Table I. Previous case reports on allogeneic DC vaccination.

| No. | Age, sex | Diagnosis | Donor characteristics (age/sex) | Pulsed with | Systemic adverse effects | | Immune response | | References |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | GVHD | Others | Tetramer | ELISPOT | |
| 1 | 24 y, F | ALL relapse | HLA-matched donor (NA/NA) | Tumor lysate | (−) | (−) | NA | NA | Fujii *et al.* [7] |
| 2 | 55 y, M | AML relapse | HLA-matched donor (NA/NA) | Tumor lysate | (−) | (−) | NA | NA | Fujii *et al.* [7] |
| 3 | 16 y, F | NHL relapse | HLA-matched donor (NA/NA) | Tumor lysate | (−) | (−) | NA | NA | Fujii *et al.* [7] |
| 4 | 34 y, M | ALL relapse | HLA-matched donor (NA/NA) | Tumor lysate | (−) | (−) | NA | NA | Fujii *et al.* [7] |
| 5 | 58 y, F | AML relapse | HLA-matched sibling (NA/NA) | $WT1_{235-243}$ peptide KLH peptide | (−) | (−) | NA | (−) (+) | Kitawaki *et al.* [8] |
| 6[a] | 12 y, F | AML relapse | HLA 7/8–matched sibling (12 y/F) | $WT1_{235-243}$ peptide | (−) | (−) | 5.56%[b] | (+) | Present case |

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; NA, not available; NHL, non-Hodgkin lymphoma; KLH, keyhole limpet hemocyanin; tetramer, tetramer assay.

[a]Present case.

[b]Shown are the maximum frequencies of the HLA-24:02–restricted WT1-tetramer$^+$ CD8$^+$ T cells within the CD8$^+$ T cells.

leukemia-specific immunotherapy. Recent clinical trials showed the feasibility and potential efficacy of WT1 peptide vaccination [9,10], or to autologous DC vaccination pulsed with WT1 peptide [3], in patients with acute myeloid leukemia or myelodysplastic syndrome. Rezvani *et al.* [11] demonstrated that the emergence of WT1-specific CD8$^+$ T cells was associated with graft-versus-leukemia effect in the patients with acute lymphoblastic leukemia after allogeneic HSCT. However, only one report was published on WT1-specific allogeneic DC vaccination [8]. A 58-year-old patient diagnosed with acute myeloid leukemia received five sessions of DC vaccines pulsed with HLA-A*24:02-restricted $WT1_{235-243}$ peptide. However, no WT1-specific immune response was detected in the patient's PBMCs by ELISPOT assay after five sessions of DC vaccination (Table I). To the best of our knowledge, we describe the first patient exhibiting a WT1-specific T-cell immune reaction after allogeneic DC vaccination.

Our patient received two sessions of DLI after the third HSCT, 5 and 10 months before the allogeneic vaccination. WT1-specific T cells have been detected in patients with multiple myeloma undergoing allogeneic HSCT and DLI [12]. Moreover, spontaneous WT1-specific T-cell responses have been reported in patients with acute myeloid leukemia [13]. Therefore, it is possible that the WT1-specific T cells were elicited by DLI or even occurred spontaneously in the patient. However, WT1-specific T cells were hardly detected at the time of initial vaccination despite two doses of DLI, whereas they were distinctly detected and increased after vaccination, which indicates that the allogeneic DC vaccination could contribute to eliciting this anti-leukemic immune response in our patient.

Interestingly, WT1-specific T-cell responses were maximal at the fourth relapse. We did not observe the loss of WT1 expression or HLA-A*24:02 allele in the relapsed leukemia cells, which could evade HLA-restricted WT1-specific T-cell response, at the time of the fourth relapse as well as the third relapse. One possible explanation for the response is that WT1-specific T cells might be merely boosted by the re-growing leukemia cells, although they were no longer able to control the disease.

Although our patient maintained less than 14 months of remission before the third HSCT, she benefited from 44 months of remission after the third HSCT with two doses of DLI and 14 DC vaccinations. These clinical observations suggest that the WT1-specific DC vaccination contributed to the longer remission after the third HSCT in our patient.

In conclusion, this report suggests that allogeneic DC vaccination is a safe, tolerable and even feasible option even for pediatric patients and pediatric donors. Because there are currently few effective therapies for patients who have a relapse after allogeneic HSCT, future trials should consider this treatment for patients with relapsing leukemia.

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# Prognostic Markers for Patient Outcome Following Vaccination with Multiple MHC Class I/II-restricted WT1 Peptide-pulsed Dendritic Cells Plus Chemotherapy for Pancreatic Cancer

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# Prognostic Markers for Patient Outcome Following Vaccination with Multiple MHC Class I/II-restricted WT1 Peptide-pulsed Dendritic Cells Plus Chemotherapy for Pancreatic Cancer

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**Abstract.** *Background/Aim: Treatment combining dendritic cells (DCs) pulsed with three types of major histocompatibility complex (MHC) class I and II (DC/WT1-I/II)-restricted Wilms' tumor 1 (WT1) peptides with chemotherapy may stabilize disease in pancreatic cancer patients. Materials and Methods: Laboratory data from seven patients with pancreatic cancer who underwent combined DC/WT1-I/II vaccination and chemotherapy were analyzed. The DC phenotypes and plasma cytokine profiles were analyzed via flow cytometry. Results: The post-treatment neutrophil to lymphocyte (N/L) ratio was a treatment-related prognostic factor for better survival. Moreover, the mean fluorescence intensities (MFIs) of human leukocyte antigen (HLA)-DR and cluster of differentiation (CD)83 on DCs were significantly increased after chemoimmunotherapy. Interestingly, interleukin (IL)-6 level in plasma was significantly increased after chemoimmunotherapy in non-super-responders. Conclusion: An increased N/L ratio, as well as HLA-DR and CD83 MFI levels may be prognostic markers of longer survival in patients with advanced pancreatic cancer who undergo chemoimmunotherapy.*

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) that can present tumor-associated antigens (TAAs) in the context of class I and II (MHC-I/II) major histocompatibility complexes (MHC) and the co-stimulatory molecules cluster of differentiation (CD)80 and CD86 (1). TAAs are recognized by $CD8^+$ cytotoxic T-lymphocytes (CTLs) in the context of MHC class I (MHC-I) molecules, whereas $CD4^+$ T-cells recognize antigenic peptides in association with MHC class II (MHC-II) molecules (1). Therefore, DCs have been pulsed with various MHC-I-restricted antigenic peptides in clinical studies. However, the antitumor effects of cancer vaccine-targeting $CD8^+$ CTLs have not been investigated as vigorously in clinical trials (2).

$CD4^+$ T-cells are required for the priming, generation and maintenance of TAA-specific $CD8^+$ CTLs (1). Moreover, $CD4^+$ T-cells play a more direct role in antitumor immunity (3, 4). The Wilms' tumor 1 (WT1) antigen is highly expressed in various types of tumors, including pancreatic cancer (5), and is an excellent TAA target for cancer vaccines (6, 7). Therefore, we recently investigated the clinical and immunological responses to DCs that were pulsed with multiple MHC class I/II-restricted WT1 peptides (DC/WT1-I/II) in combination with chemotherapy for pancreatic cancer (8).

The treatment of advanced pancreatic cancer *via* DC/WT1-I/II and chemotherapy resulted in longer survival

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*Key Words:* Cytokine, chemoimmunotherapy, dendritic cell, MHC class II, pancreatic cancer, vaccine, WT1.

among patients who exhibited positive delayed-type hypersensitivity (DTH) reactions against the WT1 peptides (8). Moreover, patients with strongly-positive DTH reactions maintained WT1-specific memory CTLs throughout the entire treatment period. Therefore, we suggested that a combination treatment involving DC/WT1-I/II and chemotherapy could lead to disease stability in patients with advanced pancreatic cancer (8). In the present study, we analyzed the prognostic markers for the outcomes of patients with pancreatic cancer who underwent chemoimmunotherapy with DC/WT1-I/II vaccination and chemotherapy.

## Materials and Methods

*Study design.* The ethics committee of the Jikei Institutional Review Board at the Jikei University School of Medicine and the clinical study committee of Jikei University Kashiwa Hospital (No. 14-60 (3209) and 21-204 (6082)) reviewed and approved this study. All 7 patients with pancreatic cancer provided written informed consent and all procedures were performed in accordance with the Helsinki Declaration. All patients underwent DC/WT1-I/II vaccination and chemotherapy. The laboratory data of patients who underwent chemoimmunotherapy (*e.g.*, C-reactive protein level (CRP), lymphocyte number, neutrophil number, neutrophil to lymphocyte (N/L) ratio) were analyzed prior to and after the treatments. The N/L ratio was defined as the ratio of the number of neutrophils to the number of lymphocytes in the blood.

*DC-WT1-I/II vaccines.* DCs were generated from peripheral blood mononuclear cells (PBMCs) that had been prepared from leukapheresis products using Ficoll-Plaque Premium (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) density gradient solution as previously described (9). The DCs were pulsed with a mixture of three WT1 peptide types that were restricted to HLA-A* 02:01, A* 02:06 (126-134: RMFPNAPYL), A*24:02 (235-243: CYTWNQMNL) and MHC-class II (332-347: KRYFKLSHLQ MHSRKH; NeoMPS Inc., City, CA, USA) (8).

*Chemoimmunotherapy.* The chemotherapeutic agent gemcitabine was intravenously administered at a dose of 1,000 mg/m$^2$ on days 1, 8 and 15 of a 28-day cycle. After the first gemcitabine administration cycle, the pancreatic cancer patients were treated with a combination of DC/WT1-I/II and gemcitabine. The DC/WT1-I/II vaccine (approximately $1\times10^7$ cells/dose) was intradermally administered biweekly. Nearly all vaccines overlapped with the standard chemotherapy (8).

*Phenotype analysis.* DCs generated from PBMCs that had been prepared from leukapheresis products were stained with the following monoclonal antibodies (mAb) for 30 min on ice: fluorescein isothiocyanate (FITC)-conjugated anti-human HLA-ABC (W6/32), CD80 (2D10), CD40 (5C3), phycoerythrin (PE)-conjugated anti-human CC chemokine receptor (CCR) 7 (150503; R&D Systems, Minneapolis, MN, USA), HLA-DR (L243), CD83 (HB 15e) and CD86 (IT2.2; BioLegend, San Diego, CA, USA). The cells were subsequently washed, fixed and analyzed using MACSQuant Analyzers (Miltenyi Biotec Inc., CA, USA) and the FlowJo analysis software (Tree Star, OR, USA).

*Plasma cytokine profiles.* The patient plasma samples collected were stored at −80°C. The stored plasma cytokine profiles were determined using the Human MACSPlex Cytokine 12 Kit, which enables the simultaneous measurement of human granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-α, IFN-γ, interleukin (IL)-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-17A and tumor necrosis factor (TNF)-α in a single immunoassay (Miltenyi Biotec Inc.) *via* MACSQuant Analyzers (Miltenyi Biotec Inc., CA, USA). The cytokine concentrations were quantified according to the manufacturer's instructions.

*ELISA.* To assess the production of IFN-γ and IL-10 in PBMCs upon stimulation with MHC-I/II-restricted WT1 peptides *in vitro*, PBMCs ($1\times10^6$ cells/ml per well) from 6 DC/WT1-I/II vaccination cycles were cultured for 6 days with 10 μg/ml WT1 class I and II peptides in the presence of 10 U/ml recombinant human (rh) IL-2 (Shionogi, Osaka, Japan) and 10 ng/ml IL-7 (Peprotech, Rocky Hill, NJ, USA). HIV env peptides were used as negative controls. The IFN-γ and IL-10 concentrations in the sample supernatants were analyzed using ELISA kits (BioLegend) according to the manufacturer's instructions.

*Statistical analysis.* The statistical analyses of the overall survival (OS) and progression-free survival (PFS) prognostic factors were performed according to the Kaplan-Meier method and evaluated using the log-rank test. The immunological parameters of the pancreatic cancer patients were evaluated in a *t*-test analysis. Values were expressed as the means±standard deviation (SD). A *p*-value of $<0.05$ was considered statistically significant.

## Results

*Patients' characteristics.* Patients with pathologically- or cytologically-confirmed, measurable, metastatic pancreatic adenocarcinoma or recurrent disease were enrolled in a non-comparative, open-label, phase I study (8). All 7 patients had stage IV disease and were treated with DC/WT1-I/II and chemotherapy. As shown in Table I, we identified 3 super-responders (OS>1 year) and 4 non-super-responders (OS≤1 year). After treatment, pancreatic cancer spread to the liver in one super-responder; however, this patient remains alive (>760 days) with a 100% Karnofsky Performance Status (KPS) after receiving treatment and has received more than 51 vaccinations. The remaining 2 super-responders with stage IV pancreatic cancer died at 582 and 717 days after the first treatment.

*Prognostic markers as indicated by laboratory data.* There were no differences between the super-responders (OS>1 year) and non-super-responders (OS≤1 year) with regard to sex, age or tumor location (Table II). We analyzed the laboratory data prior to treatment, after the first course of gemcitabine (prior to the first vaccination) and after 6-8 rounds of DC/WT1-I/II vaccination combined with gemcitabine. There was no significant difference in the lymphocyte numbers between the super-responders and non-super-responders. Moreover, the pretreatment N/L ratio of

Table I. *Patients' characteristics.*

| No. | Gender | Age (years) | Location | Metastases (base line) | Previous therapy | Number of vaccines | PFS (days) | OS (days) |
|---|---|---|---|---|---|---|---|---|
| 1 | Male | 70 | body | Peritonitis | No | 35 | 440 | 582 |
| 2 | Male | 68 | body | Liver, Lymph nodes | Ope, Cx | 46 | 208 | 717 |
| 3 | Female | 49 | head | Liver, Peritonitis, Lymph nodes | No | 7 | 26 | 133 |
| 4 | Male | 35 | body | Liver, Lymph nodes | No | 6 | 147 | 283 |
| 5 | Female | 72 | body | Peritonitis, Lymph nodes | No | 14 | 109 | 215 |
| 6 | Female | 69 | body-tail | Lymph nodes | No | 53+ | 545 | 783+ |
| 7 | Male | 39 | head | Peritonitis | No | 20 | 290 | 325 |

Ope: Operation, Cx: chemotherapy.

Table II. *Prognostic factors of OS or PFS.*

| | OS | | | PFS | | |
|---|---|---|---|---|---|---|
| | Before chemotherapy | Before vaccination | After 6-8 vaccinations | Before chemotherapy | Before vaccination | After 6-8 vaccinations |
| Sex (male/female) | 0.427 | | | 0.427 | | |
| Age (≥65/<65) | 0.207 | | | 0.464 | | |
| Ptimary tumor site (head/body-tail) | 0.583 | | | 0.953 | | |
| Lymphocyte (number/μl) (≥1000/<1000) | 0.953 | 0.197 | 0.863 | 0.646 | 0.694 | 0.207 |
| N/L ratio (≥4/<4) | 1.000 | 0.025* | 0.018* | 1.000 | 0.025* | 0.018* |
| CRP (mg/dl) (≥0.5/<0.5) | 1.000 | 0.646 | 0.863 | 1.000 | 0.583 | 0.207 |

OS: Overall survival, PFS: progression free survival, N/L: neutrophils/lymphocytes, CRP: C-reactive protein, *Statistically significant.

each group was not significantly associated with OS. Interestingly, after the first course of gemcitabine (prior to the first vaccination) and after 6-8 DC/WT1-I/II vaccinations combined with gemcitabine, the N/L ratio (<4) decreased significantly in the super-responders ($p$=0.025 and $p$=0.018, respectively; Table II and Figure 1). These results suggested that an N/L ratio <4 was a prognostic marker that correlated with OS.

*DC phenotype*. The DCs from all 7 patients displayed a characteristic phenotype comprising of HLA-ABC, HLA-DR, CD40, CD80, CD86, CD83 and CCR7 expression (Figure 2, upper panel). There were no differences in the HLA-ABC, HLA-DR, CD80, CD86, CD83 and CCR7 mean fluorescence intensities (MFIs) on DCs from super-responders (OS>1 year) *versus* non-super-responders (OS≤1 year) (Figure 3). Interestingly, the HLA-DR and CD83 MFIs were significantly increased in the DCs of the super-responders following chemoimmunotherapy (Figure 2 and Figure 4). In contrast, the CD83 MFIs in the DCs of non-super-responders decreased after therapy, although this difference was not significant ($p$=0.302).

*Plasma cytokine level profiles*. To assess the T-helper 1 (Th1) and T-helper 2 (Th2) cell-related cytokine profiles following chemoimmunotherapy, the levels of GM-CSF, IFN-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-17A and TNF-α in plasma collected after 6-8 vaccinations were immediately and simultaneously analyzed. There were no differences between the super-responders and non-super-responders in terms of these plasma cytokine levels after vaccination (data not shown). The levels of immunosuppressive cytokines, such as IL-4, IL-10 and IL-6, were higher in samples from non-super-responders (OS≤1 year) than in those from super-responders (OS>1 year), although this difference was insignificant (Figure 5A). Moreover, the levels of the Th1-stimulating cytokines IFN-α, IFN-γ and TNF-α were also higher in non-responders; again, this difference was insignificant (Figure 5A). Interestingly, the IL-6 level was significantly increased after chemoimmunotherapy in non-super-responders ($p$=0.049) (Figure 5B).

*Cytokine production by PBMCs following WT1 peptide stimulation*. To assess the cytokine profiles upon WT1 peptide stimulation *in vitro* in greater detail, PBMCs were

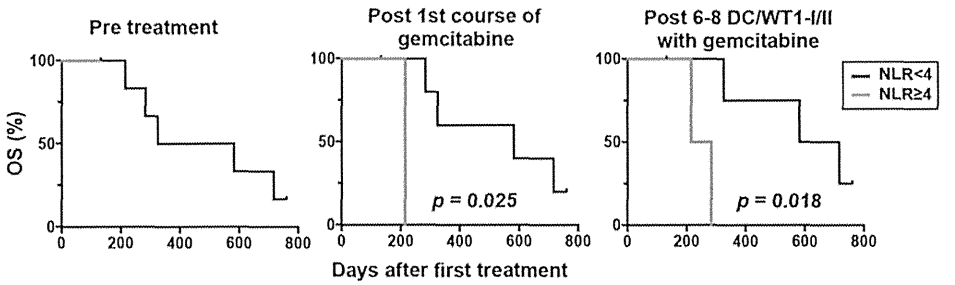


Figure 1. *Association of the neutrophil to lymphocyte ratio with overall survival. The neutrophil to lymphocyte (N/L) ratios of seven pancreatic cancer patients who received chemoimmunotherapy were analyzed prior to treatment (left panel), after the first gemcitabine course (middle panel) and after completing chemoimmunotherapy (right panel). NLR, N/L ratio.*

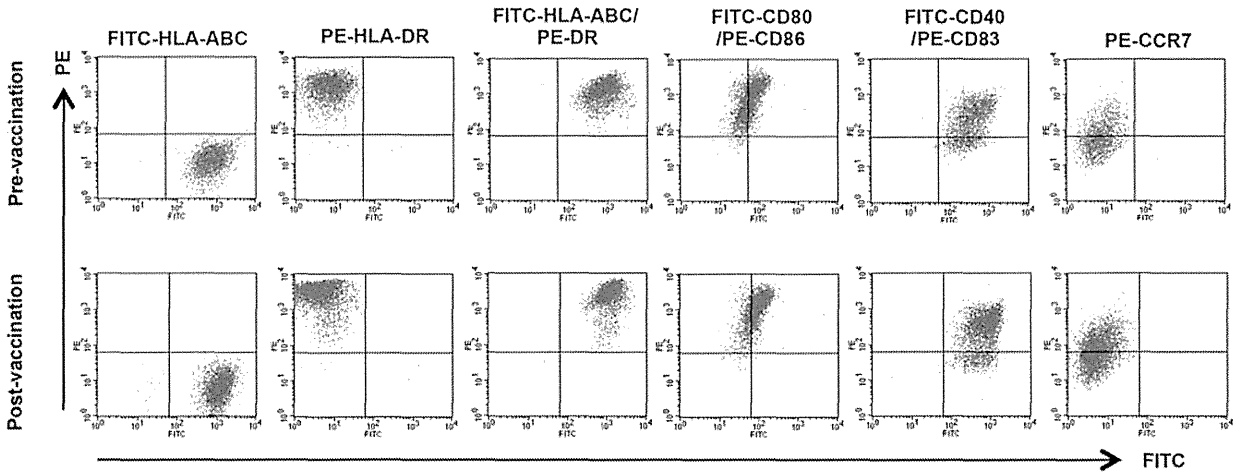


Figure 2. *Dendritic cell phenotypes. The indicated molecules expressed on the dendritic cells (DCs) from a super-responder (Patient no. 6) are shown prior to treatment and after chemoimmunotherapy.*

cultured with MHC-I- and -II-restricted WT1 peptides after which the Th1 cytokine IFN-γ and Th2 cytokine IL-10 concentrations in the supernatants were determined. In this experimental setting, no differences in the IFN-γ and IL-10 concentrations were observed between the super-responders (OS>1 year) and non-super-responders (OS≤1 year) (Figure 6). Moreover, the PBMCs produced extremely high levels of IFN-γ relative to IL-10 (Figure 6).

## Discussion

The data presented herein demonstrate that a decreased N/L ratio (<4) and increased HLA-DR and CD83 MFIs may be prognostic markers of chemoimmunotherapeutic outcome.

Results from a recent clinical trial suggest that chemotherapies, such as gemcitabine and S-1, an oral fluoropyridine, are effective chemotherapeutic agents for pancreatic cancer treatment in Japan (10). In that phase III study, the median OS was 8.8 months in the gemcitabine group, 9.7 months in the S-1 group and 10.1 months in the gemcitabine plus S-1 group. Therefore, an OS of >1 year generally indicates that the treatment was beneficial. In the present study, patients who received DC/WT1-I/II vaccinations combined with chemotherapy were classified into 2 groups: OS>1 year (super-responders) and OS≤1 year (non-super-responders). We first analyzed the pre-treatment laboratory data, including the albumin levels (data not shown), CRP levels, neutrophil numbers and lymphocyte numbers. There were no differences between the super-responders and non-super-responders in terms of these factors in our study. Our results support those from a recent report that indicated that the albumin level, CRP level, neutrophil number and lymphocyte number were not prognostic factors for the outcomes in 255 patients who had received standard

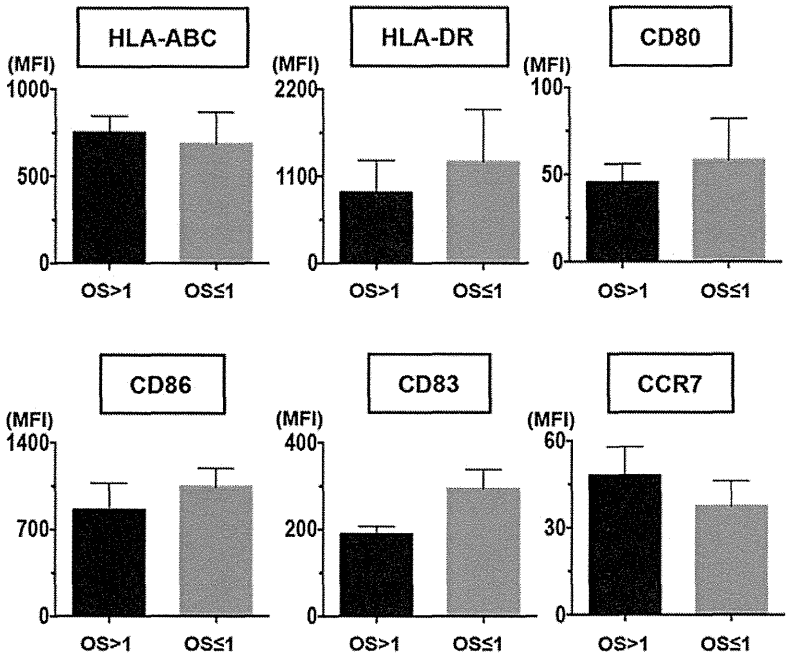


Figure 3. *The mean fluorescent intensities of surface molecules on dendritic cells. The pretreatment mean fluorescent intensities (MFIs) of HLA-ABC, HLA-DR, CD80, CD86, CD83 and CCR7 on dendritic cells (DCs) were compared between super-responders (OS>1year) and non-super-responders (OS≤1year). Values are expressed as means±SD.*

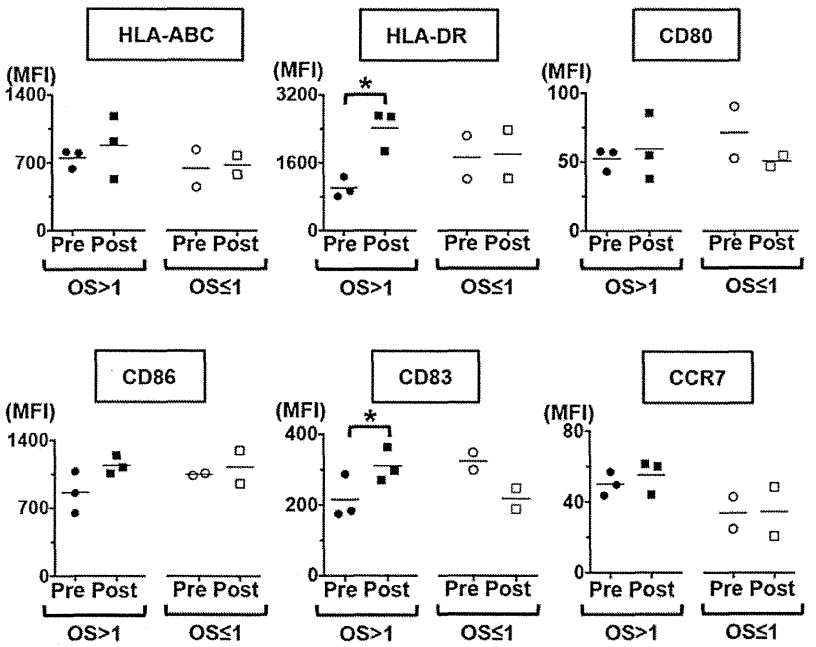


Figure 4. *The pre- and post-chemoimmunotherapy mean fluorescent intensities of surface molecules on dendritic cells. The pre- and post-chemoimmunotherapy mean fluorescent intensities (MFIs) of the indicated molecules on the surfaces of dendritic cells (DCs) were compared between super-responders (OS>1year) and non-super-responders (OS≤1year). Values are expressed as means. *p<0.05.*

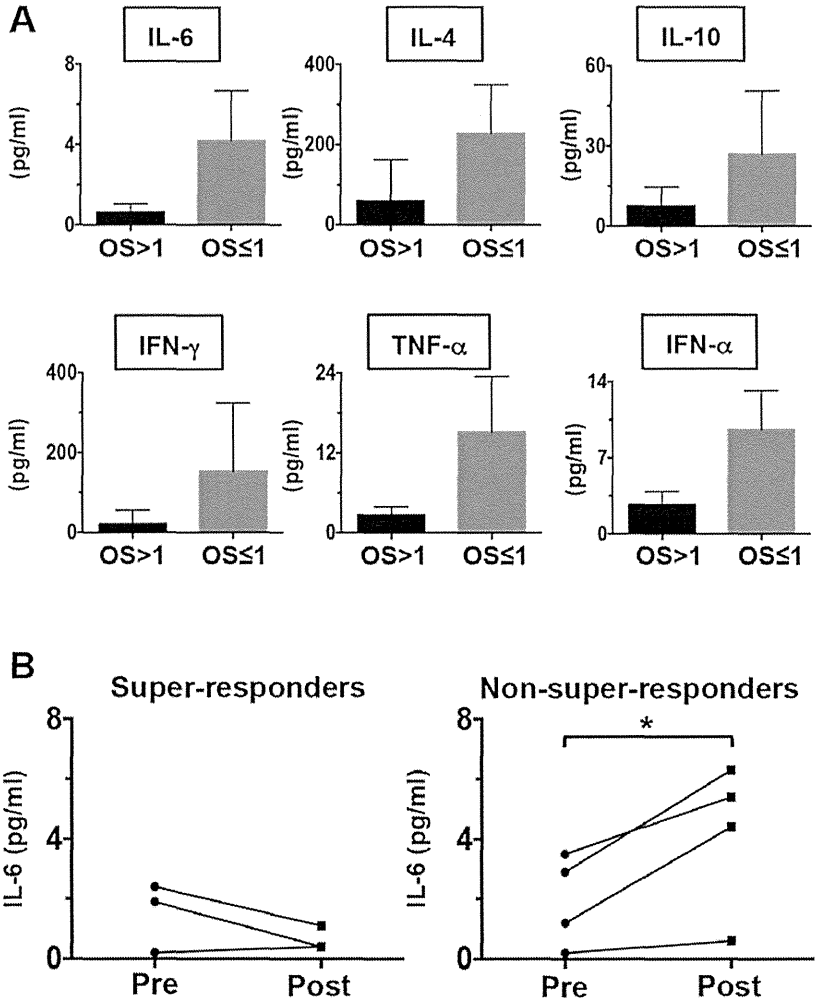


Figure 5. *Plasma cytokine profiles. A. The levels of cytokines (IL-6, IL-4, IL-10, IFN-γ, TNF-α and IFN-α) shown in plasma samples from patients who received 6-8 vaccinations and chemotherapy are compared between super-responders (OS>1 year) and non-super-responders (OS≤1 year). B. IL-6 levels in plasma samples (prior to treatment and after 6-8 vaccinations and chemotherapy) are compared between super-responders (OS>1year) and non-super-responders (OS≤1year). Values are expressed as means±SD. *p<0.05.*

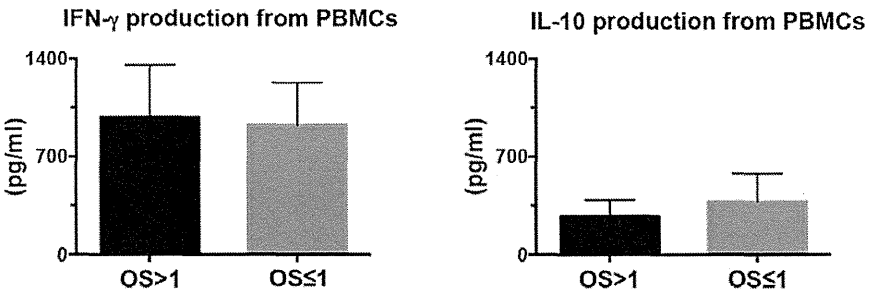


Figure 6. *IFN-γ and IL-10 production by peripheral blood mononuclear cells. IFN-γ and IL-10 production by peripheral blood mononuclear cells after 6 vaccinations is compared between super-responders (OS>1 year) and non-super-responders (OS≤1 year).*

chemotherapy combined with MHC-I-restricted peptide-pulsed DCs (11). Interestingly, a low post-treatment N/R ratio (<4) was associated with a good prognosis (OS>1 year) for pancreatic cancer patients in this study. Previously, an early reduction in the N/L ratio after effective treatment was reported to be associated with improved survival in cancer patients (12). Gemcitabine has been shown to up-regulate antigenic peptides on the HLA molecules of tumor cells (13), increase antigen cross-presentation (14) and decrease the immunosuppressive myeloid-derived suppressive cell (MDSC) (15) and regulatory T-cell (Treg) populations (16), resulting in the augmentation of antitumor immunity. The rapid decrease in the N/R ratio immediately following the initial gemcitabine course, as shown above, may have been induced by the reduced tumor-associated inflammatory and immunosuppressive responses. Moreover, the combination of DC/WT1-I/II vaccination and gemcitabine administration was also associated with additional reductions in the blood N/L ratio. A low N/L ratio was predictive of longer survival in patients with advanced pancreatic cancer who received gemcitabine (17).

The treatment of patients with advanced pancreatic cancer using DC/WT1-I/II vaccination plus gemcitabine-based chemotherapy has been associated with disease stability (8). In a clinical phase I trial, WT1-specific DTH-positive patients exhibited significant improvements in OS and PFS compared to negative controls. Moreover, all patients with strong DTH reactions were super-responders. In DC-based vaccines, autologous DCs are generated from GM-CSF- and IL-4-treated monocytes and subsequently mature through incubation with penicillin-killed and lyophilized preparations of a low-virulence *Streptococcus pyogenes* (OK-432) strain (Su) and prostaglandin E2 (PGE2). The expression levels of HLA-ABC, HLA-DR, CD80, CD86, CD83 and CCR7 on the DCs derived prior to treatment did not differ between patients, thus indicating a uniform DC quality. Interestingly, in the super-responders, the HLA-DR and CD83 expression levels increased significantly after treatment relative to pre-treatment levels. Vaccination with fusion products generated from DCs and whole tumor cells has been reported to result in DC maturation (18, 19). Our results were also consistent with reports in which the phenotypic features of DCs were found to differentiate *in vitro* following vaccination. These results suggested that the increased surface expression of DC markers after treatment indicates improved antigen-presenting function in these cells (18). Patients with advanced pancreatic cancer exhibited impaired DC function; however, gemcitabine improved DC function (20). The significantly increased levels of HLA-DR and CD83 expression on the DCs derived from patients who received DC/WT1-I/II and gemcitabine suggest that chemoimmunotherapy may restore DC function. In the super-responders, the improved DC phenotype (HLA-DR and CD83) may be associated with longer survival.

The plasma cytokine profile may be important when assessing the prognostic markers associated with chemoimmunotherapy. In this study, the pancreatic cancer patients received DC/WT1-I/II vaccines combined with chemotherapy. Therefore, we analyzed the Th1 and Th2 cytokine profiles after vaccinations. Our results revealed no differences between the cytokine profiles of super-responders and non-super-responders. The levels of the immunosuppressive cytokines IL-4, IL-10 and IL-6 were higher in non-super-responders than in super-responders, although this difference was not significant. Unexpectedly, the levels of the Th1 cytokines IFN-γ, TNF-α and IFN-α were also higher in non-super-responders; however, this difference was not significant. We also analyzed in greater detail the Th1 and Th2 responses of PBMCs upon WT1 peptide stimulation *in vitro*. Similarly, there were also no differences between the super-responders and non-super-responders in terms of IFN-γ or IL-10 production by PBMCs after 6-8 vaccinations. Interestingly, IL-6 plasma levels in non-super-responders were significantly increased after chemoimmunotherapy relative to the pretreatment levels. IL-6 is one of the major immunosuppressive cytokines, which can induce tumor progression by manipulating immune responses. Therefore, an increased IL-6 level in plasma may be associated with poor prognosis. The immune checkpoint blockade targeted-agents, such as programmed cell death protein 1(PD-1) and programmed death-ligand 1 (PD-L1) have been used to successfully treat patients with advanced melanoma (21). Therefore, it may be more important to inhibit immuno-suppressive responses than to stimulate immunity in patients with advanced pancreatic cancer. The primary limitation of our study is the relatively small size of the evaluated sample. Further studies are required to evaluate the prognostic markers of chemoimmunotherapy with DC/WT1-I/II.

## Conflicts of Interest

The Authors declare that they have no competing interests.

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# Transduction of a Novel HLA-DRB1*04:05-restricted, WT1-specific TCR Gene into Human CD4+ T Cells Confers Killing Activity Against Human Leukemia Cells

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**Abstract.** *Background/Aim: Wilms' tumor gene 1 (WT1) product is a pan-tumor-associated antigen. We previously identified WT1 protein-derived promiscuous helper peptide, $WT1_{332}$. Therefore, isolation and characterization of the $WT1_{332}$-specific T-cell receptors (TCRs) are useful to develop broadly applicable TCR gene-based adoptive immunotherapy. Materials and Methods: A novel HLA-DRB1*04:05-restricted $WT1_{332}$-specific TCR gene was cloned and transduced into human $CD4^+$ T-cells by using a lentiviral vector. Results: The $WT1_{332}$-specific TCR-transduced $CD4^+$ T-cells showed strong proliferation and Th1-cytokine production in an HLA-DRB1*04:05-restricted, $WT1_{332}$-specific manner. Furthermore, the $WT1_{332}$-specific TCR-transduced $CD4^+$ T-cells could lyse HLA-DRB1*04:05-positive, WT1-expressing leukemia cells in vitro. Conclusion: The novel TCR gene cloned here should be a promising tool to develop adoptive immunotherapy of $WT1_{332}$-specific TCR-transduced $CD4^+$ T-cells for the treatment of WT1-expressing cancer, such as leukemia.*

Wilms' tumor gene 1 (*WT1*) was isolated as a gene responsible for a childhood renal neoplasm, Wilms' tumor, and encodes a zinc finger transcription factor involved in the regulation of cell differentiation, proliferation and apoptosis (1-3). Abundant expression of the wild-type *WT1* gene has been detected in various kinds of solid tumors and hematological malignancies (4). In particular, the expression levels of *WT1* clearly correlated with disease aggressiveness and prognosis in leukemia (5, 6). Importantly, a recent study has demonstrated that chemotherapy-resistant human leukemia stem cells abundantly expressed *WT1* (7). The accumulating evidence indicated that WT1 should be a good target for treatment of leukemia. In fact, WT1-targeting immunotherapy was effective in leukemia (4, 8-10). Based on accumulated evidence, WT1 has been rated as the most promising tumor-associated antigen (TAA) among 76 TAAs (11).

A number of cancer immunotherapy approaches, such as a TAA-targeting vaccine and adoptive transfer of TAA-specific T-cells and TAA-specific T-cell receptor (TCR)-transduced T-cells, have been devoloped and conducting. However, most of the studies have been focusing on $CD8^+$ T-cells as effector cells, while the studies on $CD4^+$ T-cell as effector cells are limited. We previously identified a WT1-derived HLA class II-restricted peptide, $WT1_{332}$, which could bind to multiple HLA class II molecules and induce strong Th1 response (12-14), and reported that HLA class I(A*24:02)-restricted WT1 peptide vaccination induced $WT1_{332}$-specific $CD4^+$ T-cell responses and that the higher responses were correlated with better clinical outcome indicating the important roles of $CD4^+$ T-cell responses in anticancer immunity (15). Furthermore, we reported that HLA-DPB1*05:01-restricted, $WT1_{332}$-specfic TCR-transduced $CD4^+$ T-cells not only enhanced the induction of WT1-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) but also directly killed leukemic cells

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*in vitro* (16). These finding raised the concept that adoptive immunotherapy using HLA class II-restricted $WT1_{332}$-specfic TCR-transduced $CD4^+$ T cells should be a promising strategy for cancer treatment, especially for leukemia treatment because leukemia usually expresses HLA class II.

Major obstacles for application of the adoptive immunotherapy to broad-range population are the HLA-restriction and limited expression of tumor-associated antigens (TAAs). A way to solve these problems is cloning of various TCR repertoires that are specific for the complexes consisted of broadly-expressing TAAs (epitopes) and corresponding HLA class II molecules. Since the $WT1_{332}$ helper peptide is derived from a typical pan-TAA, WT1, and since it has the capacity to bind to multiple HLA class II molecules that are frequent in not only Asian but also Caucasian populations, cloning of a $WT1_{332}$-specific TCR gene is useful to the preparation of TCR gene sets for the adoptive immunotherapy. Herein, we cloned a novel HLA-DRB1*04:05-restricted, $WT1_{332}$-specific TCR gene and demonstrated that transduction of this TCR gene into human $CD4^+$ T-cells confers $WT1_{332}$-specific proliferative response, Th1 cytokine production and killing activity against WT1-expressing human leukemia cells. Thus, this novel $WT1_{332}$-specific TCR gene should be a promising tool to develop broadly-applicable TCR gene-based adoptive immunotherapy.

## Materials and Methods

*Cell lines.* The TCRαβ-deficient T-cell lines Jurkat 76 cell line (J76) was obtained from Dr. Hans Stauss (University College London, UK). Endogenously WT1-expressing and HLA-DRB1*04:05-positive MEG-01 (megakaryoblastic leukemia cell line) was obtained from Dr. Masaki Yasukawa (Ehime University, Ehime, Japan). Endogenously WT1-expressing and HLA-DPB1*05:01-positive C2F8 (early erythroblastic leukemia cell line) was kindly provided from Dr. Tatsuo Furukawa (Niigata University, Niigata, Japan) (17). Endogenously WT1-expressing and HLA-DRB1*04:05-negative K562 was obtained from Dr. Yoshiki Akatsuka (Fujita Health University, Aichi, Japan). Epstein-Barr virus (EBV)-transformed B cell line, B-LCL(–) and WT1-expressing B-LCL(+) were previously established from an HLA-DRB1*04:05-positive donor (13). All cell lines were cultured in RPMI1640 (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated FBS (Euro-lone, Milano, Italia) and 1% penicillin/streptomycin (Nacalai Tesque). X-VIVO™ 15 (Lonza, City, MD, USA) supplemented with 10% AB serum (Gemini, City, NC, USA) and 20 IU/ml interleukin-2 (IL-2) (kindly donated by SHIONOGI & Co., Ltd., Osaka, Japan) was used for culture of peripheral blood mononuclear cells (PBMCs) and T cells.

*Antibodies, peptides and reagents.* $WT1_{332}$ peptide (KRYFKLS HLQMHSRKH), 14 truncated $WT1_{332}$ peptides (as described in Figure 1), tumor cell lysates, Ac-IETD-Cho (granzyme B inhibitor) and blocking monoclonal antibodies (mAbs) were prepared as described previously (16). For flow cytometry analysis, the following mAbs were used: anti-CD107a-allophycocyanin (APC), anti-CD3-Pacific Blue, anti-CD4-APC-H7, anti-granzyme B-phycoerythrin (PE), anti-IL-5-PE, anti-IL-10-PE, anti-interferon (IFN) γ-PE-Cy7, anti-IFNγ-PE, anti-tumor necrosis factor (TNF) α-APC, anti-granulocyte-macrophage colony-stimulating factor (GM-CSF)-PE, anti-perforin-APC, anti-IL-17A-PE, (eBioscience, San Diego, CA, USA) and anti-IL-2-APC (BioLegend, San Diego, CA, USA).

*Generation of $WT1_{332}$-specific $CD4^+$ T cell clones.* $WT1_{332}$-specific $CD4^+$ T-cell clones were generated by using the CD154 expression assay as described previously (16, 18-20). Briefly, $WT1_{332}$-primed PBMCs were re-stimulated with the $WT1_{332}$ peptide for 6h and then $CD154^+$ $CD4^+$ T cells were single-cell sorted in 96-well plate. The sorted cells were expanded with phytohemagglutinin (PHA), IL-2 and irradiated allogeneic PBMCs. Then, established single-cell-derived $CD4^+$ T-cell clones were screened for $WT1_{332}$-specificity and used for further experiments.

*Cloning of TCR α- and β-genes and preparation of recombinant lentivirus.* HLA-DRB1*04:05-restricted, $WT1_{332}$-specific TCR α- and β-genes were amplified from a $WT1_{332}$-specific $CD4^+$ T cell clone (clone K) by using 5`-RACE (rapid amplification of cDNA ends) and separately inserted into a cloning vector as described previously (16). A TCR α-p2A-TCR β cassette was constructed and cloned into CSII-EF-MCS-IRES2-Venus lentiviral vector (kindly provided from Drs. Hiroyuki Miyoshi and Atsushi Miyawaki, RIKEN BioResource Center, Tsukuba, Japan). KOD FX DNA polymerase (Toyobo, Osaka, Japan) and the primers (as listed in Table I) were used to amplify TCRs. Recombinant lentivirus were generated by co-transfecting 293T cells with CSII-EF-MCS-IRES2-Venus encoding $WT1_{332}$-specific TCR gene or empty plasmid, pCAG-HIVgp and pCMV-VSV-G-RSV-Rev (kindly provided by Dr. H Miyoshi) as described previously (16).

*Establishment of HLA-DRB1*04:05-positive K562 cells.* cDNA was synthesized from total RNA of HLA-DRB1*04:05+ PBMCs by Super Script III (Invitrogen Life technologies, Carlsbad, CA, USA). HLA-DRA1-p2A-HLA-DRB1 cassette was constructed with primers as listed in Table I and inserted into the *Not I* and *BamH I* site of pcDNA3.1 (+) expression vector (Invitrogen Life technologies). The establishment of HLA-DRB1*04:05-positive K562 was accomplished by electroporation of the vector.

*Transduction of $WT1_{332}$-specific TCR gene into J76 cells.* Three hundred thousand J76 cells were added to a 48-well plate and incubated with $WT1_{332}$-specific TCR genes-encoding ($WT1_{332}$-TCR) or control lentivirus (mock=empty vector) in the presence of 8 μg/ml polybrene (Sigma, St. Louis, MO, USA). After 12 h of incubation, the medium was changed and transduced cells were further cultured and analyzed for the expression of CD3 molecules on their cell surface.

*Generation of $WT1_{332}$-specific TCR gene-transduced $CD4^+$ T-cells.* Freshly-isolated $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) mAbs in the presence of 40 IU/ml IL-2 for 2 days. Three hundred thousand of the activated cells were incubated in the presence of recombinant lentivirus and 8 μg/ml polybrene in a RetroNectin (TaKaRa Bio Co., Shiga, Japan)-coated 48-well plate. The plate containing the cells was centrifuged at 1,000 ×g at 33°C for 1 h. After 12 h of incubation, medium change was carried out and the cells were further incubated for 48-72 h. Then, $Venus^+$ $CD4^+$ T cells were sorted as transduced cells by FACSAria (BD Bioscience, San

Table I. *Primers employed.*

| Primers | Sequences 5 to 3' |
|---|---|
| For cloning of TCRs | |
| Cα 3'UTR-primer | CACAGGCTGTCTTACAATCTTGCAGATC |
| Cβ1 3'UTR-primer | CTCCACTTCCAGGGCTGCCTTCA |
| Cβ2 3'UTR-primer | TGACCTGGGATGGTTTTGGAGCTA |
| For construction of TCR α-p2A-TCR β cassette | |
| primer 1*(for TCR α 21.2) | CGCTCTGCGGCCGCGCCACCATGGAGACCCTCTTGGGCCTGCTTA |
| primer 1*(for TCR α 26-1.2) | CGCTCTGCGGCCGCGCCACCATGAGGCTGG |
| primer 2* | GCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGA AAACCCCGGTCCCATGAGCAACC |
| primer 3*(for TCR α 21.2) | GGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGA AGTTCGTGGCTCCGGAACCGCTGGACCAC |
| primer 3*(for TCR α 26-1.2) | GGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGA AGTTCGTGGCTCCGGAACCGCTGGACCAC |
| primer 4* | CCGGGATCCTCAGAAATCCTTTCTCTTGACCATGGCCAT |
| For cloning of HLA-DRA1/DRB1*04:05 | |
| DRA1 primer Forward | CGCTCTGGATCCGCCACCATGGCCATAAGTGGAGTCCCTGTGC |
| DRA1 primer Reverse | GGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGA AGTTCGTGGCTCCGGAACCCAGAGGCCCCCTGCGTTCTGCTGCA |
| DRB1 primer Forward | GCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAA ACCCCGGTCCCATGGTGTGTCTGAAGTTCCCTGGAG |
| DRB1 primer Reverse | CCGGCGGCCGCTCAGCTCAGGAATCCTGTTGGCTGA |

*Primer positions are indicated in Figure 1c.

Jose, CA) and re-stimulated with irradiated, $WT1_{332}$ peptide-pulsed autologous PBMCs. Mock-transduced $CD4^+$ T-cells were stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) mAbs in the presence of 40 IU/ml IL-2. One week later, the established $CD4^+$ T-cells were used for various experiments as described below. In order to maintain stably the established $CD4^+$ T-cells, they were re-stimulated with irradiated, $WT1_{332}$-pulsed autologous PBMCs every 10 days.

*Intracellular cytokine staining assay and CD107a mobilization assay.* For intracellular cytokine staining assays, $1\times10^5$ $CD4^+$ T-cells were incubated with the respective peptides in the presence of 2 μg/ml CD28/CD49d Costimulatory Reagent and 10 μg/ml Brefeldin A (Sigma) for 4 h. Intracellular staining for cytokines was performed using BD Cytofix/Cytoperm Buffer (BD Biosciences) according to the manufacture's procedures after surface staining of CD3 and CD4 molecules. The cells were analyzed with FACSAria. The data were analyzed with the FlowJo software (TreeStar, San Carlos, CA, USA).

For the CD107a mobilization assay, $1\times10^5$ $CD4^+$ T-cells were incubated with $1\times10^5$ $WT1_{332}$ peptide-pulsed or -unpulsed HLA-DRB1*04:05-positive K562 in the presence of 2 μM BD GolgiStop™ and anti-CD107a-APC mAb for 5 h. Then, the cells were harvested and intracellular cytokine staining was performed as described above.

*Proliferation assay.* The proliferative capacity was assessed using a standard [$^3$H]-thymidine incorporation assay, as described previously. In brief, $3\times10^4$ $CD4^+$ T cells were cultured with $2\times10^5$ irradiated autologous PBMCs pulsed or unpulsed with tumor lysate and WT1 peptide (20 μg/ml) for 2 days. Subsequently, [$^3$H]-thymidine (Amersham Biosciences, City, NJ, USA) was added to the cell culture and the cells were cultured for further 18 h. For the blocking assays, L243, SPVL3 and B7/21 mAbs were added to the proliferation assays at their optimal concentrations for blocking HLA-DR, -DQ and -DP, respectively.

*$^{51}$Cr release assay.* $^{51}$Cr release assays were performed as described previously (16). Briefly, target cells ($1\times10^4$ cells) labeled with $^{51}$Cr were added to wells containing varying numbers of effector cells in 96-well plates. After 16 h of incubation at 37°C, the supernatant was collected and measured for radioactivity. For granzyme B inhibition, target cells were pre-treated with 100 μM Ac-IETD-Cho, or DMSO as a control, at 37°C for 2 h and used for the experiment.

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

## Results

*Cloning of TCR genes from HLA-DRB1*04:05-restricted, $WT1_{332}$-specific $CD4^+$ T-cell clone.* In order to establish HLA-DRB1*04:05-restricted, $WT1_{332}$ helper peptide-specific $CD4^+$ T-cell clones, PBMCs from an HLA-DRB1*04:05-positive (HLA-DRB1*04:05/08:03) donor were cultured in the presence of the $WT1_{332}$ peptide for one week and $WT1_{332}$ peptide-specifically activated $CD4^+$ T-cells were single-cell sorted from the PBMCs by using the CD154 assay as described previously (16). Consequently, 32 $WT1_{332}$-specific $CD4^+$ T-cell clones were established. Clone K showed $WT1_{332}$-specific proliferative response, which was remarkably inhibited by addition of anti-

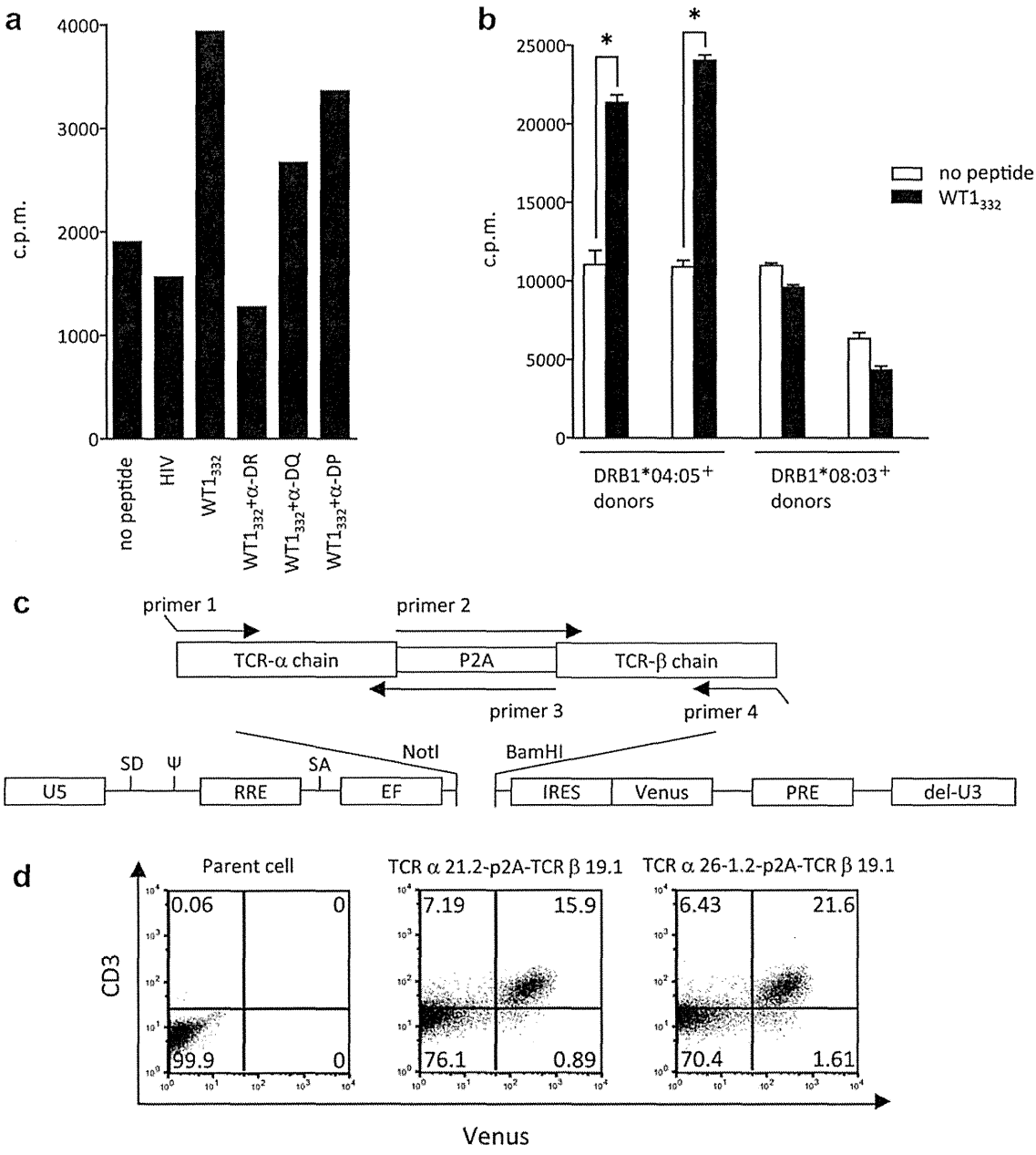


Figure 1. *Continued*

HLA-DR-blocking mAb (partial inhibition, which was frequently observed, by an anti-HLA-DQ-blocking mAb was a non-specific reaction) (Figure 1a). Furthermore, clone K strongly proliferated in response to $WT1_{332}$ peptide-pulsed allogeneic HLA-DRB1*04:05-positve PBMCs but not to $WT1_{332}$ peptide-pulsed allogeneic HLA-DRB1*08:03-positve PBMCs (Figure 1b). These results indicated that clone K was a $WT1_{332}$-specific, HLA-DRB1*04:05-restricted $CD4^+$ T cell clone.

Next, full-length TCR α-chain and β-chain cDNA derived from clone K were isolated and two α-chains (TCR α 21.2 and 26-1.2) and one β-chain (TCR β 19.1) were identified, indicating that allelic exclusion of α-chain in this clone was incomplete. Then, each α-chain was linked with the β-chain *via* the p2A peptide to definitely express both α- and β-chains and TCR α 21.2-p2A TCR β 19.1 and TCR α 26-1.2-p2A TCR β 19.1 cassettes were inserted into the lentiviral