated with WT1 peptide, then CD107a/b cell surface expression was examined. (A) Frequencies of $CD107a/b^+$ cells in WT1 $tetramer^+$ $CD8^+$ T cells in BM (closed circles) and PB (open circles). (B) Frequencies of $CD107a/b^+$ cells in effector-memory (EM) and effector (E) subsets in WT1 $tetramer^+$ $CD8^+$ T cells in BM (closed circles) and PB (open circles). Horizontal bars indicate median values of the frequencies.

6 h and analyzed for CFSE dilution in WT1 peptide-responding IFN-γ^+ $CD3^+$ $CD8^+$ cells (Fig. 4). A substantial number of IFN-γ^+ cells were detected after the stimulation with WT1 peptide (Fig. 4A), but only a few IFN-γ^+ cells were detected without the stimulation (negative control; data not shown). CFSE dilution profile of the IFN-γ^+ cells and its mean fluorescence intensity (MFI, 3008.5 [PB] *vs* 11 051 [BM]; $P < 0.05$) showed that IFN-γ^+ $CD3^+$ $CD8^+$ cells in BM were more proliferative than those in PB (Fig. 4B). These results indicated that WT1-specific $CD8^+$ T cells in BM had higher proliferative potential than those in PB.

WT1-specific $CD8^+$ T cells in BM have lower cytotoxic potential than those in PB. Cytotoxic potential of WT1 $tetramer^+$ $CD8^+$ T cells was analyzed by CD107a/b assay, because the assay was shown to strongly correlate with killing activity of CTLs.[40] BMMCs and PBMCs were stimulated with WT1 peptide or mERK irrelevant peptide, then frequencies of CD107a/b-expressing cells were examined. As shown in Figure 5A, the frequencies of CD107a/b-expressing cells in WT1 $tetramer^+$ $CD8^+$ T cells were lower in BM than in PB (median, 12.4% *vs* 33.7%; $P < 0.05$). Furthermore, CD107a/b expression on EM and E subsets of WT1 $tetramer^+$ $CD8^+$ T cells was investigated in BM and PB. As shown in Figure 5B, frequencies of CD107a/b-expressing cells in EM and E subsets of WT1 $tetramer^+$ $CD8^+$ T cells were significantly lower in BM than in PB (EM, 9.1% *vs* 25.0%, $P < 0.05$; E, 0.8% *vs* 36.7%, $P < 0.05$). These results strongly indicated that WT1-specific cytotoxic potential of WT1-specific $CD8^+$ T cells in BM was lower than that of WT1-specific $CD8^+$ T cells in PB.

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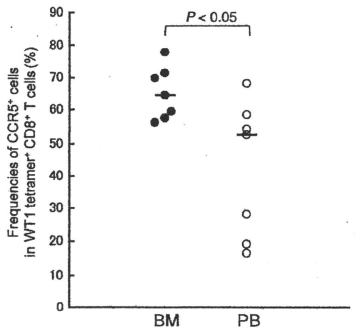

Fig. 6. Expression of chemokine receptor CCR5 on WT1 tetramer$^+$ CD8$^+$ T cells. Frequencies of CCR5$^+$ cells in WT1 tetramer$^+$ CD8$^+$ T cells in bone marrow (BM; closed circles) and peripheral blood (PB; open circles). Horizontal bars indicate median values of the frequencies.

Expression of chemokine receptor CCR5 on WT1-specific CD8$^+$ T cells higher in BM than in PB. Expression levels of chemokine receptors CCR5 and CXCR4 on WT1 tetramer$^+$ CD8$^+$ T cells were examined in BM and PB. As shown in Figure 6, frequencies of CCR5$^+$ cells in WT1 tetramer$^+$ CD8$^+$ T cells were significantly higher in BM than in PB (median, 64.3% *vs* 52.5%; $P < 0.05$). Frequencies of CXCR4$^+$ cells in WT1 tetramer$^+$ CD8$^+$ T cells tended to be higher in BM than in PB, although they were not statistically significant (median, 49.3% *vs* 35.7%, not significant) (data not shown). Ligands CCL5 and SDF-1 for chemokine receptors CCR5 and CXCR4, respectively, are highly expressed in the BM microenvironment and play an important role in interaction between the cells with these chemokine receptors and the BM microenvironment. Therefore, these results, at least in part, gave us an explanation for the preferential residence of WT1-specific CD8$^+$ T cells in BM compared to PB.

Discussion

The present study showed for the first time the characterization of WT1-specific CD8$^+$ T cells that were spontaneously induced as a result of stimulating the immune system by highly WT1-expressing tumor cells in patients with solid tumor. In comparison with WT1-specific CD8$^+$ T cells in PB, those in BM were higher in frequency, less differentiated, and more proliferative, and had less cytotoxic potential. The preferential residence of WT1-specific CD8$^+$ T cells in BM could be explained, at least in part, by the higher expression of chemokine receptors CCR5 and CXCR4 on WT 1-specific CD8$^+$ T cells in BM compared to WT1-specific CD8$^+$ T cells in PB.

Our results allowed us to consider that BM provided an important site for priming and reactivation of CD8$^+$ T cells with TAAs, that is, BM functioned as a secondary lymphoid organ. It has been reported that differentiated DCs constitutively traffic from peripheral tissues to blood and the circulating DCs home with a rather high tropism to BM, where the DCs activate naïve and resident T cells.[10–13,41] Therefore, it appeared that DCs that captured WT1 antigen, which was produced from WT1-expressing tumor cells, in peripheral sites homed to BM, presented the WT1 antigen to circulating naïve CD8$^+$ T cells and resident CD8$^+$ T cells and activated them. It then seems that the activated WT1-specific CD8$^+$ T cells differentiated into the CD8$^+$ T cells with more matured phenotypes, which finally migrated from BM to tumor sites through PB and exerted cytotoxic activity there. Others analyzed MUC-1- or Her2/neu-specific CD8$^+$ T cells that were spontaneously induced in tumor-bearing patients by using the corresponding tetramers and reported no difference in the frequencies of the CD8$^+$ T cells between BM and PB.[42,43] However, as further detailed analysis of the CD8$^+$ T cells was not done in these studies, the cause of the discrepancy between our results and theirs cannot be discussed in detail.

Concerning another aspect of BM function, a number of reports showed that BM was a pooling site of memory T cells.[12,14,16,18,41,44–49] Adoptive T cell transfer studies showed that memory T cells migrated to BM and preferentially proliferated there through the signals by cytokines such as IL-7 and IL-15.[14,16] Thus, BM plays an important role in the maintenance of memory T cells. Our present study also showed that WT1-specific effector-memory and effector CD8$^+$ T cells accumulated in BM in patients with WT1-expressing solid tumor. Furthermore, our detailed phenotype analysis showed that WT1-specific CD8$^+$ T cells in E and EM subsets in BM had unique phenotypes, such as less differentiated state, more proliferative potential, and less cytotoxic, compared to those in E and EM subsets in PB. T cells with low expression of CD57 and high expression of CD28 are considered to sustain sufficient proliferative potential and less cytotoxic potential. Conversely, high expression of CD57 and low expression of CD28 are associated with replicatively senescent T cells and clonally exhausted T cells with cytotoxic potential, respectively.[38,39,50–52] In the T cells with these characteristics, T-cell receptor management extra circles (TREC) levels were very low and telomere lengths were shortened, and hence these T cells apoptose by antigen stimulation.[39,53] Therefore, our phenotype analysis suggested that WT1-specific CD8$^+$ T cells in EM and E subsets in BM sustained more sufficient proliferative potential and less cytotoxic potential, compared to those in EM and E subsets in PB. CD107a/b assay, a functional assay to examine cytotoxicity, also revealed that WT1-specific CD8$^+$ T cells in BM had less cytotoxic potential than those in PB, consistent with the results of the phenotype analysis of CD57 and CD28 expression. In the CD107a/b assay presented here, whole BMMCs and PBMCs were used. To confirm our present results, CD107a/b assay was applied to EM and E subsets FACS-sorted from another patient. As expected, frequencies of CD107a/b-expressing cells in WT1 tetramer$^+$ CD8$^+$ T cells in purified EM and E subsets were lower in BM than in PB, confirming our results. Furthermore, proliferation assay by CFSE dilution that showed higher proliferative potential of WT1-specific CD8$^+$ T cells in BM than in PB was also compatible with the results of phenotype analysis. These results suggested that WT1-specific CD8$^+$ T cells with more proliferative and less cytotoxic potential could be maintained in the BM until they are reactivated by DCs.

WT1-specific CD8$^+$ T cells in BM expressed chemokine receptors CCR5 and CXCR4 at higher frequencies than those in PB. CCL3 and CCL5, ligands for CCR5, were expressed on BM fibroblasts, and CXCL12, a ligand for CXCR4, was produced on BM stromal cells and endothelium of BM microvessels.[54–56] Therefore, preferential homing and localization of WT1-specific CD8$^+$ T cells to BM should be ascribed to preferential interaction between chemokine receptors on the T cells and its ligands in the BM microenvironment. It appears to be reasonable to consider that downregulation of such chemokine receptors on the T cells promotes emigration of the T cells from BM to PB. These findings also allowed us to consider BM as a secondary lymphoid organ.

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