Treatment with Chemotherapy and Dendritic Cells Pulsed with Multiple Wilms' Tumor 1 (WT1)–Specific MHC Class I/II–Restricted Epitopes for Pancreatic Cancer

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Abstract

Purpose: We performed a phase I trial to investigate the safety, clinical responses, and Wilms' tumor 1 (WT1)-specific immune responses following treatment with dendritic cells (DC) pulsed with a mixture of three types of WT1 peptides, including both MHC class I and II–restricted epitopes, in combination with chemotherapy.

Experimental Design: Ten stage IV patients with pancreatic ductal adenocarcinoma (PDA) and 1 patient with intrahepatic cholangiocarcinoma (ICC) who were HLA-positive for A*02:01, A*02:06, A*24:02, DRB1*04:05, DRB1*08:03, DRB1*15:01, DRB1*15:02, DPB1*05:01, or DPB1*09:01 were enrolled. The patients received one course of gemcitabine followed by biweekly intradermal vaccinations with mature DCs pulsed with MHC class I (DC/WT1-I; 2 PDA and 1 ICC), II (DC/WT1-II; 1 PDA), or I/II–restricted WT1 peptides (DC/WT1-I/II; 7 PDA), and gemcitabine.

Results: The combination therapy was well tolerated. WT1-specific IFN-γ–producing CD4+ T cells were significantly increased following treatment with DC/WT1-I/II. WT1 peptide-specific delayed-type hypersensitivity (DTH) was detected in 4 of the 7 patients with PDA vaccinated with DC/WT1-I/II and in 0 of the 3 patients with PDA vaccinated with DC/WT1-I or DC/WT1-II. The WT1-specific DTH-positive patients showed significantly improved overall survival (OS) and progression-free survival (PFS) compared with the negative control patients. In particular, all 3 patients with PDA with strong DTH reactions had a median OS of 717 days.

Conclusions: The activation of WT1-specific immune responses by DC/WT1-I/II combined with chemotherapy may be associated with disease stability in advanced pancreatic cancer. Clin Cancer Res; 20(16); 4228–39. ©2014 AACR.

Introduction

Dendritic cells (DC) play important roles in the initiation and regulation of tumor-specific immune responses (1). Cancer cells express tumor-associated antigens (TAA) by MHC class I (MHC-I) molecules, and these antigens are recognized by CD8+ cytotoxic T lymphocytes (CTL). Therefore, DCs have been pulsed with various MHC-I peptides to induce antigen-specific immunity (1). Other groups and our laboratory have shown that antigen-specific immune responses can be induced by DCs pulsed with an MHC-I–restricted peptide in patients with pancreatic ductal adenocarcinoma (PDA; refs. 2, 3). Most DC-based vaccines have targeted only CD8+ CTLs; however, the antitumor effects of these vaccines are not as vigorous in clinical trials (2). Increasing evidence has suggested that CD8+ CTLs depend on CD4+ T cells, which provide the CD8+ CTLs with growth...
Translational Relevance

Dendritic cells (DC) have been extensively used in the development of anticancer vaccines. Most DC-based cancer vaccines have targeted only CD8+ CTLs; however, the antitumor effects of these vaccines are not as vigorous in clinical settings. CD4+ T cells play a direct role beyond assisting in the generation of antitumor immunity. In this phase I study, we investigated the safety and the clinical and immunologic responses of DCs pulsed with a mixture of three types of WT1 peptides, including MHC class I and II-restricted epitopes (DC/WT1-I/II), when used in combination with chemotherapy. Our results showed that the combination therapy induced WT1 peptide-specific delayed-type hypersensitivity in 4 of the 7 patients with pancreatic cancer, and that these responses lasted throughout long-term vaccination and were associated with clinical responses. These findings suggest that targeting CD4+ and CD8+ T cells with DC/WT1-I/II could be a promising therapy for patients with pancreatic cancer.

Factors, such as interleukin (IL)-2, and can mediate the destruction of the tumor cells (4–8). Therefore, the presentation of antigenic epitopes of both MHC class I and II (MHC-I/II) induces high affinity T cells that react with the MHC-I/II epitopes (9).

With many TAAs, the establishment of criteria for selecting particular TAAs for clinical development is important. The Wilms’ tumor gene 1 (WT1) is highly expressed in various types of malignancies, including pancreatic cancer (71%–75%; refs. 10, 11), and has been found to be both oncogenic during tumorigenesis (12) and immunogenic (13–15). Therefore, other groups and our laboratory have performed clinical studies investigating the efficacy of immunotherapies targeting WT1 using a MHC-I-restricted peptide for patients with PDA (16, 17). Recently, MHC class II (MHC-II) epitopes derived from WT1 have been made available for use in clinical trials (18, 19). We hypothesized that the use of WT1 peptides, including MHC-I/II-restricted epitopes, would result in disease stability in patients with PDA. Here, we report a phase I clinical trial in stage IV patients with PDA that investigated the safety and effects of chemotherapy treatment combined with DCs pulsed with MHC-I/II-restricted WT1 epitopes.

Materials and Methods

Study design

This phase I study was reviewed and approved by the ethics committee of the Jikei Institutional Review Board, Jikei University School of Medicine (Tokyo, Japan), and by the clinical study committee of Jikei University Kashiwa Hospital [No. 21-204 (6082)]. In addition, this study was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number, 000004063). All patients provided written informed consent, and the procedures were performed in accordance with the Helsinki Declaration. The primary endpoint was the assessment of the safety and toxicity of the combination therapy based on the Common Terminology Criteria for Adverse Events (CTCAE v.4.0). Toxicity was defined as hematologic and nonhematologic events, including at the vaccine-injection sites, during the first three courses. The secondary endpoint was the assessment of the immunologic response, tumor response, overall survival (OS), and progression-free survival (PFS) from the first treatment.

Patient population

Patients with pathologically or cytologically confirmed, measurable, metastatic pancreatic or biliary tract adenocarcinoma or with recurrent disease were eligible for this noncomparative, open-label, phase I study. All patients were required to have an HLA type of A*02:01, A*02:06, A*24:02, DRB1*04:05, DRB1*08:03, DRB1*15:01, DRB1*15:02, DPB1*05:01, or DPB1*09:01. Approximately 80% of the Japanese population has HLA types A*02:01, A*02:06, or A*24:02. Additional inclusion criteria included patients with ages between 20 and 75 years, Karnofsky performance status (KPS) of 60% to 100%, a minimum 6-month interval from the completion of any previous treatment for recurrent disease, a life expectancy of ≥3 months, and adequate organ function. The exclusion criteria were pregnancy, serious infections, severe underlying disease, severe allergic disease, and a judgment of unsuitability by the principal investigator.

Clinical responses

Computed tomography was performed every 4 weeks during the treatment protocol and every 4 to 8 weeks during the additional treatment until the disease progressed, and treatment efficacy was determined according to the Response Evaluation Criteria in Solid Tumors (RECIST). Stable disease (SD) was defined as disease that was stable for more than 8 weeks after the start of the treatment.

DC preparation

DCs were generated from peripheral blood mononuclear cells (PBMC) prepared from leukapheresis products using Ficoll-Plaque Premium (GE Healthcare, Life Science) density gradient solution, as previously described (3). Briefly, plastic-adherent monocytes were cultured in AIM-V medium (Gibco) containing granulocyte macrophage colony-stimulating factor (50 ng/mL, Primmune Corp) and IL4 (50 ng/mL, R&D Systems) for 5 days to generate immature DCs. The immature DCs were then matured by incubation with penicillin-killed and lyophilized preparations of a low virulence strain (Su) of Streptococcus pyogenes (OK-432; 10 µg/mL, Chugai Pharmaceutical) and prostaglandin E2 (PGE2; 50 ng/mL, Daiichi Fine Chemical Co, Ltd) for 24 hours. The DCs were cryopreserved until the day of administration. After thawing, the cell viability for each vaccination was confirmed to be more than 90% using Trypan blue exclusion analysis. To determine the phenotype of the DCs, the cells were incubated with the following monoclonal
antibodies (mAb): fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 (61D3, ebioscience), HLA-ABC (W6/32), CD80 (2D10), CD84 (5C3), phycoerythrin (PE)-conjugated anti-human CCR7 (150503, R&D Systems), CD11c (3.9), HLA-DR (L243), CD83 (HB 15e), and CD86 (IT2.2; BioLegend). The cells were analyzed using MACSQuant Analyzers (Miltenyi Biotec Inc.) and the FlowJo analysis software (Tree Star). The endotoxin levels and bioburden of these DCs were tested and determined to be acceptable based on saline. The DC/WT1 cells were suspended in 500 µL of saline for injection. The endotoxin levels and bioburden of these DCs were tested and determined to be acceptable based on saline.

WT1 peptide-pulsed DCs (DC/WT1)
For each vaccination, DCs were pulsed with the MHC-I, -II, or -I/II-restricted epitopes of WT1, depending on their HLA. Briefly, mature DCs were incubated with WT1 peptides restricted to HLA-A*02:01, A*02:06 (126-134: RMFPNAPYL, NeoMPS Inc.) or A*24:02 (235-243: CYTWNQMNL, NeoMPS Inc.; refs. 16, 17), and/or DRB1*04:05, DRB1*08:03, DRB1*15:01, DRB1*15:02, DPB1*05:01, or DPB1*09:01 (332-347: KRYFKSLHQMHSRKH, NeoMPS Inc.; refs. 20, 21, and unpublished data) for 30 minutes and washed with saline. The DC/WT1 cells were suspended in 500 µL of saline for injection. The endotoxin levels and bioburden of these peptides were tested and determined to be acceptable based on the GMP grade for the vaccines.

Combination therapy
Gemcitabine was intravenously administered at a dose of 1,000 mg/m² on days 1, 8, and 15 of a 28-day cycle. After the first cycle of gemcitabine administration, the patients were treated with a combination of gemcitabine and DC/WT1. The DC/WT1 vaccine (usually 1 × 10^6 cells/dose) was intradermally administered biweekly at six different sites (bilateral upper arms, lower abdomen, and femoral regions) regardless of the regimen of chemotherapy. However, nearly all vaccines overlapped with standard chemotherapy. The initial treatment protocol was planned as three courses. The patients without early progressive disease at the completion of the treatment protocol could receive additional treatment until the occurrence of disease progression, unacceptable adverse events, or withdrawal of consent.

Delayed-type hypersensitivity test
The delayed-type hypersensitivity (DTH) test was performed before treatment in all patients and after 2, 4, and 6 vaccinations; it was also performed at suitable times during the additional treatment. Briefly, 30 µg of a WT1 peptide (RMFPNAPYL, CYTWNQMNL, or KRYFKSLHQMHSRKH) in saline or saline alone was intradermally injected in the forearm, and the maximum diameter of erythema and other skin reactions, including induration, were measured after 48 hours. WT1-specific DTH positivity was defined as erythema greater than 2 mm in diameter, which was the minimum size measurable with a ruler. Moreover, we chose the value of 5-mm erythema to discriminate between weak (2-5 mm) and strong (>5 mm) DTH.

PBMC preparation for immunologic monitoring
PBMCs were obtained from the patients before chemotherapy and during vaccination. The PBMCs were prepared using a Ficoll-Plaque Plus (GE Healthcare Bio-Sciences) density gradient solution and were stored at −80 °C in Bambanker (Nippon Genetics Co., Ltd.) without serum within 24 hours of blood collection. After thawing, cell viability was confirmed to be greater than 90% using the Trypan blue exclusion assay.

Detection of WT1-specific immune responses
The cryopreserved PBMCs were thawed and cultured with 10 µg/mL WT1 class I and II peptides in the presence of recombinant human (rh) IL2 (10 U/mL; Shionogi) and IL7 (10 ng/mL; Peprotech) for 9 days. The HIV env peptides and matched isotype IgG were used as negative controls. Cells were assessed using the PE-conjugated tetramer for WT1/HLA-A*24:02 (MBL) and FITC-conjugated anti-human CD8 mAb (BioLegend). The PE-conjugated tetramer for human immunodeficiency virus (HIV env)/HLA-A*24:02 (RYLRDQQLL) was used as a negative control, and cytomegalovirus (CMV pp65)/HLA-A*24:02 (QYDPVAALF) was used as a positive control (MBL). The amount of WT1-specific CD8^+ T cells (WT1-CTLs) is shown as the percentage of the double-positive population (WT1/HLA-A*24:02 tetramer−positive CD8^+ T cells).

Detection of WT1/HLA-A*24:02−specific memory cells in CD8^+ T cells
To assess the population of WT1/HLA-A*24:02−specific memory cells in CD8^+ T cells, cryopreserved PBMCs from six vaccinations with DC/WT1-II were thawed and immediately assessed using the following mAbs: 7-amino-actinomycin D (7-AAD; ebioscience), APC-Cy7−conjugated anti-human CD3, PE-conjugated tetramer for WT1/HLA-A*24:02, FITC−conjugated anti-human CD8, PE-Cy7−conjugated anti-human CD45RA (BioLegend), APC-conjugated anti-human CCR7 (BD Biosciences), or matched isotype control IgG (BioLegend). First, CD3^+7-AAD− cells were gated, and then the lymphocytes were re-gated. The percentage of memory (CD45RA^−CCR7^+ and CD45RA^−CCR7^−) phenotypes in the total CD3^+ T-cell population was determined. Then, the number of WT1/HLA-A*24:02 tetramer−positive cells in the total memory cells was determined. Finally, the percentages of WT1/HLA-A*24:02 tetramer−positive memory cells in CD8^+ T cells were determined using MACSQuant Analyzers and the FlowJo analysis software.

ELISA
To assess the production of IFNγ or IL10 in the PBMCs, the PBMCs (1 × 10^6 cells/mL in each well) from 6 vaccinations were cultured with 10 µg/mL WT1 class I and II peptides in the presence of 10 U/mL rh IL2 and 10 ng/mL IL7 for 6 days. The HIV env peptides were used as negative controls. The supernatants from the samples were analyzed for IFNγ or IL10 using an ELISA (BioLegend) according to the manufacturer’s instructions.
Intracellular staining of IFNγ
To assess the function of the WT1-CTLs, PBMCs (1 × 10^6 cells/mL in each well) were cultured with 10 μg/mL WT1 class I and II peptides in the presence of 10 U/mL rh IL-2 and 10 ng/mL IL-7 for 9 days. The HIV env peptides and matched isotype IgG were used as negative controls. The cells (1 × 10^5 cells/50 μL in each well) were restimulated with 10 μg/mL WT1 class I and II peptides for 6 hours using a GolgiPlug kit (BD Pharmingen); the cells were then stained with FITC-conjugated anti-human CD8 mAb, APC-Cy7–conjugated anti-human CD4 mAb (eBioscience), and APC-conjugated anti-human IFN-γ mAb (BioLegend). The IFNγ-producing cells in the CD4^+ or CD8^+ T cells were analyzed using MACSQuant Analyzers and the FlowJo analysis software.

Proliferation assays
PBMCs (1 × 10^6 cells/mL in each well) were cultured with 10 μg/mL WT1 class II peptide in the presence of 10 U/mL rh IL-2 and 10 ng/mL IL-7 for 9 days. The cells were stained with FITC-conjugated anti-human CD8 mAb and APC-Cy7–conjugated anti-human CD4 mAb, and then the total CD4^+ or CD8^+ T-cell numbers were determined.

Detection of immunosuppressive cells
To assess the CD4^+CD25^+Foxp3^+ regulatory T cells (Tregs) in the CD4^+ T cells, the PBMCs were stained with FITC-conjugated anti-human CD4 mAb, PE-conjugated anti-human CD125 mAb, and then the total CD4^+ T-cell population was determined.

Detection of WT1/HLA-A^24:02–specific PD1^+ cells in CD8^+ T cells
To assess whether the WT1-CTLs were impaired by the DC/WT1-I/II vaccination, the following mAbs were used: APC-Cy7–conjugated anti-human CD3, FITC-conjugated anti-human CD8, PE-conjugated tetramer for WT1/HLA-A^24:02, and APC-conjugated anti-human CD279 [programmed death 1 (PD1); BioLegend]. The HIV env/HLA-A^24:02 peptide and matched isotype IgG were used as negative controls. First, CD3^+ cells were gated and then the lymphocytes were regated. The percentage of PD1^+ cells in the entire CD8^+ T-cell population was determined. Then, the number of WT1/HLA-A^24:02 tetramer–positive cells in the PD1^+ CD8^+ T-cell population was determined.

PD-1^+ cells in CD8^+ T cells was analyzed using MACSQuant Analyzers and the FlowJo analysis software.

Statistical analysis
OS and PFS were calculated from the date of treatment to the date of death or final follow-up and the date of disease progression, respectively. Statistical analyses of the prognostic factors of OS or PFS were performed using the Kaplan–Meier method and were evaluated using the log-rank test. Immunologic parameters in the patients after therapy were evaluated using Student’s t test for two independent groups and a one-way analysis of variance for multiple-group comparisons. A P value less than 0.05 was considered statistically significant.

Results
Patient characteristics
Between August 2011 and January 2013, 11 patients were enrolled. The patient characteristics are presented in Table 1. Ten patients had PDA and 1 patient had intrahepatic cholangiocarcinoma (ICC). First, 2 patients with PDA and 1 patient with ICC were treated with DCs pulsed with MHC-I (HLA-A^02:01, 02:06 and/or 24:02)–restricted WT1 peptides (DC/WT1-I). Then, 1 patient with PDA was treated with DCs pulsed with MHC-II (DRB1^04:05, DRB1^08:03, DRB1^15:01, DRB1^15:02, DPB1^05:01, or DPB1^09:01)–restricted WT1 peptides (DC/WT1-II; Supplementary Fig. S1). We assessed the toxicities in these 4 patients. Next, DCs pulsed with MHC-II–restricted WT1 peptides (DC/WT1-I/II) were used for the remaining 7 patients with PDA. All patients completed the initial treatment protocol (first three courses; Supplementary Fig. S2).

Toxicity
The toxicities documented within the first three courses are shown in Supplementary Table S1. During these periods, the average total gemcitabine dose was 7,400 mg/m², and the total vaccination was 3.8 times. One patient, PDA-06, with multiple liver metastases showed rapid disease progression in the liver and died of a cerebral infarction 133 days after the first treatment. Finally, PDA-06 received 10 gemcitabine (10,000 mg/m²) and 7 DC/WT1-I/II treatments. A grade 1 pulmonary fibrosis occurred in one patient (PDA-07) after a total of 12 gemcitabine (11,800 mg/m²) and 6 DC/WT1-I/II treatments. All 11 patients experienced grade 1 skin reactions at the site of vaccination. Grade 1 to 3 leukopenia and anemia thought to be caused by gemcitabine were observed in all 11 patients. Grade 1 to 3 lymphopenia and grade 2 to 3 neutropenia were also observed in 10 patients. Other major nonhematologic adverse events included grade 1 to 2 anorexia and nausea, all of which were previously reported as major adverse events associated with gemcitabine. Grade 1 to 3 hepatic transaminase elevation related to disease progression and/or hepatobiliary infection was detected in 5 patients.
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**Abbreviations:** Cx, chemotherapy; DTH -, erythema <1 mm; DTH +, erythema 2-5 mm; DTH ++, erythema >5 mm; LN, lymph nodes; N.D., not done; Op, operation.
Clinical responses

None of the 7 patients with PDA vaccinated with DC/WT1-I/II reached a complete or partial response, and 6 of these patients (85.7%) exhibited SD (Supplementary Table S2). However, 1 of the 3 PDA patients (33.3%) vaccinated with DC/WT1-I or -II exhibited SD, and the remaining 2 patients (66.7%) exhibited progressive disease (PD; Supplementary Table S2). In addition, IFNγ-positive T cells were at baseline levels before the vaccinations (data not shown). To assess the more critical role of the WT1-specific CD4+ T-cell response that is restricted by MHC class II molecules with the DC/WT1-I/II vaccine, proliferation assays were performed. Figure 2C shows that, upon stimulation with WT1 class II peptide in vitro, CD4+ T cells from DC/WT1-I/II-vaccinated patients with PDA showed significant proliferation compared with DC/WT1-I or -II vaccine. Moreover, no significant difference between the proliferation of CD4+ or CD8+ T cells before and after vaccination with DC/WT1-I or -II was observed.

Assessment of WT1-specific immune responses

No patients with PDA exhibited DTH reactivity against the WT1 peptides during pretreatment. After vaccination with DC/WT1-I/II, 4 of the 7 patients with PDA (57.1%) showed DTH positivity, and all conversions were detected during the treatment protocol (Table 1). Interestingly, the MST and the median PFS of the patients vaccinated with DC/WT1-I/II were significantly longer than those receiving the DC/WT1-I or -II vaccines (P = 0.036 and P = 0.010, respectively; Fig. 1A and B).

Analysis of WT1-specific immune responses in vitro

We first analyzed the production of IFNγ or IL10 from the PBMCs of patients vaccinated with DC/WT1-I, -I/II, or -II. After six vaccinations, all vaccines induced extremely high levels of IFNγ upon stimulation with WT1-I/II peptides in vitro compared with the levels of IL10 (Fig. 2A). Next, the percentages of the IFNγ-positive CD4+ or CD8+ T-cell populations were examined (Fig. 2B). The maximum levels of IFNγ-producing cells in the CD4+ T cells were significantly increased by vaccinations with DC/WT1-I/II compared with DC/WT1-I or -II (Supplementary Table S3). In addition, IFNγ-positive T cells were at baseline levels before the vaccinations (data not shown). To assess the more critical role of the WT1-specific CD4+ T-cell response that is restricted by MHC class II molecules with the DC/WT1-I/II vaccine, proliferation assays were performed. Figure 2C shows that, upon stimulation with WT1 class II peptide in vitro, CD4+ T cells from DC/WT1-I/II-vaccinated patients with PDA showed significant proliferation compared with DC/WT1-I or -II vaccination. Moreover, no significant difference between the proliferation of CD4+ or CD8+ T cells before and after vaccination with DC/WT1-I or -II was observed.

HLA-A*24:02-positive patients were selected to assess the induction of HLA-A*24:02–restricted WT1-CTls (Fig. 3A), as adequate HLA-A*02:01 and HLA-DRB1/DPB1 tetramers were not available. Eight patients were positive for HLA-A*24:02 among all enrolled patients. In all 8 of the HLA-A*24:02-positive patients, 2 were vaccinated with DC/WT1-I, and 6 were vaccinated with DC/WT1-I/II. Before treatment, the number of WT1-CTls was extremely low in all 8 patients. However, the WT1-CTls were induced in all 8 patients during the vaccination period (Fig. 3B). The percentage of WT1-CTls in the total CD8+ T-cell population derived from the patients vaccinated with DC/WT1-I/II was higher, but not significantly higher, than that in the patients vaccinated with DC/WT1-I after seven or eight vaccinations (Fig. 3C, left). In the DC/WT1-I group, the WT1-CTls were increased early...
after vaccination, but this increase was not maintained during the entire vaccination period (Fig. 3B left). However, the WT1-CTLs in the circulating CD8+ T-cell population derived from the DTH-positive patients vaccinated with DC/WT1-I/II were maintained during the entire vaccination period (Fig. 3B middle). We also assessed the association between survival and the WT1-specific immune responses in the HLA-A24:02-positive PDA patients vaccinated with DC/WT1-I/II. As an OS of ≥1 year generally indicates that the treatment has been beneficial (22), the patients vaccinated with DC/WT1-I/II were classified into 2 groups: OS ≥ 1 year (super-responders) and OS < 1 year (nonsuper-responders). Three super-responders, all of who received DC/WT1-I/II, were discovered. We could not detect a difference in the percentage of WT1-CTLs in the total CD8+ T-cell populations between these 2 groups after 7 or 8 vaccinations (Fig. 3C right).

Because assessing the WT1-specific memory CD8+ T cells may be more important for determining a patient’s response, the percentages of WT1/HLA-A24:02 tetramer–positive cells (CD45RA+CCR7− and CD45RA−CCR7−) in CD8+ T cells were analyzed (Fig. 4A and B). Interestingly, the association between the percentages of WT1/HLA-A24:02-specific memory cells in CD8+ T cells and the OS of super-responders was significant after six vaccinations with DC/WT1-I/II (P = 0.046; Fig. 4B). Moreover, the combined analysis of the tetramer and functional IFNγ assay confirmed that almost all the HLA-A24:02–restricted WT1-CTLs produced IFNγ (data not shown).

**Immunosuppressive factors**

The vaccination of patients with PDA with DC/WT1-I/II did not generate a significantly increased population of CD25+Foxp3+CD4+ T cells or CD14+CD11b+CD33+ PBMCs compared with the DC/WT1-I or -II vaccines (Supplementary Fig. S3). Moreover, in the patients with PDA vaccinated with DC/WT1-I/II, the super-responders exhibited a decreased percentage of both CD25+Foxp3+CD4+ T cells and CD14+CD11b+CD33+ PBMCs compared with the non–super-responders but this difference was not significant (P = 0.052 and 0.328, respectively; data not shown). Next, we assessed the population of WT1/HLA-A24:02 tetramer–positive PD1+ cells in the CD8+ T cells in DC/WT1-I/II–vaccinated PDA (Fig. 5A and B). A low percentage of WT1/HLA-A24:02 tetramer–positive PD1+ cells was observed in the circulating CD8+ T cells before therapy; however, after 10 vaccinations, the population was significantly increased in the non–super-responders compared with the super-responders (P = 0.018; Fig. 5B). In contrast, in the super-responders, there was no difference in the populations before and after 10 vaccinations (Fig. 5B).

**Discussion**

Our phase I study is the first trial to use mature DCs pulsed with a mixture of three types of WT1 peptides restricted by MHC-I/II into one site in combination with chemotherapy.

The safety profile constituted the primary end point. One patient PDA-06 with multiple liver metastases showed rapid disease progression and died of a cerebral infarction. DTH
to the WT1-I/II peptides was negative during all vaccination periods. Cerebral infarction, reported here as a severe adverse event, could be caused by the pancreatic cancer itself and/or the administration of gemcitabine, both of which are associated with a high risk of developing thrombotic disease (23). In particular, patients with PDA with metastatic disease are at the highest risk for cancer-associated thromboembolic stroke (24). The supervising Data Safety and Monitoring Board (DSMB) determined that the patient died of stroke induced by a cancer-related hypercoagulable state. Finally, the DSMB determined that the case was not related to the treatment protocol. Moreover, grade 1 pulmonary fibrosis occurred in one patient, PDA-07, after a total of 12 gemcitabine and 6 DC/WT1-I/II treatments. At that time, the drug lymphocyte stimulation test (DLST) for gemcitabine was positive. Moreover, DTH to the WT1-I/II peptides was negative during the entire vaccination period. This adverse event was considered to be multifactorial, and the DSMB determined that it was definitely related to the combination therapy, as the DLST for gemcitabine was positive. The patient continued treatment with S-1, an oral fluoropyrimidine, which is the major chemotherapy regimen for PDA in Japan (25), without additional toxicity. In all 7 enrolled patients, except for skin reactions at the local injection sites, the toxicity profiles of the DC/WT1-I/II vaccine in combination with gemcitabine were consistently similar to those of gemcitabine alone (25).

WT1 peptide–specific DTH reactivity was induced in 4 of the 7 patients with PDA vaccinated with DC/WT1-I/II; however, no patients with PDA vaccinated with DC/WT1-I or -II were DTH-positive. Moreover, in all 4 DTH-positive patients with PDA vaccinated with DC/WT1-I/II, no complete response (CR) or partial response (PR) was observed, but long-term SD was observed and exhibited prolonged survival times. Modern trial experience suggests the response ratio (RR) of gemcitabine is approximately 10% (25). Because cancer vaccines do not work as quickly as cytotoxic agents, the RECIST criteria may not adequately...

Figure 3. Induction of HLA-A24:02-restricted WT1-specific CTLs. A, dot plots of HLA-A24:02-restricted, WT1 tetramer-positive populations in CD8+ T cells derived from PDA-04 are shown before and after eight vaccinations. B, the percentage of HLA-A24:02-restricted, WT1-specific CTLs (WT1-CTLs) in CD8+ T-cell populations was analyzed after patients were vaccinated with DC/WT1-I (left) or with DC/WT1-I/II [4 DTH-positive (middle) and 2 DTH-negative (right)]. C, the percentages of WT1-CTLs in the CD8+ T-cell populations of patients treated with DC/WT1-I or DC/WT1-I/II were compared with those before vaccination (left panel). After 7 or 8 vaccinations with DC/WT1-I/II, the percentages of WT1-CTLs in the CD8+ T-cell populations in patients with an overall survival (OS) time of ≥1 year (PDA-04, -05, and -09) and <1 year (PDA-06, -08, and -10) were compared (right). The results are expressed as the mean ± SE.
capture the clinical benefit of cancer vaccines (26). The long-term SD that is shown in this study may be a unique characteristic of cancer vaccines. Interestingly, 3 of the 7 patients vaccinated with DC/WT1-I/II were strongly DTH positive during the entire treatment period. DTH is an inflammatory reaction mainly mediated by CD4\(^+\) effector-memory T cells that infiltrate the injection site of the antigen against which the immune system has been primed by the cancer vaccines (27). Our results support previous findings that showed there was a significant correlation between favorable clinical outcomes and the presence of a vaccine-related antigen-specific DTH test (27, 28). These findings suggest that the WT1-CTLs generated following vaccination with DC/WT1-I may be functionally impaired, resulting in short-lived WT1-specific immune responses (30). In contrast, all 4 DTH-positive patients maintained WT1/HLA-A\(^*\)24:02–specific tetramer-positive cells during the entire treatment period. The maintenance of the WT1-CTLs may be, at least in part, associated with prolonged survival. Moreover, a significant increase in the number of IFN\(_\gamma\)-producing cells in the CD4\(^+\) T-cell populations in patients with PDA vaccinated with DC/WT1-I/II was detected upon stimulation with WT1 class II peptide in vitro, suggesting that concurrent CD4\(^+\) T-cell activation is essential to induce functional CTLs. Furthermore, almost all of the HLA-A\(^*\)24:02–restricted WT1-CTLs produced IFN\(_\gamma\) following challenge with WT1 peptides. These findings support the hypothesis that the coactivation of WT1-specific CD4\(^+\) helper T cells augments the induction and proliferation of functional, circulating WT1-CTLs. Indeed, vaccination with DC/WT1-I/II resulted in populations of circulating functional memory CTLs that were specific for WT1 and were long-lived, lasting for the entire treatment period in the super-responders. In pancreatic cancer, long-term survivors who had been vaccinated with a mutant K-ras peptide...
designed to elicit Th responses were reported to exhibit persistent K-ras–specific memory CTLs (31). Importantly, our results also showed that the generation and maintenance of WT1-specific memory CTLs by DC/WT1-I/II were significantly linked to beneficial clinical outcomes. Therefore, vaccination with DCs pulsed with WT1 peptides for both MHC class I and II may be associated with the long-term survival of patients with PDA.

Although WT1-CTLs were generated by vaccination with DC/WT1-I/II and were detected in the circulation of the vaccinated patients in this study, these CTLs may not act against the tumor. In the tumor microenvironment, there are many immunosuppressive cells, including CD4+ CD25+ Foxp3+ Tregs and MDSCs (32). Moreover, the interaction of PD1 in activated CTLs with its ligand PDL1 in the tumor cells plays a major role in immune escape (33). After vaccination with either type of DC/WT1 vaccine, the percentages of CD25+ Foxp3+ cells in the CD4+ T-cell population and the CD14+ CD11b+ CD33+ cells in the PBMCs were not significantly changed. After 10 vaccinations with DC/WT1-I/II, the non–super-responders showed a significantly increased population of WT1/HLA-A*24:02–specific PD1+ cells in CD8+ T cells, compared with the super-responders. The low percentage of the exhausted WT1-CTLs in the super-responders may be, at least in part, associated with longer survival times. However, these results were obtained from peripheral cells. Therefore, we must understand the immune responses observed in the peripheral blood versus the responses at the tumor site. Of the 3 super-responders, we could examine PDL1 expression in 2 patients, PDA-05 and PDA-09, and detected strong PDL1 expression on both pancreatic cancer cells (data not shown). Endogenous inflammatory immune responses induced by IFNγ-producing CTLs have been shown to promote the expression of PDL1 on cancer cells (34). Therefore, the overexpression of PDL1 on tumor cells and the lower frequency of WT1/HLA-A*24:02–specific PD1+ cells in CD8+ T cells that was observed in the super-responders, compared with the non–super-responders, may be associated with long-term survival (34, 35). Indeed, an immune checkpoint blockade by antibodies targeting inhibitory immune receptors, such as PD1 and PDL1, can be used to successfully treat patients with advanced melanoma (33). Monitoring of WT1-CTLs and immunosuppressive cells at the tumor site may be directly associated with clinical responses. However, cells from the tumor site are not readily available for monitoring purposes in patients with PDA. Antigen-specific T cells obtained from DTH sites have been reported to be significantly correlated with favorable clinical outcomes (27). Therefore, the ability to assess the function of the WT1-CTLs and the immunosuppressive cells from the DTH site may serve as useful prognostic markers for survival during treatment with this combination therapy. Future studies are needed to address these issues and to identify the immune biomarkers that are capable of predicting clinical response.

In conclusion, the combined treatment of chemotherapy and DCs pulsed with a mixture of 3 types of WT1 peptides, including both MHC class I and II–restricted epitopes, was well tolerated, induced WT1-CTLs during long-term vaccination, and appeared to provide some clinical benefits to
DTH-positive patients. Further investigations are needed to validate these findings in a larger-scale clinical trial.

Disclosure of Potential Conflicts of Interest
M. Okamoto holds ownership interest in Tella, Inc. H. Sugiyama is the inventor of patents PCT/JP2012/02794 and PCT/JP2014/16336 which are held by the International Institute of Cancer Immunotherapy. No potential conflicts of interest were disclosed by the other authors.

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Treatment with Chemotherapy and Dendritic Cells Pulsed with Multiple Wilms' Tumor 1 (WT1)—Specific MHC Class I/II–Restricted Epitopes for Pancreatic Cancer

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