A Clear Correlation between WT1-specific Th Response and Clinical Response in WT1 CTL Epitope Vaccination

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Abstract. *Clinical studies of WT1-targeted cancer vaccine are being performed. However, WT1-specific Th response in cancer patients remains unclear. Using quantitative real-time RT-PCR, we investigated IFN-γ and IL-10 mRNA expression from Th cells by stimulation with helper peptide $WT1_{332}$. Seventeen patients, of whom 10 had achieved stable disease and the remaining 7 had progressive disease, were weekly vaccinated with WT1 CTL epitope (modified $WT1_{235}$) and examined for $WT1_{332}$-specific Th response. A clear correlation between $WT1_{332}$-specific Th response and clinical response was observed at 4 weeks post-vaccination. In patients who responded, a clear inverse correlation between IL-10-type and IFN-γ-type $WT1_{332}$-specific Th response was detected at pre- and 4 weeks post-vaccination, and the shift of the Th response from IL-10-type dominancy at early phase to IFN-γ-type dominancy at late phase was observed. From this study we concluded that occurrence of $WT1_{332}$-specific Th response could predict good clinical response of WT1 CTL epitope vaccination.*

The Wilms' tumor gene *WT1* was isolated as a gene responsible for a childhood renal neoplasm, Wilms' tumor (1, 2). The *WT1* gene encodes a zinc finger transcription factor, and the gene product positively or negatively regulates transcription of various kinds of genes (3). Although the *WT1* gene was first categorized as a tumor-suppressor gene (4), we and others recently demonstrated that the wild-type WT1 gene, which was highly expressed in leukemia and various kinds of solid cancers, performed an oncogenic role rather than a tumor-suppressor function in these malignancies (5-9). These findings led us to consider that the *WT1* gene product could be a promising target antigen for cancer immunotherapy. In fact, cytotoxic T lymphocyte (CTL) epitopes of WT1 protein were already identified (10, 11) and used as peptide vaccines for the treatment of malignancies in the clinical setting (12-17).

Recently, many clinical studies of cancer immunotherapy are being performed and data of tumor-specific CTLs has been accumulating. Although CTLs are the most important effectors in antigen-specific cancer immunotherapy, activation of CTLs alone often leads to only limited clinical responses (18). Activation of other kinds of immune cells, particularly CD4+ helper T (Th) cells, is also needed to obtain stronger immunological responses (19-22). However, information of tumor antigen-specific Th cell was not enough. We previously identified WT1 protein-derived helper peptide $WT1_{332}$ that could promiscuously bind to HLA-DRB1*0405, 1501, 1502 and -DPB1*0901 molecules(23, 24). It was also shown that the $WT1_{332}$ was naturally generated from WT1 protein through processing in cancer cells and dendritic cells (DCs) and preferentially induced Th1 responses *in vitro*. Therefore, this peptide should be useful for co-vaccination with CTL epitopes to induce stronger immunological responses.

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Key Words: Wilms' tumor gene (WT1), helper T cell, helper peptide, peptide vaccine, cancer immunotherapy.

In the present study, to investigate whether or not $WT1_{322}$-specific Th response is involved in clinical outcome of WT1 CTL-epitope vaccination, peripheral blood mononuclear cells (PBMCs) from patients who were treated with CTL epitope peptide vaccine were stimulated by $WT1_{332}$ and IFN-γ and IL-10 mRNA expression in Th cells which were measured using quantitative real-time RT-PCR method. Here we clearly demonstrated an apparent correlation between $WT1_{332}$-specific Th response and clinical response.

Patients and Methods

Patients and clinical study of WT1 CTL epitope peptide vaccination. In "Phase I/II clinical trial of WT1 peptide-based vaccine for the patients with malignant tumors" (ID: UMIN000002001), which was approved by the Ethical Review Boards of Faculty Medicine, Osaka University, patients with various kinds of solid cancers were intradermally injected with a 9-mer modified $WT1_{235}$ peptide (CYTWNQMNL) emulsified with Montanide ISA51 adjuvant at weekly intervals for 3 months and clinical responses were assessed by RECIST criteria. Major entry criteria were as follows: 1) expression of WT1 in cancer tissues determined by RT-PCR and/or immunohistochemistry, 2) HLA-A*2402-positivity, 3) estimated survival of >3 months, 4) performance status from 0 to 1 (Eastern Cooperative Oncology Group), 5) no severe impairment of organ function, and 6) no administration of chemotherapy, immunotherapy, immunosuppressive therapy, or radiotherapy within 4 weeks before WT1 vaccination.

WT1 peptides. A 9-mer modified $WT1_{235}$ peptide (CYTWNQMNL, 235-243) (11), in which M at position 2 (an anchor for HLA-A*2402-binding) in the natural epitope (CMTWNQMNL) was replaced by Y, was used in the phase I/II clinical study of WT1 immunotherapy. This modified peptide had been shown to induce stronger CTL activity against WT1-expressing tumor cells than the natural peptide and purchased from Neo-Multiple Peptide Systems (San Diego, CA).

$WT1_{332}$ helper peptide (KRYFKLSHLQMHSRKH, 332-347) that could induce Th cell response in the context of multiple HLA class II molecules(23) were purchased from Sigma Genosys Japan (Hokkaido, Japan).

Cell preparation and $WT1_{332}$ stimulation. Peripheral blood (PB) samples were obtained from 17 patients at pre- and post-WT1 vaccinations and from 22 healthy donors. PBMCs were separated by density centrifugation using Lymphocyte Separation Solution (nacalai tesque, Japan) and frozen until use was indicated.

For analysis of $WT1_{332}$-specific Th responses, the frozen PBMCs were thawed, washed twice with RPMI medium, suspended in X-VIVO 15 medium supplemented with 10% AB serum in 24-well plate and incubated at 37˚C in a humidified 5% CO_2 overnight. On next day, the PBMCs were harvested and washed twice, and suspended in X-VIVO 15 medium supplemented with 1% AB serum and CD28/49d Costimulatory Reagent (BD Bioscience, San Jose, CA) and put in 12×75 mm polystyrene tubes. Ten μl of water containing or non-containing 20 μg of $WT1_{332}$ peptide were added to the culture tubes and then the culture tubes were incubated at a 5˚ slant at 37˚C for 4 h in a humidified 5% CO_2 atmosphere. After the incubation, CD4+ T cells were isolated from PBMCs using BDTM IMag Human CD4 T Lymphocyte Enrichment Set (BD Bioscience) according to the manufacture's protocol. The purity of the isolated CD4+ T cells was confirmed to be more than 93% by flow cytometric analysis.

RNA isolation from CD4+ T cells and cDNA synthesis. RNA isolation from CD4+ T cells was performed with Trizol (Invitrogen, California, USA) according to the manufacturer's instruction. RNA was dissolved in water and stored at –70˚C. For cDNA synthesis, Approximately one μg of total RNA from each sample was reverse transcribed into cDNA with $Oligo(dT)_{12\text{-}18}$ primer (Invitrogen) using a SuperScript™ III first strand synthesis SuperMix kit (Invitrogen) according to the manufacturer's instructions. cDNA was stored at –80˚C until use.

Construction of plasmid cDNA standards. cDNA of IFN-γ, IL-10 and β-actin were obtained from activated lymphocytes that were cultured in the presence of PHA and IL-2 for 3 days. cDNA fractions of the IFN-γ, IL-10 and β-actin were amplified by a PCR reaction using a PCR thermocycler 9700 system (Applied Biosystems, Foster City, CA). The oligonucleotide primers used were as follows: IFN-γ forward primer, 5'-GTC CTT TGG ACC TGA TCA GC-3'; reverse primer, 5'-CCT TGA TGG TCT CCA CAC TC-3'; IL-10 forward primer, 5'-TAA GGG TTA CCT GGG TTG CC-3'; reverse primer, 5'-GCC ACC CTG ATG TCT CAG TT-3'; β-actin forward primer, 5'-CCA TCA TGA AGT GTG ACG TGG-3'; reverse primer, 5'-CGC AAC TAA GTC ATA GTC CGC-3'. The PCR reaction solution contained 1 μl cDNA template (10-100 ng), 200 μM dNTPs, 2 μM of each primer, 2 mM $MgCl_2$, 1.25 U TaqGold polymerase (Applied Biosystems, CA, USA) and 5 μl 10× Taq buffer (Applied Biosystems) and adjusted to the final volume of 25 μl by the addition of water. The PCR conditions were as follows: 9 min at 95˚C, 30 cycles of 45 s at 94˚C, 45 s at 60˚C, 45 s at 72˚C, and 7 min at 72˚C. Amplification products were purified using QIA quick PCR Purification Kit. The purified PCR amplicons were then ligated into a TA-cloning vector using T4 DNA ligase supplied with the TA Cloning® Kit. The vector-PCR product ratio was 1:1, and the ligation reaction solution consisted of a fresh PCR sample, 50 ng pCR2.1 vector, T4 DNA Ligase (4.0 U) and 10× Ligation Buffer, were adjusted to a final volume of 10 μl by the addition of water and incubated at 14˚C overnight. Two microliters of the ligation reaction samples were mixed with competent E. coli Top10 cells for transformation using the heat shock method described in the manufacturer's manual (Invitrogen). Clones containing inserts were identified by PCR analysis. Positive clones were sequenced in a Perkin Elmer ABI-Prism 377 DNA sequencer (Applied Biosystems) using ABI-prism Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems). The clones containing the correct IFN-γ, IL-10 and β-actin cDNA inserts were used for standard curve.

Quantitative real-time RT-PCR. Measurement of gene expression was performed utilizing the ABI prism 7900HT Sequence Detection System (Applied Biosystems). Primers and TaqMan probes (Applied Biosystems) were designed to span exon-intron junctions to prevent amplification of genomic DNA and to let amplicon size reduce to <150 bp for enhancement of efficiency of PCR amplification. Primer and probe sequences were as follows: IFN-γ (forward), 5'-TTT TCA GCT CTG CAT CGT TTT G-3'; IFN-γ (reverse), 5'-GCT ACA TCT GAA TGA CCT GCA TTA A-3'; IFN-γ (probe), FAM-TCT TGG CTG TTA CTG CCA GGA CCC A-TAMRA; IL-

Table I. *Patient profile*.

Pt. no.	Age (years)	Gender	Disease	DR‡	$WT1_{332}$-specific response (IFN-γ ratios/IL-10 ratios)§			Clinical response¶
					Pre	4weeks post	8weeks post	(RECIST criteria)
1	54	F	BC	4	0.53/2.62	0.77/2.94	0.68/1.08	SD
2	75	M	GBM	15	0.35/7.4	0.29/7.35	0.94/0.81	SD
3	56	M	Astrocytoma	4	0.56/3.07	0.66/3.52	0.84/1.03	SD
4	66	M	GBM	15	0.46/2.24	1.58/u.e.	n.t.	SD
5	53	M	PC	4	0.81/1.53	0.32/2.75	0.77/1.24	SD
6	40	F	Astrocytoma	4	0.7/0.39	1.69/0.34	1.65/0.09	SD
7	70	M	Renal cancer	15	1.17/0.45	1.16/0.14	1.21/0.48	SD
8	62	F	CC	15	1.12/0.86	1.44/0.3	n.t.	SD
9	52	M	Rectal cancer	15	1.4/u.e.	1.39/1.95	0.6/0.45	SD
10	69	M	GBM	15	1.01/0.83	0.79/0.54	0.93/1.4	SD
11	58	M	CC	4	0.71/0.66	0.72/3.65	n.t.	PD
12	55	M	GBM	15	0.86/1.85	1.37/1.15	1.42/4.54	PD
13	76	M	Mediastinal tumor	4	0.77/0.22	1.02/1.47	0.73/0.81	PD
14	56	F	BC	15	0.95/0.62	0.86/1.78	1.19/0.43	PD
15	54	F	BC	4	1.05/0.6	0.62/0.99	n.t.	PD
16	78	F	PC	15	1.27/0.78	1.49/0.7	1.21/0.81	PD
17	33	M	Astrocytoma	15	0.86/1.6	1.11/0.72	0.88/1.58	PD

GBM, glioblastoma multiforme; astrocytoma, anaplastic astrocytoma; CC, colon cancer; PC, pancreatic cancer; BC, breast cancer; ‡HLA-DR allele. DR4 and DR15 represent DRB1*0405 and DRB1*1502, respectively; §ratio of relevant gene mRNA copies of $WT1_{332}$-stimulated Th cells to those of unstimulated Th cells. ¶clinical response by WT1 peptide (CTL-epitope) vaccination; SD, stable disease; PD, progressive disease; n.t., not tested; u.e., unevaluated.

10 (forward), 5'-CCC CAA GCT GAG AAC CAA GAC-3'; IL-10 (reverse), 5'-GGC CTT GCT CTT GTT TTC ACA-3'; IL-10 (probe), FAM-ATC GAT GAC AGC GCC GTA GCC TCA-TAMRA; β-actin (forward), 5'-TTG CCG ACA GGA TGC AGA A-3'; β-actin (reverse), 5'-GGA CAG CGA GGC CAG GAT-3'; β-actin (probe), FAM-TCA AGA TCA TTG CTC CTC CTG AGC GC-TAMRA. To make standard curves, the plasmid solution containing respective cDNA standards was serially diluted and amplified *via* quantitative real-time RT-PCR. Quantitative real-time RT-PCR reaction was performed in a total volume of 20 μl with TaqMan Master Mix (Applied Biosystems), 900 nM primers and 250 mM probes. Thermal cycler parameters included 2 min at 50˚C, 10 min at 95˚C, 40 cycles involving denaturation at 95˚C for 15 s and annealing/extension at 60˚C for 1 min. Standard curves were prepared for each gene and extrapolation of copy number was performed for *IFN-γ, IL-10* and *β-actin*. Normalization of samples was performed by dividing the copy number of *IFN-γ* and *IL-10* by that of *β-actin*. All PCR assays were performed in duplicates and reported as the mean.

Statistical analysis. When the expression levels of IL-10 mRNA of $CD4^+$ T-cells increased or decreased more than 2-fold by the stimulation with $WT1_{332}$ helper peptide compared to no stimulation with the peptide, $WT1_{332}$-specific $CD4^+$ T-cell response was defined to be positive and classified into IL-10-Up- and -Down-type response, respectively. The cut-off value of 2-fold was used here, as previously described (25).

The Mann-Whitney *U*-test was used to determine whether there was a statistically significant difference in expression level of IFN-γ and IL-10 mRNA between patients and healthy donors. The Wilcoxon signed-ranks test was used to analyze change of expression level of *IFN-γ* and *IL-10* mRNA among pre-vaccination, 4 and 8 weeks post-vaccination. The Spearman's correlation coefficient by rank test was used to analyze correlation between expression levels of *IFN-γ* and *IL-10* mRNA in patients. The Fisher's exact probability test was used to investigate a correlation between $WT1_{332}$-specific $CD4^+$ T-cell responses and clinical responses.

Results

A clear correlation between $WT1_{332}$ helper peptide-specific Th response and clinical response. PBMCs from WT1 CTL epitope peptide-vaccinated cancer patients were obtained from 17 patients at three time points (pre-, 4 and 8 weeks post-WT1 vaccination) and stimulated with or without $WT1_{332}$ helper peptide in the presence of CD28/49d Co-stimulatory Reagent. Then Th cells were enriched and examined for both the mRNA expression levels of *IFN-γ* and *IL-10* by quantitative real-time RT-PCR methods (Table I).

$WT1_{332}$-specific Th responses were classified into two types. IL-10-Up-type: *IL-10* mRNA expression increased more than 2-fold by $WT1_{332}$ stimulation compared to no peptide stimulation. IL-10-Down-type: *IL-10* mRNA expression decreased more than 2-fold by $WT1_{332}$ stimulation compared to no peptide stimulation. The

Table II. *$WT1_{332}$-specific Th response and clinical response in cancer patients.*

Pt. No.	$WT1_{332}$-specific response			Clinical response‡ (RECIST criteria)
	pre	4w	8w	
1	Up	Up	n.s.	SD
2	Up	Up	n.s.	SD
3	UP	Up	n.s.	SD
4	Up	u.e.	n.t.	SD
5	n.s.	Up	n.s.	SD
6	Down	Down	Down	SD
7	Down	Down	Down	SD
8	n.s.	Down	n.t.	SD
9	u.e.	n.s.	Down	SD
10	n.s.	n.s.	n.s.	SD
11	n.s.	Up	n.t.	PD
12	n.s.	n.s.	Up	PD
13	Down	n.s.	n.s.	PD
14	n.s.	n.s.	Down	PD
15	n.s.	n.s.	n.t.	PD
16	n.s.	n.s.	n.s.	PD
17	n.s.	n.s.	n.s.	PD

Up, IL-10-Up-type response; Down, IL-10-Down-type response; n.s., not significant (an increase or decrease in *IL-10* mRNA expression was below 2-fold); n.t., not tested; u.e., unevaluable. ‡Clinical response by WT1 CTL epitope peptide vaccination: SD, stable disease; PD, progressive disease.

Table III. *A correlation between $WT1_{332}$-specifc Th response and WT1 CTL epitope peptide vaccination-induced clinical response.*

	Clinical response‡ Pre		
$WT1_{332}$-specific Th response†	+	–	p-Value§
+	6	1	0.054
–	3	6	
	9	7	
	4 Weeks post		
	+	–	p-Value
+	7	1	0.02
–	2	6	
	9	7	
	8 Weeks post		
	+	–	p-Value
+	3	2	0.75
–	5	3	
	8	5	

†The number of patients who showed (+) or did not show (–) an IL-10-Up- or IL-10-Down-type response; ‡The number of patients of SD (+) or PD (–) capable of evaluating $WT1_{332}$-specific Th response; §*p*-values were obtained by Fisher's exact probability test.

$WT1_{332}$-specific Th response types and clinical responses were summarized in Table II. Relationship between $WT1_{332}$-specifc Th response and clinical response was investigated (Table III). A clear correlation (p=0.02) between occurrence of $WT1_{332}$-specific Th response of either IL-10-Up- or -Down-type and clinical response was found at 4 weeks post-WT1 vaccination. At pre-WT1 vaccination, such a correlation was also observed although it was not statistically significant (p=0.059).

Thus, these results indicated that monitoring of $WT1_{332}$-specific Th responses at 4 weeks post-WT1 vaccination and probably at pre-WT1 vaccination should be useful for prediction of clinical response of WT1 CTL epitope peptide vaccination.

An inverse correlation between $WT1_{332}$-specific IL-10 and IFN-γ responses. In order to dissect the difference in $WT1_{332}$-specific Th response between responders and non-responders, the ratios of IL-10 and IFN-γ mRNA expression in Th cells by $WT1_{332}$ stimulation to those in Th cells by no peptide stimulation were plotted on two axes at three time points (Figure 1). In responders, a clear inverse correlation between *IL-10* and *IFN-γ* mRNA expression ratios was shown at pre- and 4 weeks post-WT1 vaccination (rs=–0.75 and –0.8, respectively) (Figure 1A). In contrast to responders, in non-responders, such an inverse correlation was not observed at any of the three time points (Figure 1B). Importantly, in responders, data were quite scattered for the *IL-10* axis at pre- and 4 weeks post-WT1 vaccination (mean=2.15, SD=2.19 and mean=2.2, SD=2.32, respectively), but they converged on approximately ratio=1.0 at 8 weeks post-WT1 vaccination (mean=0.80, SD=0.44). In association with a decrease in *IL-10* mRNA expression at 8 weeks post-WT1 vaccination, *IFN-γ* mRNA expression (mean=0.98, SD=0.33) slightly increased at that time point, compared to IFN-γ mRNA expression at pre- and 4 weeks post-WT1 vaccination (mean=0.75, SD=0.29 and mean=0.95, SD=0.49, respectively). On the other hand, in non-responders, data converged on nearly ratio=1.0 not only in the *IL-10* axis but also in the *IFN-γ* axis.

These results indicated that $WT1_{332}$-specific Th response was strongly induced in responders compared to non-responders. In responders, IL-10-type of $WT1_{332}$-specific Th response was dominant at pre- and 4 weeks post-WT1

vaccination, but its dominancy became weak at 8 weeks post-WT1 vaccination, leading to the disappearance of the inverse correlation at that time point. Thus, these results suggested that repeated vaccination of WT1 CTL epitope peptide gradually allowed the cytokine production to switch from IL-10 to IFN-γ in the responders, implying the shift to Th1 response by the repeated WT1 CTL epitope peptide vaccination.

Enhancement of spontaneous IFN-γ expression in Th cells in responders. Spontaneous *IFN-γ* and *IL-10* mRNA expression were measured in Th cells in the absence of *in vitro* $WT1_{332}$ stimulation (Figure 2). Spontaneous IFN-γ mRNA expression in Th cells was significantly higher in responders at pre-WT1 vaccination than in healthy donors and the higher expression continued (Figure 2A). In contrast, in non-responders the expression levels were not significantly different from those in healthy donors at any time points (Figure 2A). As for spontaneous *IL-10* mRNA expression in Th cells, no significant difference was found between healthy donors and responders, and healthy donors and non-responders (Figure 2B). These results indicated that spontaneous expression of *IFN-γ* had been increased and continued in responders, suggesting that Th cells in responders were already activated towards pro-immune state before the start of the vaccination.

Discussion

ELISPOT and intracellular cytokine staining assay are considered to be a reliable method for analysis of antigen-specific T-cell responses. However, if the frequencies of the antigen-specific T-cells such as $CD8^+$ CTLs and $CD4^+$ Th cells are extremely low, it is very difficult to measure "quantitatively" the antigen-specific responses by using these assays. In order to circumvent this difficulty, quantitative real-time RT-PCR method was often used for the analysis of cancer antigen-specific T-cell responses in peripheral blood and tumor tissue as previously described (25, 26). In the present study, we adopted this method to measure mRNA of *IFN-γ* and *IL-10* quantitatively in the Th cells for the analysis of $WT1_{332}$-specific Th cells. We demonstrated here that i) occurrence of $WT1_{332}$-specific Th responses at 4 weeks post-WT1 vaccination correlated with clinical response in responders, and ii) high levels of spontaneous IFN-γ expression were induced in responders prior to and after WT1 vaccination.

To date, there have been no reports demonstrating the correlation between TAA-specific Th response and clinical response. We demonstrate for the first time that occurrence of $WT1_{332}$-specific Th responses at 4 weeks post-WT1 vaccination clearly correlated with clinical response. These findings suggested that monitoring of $WT1_{332}$-specific Th

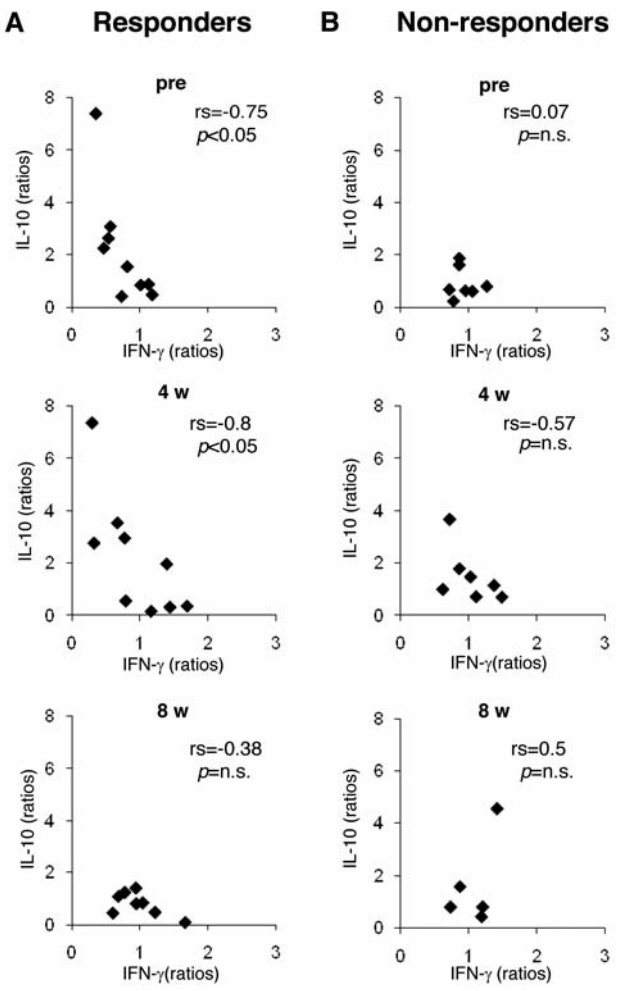

Figure 1. *An inverse correlation between $WT1_{332}$-specific IL-10 and IFN-γ mRNA expression in responders. Ratios of IL-10 and IFN-γ mRNA expression in Th cells by $WT1_{332}$ helper peptide stimulation to those in Th cells by no peptide stimulation were plotted at three time points (pre-, 4 and 8 weeks post-WT1 vaccination) in individual responders (A) and non-responders (B). The Spearman's correlation coefficient by rank test was used to analyze correlation between ratios of $WT1_{332}$-specific IL-10 and IFN-γ mRNA expression in patients.*

response was available for the prediction of clinical response by WT1 CTL epitope peptide vaccination. Unexpectedly, correlation between occurrence of $WT1_{332}$-specific Th response and clinical response disappeared at 8 weeks post-WT1 vaccination. This disappearance seemed to be due to the decrease in IL-10-type response at 8 weeks post-WT1 vaccination. $WT1_{332}$-specific *IFN-γ* mRNA expression slightly increased along with a decrease in *IL-10* mRNA expression at 8 weeks post-WT1 vaccination, indicating that the dominant response type changed from IL-10 response (pre- and 4 weeks post-WT1 vaccination) to IFN-γ response (8 weeks post-WT1 vaccination). Lack of correlation

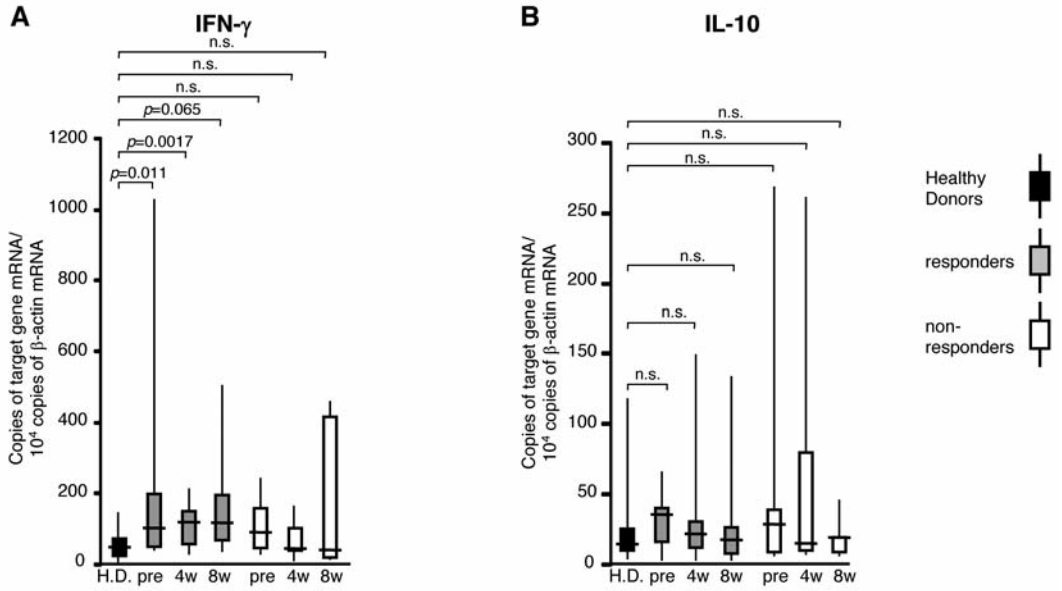

Figure 2. *Increased spontaneous IFN-γ expression in responders. Spontaneous IFN-γ (A) and IL-10 (B) mRNA expression levels in Th cells of responders and non-responders at three time points were shown in comparison with those in Th cells of healthy donors. Horizontal bars indicate median values of the expression levels. The Mann-Whitney U-test was used to determine the statistical significance of difference in the expression levels of IFN-γ and IL-10 mRNA between patients and healthy donors.*

between $WT1_{332}$-specific Th response and clinical response at 8 weeks post-WT1 vaccination should result from the time point still being a transitional stage from an IL-10 response to an IFN-γ one and that thus the IFN-γ response was not strong enough to consider the response as a positive one (it is represented as "n.s." in Table II).

We demonstrated here that IL-10-Up-type and -Down-type responses were an important factor for prediction of clinical response. It is generally thought that IL-10 is mainly produced from Tr-1 and $CD4^+CD25^+Foxp3^+$ Treg cells and that these cells facilitate the suppression of anti-tumor immunity. Recent studies reported an immunosuppressive function of IL-10 that strongly inhibited the activation of antigen-presenting cells, leading to a reduced production of pro-inflammatory mediators and resulting in diminished T-cell stimulation (27, 28). In addition, the inhibitory effects of IL-10 on the pro-inflammatory function of CTLs, Th1-type $CD4^+$ T-cells and NK cells further contribute to its immunosuppressive role. However, in striking contrast with the crucial role of IL-10 in immunosuppression, IL-10 also displays its immunostimulatory function (29, 30). IL-10 can enhance tumor rejection (31, 32), elicit tumor-reactive CTLs (33) and prevent T-cell apoptosis (34). These reports show that IL-10-producing tumor antigen-specific Th cells facilitate anti-tumor immunity in cancer patients. In the present study, the responders had higher IL-10 response, compared to the non-responders at pre- and 4 weeks post-WT1 vaccination. Afterward, the IL-10-type response declined accompanying with enhanced $WT1_{332}$-specific IFN-γ-type response. Therefore, we hypothesized that IL-10 was mainly involved in anti-tumor immunity at early phase (4 weeks post-WT1 vaccination), while at the late phase (8 weeks post-WT1 vaccination), IFN-γ mainly contributed to anti-tumor immunity. Actually, biphasic function of IL-10 has been reported using autoimmune disease model. In this model, early participation of IL-10 in the disease process provoked rapid pathogenic autoimmunity (35, 36). In contrast, the later participation of IL-10 in the disease process protected NOD mice from destructive autoimmunity (37, 38). These paradoxes evidently related to the period in which the immune system was exposed to high levels of IL-10. In human, Mocellin *et al.* previously demonstrated that tumor lesions which expressed high levels of *IL-10* mRNA at pre-vaccination showed tumor regression after the vaccination, and that during the vaccination, *IFN-γ* mRNA expression levels significantly increased in such regressing lesions, compared to non-regressing lesions (39). Taken together, these results indicated that IL-10 functioned as an immunostimulatory cytokine at early phase in WT1 immunotherapy. The immunostimulatory function of IL-10 is needed to be addressed in more detail.

It is very important to establish an immunological monitoring method to predict clinical responses in patients who are being treated with cancer vaccine. Recent report on melanoma patients vaccinated with GP100 peptide showed

that tumor progression occurred despite of induction of high levels of tumor antigen-specific $CD8^+$ T-cells that were detected by tetramer and ELISPOT assay(18). This report indicated that the detection of expanded numbers of vaccine-induced, tumor antigen-specific $CD8^+$ T-cells was not sufficient as a "surrogate marker" for vaccine efficacy. In the present study, the existence of $WT1_{332}$-specific Th responses at 4 weeks post-WT1 vaccination was predictable for occurrence of clinical response in WT1 CTL epitope peptide vaccination. Thus, Th responses should be valuable as the prediction markers for clinical efficacy of CTL epitope peptide vaccination.

Acknowledgements

We thank S. Watanabe and H. Yamamoto for the preparation of the manuscript. The excellent help of T. Umeda and Y. Hayami as a research nurse and technician, respectively, is acknowledged. This work was supported by The Charitable Trust Osaka Cancer Researcher-Found.

Conflict of Interest Disclosure

All Authors have no financial conflicts of interest to disclose.

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Received March 6, 2010
Revised April 16, 2010
Accepted April 22, 2010