Long-term Vaccine Therapy with Autologous Whole Tumor Cell-pulsed Dendritic Cells for a Patient with Recurrent Rectal Carcinoma

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Abstract. *We performed continuous dendritic cells (DCs) vaccination to treat a patient with chemotherapy-resistant recurrent rectal carcinoma and lung and bone metastases. A patient has received a total of 66 DC vaccinations at 14-day intervals for 3 years until his death. Necrotic whole tumor cells (WTC) were selected as the tumor-associated antigen source because they showed a greater capacity for DC maturation and interleukin-12 secretion than both necrotic tumor lysate alone and necrotic tumor cell fragment alone. After the sixth vaccination, both skin test and interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) response by peripheral blood T-cells to WTC-pulsed DCs were positive. Importantly, T-cell responses were positive towards DCs pulsed with several synthetic peptides including Carcinoembryonic antigen (CEA), Melanoma associated antigen (MAGE)1 and MAGE3. A biopsy specimen collected from the pelvic bone metastasis after the 6th vaccination showed marked necrotic change of the carcinoma cells, with many infiltrating mononuclear cells. The patient did not show any particular adverse reactions to vaccination such as autoimmune phenomena. Our experience of this case suggests that continuous long-term vaccination with autologous WTC-pulsed DCs can elicit in vivo T-cell response against multiple tumor-associated antigens and induce tumor regression in disease that has proven resistant to intensive chemo- or radiation therapy.*

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Although moderate advances in chemo- and radiation therapy for recurrent colon carcinoma have been made during the past decade, this type of cancer essentially remains incurable. The ineffectiveness and adverse effects of current treatment motivated us to search for alternative tumor therapies. One such alternative is immunotherapy, particularly active specific immunotherapy, which targets and eliminates cells expressing tumor-specific or tumor associated antigens (TAAs).

Dendritic cells (DCs), professional antigen-presenting cells, are critical for eliciting T-cell-mediated immune responses and provide new direction for cancer treatment (1-3). DCs loaded with various TAAs such as those present in tumor lysates (4) , tumor-derived peptides (5), major histocompatibility complex (MHC)-restricted peptides (6), tumor-derived RNA (7), or tumor-derived DNA (8), have been shown to generate anti-tumor immune responses and antitumor activity both *in vitro* and *in vivo*. Among these TAAs, synthetic peptides have been shown to induce antigen-specific antitumor immune responses in clinical studies of DC-based immunotherapy (9-13). Although peptides are useful for the establishment of known antigen-specific T-cell responses, DCs loaded with whole tumor cells (WTCs) should be more useful because they induce a wide array of responses against both known and unknown TAAs.

Recent studies have shown that autologous tumor lysate-pulsed DCs elicit T-cell responses and clinical responses *in vivo* (14-18). Berard et al demonstrated that DCs pulsed with killed melanoma cells can induce specific T-cell responses *in vitro* against several melanoma-specific TAAs such as melanoma antigen recognized by T-cells (MART)1 and GP100 (19). However, it is unclear whether WTC-pulsed DCs can elicit *in vivo* T-cell responses against certain defined TAAs in cancer patients. There are only a few reports showing data for DC vaccination for advanced cancer

patients lasting more than 6 months. Therefore, there is only limited data concerning adverse effects, especially autoimmune phenomena such as arthritis and fever of unknown origin, induced by long-term DC vaccination. The case we present here had DC vaccination 66 times over 3 years. Our results suggest that long-term continuous vaccination with WTC-pulsed DCs is safe and can induce disease stability even in a patient with chemotherapy- or radiation-resistant carcinoma. We also show that WTC-pulsed DCs may induce T-cell responses against multiple TAAs, including several defined TAAs even in patients with far-advanced carcinoma.

Patient and Methods

Patient. The patient was a 40-year-old man who underwent resection of primary rectal cancer at the Department of Surgery I, Kyushu University (Fukuoka, Japan). Five years after the operation, local recurrence in the pelvic cavity was identified and surgically removed. Following surgery, the patient underwent adjuvant systemic chemotherapy [*cis*-diamminedichloroplatinum (CDDP) plus 5-fluorouracil (5-FU)] and radiation to the pelvic space. Nevertheless, the level of carcinoembryonic antigen (CEA) gradually increased, and chest X-ray and computed tomography (CT) examinations revealed multiple bilateral lung metastases. Thereafter, he received two courses of second line chemotherapy (CDDP plus irinotecan hydrochloride), but the lung metastases rapidly increased in size and number, and a new metastatic bone mass was found in the right pelvic region. Believing the patient required additional adjuvant therapy including immunotherapy, we had collected peripheral blood mononuclear cells (PBMCs) with apheresis and stored them at −80˚C before he underwent the intensive chemotherapy. After receiving 66 DC vaccinations over 3 years, the patient died of respiratory failure due to rapidly increased lung metastasis.

Establishment of autologous tumor cell line. A carcinoma specimen was collected by CT-guided biopsy from the pelvic metastatic mass. The tumor specimen was minced with scalpels and passed through metal meshes of decreasing pore size. Cells were collected and cultured in serum-free enriched culture media (EBM2, Sanko Jyunyaku, Tokyo, Japan) containing several growth factor including 1 ng/ml of basic fibroblast-growth factor, 1 ng/ml of epidermal growth factors, and 5 μg/ml of insulin. To avoid any decrease in TAAs, no chemical digestion was performed. After the fifth passage, the autologous tumor cell line was established. Tumor cells were then maintained in serum-free enriched medium supplemented with 1% human albumin.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was obtained from the tumor cell line by acid guanidine thiocyanate-phenol-chloroform extraction (20). The cDNA was synthesized from 3.0 μg of total RNA and amplified by polymerase chain reaction (PCR) as previously described (21). The presence of melanoma associated antigen (MAGE)1, MAGE3, HER2/neu, and CEA cDNA was detected in four separate reactions with oligonucleotide primers. Thermal cycler parameters included a 5-min initiation at 95˚C and 33 cycles of 1 min at 95˚C for denaturation. Annealing temperature was at 58˚C for *GAPDH, CEA, HER2/neu*, and 72˚C for *MAGE1* and *MAGE3*. Primers used for PCR were as follows: 5'CCACCCATGGCAAATTCCATGGCA, 3'TCTAGACGGCAGGTCAGGTCCACC for *GAPDH* (22); 5'CGG CCG AAG GAA CCT GAC CCA G, 3'GCT GGA ACC CTC ACT GGG TTG CC for *MAGE1* (23); 5'TGG AGG ACC AGA GGC CCC C, 3'GGA CGA TTA TCA GGA GGC CTG C for *MAGE3* (23); 5'CGG GAG ATC CCT GAC CTG CTG GAA, 3'CTG CTG GGG TAC CAG ATA CTC CTC for *HER2/neu* (24); and 5'GAT TGA TGG GAA CAT CCA GC, 3'CAG TAG CCA AGT TAG AG ACA for *CEA* (25).

HLA typing and synthetic peptides. According to standard HLA serotyping, the patient was type HLA-A2, A24. The following HLA-A24-binding peptides were obtained from Takara (Osaka, Japan): FluNP.38(RFYIQMCTEL), MAGE1(NYKHCFPEI), MAGE3 (IMPKAGLLI), HER2/neu (RWGLLLALL) and CEA625 (TYACFVSNL). Peptides were dissolved in dimethylsulfoxide at 2 mg/ml and stored at −80˚C. From this stock solution, peptides were dissolved in RPMI-1640 medium (HyMedia, Nipro,Tokyo, Japan) at 200 μg/ml immediately before use.

Preparation of necrotic tumor cells. Tumor cells were harvested with a rubber policeman and resuspended in serum-free RPMI 1640 medium and subjected to two cycles of freezing (liquid nitrogen) and thawing (37˚C water-bath) for the dissociation of cell clusters. The dissociated tumor cells were counted with a cell counter (PD-500; Sysmex, Kobe, Japan). The cell density was adjusted to 10^6/ml.

Tumor cells were resuspended in 2 ml of RPMI-1640 and lysed by five freeze-thaw cycles, and then divided between two tubes. Total cell disruption was microscopically validated using trypan blue staining. These lysed cells were used as necrotic WTCs. After centrifugation of the WTCs at 15,000×g (10 min at 4˚C), supernatant was recovered, then the pellet of the cell fragments (NCF) was resuspended in 2 ml of RPMI-1640. The effects of WTCs, supernatant, and NCF on interleukin-12 (IL-12) production and maturation of the autologous immature DCs were examined by enzyme-linked immunosorbent assay (ELISA) and fluorescence-activating cell sorter (FACS), respectively.

Generation of WTC-pulsed DCs. The generation of DCs and the preparation of vaccine for clinical use were approved by the Ethics Committee at Kyusyu University. Cell culture and antigen pulsing were performed in the Cell Therapy Laboratory at the Collaborative Research Center of Kyusyu University, which is a facility that operates under good manufacturing practice requirements. The patient underwent leukapheresis with a COBE spectrum apheresis system (GAMBRO BCT, Inc, CO, USA) three times. The PBMCs recovered from frozen leukapheresis products were resuspended in GMP-grade RPMI-1640 (Hy-Media) with 1% human albumin containing 4×10^6 cells/ml, and 500 μl of cell suspension was added to each well of 24-well culture plates. The plates were incubated in 5% CO_2 at 37˚C, and after 4 h, the nonadherent cells were gently removed. The adherent cells were cultured for 7 days in Hy-Media containing 1% human albumin, 100 ng/ml of recombinant human granulocyte/monocyte colony-stimulating factor (GM-CSF; North China Pharmaceutical group Corporation-GeneTech, China) and 50 ng/ml of recombinant human interleukin-4 (IL-4; Osteogenetics, Wuerzburg, Germany). After 7 days, the DCs were harvested by gentle pipetting. A total of 2 to 20×10^6 immature DCs was obtained per preparation from 2 to 20×10^7 PBMCs. Two days before each DC vaccination, immature DC

cultures were incubated with necrotic WTCs overnight (DC:tumor=5 to 10:1). Cells were further cultured in the presence of 20% monocyte-conditioned medium (MCM) for DC maturation as previously described (26). For DC maturation in the late stage of this study (6 months after initial vaccination), we used OK-432, a streptococcal preparation-induced PBMC supernatant that contains tumor necrosis factor (TNF)-α, IL-1β, IL-6, interferon (IFN)-γ, and IL-12 as the maturation factor (examined and verified by preliminary experiments as a potent DC maturation factor).

Procedure for DC vaccine. The patient received 2 to 30×10^6 WTC-pulsed mature DCs suspended in 2 ml of 1% human serum albumin-containing saline subcutaneously in the left supraclavicular lesion. A total of 66 vaccinations were administered until his death. DC vaccination was usually performed in the outpatient clinic.

FACS analysis. DCs (1×10^5) were suspended in 100 μl of diluted fluorescein-isothiocyanate or phycoerythrin-conjugated monoclonal antibodies (CD40, CD80, CD83, CD86, Class I, and HLA-DR; Becton Dickinson, CA, USA). They were incubated for 45 min at 4˚C in the dark and then washed twice with FACS buffer (phosphate-buffered saline (PBS)/1% bovine serum albumin/0.1% NaN_3). They were resupended in 500 μl in FACS buffer, and were assayed using flow cytometry (FACS Caliber; Becton Dickinson) and the data were analyzed with CellQuest v3.2.1f1 (Becton Dickinson).

ELISA. The concentrations of IL-12 in the supernatants of DC cultures were measured with an ELISA specific to IL-12 p40 according to the manufacturer's instructions (Biosource, CA,USA). The limit of detection of the assay was 10 pg/ml.

Immune monitoring and enzyme-linked immunospot (ELISPOT) assay. One million WTC-pulsed DCs were injected intradermally in the forearm every 4 weeks. Forty-eight hours after each injection, the erytherma (diameter) and induration at the injection site were measured and assessed for delayed-type hypersensitivity (DTH) response.

PBMCs were obtained every 4 weeks and assayed for immune monitoring, and aliquots of PBMCs were cryopreserved in serum-free frozen media (Cell-Banker II, Diaclone, Tokyo, Japan). Nonadherent PBMCs, the T-cell source, were suspended in 1% albumin RPMI at 5×10^5 cells/ml for ELISPOT assay. In some experiments, $CD4^+$ and $CD8^+$ T lymphocytes were positively isolated from PBMCs using immunomagnetic $CD4^+$/$CD8^+$ microbeads (DYNAL; ASA, Oslo, Norway). ELISPOT assays were performed using IFN-γ ELISPOT assay kit according to the manufacturer's recommended protocol (Diaclone Research, Besancon, France). Briefly, 96-well plates with PVDF membrane (Millipore Bedford, MA,USA) were coated overnight at 4˚C with anti-IFN-γ captured monoclonal antibody (mAb). The next day the mAb was washed away, and the plate was blocked with 2% skin-milk–PBS for 2 h at room temperature. DCs and PBMCs were added to the wells and incubated for 15 h at 37˚C. The cells were washed with PBS containing 0.1% Tween-20, and 1 μg/ml secondory anti-IFN-γ mAb was added for 2 h. The plates were washed with 0.1% Tween-20 and incubated with avidin-biotynylated alkaline phosphatase (ALP) for 1 h at room temperature. The assay was developed with the addition of nitro blue tetrazolium (NBT) solution for 20 min at room temperature. The spots formed were counted with a stereomicroscope. In some experiments, immature DCs were matured with TNF-α (100 U/ml) and prostaglandin E2 (1 μg/ml) for 24 h, then pulsed with HLA-A24 restricted peptides (CEA, HER2/neu, MAGE1, MAGE2, or MAGE3, Takara Bio Inc, Shiga, Japan). Nonadherent PBMCs or purified $CD8^+$ T-cells were cocultured with the peptide-pulsed mature DCs for 15 h, and IFN-γ ELISPOT assay was performed as described above.

Cytological and histological examination. Before vaccination and 4 months after the initial DC vaccination, carcinoma needle-biopsied specimens were obtained from a pelvic metastatic mass under CT guidance. Specimens were stained with Papanicolaou solution, and cytological examination was performed. Punch biopsy (10-mm diameter) was performed to obtain skin tissue from the DTH-positive skin area. These specimens were fixed with buffered formalin methanol for histological analysis. Paraffin tissue sections were cut at 5 μm, heat-fixed momentarily, and stained with hematoxylin-eosin stain or immunologically stained with CD8 and HLA-DR mAbs (eBioscience, San Diego, CA, USA).

Statistical analysis. An unpaired two-tailed Student's *t*-test was used for statistical analysis. A *p*-value of <0.05 was considered significant.

Results

Characterization of the established autologous tumor cell line. The patient's tumor tissue was biopsied from the metastatic pelvic mass under CT guidance (Figure 1A). The biopsy specimen was passaged ten times to obtain approximately 10^8 cells. The established cell line exhibited colonized but not single cell morphology (Figure 1B). The cell line was identified by RT-PCR detection of mRNA expression of the TAAs characteristic of colon carcinomas. We selected CEA, MAGE1, MAGE3, and HER2/neu as TAAs because they are representative TAAs for malignant epithelial tumors. All RT-PCR primers were designed to delete genomic DNA contamination. The established cell line expressed all four of these TAAs at the mRNA level (Figure 1C). Immunohistochemical analysis of the original tumor specimen revealed strong expression of CEA and weak expression of MAGEs and HER2/neu at the protein level (data not shown).

Characterization of autologous WTC-pulsed DCs. Immature DCs were generated in serum-free enriched medium from cryopreserved PBMCs collected by leukapheresis before the DC vaccine therapy as described in the Materials and Methods section. Immature DCs were pulsed with freeze-thaw necrotic WTC to allow phagocytosis of WTC. Pulsation conditions were established through preliminary experiments. In this study, the ratio of immature DCs to tumor cells was 5 to 10:1 and pulsation time was 12 h (27). After pulsation with WTCs, dendrite-forming and floating immature DCs (Figure 2A) changed morphologically to spindle-shaped adherent DCs (Figure 2B). WTC-pulsed DCs were further matured with 20% MCM for 48 h. FACS analysis of WTC-pulsed DCs nine days after the initiation of culture showed that more than 90% of

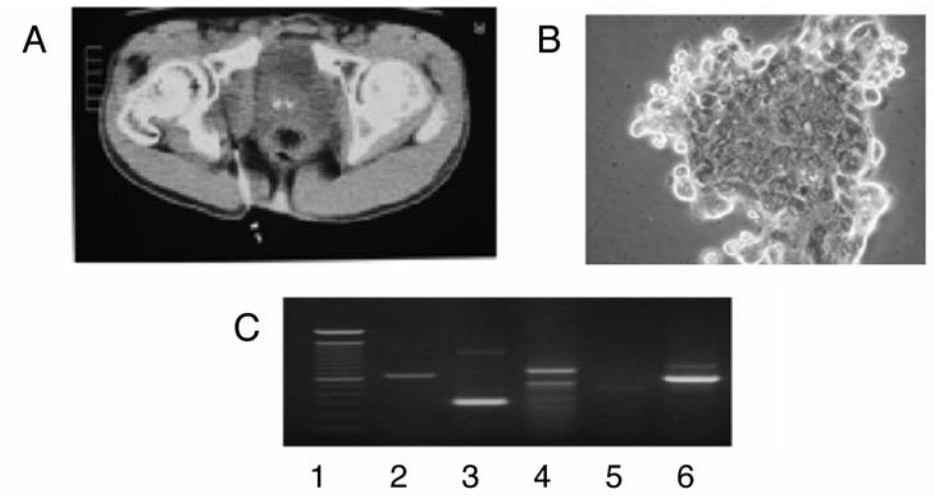

Figure 1. *Establishment and characterization of an autologous tumor cell line for DC therapy. A: Pelvic bone metastasis and CT-guided tumor biopsy. The biopsy needle inserted into the tumor is shown. B: Morphology of the autologous tumor cells under inverted microscopy (×200). C: mRNA profiles of tumor cells. Lane 1: Molecular size marker, 2: GAPDH, 3: MAGE1, 4: MAGE3, 5: HER2/neu, 6: GAPDH.*

DCs were larger in size and expressed a characteristic phenotype: HLA-ABC^{high}, HLA-DR^{high}, CD40^{high}, CD80^{high}, CD86^{high}, CD83^{+}, CD14^{-} (Figure 3). These features were stable despite the removal of GM-CSF, IL-4, and MCM for at least two more days of culture.

Effects of subcellular fractions derived from freeze-thaw necrotic tumor cells on maturation and activation of immature DCs. To induce potent antigen-presenting DCs, we examined which fraction derived from necrotic tumor cells the most effectively induced expression of co-stimulatory molecules and T cell-stimulating DC activity. Firstly, we prepared three fractions including supernatant, NCF, and WTCs from autologous tumor cells, as described in the Materials and Methods. Immature DCs were pulsed with each fraction for 12 h and further cultured in the presence or absence of 20% MCM for 48 h. As shown in Figure 4A, WTCs induced the largest amount of IL-12 secretion in immature DCs. Not only IL-12 secretion from DCs but also co-stimulatory molecule expression play an important role in DC-mediated T-cell stimulation. As shown in Figure 4B, WTCs induced the greatest expression of CD40, HLA-DR, and CD83, a maturation marker. Therefore, we chose WTCs as the pulsation source for TAAs in this case.

We used IFN-γ ELISPOT analysis to determine the effectiveness of WTCs for induction of T-cell response. Freshly collected peripheral blood nonadherent cells as T-cell source obtained during DC vaccine therapy were co-cultured with immature DCs pulsed with each cell fraction of necrotic tumor cells for 12 h and further cultured with or without 20% MCM for 48 h. Possible generation of TAA-specific T-cells was found after the DC vaccination. WTCs produced the largest number of TAA-specific T-cell responses in co-culture of DC and nonadherent PBMCs (Figure 4C), as measured by IFN-γ ELISPOT. Thus we confirmed that WTC-pulsed DCs induced the greatest T-cell stimulation.

Induction of multiple TAA-specific T-cell responses. To determine whether DC vaccination could induce T-cell responses against established TAAs *in vivo*, we obtained PBMCs after the eighth DC vaccination, and both CD4^{+} and CD8^{+} T-cells were purified as described in the Materials and Methods. T-Cells were co-cultured for 15 h with WTC-pulsed mature DCs or mature DCs pulsed with each of the four kinds of synthetic peptides and IFN-γ-producing activity was determined by ELISPOT assay. As shown in Figure 5, the largest number of IFN-γ producing T-cells was induced with WTC-pulsed mature DCs and a significant increase in IFN-γ production from T-cells was detected in CEA- and MAGE1-pulsed mature DCs. Although the increase was not significant, IFN-γ production was also elevated in T-cells co-cultured with MAGE3, and with HER2/neu-pulsed mature DCs. CD4^{+} or CD8^{+} selection showed that both types of T-cells responded in a similar manner to the WTC-pulsed DCs (Figure 6A and 6B). Interestingly, nonadherent PBMCs, freshly obtained from the patient after immunization were able to elicit an antitumor effect (Figure 7). These results indicate that WTC-pulsed DC vaccine were able to elicit *in vivo* T-cell responses against multiple TAAs including CEA and MAGE1.

Immunohistopathological findings of biopsied DTH-positive skin tissue. Skin tissue was obtained from a DTH-positive site after the tenth DC vaccination and examined immuno-histopathologically (Figure 8). The biopsy specimen showed

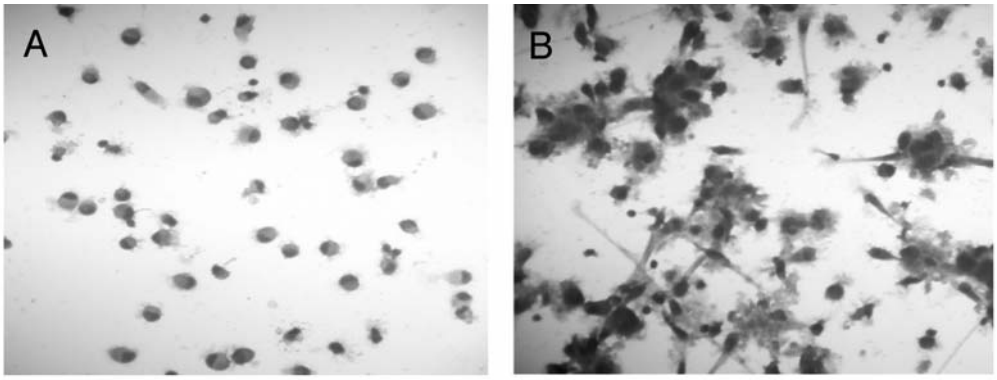

Figure 2. *Giemsa staining of DC phagocytosis of necrotic whole tumor cells (WTCs). Immature DCs (7 days culture) were pulsed with freeze-thaw WTCs (DC:WTC=5:1), then incubated overnight in a 48-well culture plate. Plates were gently washed once with warm medium, dried, and fixed with methanol. Cells were fixed and stained with Giemsa solution and observed under a phase-contrast microscope. A: No pulse, control immature DCs. B: Tumor-captured DCs. Note the morphological changes of tumor-phagocytozed DCs (×200).*

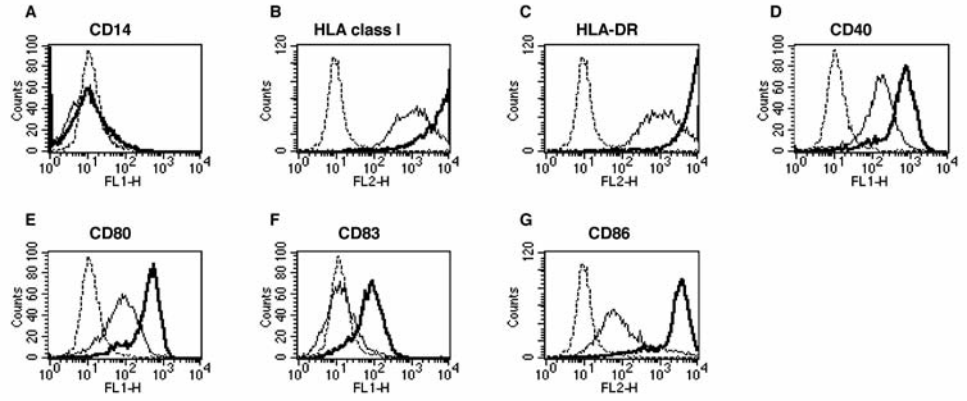

Figure 3. *Flow cytometric analysis of HLA antigens and co-stimulatory molecules of immature dendritic cells (DCs) and whole tumor cell (WTC)-pulsed matured DCs. A:CD14, B:HLA-ABC, C:HLA-DR, D:CD40, E:CD80, F:CD83, G:CD86. In each histogram, the dotted line indicates the negative control, the thin line indicates immature DCs, and the thick line indicates WTC-pulsed mature DCs.*

characteristic perivascular mononuclear cell infiltration, indicating induction of the classic DTH reaction. Most of the infiltrating cells were $CD8^+$, $HLA\text{-}DR^+$, and $CD4^-$.

Time course study of metastatic lesions and cytological findings of pelvic metastatic mass. Six months after the initial DC vaccination, we noticed a slight decrease in the size of a pelvic bone metastatic mass in which calcification occurred (Figure 9). A needle biopsy was performed to obtain a specimen from the pelvic mass for cytological examination before and after the start of DC vaccination. Cytological examination of a specimen collected before vaccination revealed many viable adenocarcinoma cells without mononuclear cell infiltration (Figure 10A). Cytological examination after the sixth DC vaccination, however, revealed only a few viable carcinoma cells with slight lymphocyte infiltration (Figure 10B). The DC vaccinations were performed at an outpatient clinic except during a short-term hospitalization for pain control.

Adverse effects of DC vaccination. A summary of the adverse reactions appears in Table I; none were severe. Low-grade fever (grade 1) occurred twice after DC vaccination. Edematous erythema at the intradermal DC injection site was seen once immediately after the DC injection. This local edematous erythema was macroscopically similar to a type I allergic reaction, and moderate serum IgE elevation was also found. However, the patient had no particular subjective complaints, and the erythema disappeared within 1 hour without treatment. One day later, the serum IgE level also decreased to within normal range. There was no clinical manifestation of autoimmune disease. Serum rheumatoid factor, antinuclear antibody, and

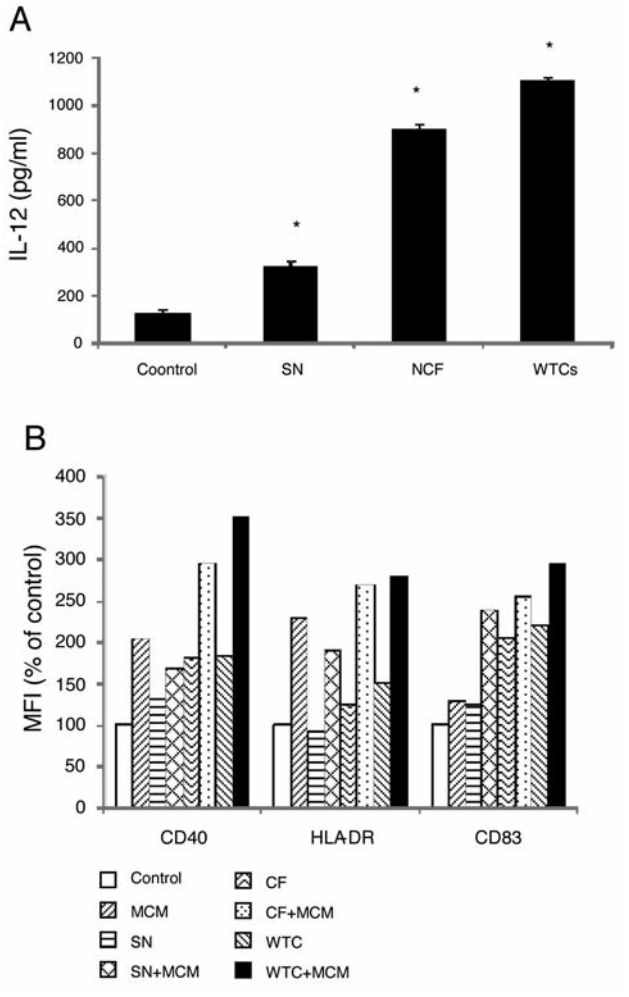

Figure 4. *Effects of various fractions of tumor lysate on IL-12 production and maturation of immature DCs. Freeze–thaw necrotic autologous WTCs (1×10^6/ml) were divided into three groups: lysate alone (supernatant; SN), cell fragment (NCF), or both (WTCs) as described in the Materials and Methods. A: SN, NCFs, or WTCs were added to immature DCs. DCs were cultured for 12 h and the supernatant was assayed for IL-12 concentration by ELISA. The graph shows mean values±SD. *p<0.05 Compared to control. B: Effects of different fractions of necrotic tumor cells with or without 20% monocyte-conditioned medium (MCM) on surface antigen expression (CD40, HLA-DR, and CD83) of immature DCs were analyzed by FACS analysis. C: DCs pulsed with each fraction of necrotic tumor cells with or without 20% MCM were co-cultured with fresh autologous nonadherent PBMCs obtained from the patient after DC immunization (DC: fresh nonadherent PBMCs=1:5). Resulting spots were developed and counted as decribed in the Materials and Methods. Each bar represents the mean number of duplicate spots per 5×10^4 nonadherent PBMCs. Results were confirmed in three independent experiments and representative data from these experiments are shown.*

antithyroglobulin antibody were all negative throughout the course of vaccine therapy. The serum CH-50 level, which can indicate complement activation, slightly increased.

Discussion

We showed that autologous necrotic WTC-pulsed DC vaccination can elicit a stable *in vivo* T cell response to multiple TAAs, including CEA and MAGEs, in a patient with recurrent rectal cancer. Induction of the CEA- and MAGE-1- specific T cells was determined by ELISPOT assay of freshly isolated PBMC-derived T cells and synthetic peptide-pulsed DCs. The development of T cell response to multiple defined tumor antigens on WTC-pulsed DCs was also associated with an increase in DTH responses. We offer the evidence that autologous necrotic WTC-pulsed DCs can induce T cell responses against multiple defined TAAs *in vivo*. To the best of our knowledge, ours is one of the longest study of autologous WTC-pulsed DCs vaccine therapy for a cancer patient.

There are many reports of possible sources of TAAs for pulsing DCs. Recent studies revealed that, in comparison to apoptotic cells, necrotic tumor cells induce significantly greater maturation and activation in DC (28, 29). In fact, apoptotic tumor cells may induce tolerance in DC-based immunity (30). Accordingly, we selected necrotic WTCs as a source of TAAs for pulsing DCs, hypothesizing that necrotic tumor cells or their components, which contain both known and unknown TAAs, should lessen the potential for tumor escape through the generation of a larger repertoire of cytotoxic T cells.

The use of WTCs as a vaccine component has the potential disadvantage that it might induce pathologic autoimmune reactivity to normal tissue antigens as a result of the processing and presentation of housekeeping or lineage-associated epitopes by autologous DCs. We did not observe clinical autoimmune disease or other systemic toxicity in response to the vaccine other than low-grade fever. Although our experience is consistent with that in

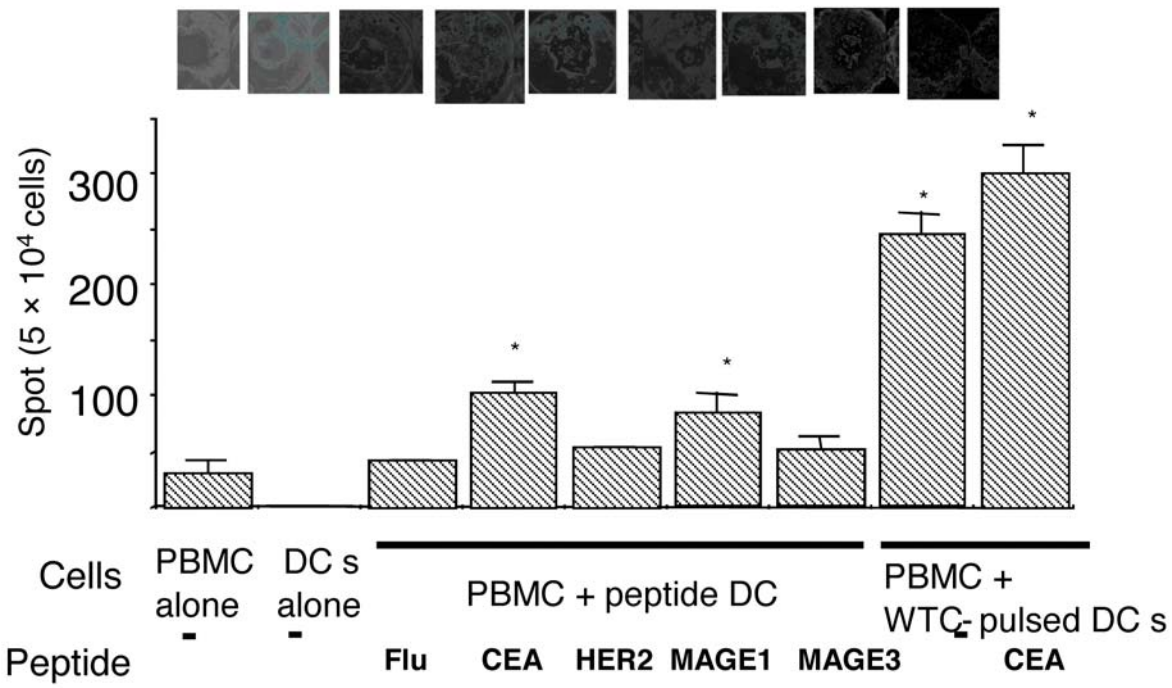

Figure 5. *Induction of T cell responses against peptide-pulsed matured dendritic cells (DCs) or WTC –pulsed DCs 8 months after vaccination. Nonadherent PBMCs freshly obtained from the patient after immunization were cultured for 15 h with mature DCs (DC:PBMCs, 1:5) pulsed without or with synthetic peptides (Flu, CEA, HER2, MAGE1, MAGE3), or WTC alone, or WTC and CEA peptide. IFN-γ-producing cells were quantified using ELISPOT assay. The graph shows the mean±SD. *p<0.05 Compared to PBMCs alone.*

other DC-based immunotherapy trials, monitoring for autoimmune disease should be continued in future studies.

One important issue for DC vaccination is the dose of mature DCs. Only a few studies have revealed dose-dependent efficacy of DC vaccination. Dhodopker *et al*. demonstrated that vaccination with 2×10^6 mature DCs was effective for induction of specific T-cell responses (31). In this study, when we escalated the DC dose from 2×10^6 to 10×10^6 per vaccination, we observed continuous strong DTH responses and ELISPOT IFN-γ responses of peripheral blood T-cells without increasing adverse effects. Since not only the quantity but also the quality of the DC vaccine is important for immunization and clinical effectiveness of the therapy, quality control of each DC vaccine should also be considered. We checked the quality of each vaccination by FACS analysis of co-stimulatory molecule expression ($CD14^-$, $CD83^{high}$, and $HLA\text{-}DR^{high}$) and *in vitro* ELISPOT analysis of T-cell response to DC.

An important aspect of immunotherapy is the need for relevant immunological monitoring. DTH testing was used as a surrogate marker for this purpose in our study. We microscopically examined the DTH response induced by the DC vaccine. Histological analysis revealed that the local injection of WTC-pulsed DCs induced not only an increased density of dermal DCs but also the perivascular infiltration of T lymphocytes. To provide additional immunological monitoring, we measured fresh peripheral blood lymphocyte response to tumor antigen expressed on DCs. We used IFN-γ ELISPOT assay as the surrogate marker and found that WTC-pulsed DC vaccination induced *in vivo* T-cell response

Table I. *Summary of adverse reactions to DC vaccination.*

Event	n
Adverse reactions	
Symptoms	
Fever	7
Fatigue	2
Rash	1
Erythema	2
Laboratory data	
Increase in serum levels of:	
Thyroglobulin antibodies	0
Anti-nuclear antibodies	0
Anti-DNA antibodies	0
Rheumatoid factor	0
IgE	2
CH-50	2

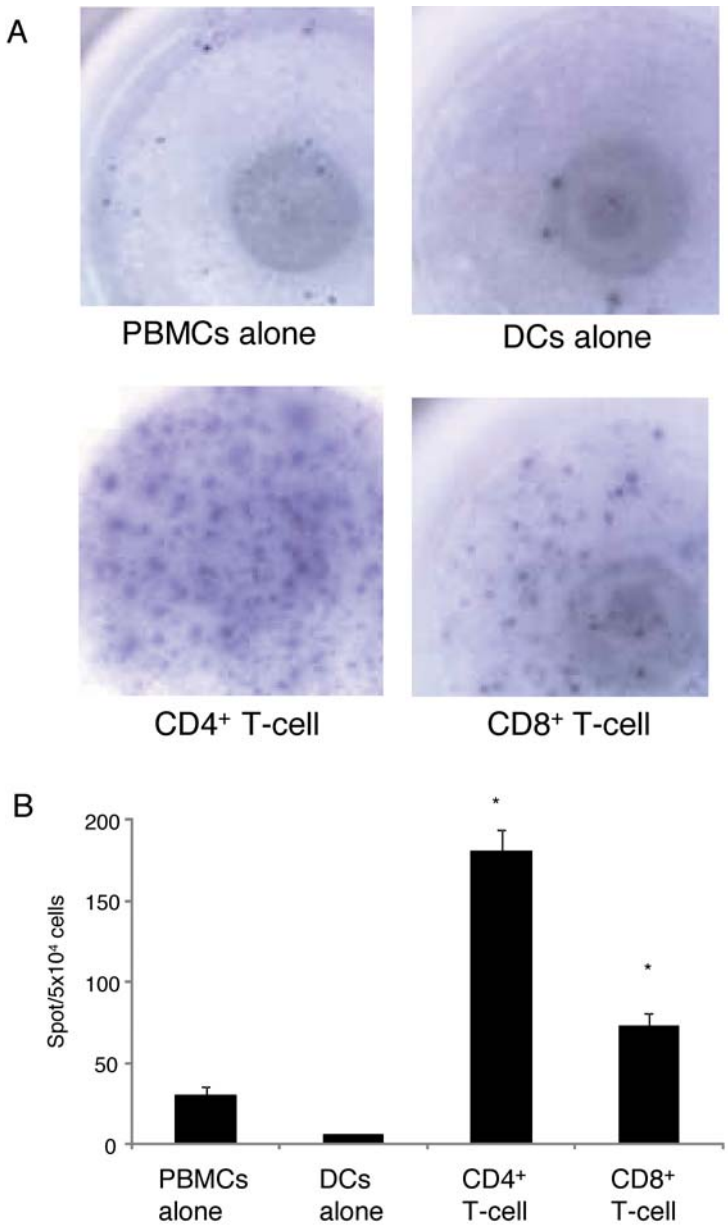

Figure 6. *Activated CD4⁺ or CD8⁺ T-cells were induced by WTC-pulsed DCs. A: Purified CD4⁺ T-cells or purified CD8⁺ T-cells were cultured with WTC-pulsed DCs for 15 h. IFN-γ-producing cells were detected using ELISPOT assay. B: The result is shown in a quantitative manner. The graph shows the mean±SD. *p<0.05 Compared to PBMCs alone.*

to more than one defined epitope such as CEA. Recent studies have shown that T-cell response to tumor antigen measured by IFN-γ release appears to be associated with conditions that favor tumor regression (32).

Conclusion

Long-term WTC-pulsed DC vaccination was performed safely, elicited a possible specific T-cell response against multiple TAAs, and induced clinical responses including long-term disease stability, in a patient with chemotherapy-resistant recurrent rectal carcinoma with lung and bone metastases.

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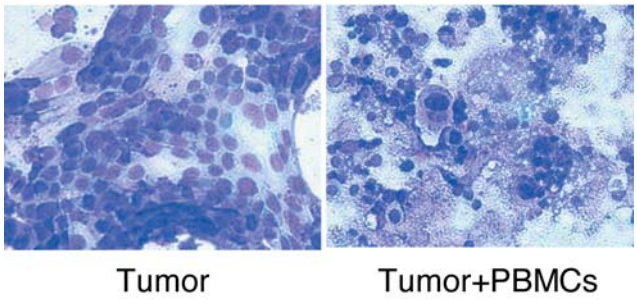

Figure 7. *Nonadherent PBMCs freshly obtained from the patient after immunization can elicit an anti-tumor effect. Nonadherent PBMCs alone were co-cultured with autologous tumor cells. After 15 h of culture, cells were fixed and stained with Giemsa solution and observed under a phase-contrast microscope (×200).*

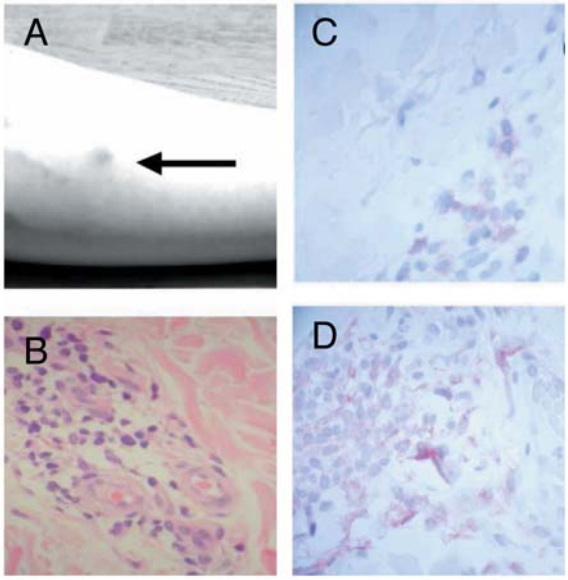

Figure 8. *Macroscopic and microscopic appearances of tumor lysates-pulsed DC vaccination site. A: Macroscopic findings of local reaction to tumor lysate-pulsed DCs at the intradermal vaccination site (72 h after vaccination). Erythema at the intradermal vaccination site is shown (12 mm in diameter). B: Photomicrograph of a representative skin biopsy specimen obtained from the intradermal injection site of tumor lysate-pulsed DCs. There is a pleomorphic, perivascular infiltrate of inflammatory cells consistent with a delayed-type hypersensitivity (DTH) response (HE staining). Immunostaining of CD8-positive T-cells (C) and HLA-DR-positive cells (D) consistent with a DTH response is shown (×200).*

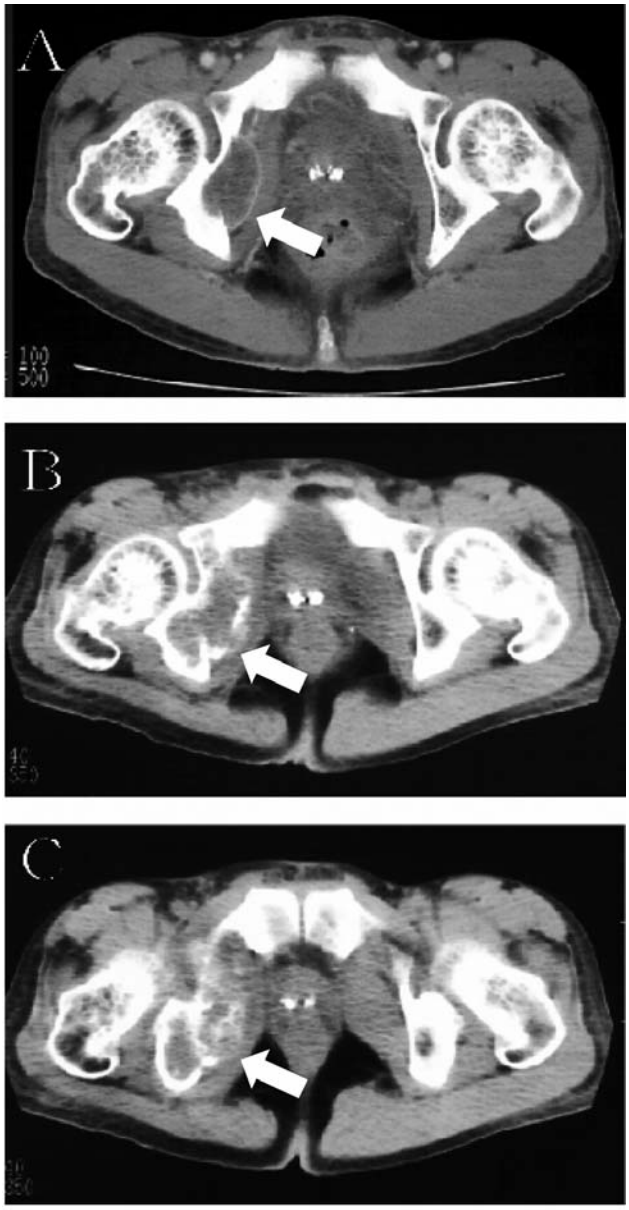

Figure 9. *Computed tomography (CT) of pelvic bone metastasis (arrows): before vaccination (A), 4 months after vaccination (B), 8 months after vaccination (C). Note the increase in calcification of the pelvic tumor at lower left of each CT scan (B and C).*

Declaration of Interest

The Authors report no conflicts of interest. The Authors alone are responsible for the content and writing of the paper.

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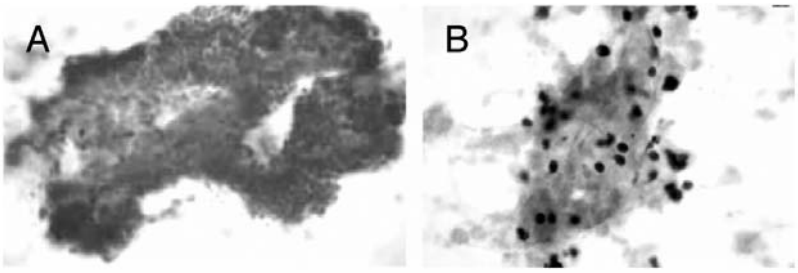

Figure 10. *Papanicolaou staining of tumor specimens aspirated under CT guidance: before vaccination (A) and 4 months after initial vaccination (B) (×200).*

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