CLINICAL TRIAL REPORT

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Phase I/II clinical trial of a Wilms' tumor 1-targeted dendritic cell vaccination-based immunotherapy in patients with advanced cancer

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Abstract
Dendritic cell (DC)-based immunotherapies have been created for a broad expanse of cancers, and DC vaccines prepared with Wilms' tumor protein 1 (WT1) peptides have shown great therapeutic efficacy in these diseases. In this paper, we report the results of a phase I/II study of a DC-based vaccination for advanced breast, ovarian, and gastric cancers, and we offer evidence that patients can be effectively vaccinated with autologous DCs pulsed with WT1 peptide. There were ten patients who took part in this clinical study; they were treated biweekly with a WT1 peptide-pulsed DC vaccination, with toxicity and clinical and immunological responses as the principal endpoints. All of the adverse events to DC vaccinations were tolerable under an adjuvant setting. The clinical response was stable disease in seven patients. Karnofsky Performance Scale scores were enhanced, and computed tomography scans revealed tumor shrinkage in three of seven patients. Human leukocyte antigen (HLA)/WT1-tetramer and cytoplasmic IFN-γ assays were used to examine the induction of a WT-1-specific immune response. The immunological responses to DC vaccination were significantly correlated with fewer myeloid-derived suppressor cells ($P = 0.045$) in the pretreated peripheral blood. These outcomes offered initial clinical evidence that the WT1 peptide-pulsed DC vaccination is a potential treatment for advanced cancer.

Keywords Dendritic cell · WT1 · Tumor-associated antigens · Cytotoxic T lymphocytes · Immunotherapy

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Abbreviations

CTLs	Cytotoxic T lymphocytes
DC	Dendritic cell
ELISPOT	Enzyme-linked immuno spot
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN	Interferon
KPS	Karnofsky Performance Status
MDSCs	Myeloid-derived suppressor cells
PBMCs	Peripheral blood mononuclear cells
RECIST	Response Evaluation Criteria in Solid Tumors
TAA	Tumor-associated antigens
Treg	Regulatory cells
WT1	Wilms tumor protein 1

Introduction

As we continue to develop and enhance tumor immunotherapies, research has concentrated on particular immune reactions against tumor-associated antigens (TAAs) and their epitopes, which are identified by human leukocyte antigen (HLA)

class-I-restricted cytotoxic T lymphocytes (CTLs) from different malignant neoplasms [1]. Of the currently determined TAAs, Wilms' tumor gene product WT1 is noted as a causative gene of Wilms' childhood renal tumors [2]. The *WT1* gene encodes a zinc finger transcription factor that is overexpressed in numerous hematological malignancies and solid tumors [3–5]. Since WT1 is an intracellular protein that is not targeted by conventional monoclonal antibody therapies, generating WT1-specific CTL responses that recognize peptides presented on cell surfaces by HLA class-I molecules is a major goal for this therapy [6–8]. High WT1 is linked to poor prognosis for patients with various hematological malignancies and solid tumors, such as breast cancer [9, 10], ovarian cancer, and gastric adenocarcinoma [11–13]; with general 5-year survival rates in these cancers of approximately 90%, 47%, and 30%, respectively [14]. Therefore, WT1 could be a target antigen for immunotherapy for these types of cancers. WT1 is an appealing vaccine candidate due to its limited expression and its oncogenic functions.

A prior WT1 peptide vaccine was used for different solid tumors, [15, 16] and vaccination with dendritic cells (DCs) prepared with TAAs may be potential immunotherapeutics for carcinoma patients [17, 18]. Antigen-presenting cell-based immunotherapies with active DCs have been documented to prompt immunity, and while DC-based treatments against TAAs are "anti-idiotypic vaccines", they are conceptually identical to other strategies with autologous monocyte-derived mature DCs to promote a tumor-specific immune reaction [19]. Further, the expression of cancer-associated antigens with HLA class-I and II antigens in tumor tissues could provide immunotherapy against cancers [20, 21]. Specifically, HLA-limited modified WT1 peptide, revealed by HLA-A*24:02 (amino acids 235–243 CYTWNQML), in which Y was substituted for M at amino acid position 2 of the natural WT1 peptide, were used for DC vaccines. The modified WT1 peptide elicits stronger tumor-recognizing cytotoxic T-lymphocyte activity natural peptides and it induces immunological and clinical reactions [22, 23].

This novel DC-based vaccination strategy could be a beneficial therapy for advanced cancer [24–33]. Immunotherapies utilizing CTLs are central cancer immunotherapy [34]. We performed a phase I/II clinical study to examine the feasibility and efficacy of WT1-pulsed "mature" DC vaccinations in extremely pretreated metastatic breast, ovarian, and gastric cancer patients. We established the feasibility and capability of prompting WT1-specific CTLs, which led to cancer regression without the destruction of healthy tissues in the clinic.

Materials and methods

Objectives

This trial was a I/II clinical study. The planned sample size was ten. The primary endpoint was adverse events graded according to Common Terminology Criteria for Adverse Events (version 4.0). The secondary endpoint was overall survival. The duration of overall survival was defined as the date patients' informed consent was obtained to the date of death. Eligibility: patients aged 20–70 with gastric, ovarian or breast cancer were eligible if their disease was resistant to conventional chemotherapy or surgery. Other inclusion criteria were: (1) HLA-A*2402-positivity, (2) a score of 0 or 1 on the Eastern Cooperative Oncology Group performance scale; (3) estimated survival of > 2 months; (4) lesion that could be evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST); (5) no immediate allergy to the WT1 peptide; (6) no severe impairment of organ function, including hematologic, hepatic, renal and cardiac function.

Follow-up was conducted with clinical examinations to determine the efficacy of vaccine given together with typical neoadjuvant treatment. The Karnofsky Performance Scale was utilized to evaluate each patient at every visit, and tumor regression was determined according to RECIST to measure initial safety and immunogenicity data for the neoadjuvant therapy. Patients continued therapy unless progressive disease occurred, or they experienced unacceptable toxicity requiring cessation of treatment. If there was a change in medical status (including pregnancy), compromised patient safety, withdrawn consent, non-compliance, or a loss to follow-up, the treatment was stopped.

DC vaccination preparation

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from patients, and DCs were generated from monocytes isolated with the MagCellect Human CD14$^+$ Cell Isolation Kit (R&D Systems). The PBMCs were incubated at 37 °C, and adherent cells (composed of 96.1% ± 2.6% CD14$^+$ monocytes) were cultured in AIM-V medium containing granulocyte–macrophage colony-stimulating factor (1000 units/mL; PeproTech) and IL-4 (500 units/mL; R&D Systems) to generate immature DCs. After 6 days of incubation at 37 °C, the DC maturing agent TNF-α (500 units/mL; PeproTech) was added to the culture for 48 h. DC vaccine preparation is described in the literature [35]. Briefly, mature DCs were incubated with 100 μg/mL WT1 peptide for 30 min and washed with saline. The WT1 peptide (a modified-type, HLA-A*2402-restricted, 9-mer WT1 peptide residues 235–243: CYTWNQMNL) of good

Table 1 Summary of patient characteristics and clinical outcomes of DC-based immunotherapy

Patient no.	Age (years) sex	Dx	Clinical stage	Previous therapy		KPS score (%)	Clinical response after first course		Clinical response after final session		Immunological responses (IR)	Overall survival (mos)	Survival status
				Tx (no. of cycles)	Additional Tx		RECIST	Clinical effect	RECIST	Clinical effect			
1	56, F	Breast	III	CT_X (20)	–	70	SD	Yes	SD PR PD	Yes	+	15.3	Deceased
2	45, F	Breast	IV	CT_X (17)	–	50	SD	Yes	SD	Yes	–	>23.2	Alive
3	37, F	Breast	IV	CT_X (10), HT_X (2)	–	60	SD	Yes	PR	Yes	+	>22.6	Alive
4	57, F	Breast	III	CT_X (19)	–	70	SD	Yes	SD	Yes	–	>17.2	Alive
5	50, F	Ovarian	IV	CT_X (16)	Ovariectomy between second and third vaccination	70	SD	Yes	PD	No	–	13.1	Deceased
6	39, F	Ovarian	III	CT_X (12)	–	80	PD	No	PD	No	–	9.3	Deceased
7	59, F	Ovarian	IV	CT_X (10)	–	50	SD	Yes	SD	Yes	+	>20.1	Alive
8	42, M	Gastric	IV	CT_X (13)	–	70	SD	Yes	PD	Yes	+	16.7	Deceased
9	67, M	Gastric	III	CT_X (12)	–	70	PD	No	PD	No	–	12.3	Deceased
10	56, F	Gastric	IV	CT_X (16)	–	60	PD	No	PD	No	–	11.6	Deceased

Dx diagnosis, Tx treatment, CT_X chemotherapy treatment, HT_X Hormones treatment, KPS Karnofsky Performance Status, – no further treatment

manufacturing practice grade was purchased from Multiple Peptide Systems (San Diego CA, USA) as lyophilized peptides.

Based on quality criteria for DC vaccines, the phenotypes CD11c+, CD40+, CD80+, CD83+, CD86+, CCR7+, and HLA-DR + were established as mature DCs. For every DC vaccination, about $1–2 \times 10^7$ DCs with OK-432 (1–2 KE, Chugai Pharmaceutical), a streptococcal primer, were injected intradermally into bilateral axillary parts on day 8 and at biweekly intervals for at least 5 sessions (1 course) based on detailed techniques for the clinical use of Cellular and Tissue-Based Products Manufacturing Products throughout a single chemotherapy regimen. If a positive response to treatment or no adverse effects were noted following a single vaccination course, more vaccinations were administered with the patient's informed consent.

WT1 peptide/HLA-A*2402 tetramer assay

WT1-specific CTLs in peripheral blood were evaluated based on HLA tetramers (Medical & Biological Laboratories Co., Ltd) before and after the course of DC vaccination. Outcomes were regarded as positive when CD3-positive, CD8-positive, and WT1/HLA-A24 tetramer-positive cell populations were discovered with the cultured cells. HIVenv/HLA-A24 tetramer-positive cells were utilized as the negative controls.

WT1-specific interferon-γ enzyme-linked immunospot assay

The IFN-γ ELISPOT assay was conducted as detailed previously. PBMCs were established as specifically sensitized when spots suggesting IFN-γ release in reaction to the WT1 peptide were twice that of the reaction to HIVenv peptide-pulsed stimulator cells in the ELISPOT assay.

Surface marker analysis

PBMCs were incubated with fluorescent-conjugated monoclonal antibodies for 40 min at 4 °C in the dark. After surface staining, cells were washed and fixed with stabilizing fixative (BD Biosciences) and determined with the fluorescent-activated cell sorting Calibur and CellQuest software (BD Biosciences).

Statistical analysis

Data are shown as mean values $\pm$ SD. Statistical significance was evaluated with GraphPad Prism 5.0 (GraphPad Software, Inc.), using a Student's t test or one- and two-way analysis of variance (ANOVA), followed by Tukey's and Bonferroni's post-hoc tests, as indicated. $P < 0.05$ was considered statistically significant.

Table 2 Immunological monitoring data in patients who received WT1-pulsed DC vaccination

Patient no.	WT1 tetramer, %		ELISpot assay (spots/1×10^5 PBMCs)	
	Pre-therapy	Post-therapy	Pre-therapy	Post-therapy
1	0.17	1.21	17	113
2	0.12	0.23	98	61
3	0.68	5.67	6	390
4	0.78	1.03	23	211
5	0.56	3.31	46	256
6	0.29	11.01	9	13
7	0.22	16.72	4	506
8	0.30	19.81	12	106
9	0.57	1.21	121	136
10	0.37	1.56	106	70

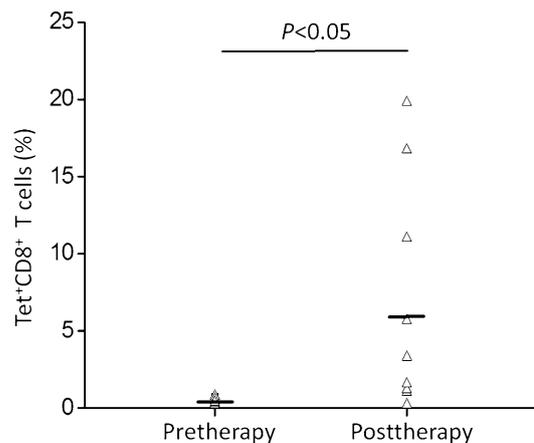*ELISpot* enzyme-linked immunospot, *PBMCs* peripheral blood mononuclear cells

Fig. 1 Frequency of WT1-specific CTLs before and after DC vaccination in the tetramer assay. The black horizontal bar shows the median. Differences between values before and after WT1 vaccination were statistically significant ($P < 0.05$)

Results

Patient characteristics

Patient characteristics are shown in Table 1, and specifics of the immunological results for every one of the patients following vaccinations are shown in Table 2. We noted no Grade 3 or greater National Cancer Institute Common Toxicity Criteria toxicities linked to DC vaccination. Every one of the 10 enrolled patients had a local inflammatory reaction with mild erythema at the vaccination injection site within several days. There were four patients who experienced postvaccination fever, and this continued for a few hours following the initial DC vaccination. Fatigue was observed in two patients.

Fig. 2 WT1-specific CTLs induced by DC vaccination. **a** Upper panel, WT1-peptide/HLA-A*2402 tetramer assays. Percentages represent the frequency of tetramer+CD8+ T cells. Cases 1, 3, 7, and 8 show a significant increase in the frequency of tetramer+CD8+ T cells after DC vaccination compared to pre-therapy (*$P < 0.05$). **b** Lower panel, IFN-γ-producing clones in ELISPOT assays with WT1 peptide. Cases 1, 3, 7, and 8 show a significant increase in the response in the ELISPOT after DC vaccination compared to pre-therapy values (*$P < 0.05$)
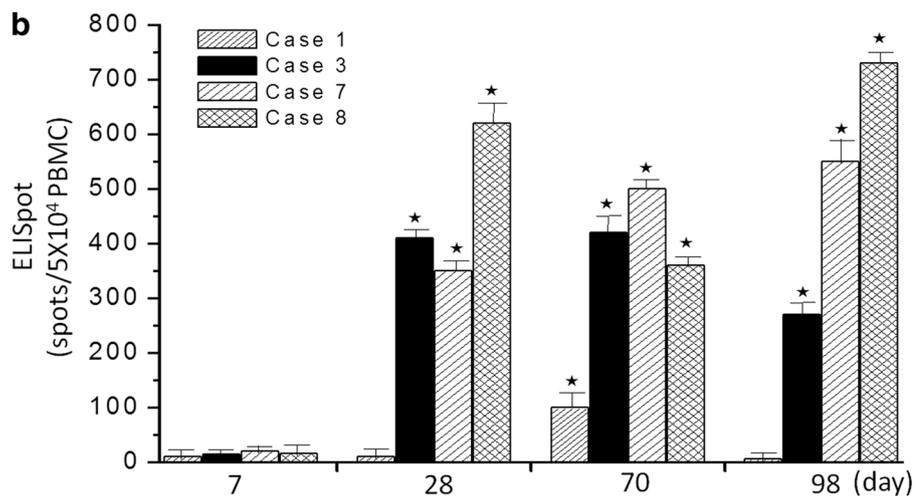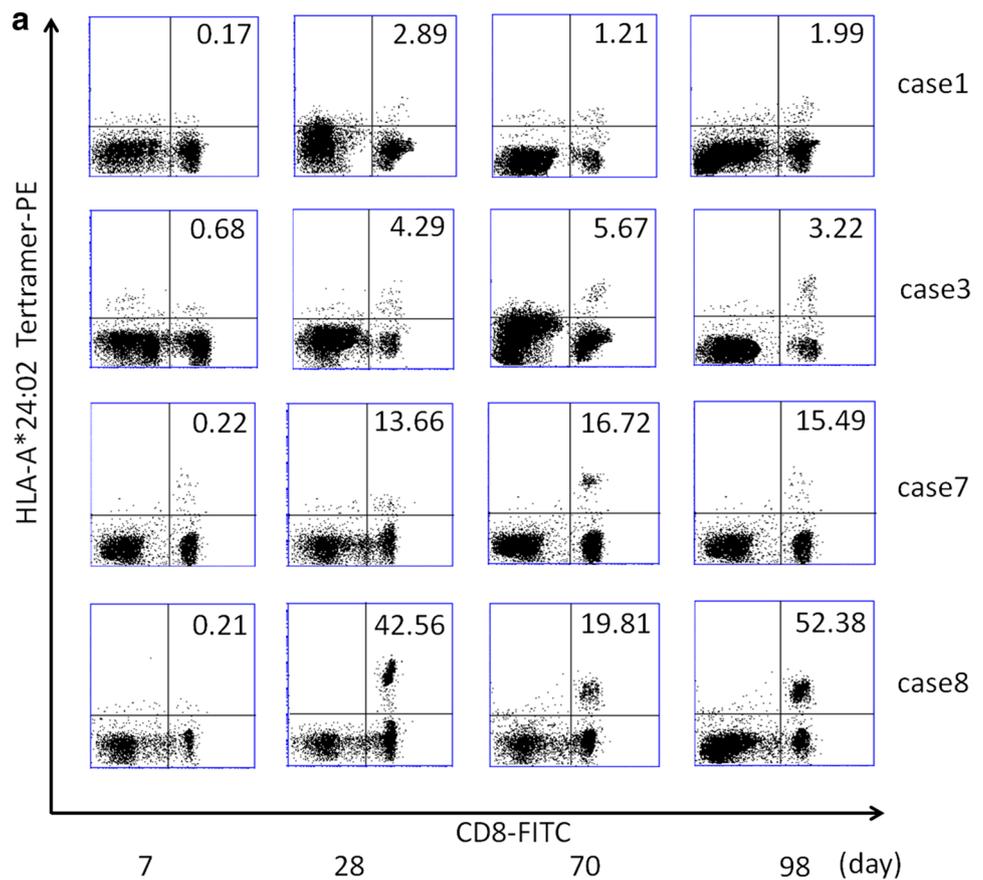
Immunological responses

WT1-pulsed DC vaccination therapy was assessed with a HLA/WT1-tetramer and cytoplasmic IFN-γ assays with PBMCs. Levels of tetramer-positive WT1-specific CTLs were significantly raised following DC vaccination ($P < 0.05$; Fig. 1). An ELISPOT assay showed that WT1-specific CTL responses were enhanced in eight patients (Table 2). Immunological responses data appear in Table 2. There were four patients (cases 1, 3, 7, and 8) who had stronger reactions to HLA-tetramer and IFN-γ ELISPOT assays following DC vaccination ($P < 0.05$; Fig. 2).
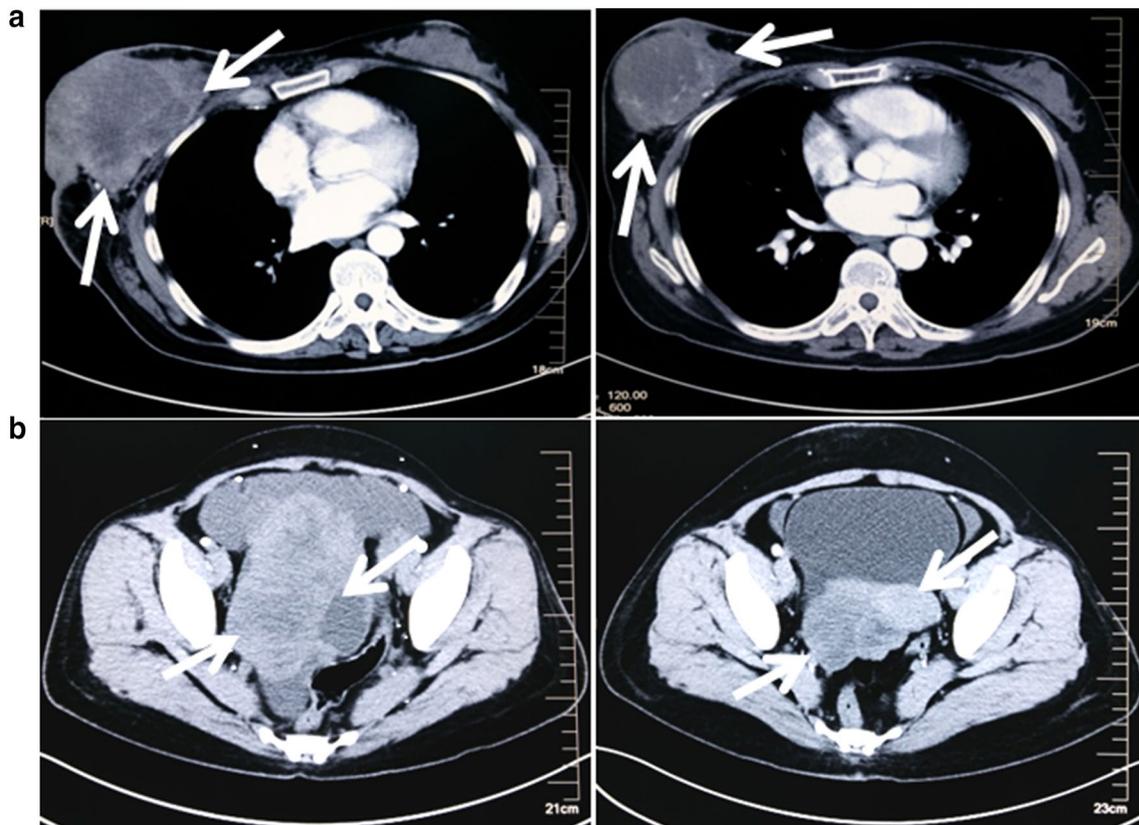
Fig. 3 Tumor regression occurred in patient 3 **a** with metastatic breast cancer and in patient 7 **b** with ovarian cancer during the course of DC vaccination. (Left) Before and (right) after DC vaccination, respectively. Arrows indicate the presumed location of the tumor

Clinical outcomes

Data for each treatment group appear in Table 1. At the conclusion of the initial DC vaccination, seven patients had stable disease and three patients had PD. Every individual with stable disease was treated with WT1-pulsed DC vaccination. For three of the seven patients with stable disease, their Karnofsky Performance Scale scores improved from 50 to 60% (patients 2 and 7) and from 60 to 70% (patient 3). Contrast-enhanced computed tomography scans revealed a regression of metastatic tumors for patient 3 (Fig. 3a), and patient 7 had tumor shrinkage (Fig. 3b) following DC vaccination. A partial response was observed throughout ongoing vaccination following the initial session. The maximum follow-up was 26 months following the initial DC vaccination; at that time, six patients had died.

To determine the factors that were predictive of immune reactions to DC vaccination, we examined immune cell subsets in pretreated peripheral blood, utilizing cytometry-based comprehensive leukocyte immunophenotyping. Figure 4 shows that immunological responses were significantly correlated with fewer myeloid-derived suppressor cells (MDSCs) ($CD14^-CD11b^+CD33^+$)

($P = 0.045$), and there were no differences in the percentages of Treg ($CD25^+Foxp3^+CD4^+$) cells ($P = 0.295$), the total $CD14^+CD11b^+$ ($P = 0.11$), and $CD14^+CD11b^-$ cells ($P = 0.36$) between immunological response-positive and immunological response-negative patients. Thus, DC vaccination may be effective for patients with fewer MDSCs and fewer Tregs prior to treatment. In contrast, there was no difference in total lymphocytes observed in the 10 evaluable patients.

Discussion

Over the last decade, DCs have emerged as key players in the initiation of anti-tumor immunity [36, 37]. Induction of potent tumor-specific cytotoxic CD8 + T-cell responses after peptide-based vaccination is the foundation of immune-based therapies for cancer patients [38, 39]. Here, we provide evidence that vaccination with WT1 peptide-pulsed DCs can be used for patients with breast, ovarian, and gastric cancers, and the DCs induce immunological response directed toward less immunogenic tumors. Patients were eligible if the disease was resistant
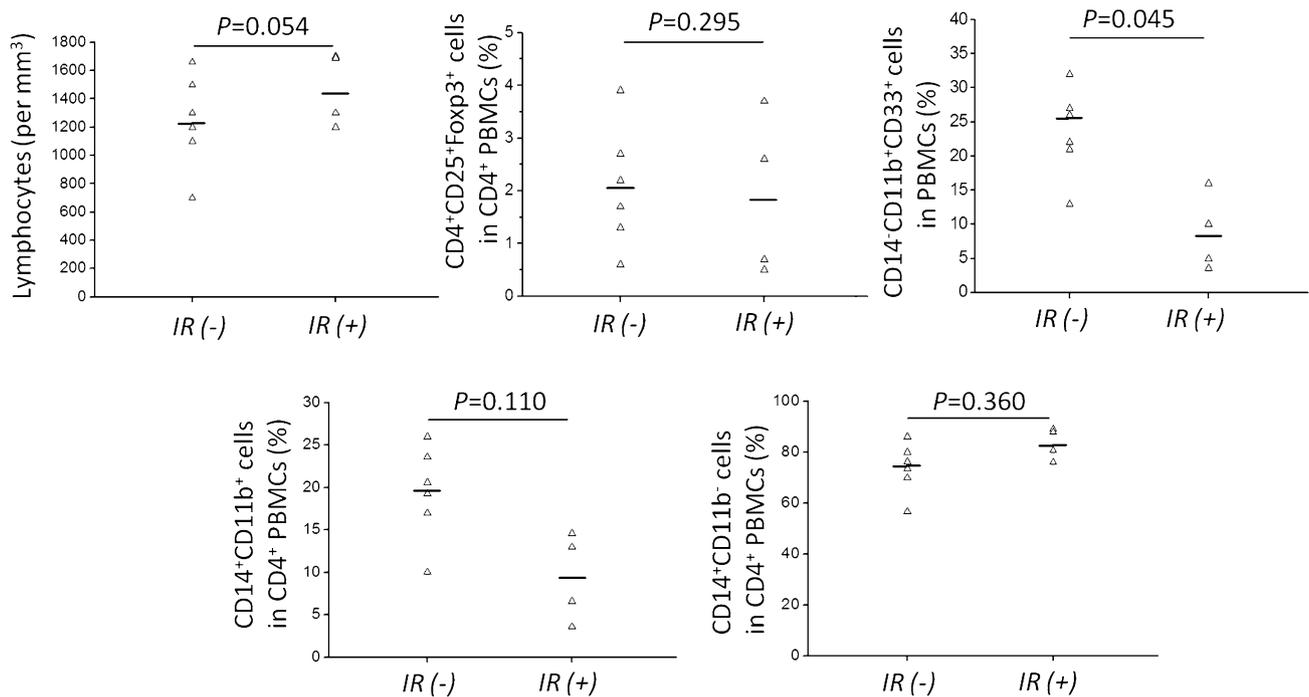
Fig. 4 Pretreatment total lymphocyte numbers and the frequency of various circulating lymphocyte phenotypes in the peripheral blood of all patients. The immunological responses were significantly cor-

related with fewer myeloid-derived suppressor cells (MDSCs). MDSCs were identified as CD14$^-$CD11b$^+$CD33$^+$. IR, immunological responses

to conventional chemotherapy, radiotherapy, or hormonal therapy (breast cancer). Patients declining such treatments but requesting DC vaccine therapy were also eligible. Clinical responses observed in this phase I/II clinical study were satisfactory. Partial response was reached in two of the ten patients throughout ongoing vaccination following the initial session. Stable disease was achieved following the first course in seven of the patients who were given DC vaccinations. Two of the patients had positive clinical and radiological reactions based on RECIST throughout the clinical course. Noted toxicities were all Grade 1 or 2 based on the National Cancer Institute Common Toxicity Criteria. Further, no autoimmune reactivity was noted in the patients who received WT1-pulsed DC vaccinations. Therefore, vaccination therapy utilizing DCs pulsed with WT1-derived peptides is well tolerated, safe, and practical for the treatment of solid tumors.

Efficacious anti-tumor immunity in humans has been associated with the presence of CD8$^+$ T-cell uptake of tumor neoantigens, a class of MHC-I-bound peptides that is highly immunogenic and not present in normal tissues. Since tumor neoantigens are unique to patients, identification of tumor-specific shared targets across a range of cancers is needed. At the National Cancer Institute, WT1 was reported to be a promising target due to its abundance and broad expression in many cancer types as well as its limited expression in normal organs [2].

Clinical studies of cancer immunotherapies targeting WT1 in leukemia and solid tumors indicate that WT1-specific CTL reactions can be stimulated, and clinical reactions have been assessed in several patients. The data reveal that WT1-specific CTLs attack WT1-expressing tumor cells and transformed stem cells, but they do not destroy healthy cells [40]. Induction and activation of WT1-specific CTLs are attained via cross priming mediated by DCs, which subsequently prompts peptides to be presented to HLA class-I (HLA-I) molecules (cross-presentation) [41, 42]. HLA-A*24:02-restricted altered WT1 peptides could improve cancer immunity [43–45]. Here, we report that the antigen-specific response in four patients was elicited by WT1-pulsed DC immunizations. Then, HLA-tetramer and cytoplasmic IFN-γ assays were used to evaluate the data, and the patients tested positive in both assays. In addition, immunological reactions corresponded with several MDSCs in pretreated peripheral blood.

In the tumor microenvironment, there are many immunosuppressive cells, including CD25$^+$Foxp3$^+$CD4$^+$ Tregs [46] and MDSCs [46]. Further, MDSC-repressed anti-tumor immunity is linked to poor outcomes in cancer patients [47–49]. Thus, limited MDSCs and fewer

CD4$^+$CD25$^+$Foxp3$^+$ Tregs prior to treatment could be useful markers for DC vaccination efficacy in cancer patients. Therefore, the evaluation of reactions to DC vaccinations could be predicted from immunological variables that correspond with the induction of immune reactions and antitumor impact. However, standardization of DC vaccine-manufacturing technology and investigation of the quality and immunological efficacy are needed for DC-based vaccination therapy to be used routinely.

In summary, DCs pulsed with WT1 peptide antigens could be helpful for certain patients; however, the efficacy and safety of DC vaccinations need to be assessed in bigger populations and in prospectively designed studies. This evaluation may lay a foundation for future studies that examine treatment of other tumors expressing WT1 peptides.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This trial was approved by the Ethics Committee of the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, and was registered with the Chinese Clinical Trials Registry (ChiCTR-IPR-15005923).

Informed consent Written informed consent was acquired from each patient in the study.

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