EXPERT REVIEWS

Vaccination strategies to improve outcome of hematopoietic stem cell transplant in leukemia patients: early evidence and future prospects

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Allogeneic hematopoietic stem cell transplantation (HSCT) has largely improved the prognosis of leukemia patients. However, relapse is still a major concern. One promising option for the prevention of relapse is vaccination therapy. The post allogeneic HSCT period provides a unique platform for vaccination, because tumor burden is minimal, lymphopenic condition allows for rapid expansion of cytotoxic T cells (CTLs), donor-derived CTLs are not exhausted and inflammatory condition is caused by allo reactions. Tumor cells, dendritic cells and peptides have been used as vaccines targeting leukemia-associated antigens or minor histocompatibility antigens. Clinical trials with several types of vaccines for post-HSCT patients showed that the vaccination induced immunological response and might benefit patients with minimal residual disease, while their effect in patients with advanced disease were limited. To enhance the effect, vaccination in combination with other immune-modulatory drugs such as checkpoint antibodies is now being considered.

KEYWORDS: allogeneic hematopoietic stem cell transplantation • immunotherapy • leukemia • vaccine • WT1

The advances in anti-leukemia drugs including chemotherapeutic drugs or molecular targeting drugs have largely improved the prognosis of leukemia patients. However, leukemia patients who have not been cured by drug therapy alone require allogeneic hematopoietic stem cell transplantation (HSCT). Advances in supportive therapies and introduction of new immunosuppressive drugs have reduced transplant-related mortality and largely improved the prognosis of leukemia patients who underwent allogeneic HSCT. However, relapse still remains a major concern, especially in allogeneic HSCT at nonremission states. Innovative therapeutic approaches for the eradication of residual leukemic cells after allogeneic HSCT are required. In allogeneic HSCT, chemotherapy-resistant leukemia cells can be eliminated as a result of immunological rejection of recipient leukemia cells by donor T cells, known as the graft-versus-leukemia (GVL) effect [1]. Success of donor lymphocyte infusion [2], including infusion of donor lymphocytes activated by IL2 [3] or stimulated with alloantigens [4], highlights the importance of GVL effects in the elimination of residual leukemia cells after HSCT. It is thus obviously important to exploit the GVL effect while minimizing graft-versus-host disease (GVHD), and vaccination is one of the promising strategies for it. In this review, we first summarize the results of clinical trials of cancer vaccines for hematological malignancies. Next, we discuss the advantages of the immunological milieu after allogeneic HSCT for immunotherapy. Finally, the clinical studies of vaccination after allogeneic HSCT are reviewed and the future prospects are described.

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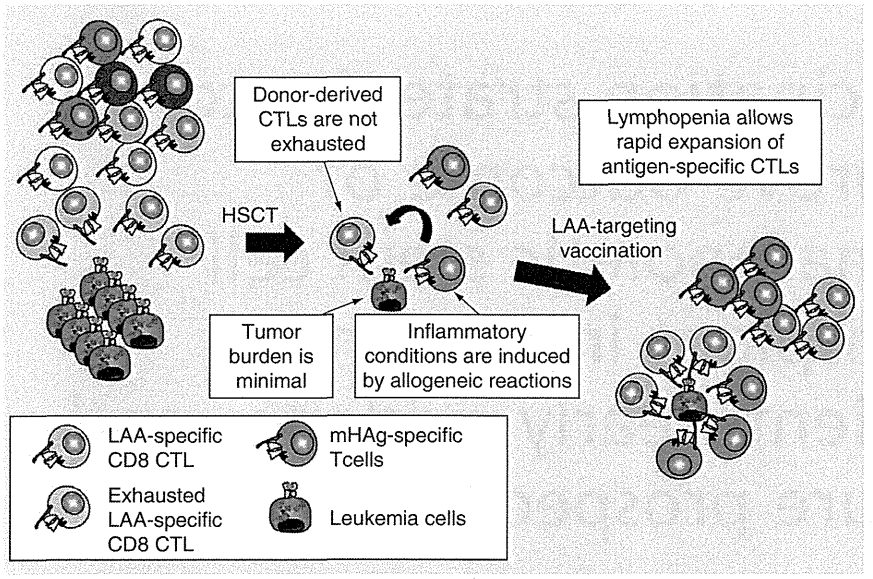

Figure 1. Post allogeneic hematopoietic stem cell transplantation period provides a unique platform for vaccination. (Left) Before HSCT, especially in nonremission states, some LAA-specific CTLs may be exhausted. (Center) After HSCT, the numbers of both leukemia and T lymphocytes decreased. (Right) Vaccination may induce selective expansion of LAA-specific CTLs. Favorable conditions for CTL expansion/activation observed after allogeneic HSCT are described in the boxes.
CTL: Cytotoxic T cells; HSCT: Hematopoietic stem cell transplantation; LAA: Leukemia-associated antigen; mHag: Minor histocompatibility antigens.

Vaccines for hematological malignancies

Allogeneic tumor cells expressing granulocyte-macrophage colony-stimulating factor (GM-CSF [GVAX]) were tested as a vaccine in several types of cancers, while their effects in patients with advanced cancer were controversial [5–7]. Qin *et al.* immunized chronic myeloid leukemia (CML) patients with K562 cells expressing GM-CSF and showed that decreased residual tumor burden following immunotherapy was associated with the induction of high-titer IgG antibodies against multiple leukemia-associated antigens (LAAs) [8]. Borello *et al.* immunized pre-auto HSCT patients with autologous leukemia cells admixed with GM-CSF-secreting K562 cells [9]. A decrease in Wilms tumor 1 (WT1) transcripts in blood was noted in 69% of patients after immunotherapy and was associated with longer 3-year relapse-free survival (61% in the immunized group vs 0% in the nonimmunized group).

Dendritic cell (DC)-based vaccines have also been widely used. Leukemic DCs generated from peripheral blood of CML patients could elicit tumor-reactive T-cell response [10]. DC fusions with leukemia cells or DCs loaded with tumor cell lysates also showed high potential for induction of tumor immune response [11]. In addition, vaccination with WT1 mRNA-electroporated DCs was reported to induce molecular remission in acute myeloid leukemia (AML) patients [12].

LAA-derived peptide [13] and DNA [14] have been used as vaccines in combination with adjuvants. BCR-ABL for Philadelphia-chromosome–positive leukemia [15], and over- or aberrantly expressed LAAs such as proteinase 3 [16,17], WT1 [18,19] and PRAME [20] have been used as targets in these trials. The reciprocal t(9; 22) translocation associated with the presence of a unique BCR-ABL fusion protein makes this protein an ideal target for an immunological attack on leukemic cells. It has been proven that BCR-ABL breakpoint-derived peptides bind to human leukocyte antigen (HLA) molecules and have the potential to elicit a peptide-specific T-cell response [21,22]. It was further shown that a BCR-ABL-derived peptide vaccine could induce an antigen-specific T-cell response [23] and stable and complete cytogenetic remission in a small subpopulation of CML patients [24]. Despite these promising results, BCR-ABL-derived peptides have not been proven to improve the prognosis of CML patients significantly. Peptide sequences for the cytotoxic T cell (CTL) epitopes, which are restricted within the region of the junction of BCR and ABL, might not be immunogenic enough.

WT1 is overexpressed in most types of acute and chronic leukemia, and thus one of the most promising targets for immunotherapy against leukemia. We and several other researchers demonstrated the safety and immunogenicity of the WT1 peptide vaccine in patients with hematological malignancies and solid cancers [25–29]. Several groups have confirmed immune responses induced by vaccination with WT1 peptide in patients with AML [30–32]. In these studies, not only immunological responses but also clinical responses (including stable disease and reduced expression of tumor markers) were observed in a substantial portion of evaluable patients. Regression of minimal residual disease in leukemia patients who received repeated vaccination with the WT1-derived peptide was also reported [33].

These studies demonstrate that tumor cell, DC and peptide vaccines have the potential to induce T-cell response. However, it is still unclear whether these vaccines can significantly benefit patients, and this needs to be tested in randomized clinical trials.

The post allogeneic transplant period provides a unique platform for vaccination

Immunological milieu after allogeneic HSCT is largely different from that after chemotherapy. There are several reasons for considering the use of immunotherapy, including vaccines, after allogeneic HSCT (Figure 1).

Minor histocompatibility antigens as an ideal target for vaccination

After allogeneic HSCT, but not chemotherapy or autologous HSCT, minor histocompatibility antigens (mHags) can be

targets for immunotherapy [34]. mHags were originally identified as antigens causing graft rejection or GVHD in HLA-matched allogeneic transplantation [35]. Molecular identification has revealed that most mHags are major histocompatibility complex (MHC)-bound short peptide fragments encoded by genes which are polymorphic as a result of single nucleotide polymorphism [35]. HA-1 and HA-2, which are mHags expressed only on hematopoietic cells, are ideal targets for immunotherapy after allogeneic HSCT. An inverse relationship between circulating T cells directed against mHags and minimal residual disease was observed in leukemia patients after allogeneic HSCT [36].

While many promising preclinical results have shown that mHags constitute excellent targets for boosting GVL effect after allogeneic HSCT [35,37–40], only a few clinical trials of immunotherapy targeting mHags were reported. Warren *et al.* reported a clinical trial of adoptive transfer of donor T cells that recognized recipient mHags for the treatment of leukemic relapse after allogeneic HSCT [41]. Seven patients with recurrent leukemia after HLA-matched allogeneic HSCT were treated with infusions of donor-derived, *ex vivo*-expanded $CD8^+$ CTL clones specific for recipient mHags. Pulmonary toxicity was observed in three patients, severe in one of them, and correlated with the level of expression of the mHag-encoding genes in lung tissue. Adoptively transferred CTLs persisted in the blood up to 21 days after infusion, and five patients attained complete but transient remission after therapy. Meij *et al.* transferred HA-1-specific $CD8^+$ T-cell lines for three leukemia patients who had suffered relapse after allogeneic HSCT [42]. No toxicity was detected after the infusion. However, no clear clinical responses were observed in the patients. One possible explanation for the lack of the effects in this study might be that the numbers of infused cells were lower than those in the study by Warren *et al.*

Tumor burden is minimal after allogeneic HSCT

The ratio between the targets and effector cells is vital for immune-mediated cancer therapy. Even if cancer vaccines can elicit significant T-cell response to tumor antigens, the numbers of antigen-specific CTLs may not be enough to compete with large numbers of tumor cells. Some clinical studies with cancer vaccines demonstrated significant potential to elicit T-cell response but did not show clinical benefits. However, it should be noted that patients suffered major relapse events in most of the studies. This suggests that patients with minimal tumor burden might be appropriate targets for immunotherapy. Since high-dose chemotherapy and/or total body irradiation before transplant and GVL effect after transplant minimize leukemia burden, the post-transplant period is appropriate for immunotherapy.

Lymphopenia allows for rapid expansion of antigen-specific CTLs

Most LAAs are self-antigens and the affinities of T-cell receptors to the LAA-derived peptides are low. Severe lymphopenia induced after allogeneic HSCT lowers the activation threshold of antigen-specific T cells and promotes thymic-independent homeostatic T-cell proliferation [43–47]. The homeostatic expansion of T cells facilitates and enhances T-cell proliferation in response to low-affinity self-antigens [44]. Preclinical murine studies have proven that lymphodepletion enhances the effect of vaccination [48,49]. In humans, the most direct evidence for the role of homeostatic T-cell proliferation in tumor eradication was demonstrated in the clinical trials of adoptive T-cell transfer to melanoma patients. Autologous tumor-infiltrating lymphocytes directed against overexpressed melanoma self-antigens were expanded *in vitro* and transferred into patients after conditioning with total body irradiation, fludarabine and cyclophosphamide. The transferred T-cell clones then multiplied massively and induced sustained regression of melanoma in 50% of cases [50]. This finding was further confirmed in clinical trials using genetically engineered peripheral blood lymphocytes carrying T-cell receptor chains specific for a melanoma antigen [51].

In the first few months after transplantation, the T-cell repertoire is oligoclonal, with skewing of the T cells toward host, tumor and viral antigens [47], although their global immune functions are severely impaired. Consistent with this finding, extremely rapid multiplication of clonal T cells targeting host cells was observed in severe acute GVHD patients early after allogeneic HSCT [52]. Because reconstitution of the T-cell compartment in lymphopenic hosts is regulated by peptides occupying MHC class I and II molecules at the time of T-cell recovery [53], there may be an opportunity to skew the T-cell repertoire by engaging the available MHC class I and class II molecules with peptides of specific interest. Thus, vaccination with mHag or LAA peptide that can bind to MHC class I and class II molecules may induce specific expansion of mHag or LAA-reactive T cells. These considerations imply that the first few months after transplantation might be an appropriate time frame for vaccination with mHag or LAA-derived peptides to induce T-cell response (Figure 2).

Donor-derived antigen-specific CTLs are not exhausted

T cells isolated from human tumors as well as experimental tumor models share many phenotypic and functional characteristics with those of exhausted T cells in chronic infections. Tumor-infiltrating CD8 T lymphocytes (TILs) were impaired in the production of effector cytokines and expressed inhibitory receptors including PD-1, LAG-3, 2B4, TIM-3 and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). In addition, changes in signaling pathways similar to exhausted T cells in chronic infection models were observed in TILs [54]. Based on these findings, it has been commonly assumed that T cells in progressive cancers exhibit an exhausted state due to a high tumor-antigen load and immunosuppressive factors in the tumor microenvironment [55]. In contrast, donor-derived LAA-specific T cells in allogeneic HSCT have not been exposed much to LAAs, and thus are not likely to be exhausted.

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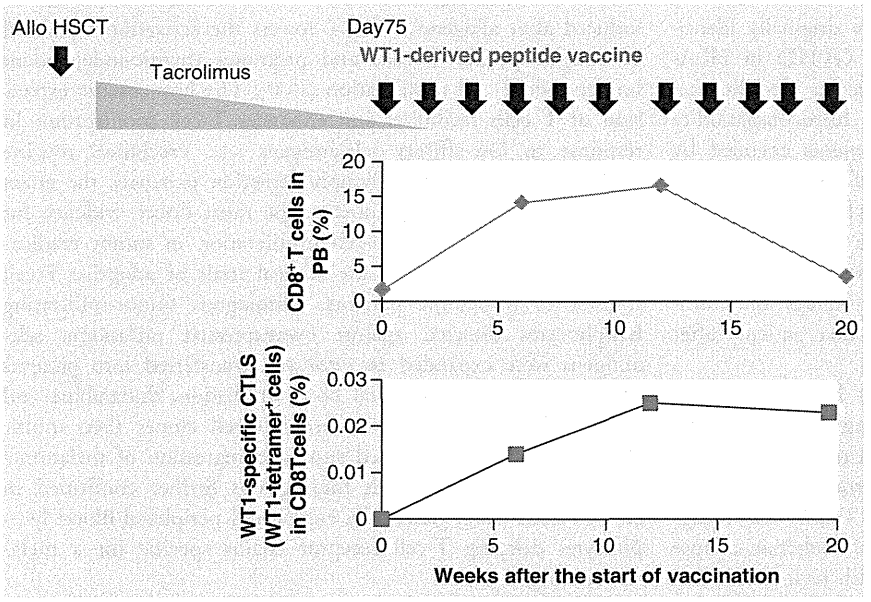

Figure 2. Leukemia-associated antigen-targeting vaccination and homeostatic T-cell expansion after hematopoietic stem cell transplantation may work synergistically and induce extensive expansion of leukemia-associated antigen-specific cytotoxic T cells. A representative AML case who was vaccinated with the WT1-derived peptide after allogeneic HSCT is shown. The graphs show the percentages of $CD8^+$ T cells in peripheral blood and those of WT1-specific CTLs in $CD8^+$ T cells. Since the patient had active disease before HSCT, tacrolimus was rapidly tapered off. WT1 peptide vaccine administration was started at day 75 after HSCT. Along with an increase in $CD8^+$ T cells, WT1-specific CTLs extensively expanded.
CTL: Cytotoxic T cells; HSCT: Hematopoietic stem cell transplantation; LAA: Leukemia-associated antigen; PB: Peripheral blood; WT1: Wilms tumor 1.

Inflammatory conditions are induced by allogeneic reactions

It is known that the GVL effect in syngeneic HSCT is not as strong as that in allogeneic HSCT, suggesting that LAAs alone are not enough to induce GVL after allogeneic HSCT. In allogeneic HSCT, inflammatory conditions caused by GVL and GVHD response targeting mHags may activate antigen-presenting cells and facilitate T-cell response to LAAs. Such inflammatory condition is preferable for vaccination therapies.

Cancer vaccine after allogeneic HSCT

A temporal inverse relationship between circulating T cells directed against mHags or LAAs and minimal residual disease was reported in patients with acute and chronic leukemia after allogeneic HSCT [36,56,57]. This suggests that GVL effects might be further enhanced by vaccination targeting mHags or LAAs [58]. However, only a small number of clinical trials of vaccination was reported in patients who had undergone HSCT, and at least to our knowledge, all the studies were pilot or Phase I studies. The results of clinical studies that have been published are listed in Table 1. The strategies for vaccination were variable. Tumor cells, DCs or peptides were used as vaccines. Not only trials for leukemia patients but also ones for other hematological malignancies are included in the list.

Tumor cell vaccine

Rousseau *et al.* used a recipient-derived tumor cell vaccine for leukemia patients including those who had undergone allogeneic HSCT. The vaccine consisted of leukemic blasts admixed with skin fibroblasts transduced with adenoviral vectors encoding human IL-2 and hCD40L [59]. Ten patients with high-risk acute myeloid (n = 4) or lymphoblastic (n = 6) leukemia in cytological remission (after allogeneic HSCT [n = 9] or chemotherapy alone [n = 1]) received the vaccine. Immunization produced a 10- to 890-fold increase in the frequencies of MHC-restricted T cells reactive against recipient-derived blasts. Eight patients remained disease free for 27–62 months after treatment. Ho *et al.* conducted a Phase I clinical trial in which high-risk acute myeloid leukemia or myelodysplasia patients were immunized with irradiated, autologous, GM-CSF-secreting tumor cells early after allogeneic nonmyeloablative HSCT [60]. While the frequencies of acute and chronic GVHD did not increase, nine of the 10 subjects who completed the vaccination schedule attained long-lasting complete remissions (CMs) during a median follow-up of 26 months. Burkhardt *et al.* performed a prospective clinical trial to test whether vaccination with whole leukemia cells early after transplantation facilitates the expansion of leukemia-reactive T cells and thereby enhances antitumor immunity [61]. Eighteen patients with advanced chronic lymphocytic leukemia received up to six vaccines starting between 30 and 45 days after transplantation. Each vaccine consisted of irradiated autologous tumor cells admixed with GM-CSF-secreting bystander cells. The estimated 2-year progression-free and overall survival rates of vaccinated subjects were 82 and 88%, respectively. $CD8^+$ T cells from vaccinated patients consistently reacted against autologous tumor cells but not allogeneic antigen-bearing recipient cells. All of these trials using tumor cell vaccines showed promising results and at least demonstrated safety. The benefit for patients' survival will have to be tested in randomized studies.

DC vaccine

Bendandi *et al.* conducted a pilot study for vaccination with DCs pulsed with idiotype Id)-derived peptide in multiple myeloma patients after allogeneic HSCT [62]. Four patients received Id-derived peptide- and keyhole limpet hemocyanin (KLH)-pulsed, donor-derived DC vaccination after disease relapse/progression following reduced intensity conditioning allogeneic HSCT and failure of rescue therapy with donor lymphocyte

Table 1. Clinical trials of vaccination after allogeneic hematopoietic stem cell transplantation.

Study (Year)	Phase of trial	Patients (n)	Disease	Antigen/adjuvant	Response	Ref.
Tumor cell vaccine						
Rousseau *et al.* (2006)	Pilot	9	AML/ALL (CR)	Autologous leukemia blasts/ fibroblasts expressing IL2 and CD40L	7 CR	[59]
Ho *et al.* (2009)	I	15	MDS/AML (CR)	Autologous GM-CSF-secreting tumor cells	9 CR	[60]
Burkhardt *et al.* (2013)	Pilot	18	Chronic lymphocytic leukemia (non-CR)	Autologous tumor cells/GM-CSF-secreting bystander cells	2-year PFS 82%, OS 88%	[61]
DC vaccine						
Bendandi *et al.* (2006)	Pilot	4	Multiple myeloma (non-CR)	Idiotype peptide/keyhole limpet hemocyanin (DC)	1 SD, 3 PD	[62]
Kitawaki *et al.* (2008)	Pilot	1	AML (non-CR)	WT1 peptide/keyhole limpet hemocyanin (DC)	1 PD	[63]
Peptide vaccine						
Hashii *et al.* (2012)	I	3	ALL/AML (CR)	WT1 peptide/ Montanide ISA 51	2 CR, 1 PD	[65]
Maeda *et al.* (2013)	I	3	AML (CR)	WT1 peptide/ Montanide ISA 51	3 CR	[66]
		2	AML/ALL (molecular relapse)	WT1 peptide/Montanide ISA 51	2 CR	
		4	AML/ALL/ multiple myeloma (non-CR)	WT1 peptide/Montanide ISA 51	4 PD	

AML: Acute myeloid leukemia; ALL: Acute lymphocytic leukemia; CR: Complete remission; DC: Dendritic cells; GM-CSF: Granulocyte-macrophage colony-stimulating factor; MDS: Myelodysplastic syndrome; OS: Overall survival; PD: Progressive diesease; PFS: Progression free survival; SD: Stable disease; WT1: Wilms tumor 1.

infusion or chemotherapy. Id-KLH-specific T-cell response was detected *in vitro*. Two patients showed a transient response. However, three patients including one responder ultimately suffered disease progression. Kitawaki *et al.* also reported DC vaccination for an AML patient after allogeneic HSCT [63]. An AML patient received WT1 peptide- and KLH-pulsed donor-derived DC vaccination for relapse after allogeneic HSCT. In this patient, immune responses to the naive antigen KLH, but not to WT1, were detected. Leukemia gradually progressed despite vaccination. These studies proved the safety of DC vaccination after allogeneic HSCT. However, its clinical benefits were limited for patients with advanced disease. The effect of DC vaccines for preventing relapse after HSCT needs to be tested in the future.

Peptide vaccine

LAAs such as proteinase 3 or WT1 were likely to be one of the target antigens for GVL [57,64]. Thus, it is reasonable to hypothesize that the GVL effect may be boosted by using peptide vaccines targeting LAAs. Hashii *et al.* reported the results of WT1-derived peptide vaccination for three pediatric leukemia patients with high risk factors for relapse after allogeneic HSCT [65]. HLA-A*2402-restricted, 9mer-modified WT1 peptide (a.a.235–243 CYTWNQML) emulsified in Montanide ISA 51 adjuvant was weekly injected. Vaccinations were started between 41 and 173 days post-stem cell transplantation. Reduced WT1 mRNA levels in bone marrow and increased WT1-specific CTLs were observed in all three cases. Two of the three cases have remained in CR for 33.5 and 40.3 months from HSCT. In one case, disease recurred on day 201 after the start of vaccination, while the frequencies of WT1-specific CTLs in peripheral blood $CD8^+$ T cells increased to 0.85%. In this case, loss of HLA expression on leukemic cells was detected, which implied immunological escape of the leukemic cells. Our group recently reported the results of a Phase I study of WT1 peptide vaccination in adult hematological malignancy patients with recurrent disease or high risk factors for relapse after HSCT [66]. The study design was almost identical to that used by Hashii *et al.* Of the nine patients enrolled, three were in CR, two were in molecular relapse and the remaining four were suffering from hematological relapse. We confirmed that

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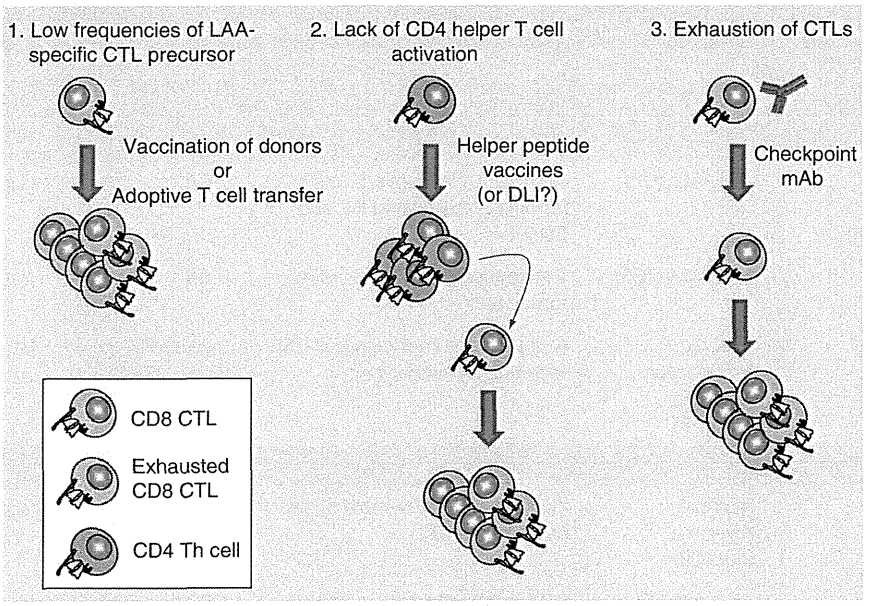

Figure 3. Strategies to improve the effect of vaccination therapies after allogeneic hematopoietic stem cell transplantation. The problems in the current vaccination therapies after HSCT and the strategies to resolve them are listed.
CTL: Cytotoxic T cells; DLI: Donor lymphocyte infusion; HSCT: Hematopoietic stem cell transplantation; LAA: Leukemia-associated antigen; mAb: Monoclonal antibody.

an antigen-specific CTL response could be elicited even in the patients who were given immunosuppressive drugs such as tacrolimus or prednisolone. Three patients in molecular CR remained in CR for 2 years, while disease gradually progressed in patients with hematologically relapsed disease. Two patients with molecular residual disease achieved CR after the start of vaccination.

These reports show that tumor cell vaccines, DC vaccines and peptide vaccines can induce antigen-specific T-cell response in post allogeneic HSCT patients, even in patients who are given immunosuppressive drugs; the results of the trials using tumor cell vaccines and peptide vaccines look promising and it may be better to test the effect of vaccination for the prevention of relapse rather than for the treatment of advanced disease. To clarify whether vaccination prevents disease relapse and benefits patients, well-controlled randomized trials should be conducted.

Future direction

As described above, vaccination therapy has potential to work synergistically with the GVL effect and is expected to be a good tool to prevent relapse after allogeneic HSCT. However, the efficacy of vaccination alone is so far limited in patients with advanced disease. Several trials are being conducted or will be tested to improve the effect of vaccination after allogeneic HSCT (Figure 3).

Donor vaccination

In allogeneic HSCT, the donors can be vaccinated before hematopoietic stem cell collection. This approach has the advantage that vaccine-primed lymphocytes are collected from a healthy donor with a healthy immune system, rather than from patients with reduced immunity. In addition, T cells from donors should not be tolerant to tumor antigens. Kwak *et al.* first showed that tumor-specific T cells could be safely induced in a healthy donor and transferred to the recipient after allogeneic HSCT [67]. These investigators immunized an HLA-matched donor with a patient-derived Id vaccine before hematopoietic stem cell collection and demonstrated that Id-specific T cells could be successfully transferred post transplantation, which was shown to be associated with a significant reduction in the serum M protein levels. Foglietta *et al.* recently immunized 10 HLA-matched sibling donors with recipient-derived clonal myeloma Id conjugated with KLH [68]. After treatment, eight of the 10 myeloma recipients showed persistent Id-specific immune responses and five of them showed improvement in disease status. These results provide proof of principle that tumor antigen-specific immunity can be safely induced in HSCT donors and passively transferred to recipients.

Combination with donor lymphocyte infusion

Donor lymphocyte infusion (DLI) is widely used as a treatment for relapse after allogeneic HSCT [2]. Since major effectors of DLI are likely to be mHags or LAA-specific CTLs, it is reasonable to expand them by vaccination after DLI. In addition, Bachireddy *et al.* recently suggested an interesting effect of DLI [69]. They analyzed the characteristics of cells from 29 patients categorized according to their response to DLI and found that gene transcription profiles before DLI showed evidence of 'exhaustion' in T cells from the marrow of only responders and that response after DLI was associated with downregulated expression of these genes. These results suggest that the infused donor CD4 T cells might eliminate recipient leukemia cells by reversing exhaustion in donor CD8 T cells that had previously infiltrated the marrow.

Activation of CD4 helper T cells

CD4 helper T cells are not needed for the primary expansion and differentiation of CD8 T cytotoxic effectors but are necessary for the secondary expansion of these effectors. The importance of CD4 helper T cells in tumor immunity has been emphasized. Tran *et al.* recently demonstrated an excellent evidence showing the essential role of CD4 helper T cells in tumor immunity in a clinical study [70]. They showed that TIL from a patient with metastatic cholangiocarcinoma contained

$CD4^+$ T cells recognizing a mutation in erbb2 interacting protein and that adoptive transfer mutation-reactive CD4 T cells induced tumor regression. To activate WT1-specific CD4 T cells together with CD8 CTLs, a polyvalent vaccine composed of longer synthetic peptides was developed to induce stronger WT1-specific $CD8^+$ T-cell responses with $CD4^+$ T-cell responses across several HLA types and to support long-lasting immunity [32]. Our group also developed a strategy to enhance WT1-specific CD4 T-cell response [71,72]. The clinical benefits of CD4-directed vaccines after allogeneic HSCT have not been evaluated and should be tested in the near future, while the influence of CD4 activation to GVHD should be assessed very carefully.

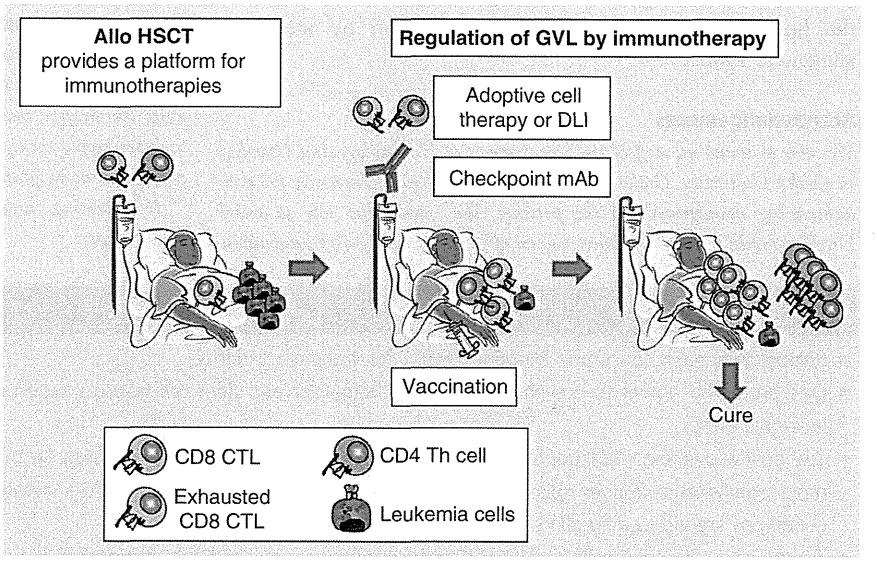

Figure 4. Allogeneic hematopoietic stem cell transplantation provides a platform for efficient immunotherapies. Schemes show the concept of immune-mediated leukemia therapy in the future. Allogeneic HSCT is not necessarily a curative therapy, but can provide a platform for immunotherapies by refreshing the immune system. Several types of immunotherapy, including vaccination, can be efficiently performed on this platform.
CTL: Cytotoxic T cells; DLI: Donor lymphocyte infusion; GVL: Graft-versus-leukemia; HSCT: Hematopoietic stem cell transplantation; LAA: Leukemia-associated antigen; mAb: Monoclonal antibody.

Combination with immune checkpoint antibody

CTLs infiltrating melanoma tumors became exhausted [54]. In leukemia models, exhaustion of CTLs in advanced disease was also reported [73]. However, recent success of checkpoint antibodies clearly demonstrate that exhausted status of CTLs is reversible. It is reasonable to test the synergistic effect of vaccination and checkpoint antibodies. CTLA-4 is a key negative regulator of T-cell activation and proliferation. Bashey *et al.* conducted a Phase I clinical trial of ipilimumab, which is an antagonist for CTLA-4, in patients with relapsed malignancy following allogeneic HSCT [74,75]. In this report, only a single infusion was administered to patients mainly for measuring the effect of ipilimumab on the number of T-cell subpopulations. Further studies are needed to know the benefit and side effects of ipilimumab after allogeneic HSCT. Programmed cell death 1, which is another coinhibitory receptor that impairs T-cell function, is highly expressed on intratumoral T cells. The safety and efficacy of anti-programmed cell death 1 antibody pidilizumab has been demonstrated in the treatment of several kinds of tumors including lymphoma [76]. The use of pidilizumab after HSCT has not been reported yet. The synergistic effect of vaccination and the checkpoint antibodies is expected, while the influence of the antibodies on GVHD should be assessed very carefully.

Expert commentary

Several evidences demonstrate that vaccination with tumor cells, DCs or mHags or LAA-derived peptides can elicit immune response in patients who underwent allogeneic HSCT. Some preliminary results suggested that vaccination including tumor cell vaccines and peptide vaccines might have the potential to prevent relapse after allogeneic HSCT. Randomized clinical trials with more patients will be needed to confirm the effect of the vaccination. Start of vaccination at early time point after allogeneic HSCT may be preferable to expand target specific T cells efficiently. However, various complications, including acute GVHD, are frequently observed within a few weeks after allogeneic HSCT, indicating that careful designing of clinical trials is needed. In contrast, the effect of vaccination therapy is limited in patients with advanced disease. Further improvement, for example, by combining with other immune-modulating drugs, will be necessary.

Five-year view

During the next 5 years, the benefits and side effects of checkpoint antibodies for leukemia patients will be clarified. Other checkpoint antibodies and immune-modulating drugs may become available for clinical use. Checkpoint antibodies are reported to be very effective in some types of cancer patients, but their effect is not specific to tumor immunity and activates the immune system globally. In contrast, vaccination activates specifically anti-leukemia immunity and will thus remain an important option of immunotherapy. Allogeneic HSCT has been considered to be a final tool for the cure of drug-resistant leukemia. However, along with the progress of transplant immunology and tumor immunology, allogeneic HSCT has now become a tool for preparing a platform for immunotherapy (Figure 4). Several types of immunotherapy can be efficiently performed after

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the host immune system has been refreshed by means of allogeneic HSCT.

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Key issues

- Relapse is a major concern in allogeneic HSCT for leukemia patients.
- One promising option to prevent relapse after hematopoietic stem cell transplantation (HSCT) is vaccination to boost the graft-versus-leukemia effect.
- The post allogeneic HSCT period provides a unique platform for vaccination because tumor burden is minimal; lymphopenia allows for rapid expansion of antigen-specific cytotoxic T cells; donor-derived antigen-specific cytotoxic T cells are not exhausted and inflammatory conditions are induced by allo reactions.
- Tumor cells, dendritic cells and LAA-derived peptides were used for vaccination and were shown to induce T-cell response even in patients who were administered with immunosuppressive drugs.
- Some preliminary reports suggest that vaccination therapies may have the potential to prevent relapse after allogeneic HSCT.
- Randomized trials will be needed to confirm the effect of vaccines for the prevention of relapse after HSCT.
- The effect of vaccination is limited in patients with advanced disease.
- To improve the efficacy of vaccination therapy, combination with other types of immunotherapy such as immune checkpoint antibodies should be considered.

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Cancer Therapy: Clinical

Clinical Cancer Research

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 IFNγ-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

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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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 $\geq$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

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antibodies (mAb): fluorescein isothiocynate (FITC)-conjugated anti-human CD14 (61D3, eBioscience), HLA-ABC (W6/32), CD80 (2D10), CD40 (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 the DCs were tested at the SRL Inc.. A general mycoplasma test was performed using a PCR ELISA Kit (Roche Applied Science).

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: KRYFKLSHLQMHSRKH, 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/m^2 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 1×10^7 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 KRYFKLSHLQMHSRKH) 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$^+$) in the total 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-I/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 entire CD3+CD8$^+$ 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.

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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 IL2 and 10 ng/mL IL7 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 IL7 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 $CD4^+CD25^+$forkhead box p3 $(Foxp3)^+$ regulatory T cells (Tregs) in the $CD4^+$ T cells, the PBMCs were stained with FITC-conjugated anti-human CD4 mAb, APC-conjugated anti-human CD25 mAb, and PE-conjugated anti-human Foxp3 mAb or matched isotype control IgG using the Human Primate Regulatory T-Cell Staining Kit (eBioscience) according to the manufacturer's instructions. The $CD4^+$ T cells were analyzed for CD25 and Foxp3 expression. For the analysis of the myeloid-derived suppressor cells (MDSC), the PBMCs were stained with 7-AAD, PE-conjugated anti-human CD14 mAb, FITC-conjugated anti-human CD11c mAb, APC-conjugated CD33 mAb (BioLegend), or the matched isotype control IgG. The $CD14\text{-}CD11b^+CD33^+$ populations in the live PBMCs were defined as MDSCs. The cells were analyzed using MACSQuant Analyzers and the MACSQuantify Software (Miltenyi Biotec Inc).

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. Finally, the population of WT1/HLA-A*24:02 tetramer–positive $PD\text{-}1^+$ 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-I/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/m^2, 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/m^2) 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/m^2) and 6 DC/WT1-I/II treatments. All 11 patients experienced grade 1 skin reactions at the site of vaccination. Grade 1 to 3 leukocytopenia 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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Table 1. Patient characteristics

Patient	Sex	Age, y	UICC stage	Location	Metastases	Prior systemic treatment	HLA genotype						Vaccine	Times	WT1 peptide–specific DTH													
							HLA-A		DRB1		DPB1				A*02:01 Vaccine times					A*24:02 Vaccine times					DRB1/DPB1 Vaccine times				
															0	2	4	6	8	0	2	4	6	8	0	2	4	6	8
PDA-01	M	41	IV	PDA (body–tail)	Bone, LN	No	02:01	—	08:03	12:01	02:02	15:01	DC/WT1-I	19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PDA-02	M	69	IV	PDA (body–tail)	Lung, peritonitis, LN	No	24:02	26:01	12:01	15:02	05:01	09:01	DC/WT1-I	11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PDA-03	M	73	IV	PDA (head)	Peritonitis	No	26:03	33:03	08:02	08:03	02:02	05:01	DC/WT1-II	13	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ICC-01	F	58	IV	ICC	Peritonitis, LN	No	02:01	24:02	04:05	15:02	09:01	—	DC/WT1-I	15	–	+	+	+	+	–	+	++	++	++	–	–	–	–	–
PDA-04	M	70	IV	PDA (body)	Peritonitis	No	02:01	24:02	04:05	15:02	05:01	09:01	DC/WT1-I/II	35	–	+	++	++	++	–	+	++	++	++	–	+	++	++	++
PDA-05	M	68	IV	PDA (body)	Liver, LN	Ope, Cx	24:02	33:03	08:03	13:02	02:02	04:01	DC/WT1-I/II	46	–	–	–	–	–	–	+	++	++	++	–	+	++	++	++
PDA-06	F	49	IV	PDA (head)	Liver, peritonitis, LN	No	02:01	24:02	04:05	09:01	02:02	05:01	DC/WT1-I/II	7	–	–	–	–	N.D.	–	–	–	–	N.D.	–	–	–	–	N.D.
PDA-07	M	35	IV	PDA (body)	Liver, LN	No	02:01	—	09:01	15:01	02:01	05:01	DC/WT1-I/II	6	–	–	–	–	N.D.	–	–	–	–	N.D.	–	–	–	–	N.D.
PDA-08	F	72	IV	PDA (body)	Peritonitis, LN	No	02:06	24:02	08:02	12:01	02:01	05:01	DC/WT1-I/II	14	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PDA-09	F	69	IV	PDA (body–tail)	LN	No	24:02	33:03	13:02	15:01	04:01	13:01	DC/WT1-I/II	44+	–	–	–	–	–	–	+	++	++	++	–	+	++	++	++
PDA-10	M	39	IV	PDA (head–body)	Peritonitis	Ope	02:10	24:02	15:01	15:02	02:02	09:01	DC/WT1-I/II	20	–	–	–	–	–	–	–	+	+	+	–	–	+	+	+

Abbreviations: Cx, chemotherapy; DTH -, erythema <1 mm; DTH +, erythema 2- 5 mm; DTH ++, erythema > 5 mm; LN, lymph nodes; N.D., not done; Ope, operation.

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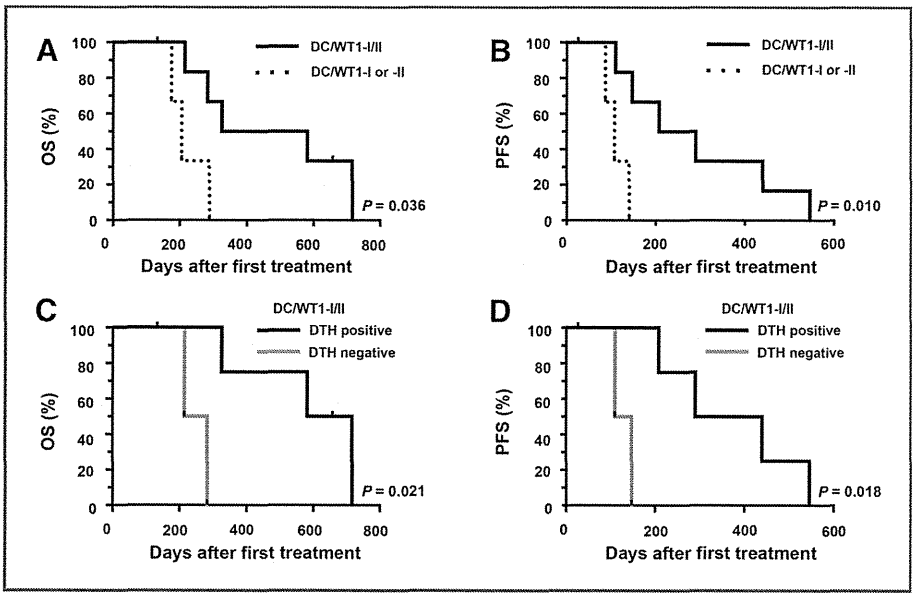

Figure 1. OS and PFS in patients with PDA. A, Kaplan–Meier estimates of OS for patients with PDA who were treated with DCs pulsed with WT1 MHC class I (DC/WT1-I) or -II (DC/WT1-II; $n = 3$) or -I/II peptides (DC/WT1-I/II; $n = 7$). B, PFS in patients with PDA who received DC/WT1-I or -II ($n = 3$) or DC/WT1-I/II ($n = 7$). C, OS in delayed type hypersensitivity (DTH)-positive ($n = 4$) or -negative ($n = 3$) PDA patients who received DC/WT1-I/II. D, PFS in DTH-positive ($n = 4$) or -negative ($n = 3$) patients with PDA who received DC/WT1-I/II.

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). The median survival time (MST) and the median PFS of the patients with PDA 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).

Assessment of WT1-specific immune responses *in vivo*

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). However, DTH positivity was not detected in all 3 patients with PDA vaccinated with DC/WT1-I or -II (Table 1). Interestingly, the MST and the median PFS of the DTH-positive PDA patients vaccinated with DC/WT1-I/II were significantly longer than that of the DTH-negative PDA patients ($P = 0.021$ and $P = 0.018$, respectively; Fig. 1C and D). Moreover, 3 patients with PDA who were vaccinated with DC/WT1-I/II were strongly DTH-positive during the entire treatment period and had an MST of 717 days and a median PFS of 440 days (Supplementary Table S2).

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, -II, or -I/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

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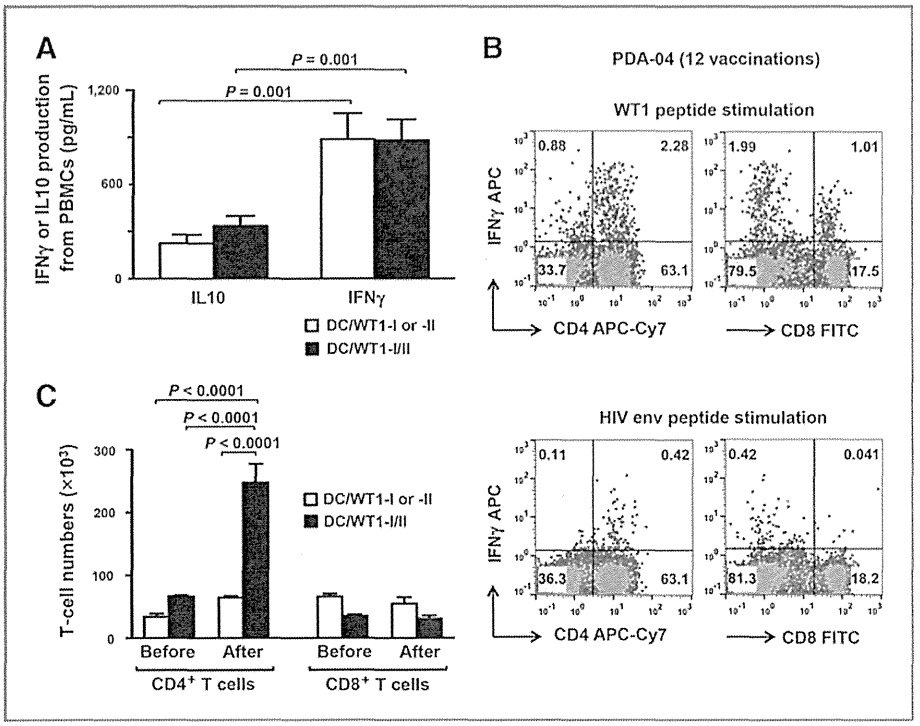

Figure 2. $CD4^+$ T-cell responses after vaccination with DC/WT1-I/II. A, The production of IFNγ or IL10 in peripheral blood mononuclear cells (PBMC) after six vaccinations with DC/WT1-I or -II ($n = 4$) or DC/WT1-I/II ($n = 7$) following stimulation with WT1 class I and II peptides was assessed. B, dot blots of PBMCs (PDA-04) analyzed for the IFNγ-producing cells in the $CD4^+$ and $CD8^+$ T cells upon stimulation with WT1 class I and II peptides *in vitro* are shown. HIV env peptides and matched isotype IgG were used as controls. C, $CD4^+$ and $CD8^+$ T-cell numbers upon stimulation of PBMCs from vaccinated patients [DC/WT1-I or -II ($n = 4$) or DC/WT1-I/II ($n = 7$)] with WT1 class II peptide *in vitro*. The results are expressed as the mean ± SE.

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-A*24:02-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-A*24:02 tetramer–positive memory ($CD45RA^-CCR7^+$ and $CD45RA^-CCR7^-$) cells in $CD8^+$ T cells were analyzed (Fig. 4A and B). Interestingly, the association between the percentages of WT1/HLA-A*24:02-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-A*24:02–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-A*24:02 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-A*24:02 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

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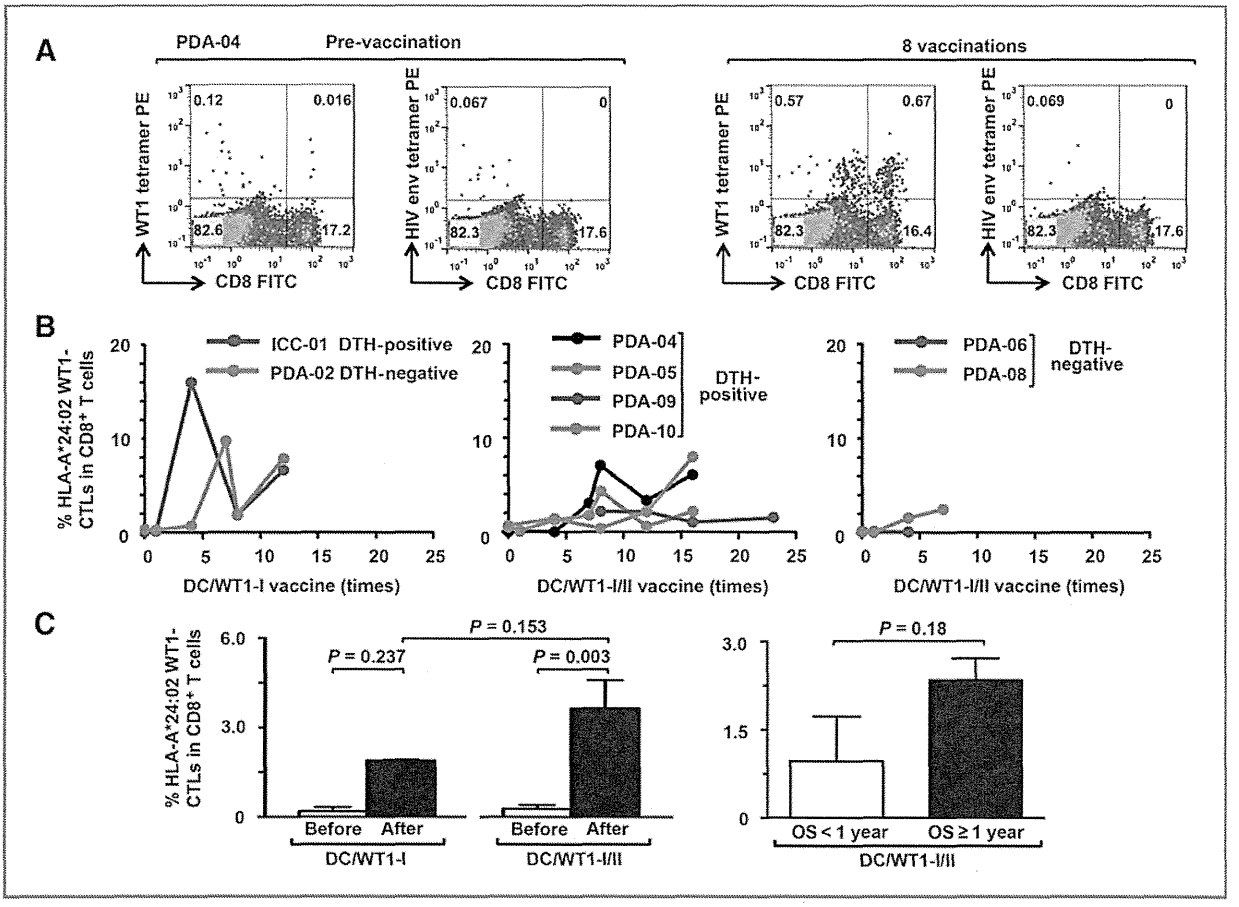

Figure 3. Induction of HLA-A*24:02–restricted WT1-specific CTLs. A, dot plots of HLA-A*24:02–restricted, WT1 tetramer-positive populations in $CD8^+$ T cells derived from PDA-04 are shown before and after eight vaccinations. B, the percentage of HLA-A*24:02–restricted, WT1-specific CTLs (WT1-CTLs) in $CD8^+$ 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.

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

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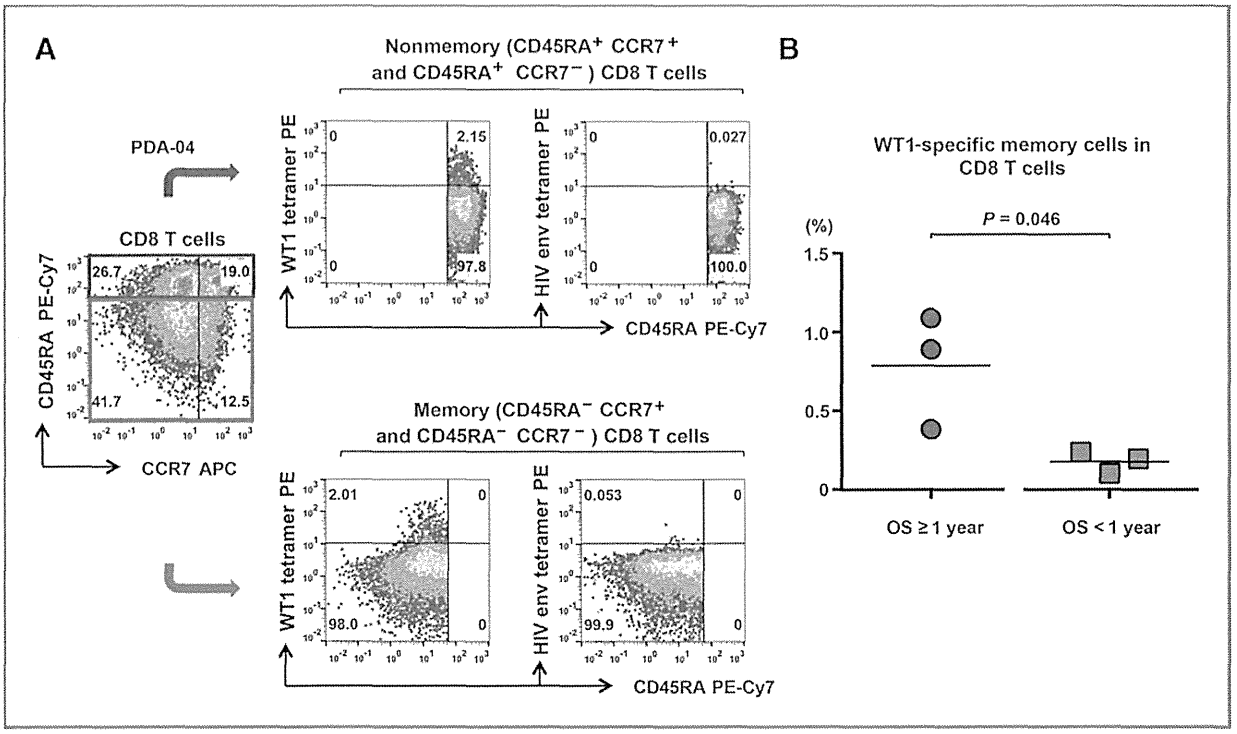

Figure 4. WT1/HLA-A*24:02–specific memory cells in $CD8^+$ T cells. A, dot plots of the memory ($CD45RA^-CCR7^+$ and $CD45RA^-CCR7^-$) and non-memory ($CD45RA^+CCR7^+$ and $CD45RA^+CCR7^-$) phenotypes in $CD3^+CD8^+$ T cells from PDA-04 treated with six vaccinations of DC/WT1-I/II are shown (left). The WT1/HLA-A*24:02$^+$ memory (bottom) and non-memory (top) cells in the $CD3^+CD8^+$ T cells are shown. The HIV env tetramer was used as a control. B, after six vaccinations with DC/WT1-I/II, the percentages of WT1/HLA-A*24:02$^+$ memory cells in the $CD3^+CD8^+$ T cells between patients with an OS time ≥1 year [PDA-04, -05, and -09 (blue) and those with an OS time <1 year (PDA-06, -08, and -10 (red)] were compared.

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 effective cancer vaccines not only stimulate CTL activity but also maintain long-term memory T cells capable of mounting functional antitumor immune responses to secondary antigenic peptide challenges.

Assessing whether WT1-CTLs can be induced by the combination therapy is essential. We used the HLA-A*24:02 tetramer to detect WT1-CTLs because adequate HLA-A*02:01 and HLA-DRB1/DPB1 tetramers were not available. In all 11 enrolled patients, 8 patients were positive for HLA-A*24:02. The patients vaccinated with DC/WT1-I showed more than 10% WT1/HLA-A*24:02-specific tetramer-positive cells in the $CD8^+$ T-cell population shortly after vaccination, suggesting that the DC/WT1-I vaccine induced the expansion of the self-restricted WT1-CTLs that originated from preexisting memory $CD8^+$ T cells (29). Importantly, the CTLs induced by the DC/WT1-I vaccine were not maintained for the entire duration of the treatment protocol. 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 IFNγ-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 IFNγ 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