Cancer Therapy: Clinical

Phase I Study of Immunization with Dendritic Cells Modified with Fowlpox Encoding Carcinoembryonic Antigen and Costimulatory Molecules

Michael A. Morse,[1] Timothy M. Clay,[1] Amy C. Hobeika,[1] Takuya Osada,[1] Shubi Khan,[1] Stephen Chui,[1] Donna Niedzwiecki,[1] Dennis Panicali,[2] Jeffrey Schlom,[3] and H. Kim Lyerly[1]

Abstract

Purpose: To determine the safety and immunologic and clinical efficacy of a dendritic cell vaccine modified to hyperexpress costimulatory molecules and tumor antigen.

Experimental Design: In this phase I study, we administered one or two cycles of four triweekly s.c./intradermal injections of *ex vivo* generated dendritic cells modified with a recombinant fowlpox vector encoding carcinoembryonic antigen (CEA) and a triad of costimulatory molecules [rF-CEA(6D)-TRICOM]. Controls consisted of immature dendritic cells loaded with tetanus toxoid and a HLA A2− restricted peptide derived from cytomegalovirus pp65 protein.

Results: Fourteen patients (11 with colorectal cancer and 3 with non–small cell lung cancer) were enrolled and 12 completed at least one cycle of immunization. There were no grade 3/4 toxicities directly referable to the immunizations. One patient had a decrease in the CEA level from 46 to 6.8 and a minor regression in adenopathy that occurred several months after completion of the immunizations. Five other patients were stable through at least one cycle of immunization (3 months). Direct analysis of peripheral blood mononuclear cells using the ELISpot assay showed an increase in the frequency of CEA-specific T cells in 10 patients (range, 10-541 CEA-specific cells/10^5 peripheral blood mononuclear cells). There was a trend for a greater peak frequency of CEA-specific T cells among those with either a minor response or a stable disease following at least one cycle of therapy. A second cycle was not associated with higher T-cell frequencies. Cytokine flow cytometry showed CEA-specific immune response among both CD4$^+$ and CD8$^+$ T cells in all immune responders.

Conclusion: This immunization strategy is safe and activates potent CEA-specific immune responses.

A common goal of cancer vaccines in development is the activation of high levels of antigen-specific T cells. Because of the crucial role of dendritic cells in adaptive immunity and their potent activity in animal tumor models, numerous pilot studies have evaluated immunotherapy with dendritic cells loaded with antigen in the form of peptide, protein, DNA, mRNA, tumor lysates, tumor fusions, and viral vectors (1). Among the many tumor antigens described, the model antigen, carcinoembryonic antigen (CEA), is of particular interest because it is widely expressed in gastrointestinal, lung, breast, and other malignancies. We have previously performed phase I studies of immature, monocyte-derived dendritic cells loaded with the CEA peptide

(CAP-1; ref. 2) and with the mRNA encoding CEA (3) and measured low frequencies of CEA-specific T-cell responses in a few patients. We hypothesized that this modest activity was due to the use of immature dendritic cells with lower levels of costimulatory and adhesion molecules necessary for T-cell activation (4–7), poor expression and presentation of the tumor epitopes by the dendritic cells loaded with mRNA (which may be rapidly degraded on uptake by dendritic cells), and lack of HLA class II peptides needed to activate T-cell help. To overcome these limitations, we have been studying strategies for genetic modification of dendritic cells with viral vectors encoding full length tumor antigens and costimulatory molecules.

Viral gene delivery into dendritic cells has been successfully done using poxviruses, herpes simplex, adenovirus, retrovirus, lentivirus, and adeno-associated virus (reviewed in ref. 8). Recombinant attenuated poxviruses (vaccinia, fowlpox, and canarypox) are notable for their ability to accept and express multiple transgenes and for their efficient transfection of most mammalian cells (9). Because these vectors do not integrate into the genome, there is no risk of insertional mutagenesis. Vaccinia is replication competent and elicits potent inflammatory responses. Fowlpox vectors cannot replicate in mammalian cells but express their transgene products for 14 to 21 days. Both have been engineered to express tumor antigen, such as CEA, and have been tested in murine (10) and human (11, 12)

Cancer Therapy: Clinical

studies. Recent modifications to these poxvectors encoding CEA have included the insertion of three genes encoding molecules important for providing the second signal for T-cell activation through different but collaborative pathways: CD80 (B7.1), which interacts with CD28; CD54 (intercellular adhesion molecule-1), which interacts with the CD11a/CD18 (leukocyte function-associated antigen-1/β_2-integrin) complex; and CD58 (leukocyte function-associated antigen-3), which interacts with CD2 (13, 14). Finally, poxvectors have been engineered to express a modified CEA [called CEA(6D)], which contains an agonist epitope of $CEA_{605-613}$ with aspartate substituting for asparagine at position 610 that enhances recognition by the T-cell receptor without any change in binding to HLA A2 (15). Immunization of patients with CEA-expressing malignancies using rF-CEA(6D)-TRICOM and rV-CEA(6D)-TRICOM has resulted in greater CEA-specific immune responses than observed previously with other CEA poxvector vaccines (16). Furthermore, modification of dendritic cells to hyperexpress one or more costimulatory molecules results in more efficient activation of antigen-specific T cells than antigen-loaded dendritic cells alone (17–19). We therefore hypothesized that modification of dendritic cells with poxvectors expressing CEA(6D) and TRICOM to hyperexpress costimulatory molecules and CEA would produce particularly potent immune responses in cancer patients by improving the functional activity of the dendritic cells. We now report the phase I clinical trial testing the safety and clinical and immunologic efficacy of dendritic cells modified with rF-CEA(6D)-TRICOM in patients with CEA-expressing malignancies.

Materials and Methods

Patients. Participants were recruited from the medical and surgical oncology clinics of Duke University Medical Center (Durham, NC) and provided signed informed consent approved by the Duke University Medical Center Institutional Review Board before enrollment. The study was done under a Food and Drug Administration–approved Investigational New Drug Exemption. Participants were required to have a metastatic cancer expressing CEA as defined by immunohistochemical analysis or elevated CEA in peripheral blood, adequate hematologic (WBC >4.0), renal (creatinine <2.0), and hepatic (bilirubin <1.5) functions. They were excluded if they had had chemotherapy, radiation therapy, or immunotherapy within the prior 4 weeks; if they have history of autoimmune disease, including inflammatory bowel disease, presence of an active acute or chronic infection, HIV, or viral hepatitis; and if they had used immunosuppressives, such as prednisone in the preceding 4 weeks.

Materials for vaccine generation. rF-CEA(6D)-TRICOM [manufactured by Therion Biologics Corp., Cambridge, MA, and supplied by Cancer Treatment Evaluation Program, National Cancer Institute, Bethesda, MD)] is a recombinant fowlpox virus containing genes for human CEA and the three costimulatory molecules B7.1, intercellular adhesion molecule-1, and leukocyte function-associated antigen-3. The *CEA* gene has a single amino acid substitution (aspartate substituting for asparagine at position 610) in the HLA A2–restricted immunodominant 9-mer epitope $CEA_{605-613}$ [designated CAP-1(6D)], which binds to T-cell receptors with enhanced affinity and has been shown to induce CTLs *in vitro* more efficiently than the native epitope. All four genes were inserted into an attenuated, live, plaque-purified isolate from the Poxvac-TC strain of fowlpox virus, which was used as the parental virus for this recombinant vaccine. rF-CEA(6D)-TRICOM was supplied in vials containing 0.3 mL of the vaccine at a final viral concentration titer of 1.4×10^9 plaque-forming units/mL formulated in PBS containing 10% glycerol (total vial contents, 4.2×10^8 plaque-forming units).

Generation of vaccines. Patients underwent a 4-hour peripheral blood leukapheresis (processing of 12 liters of blood) and the leukapheresis product was separated by density gradient centrifugation over Ficoll in a cell separator (Cobe BCT, Inc., Lakewood, CO) to obtain peripheral blood mononuclear cells (PBMC). Three quarters of the PBMCs were used to generate dendritic cells and the remainder of the PBMC was cryopreserved for baseline immunologic studies.

PBMCs were resuspended in serum-free AIM V medium (Life Technologies, Grand Island, NY) at 6×10^6 cells/mL and plated in T225 tissue culture flasks (60 mL/flask). The flasks were incubated in 5% CO_2 at 37°C, and after 2 hours, the nonadherent cells were gently resuspended by rocking the flasks and removed. The adherent cells were then cultured in AIM V medium containing recombinant human granulocyte macrophage colony-stimulating factor (800 units/mL) and recombinant human interleukin-4 (500 units/mL; R&D Systems, Minneapolis, MN) for 7 days to generate dendritic cells. After 7 days, the dendritic cells were harvested by vigorous washing from the flasks. The result was a cell product that was ∼40% to 70% dendritic cells, with the remainder of the cells bystander lymphocytes.

For vaccines that were to be administered fresh, the dendritic cell preparations were washed and resuspended at 5×10^6 cells in 0.5 mL saline. They were then mixed with the rF-CEA(6D)-TRICOM (25×10^6 particles) in a total volume of 1 mL saline for at least 30 minutes before administration. Control immunizations consisted of dendritic cells (5×10^6) that were mixed with tetanus toxoid (4 Lf; tetanus toxoid for booster use, 4 Lf/0.5 mL, Aventis Pasteur, Swiftwater, PA) or the HLA A2–restricted immunodominant peptide of cytomegalovirus (CMV) pp65 protein (CMV-pp65$_{495-503}$ NLVPMVATV, 500 μg, produced by Enzyme Systems Products, Dublin, CA). All cellular products were required to pass lot release criteria consisting of expression of HLA-DR and CD86 in at least 50% of the (large, dendritic) cells, viability >70%, and no evidence of bacterial or fungal contamination.

For vaccines administered following cryopreservation of the dendritic cells, one to three vials of dendritic cell product were thawed, washed, and allowed to recover overnight in AIM V. The next day, they were harvested and loaded with antigen as described above.

Protocol schema and patient treatment. This phase I study enrolled patients into two sequential cohorts. In the first cohort, six patients received one cycle of the dendritic cell vaccines. A cycle consisted of the leukapheresis, generation of dendritic cells, loading the dendritic cells with antigen, and administration of the fresh dendritic cells followed by three triweekly immunizations with previously cryopreserved dendritic cells. At each visit for immunization, all three dendritic cell preparations [dendritic cells + rF-CEA(6D)-TRICOM, dendritic cells + tetanus toxoid, and dendritic cells + CMV] were administered. Immunizations were given as a combination of intradermal (0.1 mL) and s.c. (0.9 mL) into the same limb (predominantly the right thigh), and injection sites were separated by 10 cm from each other.

The study protocol permitted escalation to the second cohort if no more than one of the first six patients experienced a dose-limiting toxicity related to the vaccine. In this cohort, patients received two cycles of immunization. In the 2 weeks between each cycle, patients were evaluated to be certain they did not have progressive disease. Those who had progressed on imaging studies did not receive the second cycle of vaccinations.

Clinical activity was assessed by applying the RECIST criteria to computed tomography or magnetic resonance imaging scans obtained before and after each cycle of immunization.

Detection of skin reactivity at injection sites. The diameter of erythema and induration at each dendritic cell vaccine site was measured 48 hours after each immunization.

In vitro *immunologic assays.* Peripheral blood was drawn before each immunization and at the completion of the final immunization, and the fresh PBMCs were analyzed for antigen-specific reactivity without any additional stimulation in the following assays.

IFN-γ ELISpot assay. Multiscreen-HA 96-well plates (Millipore, Bedford, MA) were coated overnight at 4°C with 100 μL/well of 10 μg/mL

mouse anti-human IFN-γ monoclonal antibody (DiaPharma Group, Inc., West Chester, OH) in Dulbecco's PBS (DPBS; Life Technologies, Gaithersburg, MD). The plates were washed thrice for 5 minutes each with 150 μL DPBS per well and blocked with 150 μL/well of RPMI 1640, 10% human AB serum, 25 mmol/L HEPES, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine for 1 hour at 37°C in 5% CO_2. PBMCs were plated at 100,000, 50,000, or 25,000 PBMCs per well in the presence of rF-CEA(6D)-TRICOM (multiplicity of infection 10), rF-wild-type (multiplicity of infection 10) as a control for any anti-fowlpox responses, CAP-1 peptide (1 μg/mL), CAP-1 mix (overlapping peptides spanning entire CEA molecule, 1,360 μg/mL), CMV-pp65$_{495\text{-}503}$ (1 μg/mL), CMV peptide mix (1:800 final dilution, ~1.75 μg/mL of each peptide, PharMingen, San Diego, CA), HIV peptide mix (1:800 final dilution) as a peptide control, tetanus toxoid without preservative (5 Lf/mL, University of Massachusetts Medical School, Jamaica Plain, MA), pokeweed mitogen (Sigma Chemical, St. Louis, MO, 2 μg/mL), or medium in a total volume of 200 μL/mL replicates of six. The plates were incubated for 18 to 20 hours at 37°C, 5% CO_2. The plates were washed with 0.05% Tween/DPBS using a Tecan 96PW plate washer (Tecan, Research Triangle Park, NC). Mouse anti-human IFN-γ biotinylated monoclonal antibodies (100 μL, DiaPharma Group) at 1 μg/mL in DPBS were added to each well and the plates were incubated for 2 hours at 37°C, 5% CO_2. After the plates were washed, Vectastain ABC peroxidase (Vector Laboratories, Inc., Burlingame, CA) was added at 100 μL/well for 1 hour at room temperature. The plates were washed for the last time with 0.05% Tween/DPBS followed by DPBS. Color was developed using 100 μL/ well of 3-amino-9-ethyl-carbazole (Sigma Chemical) reconstituted in an acetate buffer for 4 minutes at room temperature in the dark. Color development was stopped with deionized running water.

Basins were removed from the plates and the membranes were dried overnight in the dark. Membranes were removed using sealing tape (Millipore) and the number of spots per well was determined by the color "footprint" on the well membrane, indicating the presence of an IFN-γ-secreting cell. Membranes were read using the KS ELISpot Automated Reader System with the KS ELISpot version 4.2 software (Carl Zeiss, Inc., Thornwood, NY). The mean number of spots from the six replicate wells at each dilution was reported for the response to the each test antigen. The analyses in this article are based on the number of spots detected for wells containing 1×10^5 responder PBMCs, which is the dilution that yielded the highest ratio of spots per PBMC.

We defined a positive immune response by ELISpot as described at the 2002 Society of Biologic Therapy Workshop on "Immunologic Monitoring of Cancer Vaccine Therapy": a T-cell response is considered positive if the mean number of spots in six wells with antigen exceeds the number of spots in six control wells by 10 and the difference between single values of the six wells containing antigen and the six control wells is statistically significant at a level of $P \leq 0.05$ using the Student's t test (20).

Intracellular cytokine staining. PBMCs (2×10^6-4×10^6/mL) were stimulated with the same antigens in the same concentrations as described for the ELISpot assay for 6 hours at 37°C in the presence of 10 μg/mL brefelden A (Sigma Chemical) and 1 μg anti-CD28 (BD Biosciences, San Jose, CA) per 10^6 PBMCs. The exception was that PBMCs were also stimulated with rF-CEA(6D)-TRICOM (multiplicity of infection 1) and rF-wild-type (multiplicity of infection 1) as a control for any anti-fowlpox responses for 12 hours in the presence of 1 μg anti-CD28 per 10^6 PBMCs. They were cultured for an additional 6 hours in the presence of 10 μg/mL brefelden A. Afterward, the stimulated cells were washed with 1% bovine serum albumin/PBS, fixed with 1% paraformaldehyde, and permeabilized using fluorescence-activated cell sorting permeabilizing solution (BD Biosciences). Permeabilized cells were then stained with IFN-γ-FITC, CD69-PE, CD8-PerCP, and CD4-APC for 30 minutes at room temperature. The percentage of IFN-γ/CD69 double-positive CD8$^+$ T cells was determined within forward scatter^{low}/side scatter^{low} CD8$^+$ or CD4$^+$ cells.

Proliferation assay. PBMCs were cultured at 1×10^5 cells per well of a 96-well plate in the presence of medium alone, CAP-1 peptide (25, 5,

and 1 μg/mL), CMV peptide (25, 5, and 1 μg/mL), CMV peptide mix (340, 68, and 13.6 μg/mL; BD Biosciences), HIV peptide mix (340, 68, and 13.6 μg/mL; BD Biosciences), tetanus toxoid (25, 5, and 1 Lf/mL), fowlpox TRICOM-CEA (2.5×10^6, 5×10^5, and 1×10^5 plaque-forming units/mL), fowlpox wild-type (2.5×10^6, 5×10^5, and 1×10^5 plaque-forming units/mL), or pokeweed mitogen (0.5, 0.1, and 0.02 μg/mL) in a total volume of 200 μL for 4 days. Cells were pulsed with [^{3}H]thymidine (1 μCi) for 18 to 24 hours and harvested on an automated cell harvester (Tomtec, Turku, Finland). [^{3}H]thymidine incorporation was determined using a MicroBeta Plus scintillation counter (Wallac, Turku, Finland). Data are presented as mean stimulation index ± SD. Stimulation index was determined by dividing the mean counts per minute of wells incubated with antigen by the mean counts per minute of medium alone.

MHC-peptide tetramer binding analysis. Peptide-MHC tetramer staining was done on fresh PBMCs of HLA A2$^+$ patients using 10^6 cells per individual tetramer analysis. Cells were stained for 60 minutes at room temperature in a total volume of 25 μL of 1% bovine serum albumin/PBS containing the following fluorochrome-conjugated antibodies: CD3-FITC, CD8-PerCP, a cocktail of CD4, CD14, and CD19-APC, and HLA A2$^+$ tetramer-PE of interest (CAP-1 and CMV-pp65). Cells were washed using 1% bovine serum albumin/ PBS and analyzed by multivariable flow cytometry. About 25,000 gated events were collected. Tetramer-positive cells were identified by gating on the population of cells that were forward scatter^{low}/side scatterlow CD4$^-$CD14$^-$CD19$^-$CD3$^+$. These cells were analyzed for their dual expression of CD8 and T-cell receptor recognized by the tetramer.

Statistical analysis. The primary end point of this trial was safety. A traditional phase I design was employed. Dose-limiting toxicity was defined as any major organ toxicity of grade ≥3 attributable to treatment. The Wilcoxon rank-sum test was used to compare the magnitude of the immune response between patients with stable disease or minor response and those with progressive disease. Student's t test was used to compare the expression of CEA, CD54, CD58, and CD80 between fresh and cryopreserved dendritic cells following infection with rF-CEA(6D)-TRICOM.

Results

Patient characteristics. Patients with advanced CEA-expressing malignancies were enrolled in this protocol. As indicated in Table 1, patients had colorectal cancer (n = 11) or non–small cell lung cancer (n = 3) and all had extensive metastatic disease and had failed at least two prior chemotherapeutic regimens.

Vaccine characteristics. We were able to generate an adequate number of dendritic cells that satisfied all the Food and Drug Administration–mandated lot release tests from all patients. To determine if the final dendritic cell product [dendritic cells + rF-CEA(6D)-TRICOM] expressed CEA and higher levels of the costimulatory molecules, we did phenotypic analysis on a small aliquot of the final product (excess cells removed from the final product just before immunization of the patient) after it had been allowed to culture overnight to

Table 1. Patient characteristics (*N* = 14)

Male/female	7/7
Caucasian/Asian	13/1
Colorectal cancer/non – small cell lung cancer	11/3
Sites of metastases (median no.)	2
Prior chemotherapies, median (range)	2 (1-4)
Karnofsky performance status (%)	80-90

permit demonstration of transgene expression. We assumed that this served as a surrogate for the transgene expression in the dendritic cells actually administered to the patient. About half of the dendritic cells were modified to express CEA and similar numbers also had up-regulated CD54, CD58, and CD80 as detected by flow cytometric assessment using fluorescently labeled anti-CD54, anti-CD58, and anti-CD80 antibodies. The dendritic cells that were exposed to the rF-CEA(6D)-TRICOM when fresh tended to express the transgenes (e.g., mean $\pm$ SD, CEA$^+$ 57 $\pm$ 12%, CD80 35 $\pm$ 12%, CD54 77 $\pm$ 22%, and CD58 66 $\pm$ 4%) to a greater extent than the previously cryopreserved dendritic cell [mean $\pm$ SD, CEA$^+$ 34 $\pm$ 17% (P = 0.03), CD80 27 $\pm$ 14%, CD54 45 $\pm$ 21%, and CD58 45 $\pm$ 20%]. These data are consistent with our expectation that the dendritic cells used for the immunizations would express high levels of CEA and costimulatory molecules as high or higher than observed following cytokine-induced maturation. In contrast, the dendritic cells loaded with tetanus toxoid and CMV, because they were not further modified, expressed lower levels of the CD54 and CD58 and little CD80 consistent with immature dendritic cells (data not shown).

Tolerability of therapy. Overall, the immunizations were well tolerated. In the first cohort of six patients, five completed all four immunizations. One patient discontinued participation due to pancreatitis following the second immunization. This adverse event was judged to be related to progression of disease with impingement of a tumor mass on the pancreatic duct. A second patient was hospitalized for a generalized decrease in performance status and severe fatigue within 1 week of completing all four immunizations. This was also judged to be due to progression of disease. In the second cohort of eight patients, one patient discontinued participation after one immunization for personal reasons, two patients terminated participation after four immunizations due to progression of disease, one patient terminated after five immunizations due to progression of disease resulting in death, and four patients completed all the immunizations. The following grade 3/4 adverse events were reported: abdominal pain (1), other pain (1), nausea (1), fatigue (1), pancreatitis (1), and dyspnea (1). These were all judged to be unrelated to the vaccine.

Clinical efficacy. In the first cohort of six patients, one remained stable and five had progressive disease. One of the patients who initially had progressive disease later experienced a decrease in the CEA level from 46 to 6.8 and a minor regression in retroperitoneal and supraclavicular adenopathy that occurred 3 months after completion of the immunizations. During the intervening 3 months, he had received IFN-2b s.c. In the second cohort, one patient was not evaluable, having left the study for personal reasons after the first injection. Of seven evaluable patients, four remained stable after one cycle of immunizations, and of these, one continued to have stable disease after the second cycle. The remainder of patients had progressive disease.

Clinical immunologic results. We also evaluated delayed-type hypersensitivity (DTH) reactivity at the dendritic cell intradermal injection sites and reported the means of the longest diameter for the first set of injections (considered the baseline) and the maximum diameter achieved for subsequent immunizations (Table 2). The diameter of erythema at the dendritic cells + rF-CEA(6D)-TRICOM site increased following the first immunization. There were also trends for greater DTH diameter at the dendritic cells + tetanus toxoid and

dendritic cells + CMV injection sites after the first immunization. The time point for the maximum response varied across the patients, and in some instances, the first immunization resulted in the greatest DTH diameter. When the difference in the diameter of the DTH reaction at the dendritic cells + rF-CEA(6D)-TRICOM injection site between the first (baseline) injection and the time of the maximum DTH reaction was compared for patients who had stable disease or minor response (clinical benefit) and those with progressive disease, there was a greater increment for the patients with clinical benefit. The changes in DTH reactivity between the first and the maximum reactions were not significantly different for the dendritic cells + tetanus toxoid and dendritic cells + CMV.

In vitro immunologic analysis of carcinoembryonic antigen–specific T-cell response by ELISpot. PBMCs were analyzed fresh before each immunization and after all immunizations. Using a definition of immune response proposed by the Immunotherapy Working Group, 13 of 14 evaluable patients had a CEA-specific immune response at some point during their immunizations (Fig. 1). The detection of this immune response required testing with the rF-CEA(6D)-TRICOM as the *in vitro* stimulator compared with the rF-wild-type. Although the better control would have been a recombinant fowlpox encoding an irrelevant antigen and the TRICOM molecules, one was not available for our studies. We did not observe a CAP-1-specific immune response nor a response against a cocktail of overlapping peptides derived from CEA. Comparison of the ELISpot result for patients with clinical benefit versus those with progressive disease shows a numerical difference in the

---

Table 2. Diameter of erythema at dendritic cell injection sites (mean $\pm$ SD)

(A) Longest diameter of erythema at dendritic cell injection site after first injection and at the time point with maximum reactivity

	First	Maximum
CEA (mm)	40 $\pm$ 14	59 $\pm$ 25*
Tet	34 $\pm$ 35	56 $\pm$ 41
CMV	7 $\pm$ 12	17 $\pm$ 18

(B) Average change of diameter of erythema at dendritic cell injection site between first injection and maximum reactivity for patients with stable disease versus progressive disease

	Stable disease	Progressive disease
ΔCEA (mm)	34 $\pm$ 24	7 $\pm$ 13†
ΔTet	28 $\pm$ 31	19 $\pm$ 20
ΔCMV	12 $\pm$ 13	10 $\pm$ 15

NOTE: Δ refers to the difference between the diameter at the first injection site and the time of maximum DTH response.
*$P < 0.05$ for comparison of diameter of response at dendritic cells + rF-CEA(6D)-TRICOM injection site for the first injection and at the maximal response using Student's t test.
†$P < 0.05$ for the comparison of patients with stable disease/minor response and those with progressive disease using Student's t test.

Fig. 1. Enumeration of CEA-specific T cells by ELISpot assay for all patients. PBMCs (1×10^5) from before immunization and at various time points during the immunizations were cultured with either rF-CEA(6D)-TRICOM or rF-wild-type as a control and the number of cells secreting IFN-γ was determined. Number of responding T cells against rF-CEA(6D)-TRICOM at baseline (*gray columns*), at the time of maximum response (*black columns*) against CEA, and the simultaneously measured response against rF-wild-type (*white columns*). Grouped by clinical response [stable disease/minor response (*MR/SD*) versus progressive disease (*PD*)].
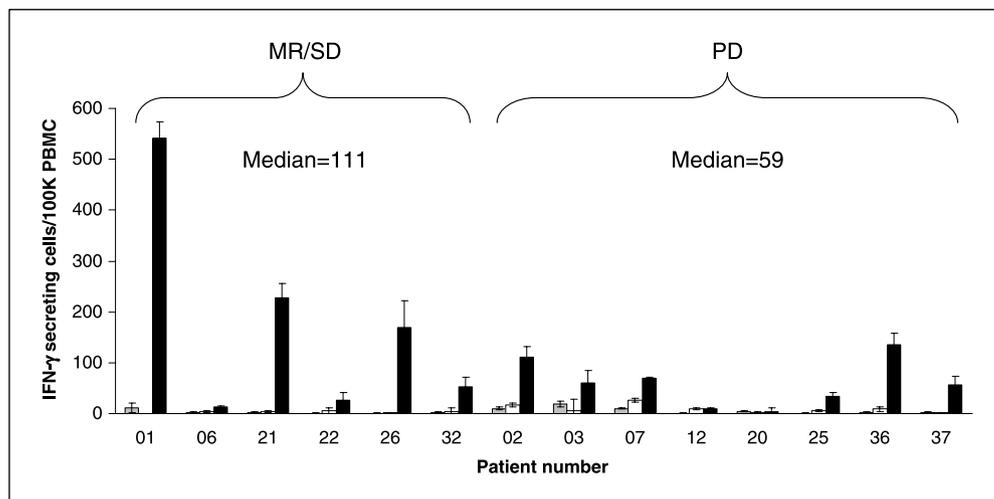
median number of CEA-specific T cells (median 111 versus 59, respectively), which was nonsignificant (P = 0.2; Fig. 1). Figure 2 shows the level of immune response at each time point for three individuals who completed two cycles of immunizations. The CEA-specific immune response increased during the first cycle but did not increase with the second cycle of therapy (except in one patient whose data are not shown). No increase in T cells specific for tetanus toxoid or CMV were observed by ELISpot (data not shown). In particular, tetanus toxoid gave results near 0 in all instances. In CMV seropositive patients, ELISpot could detect CMV-specific T cells but no increase was seen following immunization.

Activation of T-cell help by immunization. To characterize whether the CD4$^+$ and/or CD8$^+$ T cells were activated by immunization, we did cytokine flow cytometry of PBMC from each time point. All 10 of the evaluable patients experienced an increase of CD4$^+$ and CD8$^+$ CEA-specific T cells (Fig. 3). Again, it was noted that a second cycle of immunization did not increase the immune response over that seen during the first cycle of immunization, except in one patient. No increase in the response to tetanus toxoid and CMV was observed by cytokine flow cytometry (data not shown).

Proliferative responses to immunization. Although ELISpot and cytokine flow cytometry are sensitive assays for immune response, we thought that they may not be able to detect an immune response against the protein antigen tetanus toxoid due to poor presentation in the particular assays. Therefore, we also did proliferation assays that allow a period of several days during which presentation of antigen to T cells can be maximized. We determined the stimulation index for PBMCs obtained at each time point in response to CEA, tetanus toxoid, or CMV (Fig. 4). Prominent increases in the stimulation index were observed against the rF-CEA(6D)-TRICOM with only a small change observed against rF-wild-type. Furthermore, statistically significant increases in stimulation index were observed for CEA protein and CMV-pp65 peptide. The stimulation index against tetanus toxoid was high at baseline and did not increase after immunization.

Tetramer analysis. Tetramers designed to recognize the T-cell epitope CAP-1 and the immunodominant CMV-pp65 peptide were used to identify T cells specific for these epitopes. No
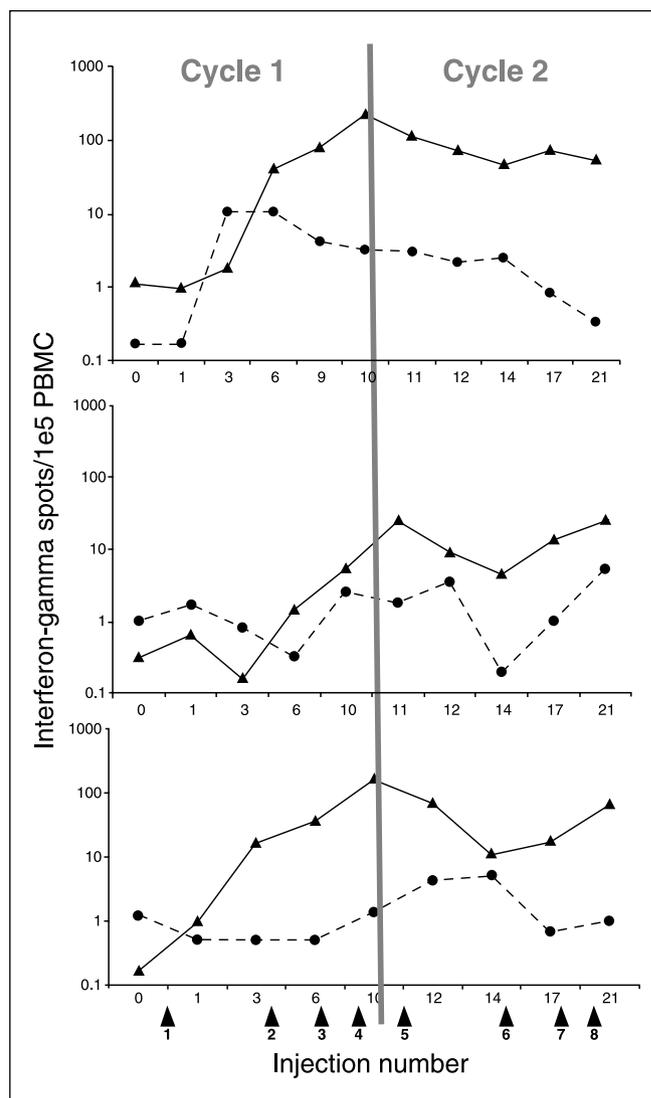
Fig. 2. ELISpot results at each time point for three patients who received both cycles of immunization. Number of cells producing IFN-γ in response to either rF-CEA(6D)-TRICOM (▲) or rF-wild-type (●) at each of the immunizations and following all immunizations.
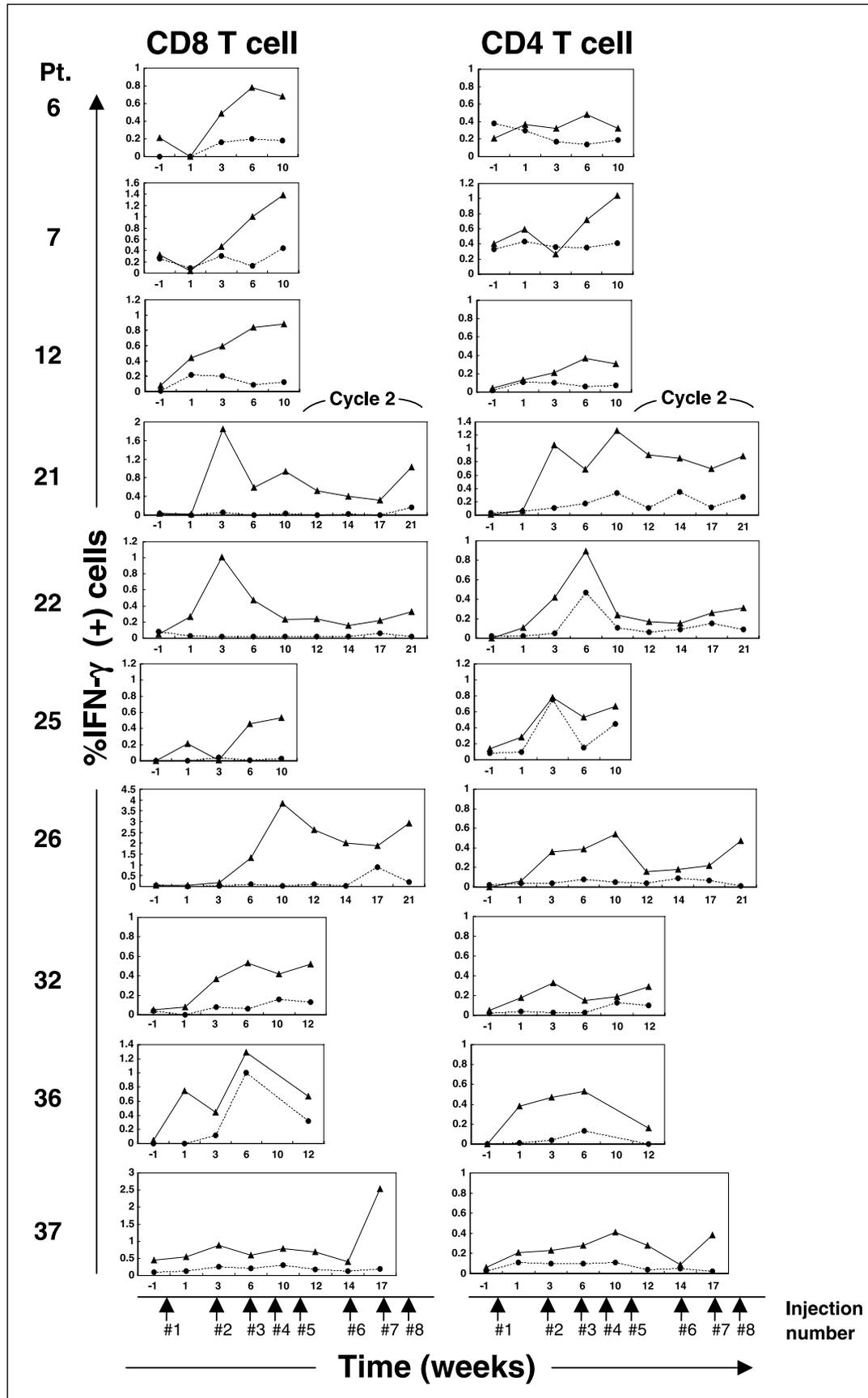
Fig. 3. Cytokine flow cytometry results at each time point. PBMCs were incubated with either rF-CEA(6D)-TRICOM (▲) or rF-wild-type (●) and analyzed for intracellular production of IFN-γ by flow cytometry. Percentage of CD4+ or CD8+CD69+ IFN-γ + T cells among all cells.
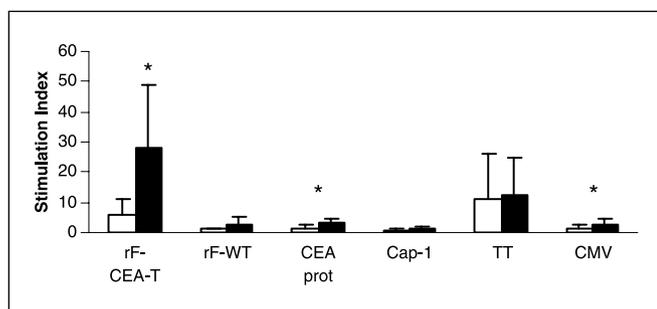
Fig. 4. Proliferative response of PBMC before immunization and at maximum immunologic response. PBMCs were incubated with either rF-CEA(6D)-TRICOM (*rF-CEA-T*), rF-wild-type (*rF-WT*), CEA protein (*CEA prot*), CAP-1 peptide (*Cap-1*), tetanus toxoid (*TT*), or CMV-pp65 peptide (*CMV*), and after 96 hours, the proliferation was measured by [³H]thymidine incorporation. Stimulation index (counts per minute for experimental well/counts per minute of unstimulated PBMCs) was calculated. Results for baseline (*white columns*) and maximum stimulation index (*black columns*) for each antigen. *, $P < 0.05$.

CAP-1-specific T cells were identifiable above background either before or after immunization. CMV-specific T cells could be identified in CMV seropositive HLA A2+ patients using the tetramer, but no increments in the number of detectable T cells could be detected after immunization (data not shown).

Discussion

Dendritic cell–based vaccines have shown promise as a means for activating anticancer immune responses (21). Several issues remain to be resolved to attain the highest level of immune response. One is the importance of a mature phenotype exemplified by up-regulated expression of costimulatory molecules (such as CD80). Immature dendritic cells may inhibit immune responses (22), whereas mature dendritic cells augment immune responses (23). In our previous experience with dendritic cells loaded with peptide (2) and mRNA (3), we observed low levels of immune response. Similarly, in the present phase I study, using ELISpot and cytokine flow cytometry, we could not detect activation of tetanus or CMV-specific immune responses using immature dendritic cells as the stimulator. In contrast, using dendritic cells with up-regulated expression of costimulatory molecules, we observed higher levels of CEA-specific immune responses than observed previously. It is true that the dendritic cells used in our study do not have other hallmarks of "mature" dendritic cells, such as CD83 expression, but the minimal, necessary components of a "mature" dendritic cells required for augmented stimulatory activity have not been completely clarified. Furthermore, when we previously compared immature dendritic cells and dendritic cells matured with a cytokine cocktail of CD40-ligand and IFN-γ, we did not observe a significant increase in antigen-specific T-cell responses.[4] In that study, the up-regulation of costimulatory molecules was not as efficient in the present study. We therefore conclude that modification of dendritic cells to achieve high levels of costimulatory molecules results in stronger activation of T cells.

Another important issue for the development of dendritic cell–based vaccines is the mechanism of delivery of antigen.

Dendritic cells may be loaded with peptides, protein, mRNA, DNA, viral vectors, and tumor lysates and may be fused with tumor cells. In our previous experience loading dendritic cells with the mRNA encoding CEA, we could not detect significant levels of CEA within the cell. In the present study, we administered dendritic cells that expressed high levels of CEA that should ensure an adequate amount of the protein for processing and presentation. The detection of both CD4+ and CD8+ CEA-specific T-cell responses by cytokine flow cytometry indicates that the CEA produced after rF-CEA(6D)-TRICOM infection of the dendritic cell is directed to both class I and II pathways. We believe that this viral vector delivery strategy represents one of the most efficient methods for ensuring antigen presentation by the dendritic cell. Although we did not directly compare viral vector-loaded dendritic cells with protein-loaded dendritic cells, the viral vectors have several advantages over protein loading: (*a*) Viral vectors are generally easier to produce in large quantity. (*b*) By producing the protein of interest endogenously in the dendritic cells, they are more likely to result in class I presentation. (*c*) Other molecules can be transferred at the same time as the antigen of interest. Whether other poxvector approaches would have yielded different results is a matter of conjecture. Schlom et al. have also tested infection of dendritic cells with vaccinia encoding CEA, and although CEA expression may be higher, vaccinia vectors are more complicated to use from a regulatory standpoint because of their risk of productive infection in humans. The other major pox vector that has been tested is avipox (ALVAC). Tsang et al. (19) showed that when avipox-CEA/B7.1 was used to infect human dendritic cells, ~75% of the dendritic cells expressed CEA. They showed that these dendritic cells could activate >30-fold more CEA-specific T cells *in vitro* than dendritic cells infected with avipox-CEA, indicating the significant activity achieved by up-regulating costimulatory molecules on the dendritic cells.

A third important issue for dendritic cell vaccine development is whether fresh or previously cryopreserved products should be used for immunization. From a regulatory standpoint, production of a batch of dendritic cells that can be cryopreserved, tested for compliance with lot release criteria, and then used as needed is preferable. From a functional standpoint, cryopreserved dendritic cells tend to have a lower viability than fresh dendritic cells. We observed modestly lower transgene expression in the previously cryopreserved dendritic cells than fresh dendritic cells following exposure to the rF-CEA(6D)-TRICOM. We do not believe that this had a meaningful impact on the immunologic response in this study because we observed a mounting CEA-specific immune response through the first cycle of immunization (first dose fresh followed by three doses of previously cryopreserved dendritic cells) in most patients.

A fourth issue for vaccine development in general is the frequency and number of immunizations required for clinical and immunologic activity. Surprisingly, we did not find an increase in the CEA-specific immune response in the second cycle of immunizations beyond what had been achieved in the first cycle. Whether we would have seen a decrement in the immune response without further immunization is not clear because we did not have the opportunity to follow the long-term immune response in most patients who completed one cycle of immunization only. In one patient in the first cohort, an ELISpot assay done ~3 months after completion of immunization showed minimal CEA-specific immunity. A possible

explanation for the lack of continued increase in the CEA-specific immune response with a second cycle of immunization could be the development of a regulatory T-cell response that could dampen any further increases in CEA-specific immunity. Animal models have shown that elimination of regulatory T cells could enhance effector T-cell responses. This concept requires further testing in subsequent studies.

A fifth issue is how best to monitor the development of an immune response. We did four different *in vitro* assays to detect immune responses directly from the PBMCs, but we used ELISpot as the primary assay because of its relative simplicity and sensitivity. In this assay, antigen is added to PBMCs that must take up and present the antigen to T cells. Although the simplest antigen to add in the studies would be peptide fragments of the antigen of interest, they may not be efficiently presented when they are merely added to the assay well. This may explain why we did not see any responses against the CAP-1 mix or the CAP-1 peptide in the ELISpot assay. Another possibility is that the dominant epitope presented by the dendritic cells is not included within the peptide mix. Although the peptide mix contains every epitope available in the linear sequence of CEA, it is possible that altered splicing of the CEA protein could produce unexpected epitopes. Indeed, Hanada et al. (24) identified a T-cell line cloned from renal cell carcinoma infiltrating lymphocytes, which recognized a peptide generated by protein splicing of carboxyl-terminal and amino-terminal residues.

Because we did not detect responses against the peptides, we instead used the vector rF-CEA(6D)-TRICOM as the "antigen" of interest because it can infect antigen-presenting cells and efficiently produce the CEA antigen for processing and presentation. To control for fowlpox-specific immune responses, we used rF-wild-type. We did not have available the most relevant control, which would be a fowlpox vector encoding an irrelevant molecule and TRICOM. It is possible that the TRICOM molecules are the reason for the better ability to detect an immune response by engaging the T-cell signaling that leads to cytokine expression. If this is so, then it is possible that we underestimated the control, anti-fowlpox response, for the rF-wild-type. For future studies, we are developing more relevant controls and methods for eliminating other potentially confounding effects on immune response measurement.

A final issue addressed by our study is whether immune response correlates with clinical outcome. We observed that the patients with either a minor response or a stable disease through at least one cycle of therapy had a trend toward a higher number of T cells responding to CEA in the ELISpot assay. In addition, patients with stable disease or minor response had a greater increment in DTH reactivity at the dendritic cells + rF-CEA(6D)-TRICOM injection site than those with progressive disease, whereas augmentation of DTH reactivity for the dendritic cells + tetanus toxoid and dendritic cells + CMV did not differ between those with and those without clinical benefit. Although it is possible that other factors could explain an association between clinical benefit and higher CEA-specific immune responses, we believe that these data are hypothesis generating and support attempts to create cancer vaccines with greater stimulatory activity to reach a goal of activating antigen-specific T cells into the range of that seen against viral infections.

Acknowledgments

References

1. Mosca PJ, Clay TM, Lyerly HK, Morse MA. Current status of dendritic cell immunotherapy of malignancies. Int Rev Immunol 2003;22:1–27.
2. Morse MA, Deng Y, Hull S, et al. A phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. Clin Cancer Res 1999;5:1331–8.
3. Morse MA, Nair SK, Mosca PJ, et al. Immunotherapy with autologous, human dendritic cells transfected with carcinoembryonic antigen mRNA. Cancer Invest 2003;21:341–9.
4. Morse MA, Mosca PJ, Clay TM, Lyerly HK. Dendritic cell maturation in active immunotherapy strategies. Expert Opin Biol Ther 2002;2:35–43.
5. O'Doherty U, Peng M, Gezelter S, et al. Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. Immunology 1994;82:487–93.
6. Larsen CP, Ritchie SC, Pearson TC, Linsley PS, Lowry RP. Functional expression of the costimulatory molecule, B7/BB1, on murine dendritic cell populations. J Exp Med 1992;176:1215–20.
7. Mosca PJ, Hobeika AC, Clay TM, et al. A subset of human monocyte-derived dendritic cells expresses high levels of IL-12 in response to combined CD40 ligand and IFNγ treatment. Blood 2000;96:3499–504.
8. Wysocki P, Grabarczyk P, Mackiewicz-Wysocka M, Kowalczyk D, Mackiewicz A. Genetically modified dendritic cells—a new, promising cancer treatment strategy? Expert Opin Biol Ther 2002;2:835–45.
9. Moss B. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci U S A 1996;93:11341–8.
10. Kass E, Schlom J, Thompson J, et al. Induction of protective host immunity to carcinoembryonic antigen (CEA) a self-antigen in CEA transgenic mice, by immunizing with a recombinant vaccinia-CEA virus. Cancer Res 1999;59:676–83.
11. Conry RM, Khazaeli MB, Saleh MN, et al. Phase I trial of a recombinant vaccinia virus encoding carcinoembryonic antigen in metastatic adenocarcinoma: comparison of intradermal versus subcutaneous administration. Clin Cancer Res 1999;5:2330–7.
12. Marshal J, Hawkins MJ, Tsang KY, et al. Phase I study in cancer patients of a replication-defective avipox recombinant vaccine that expresses human carcinoembryonic antigen. J Clin Oncol 1999;17:332–7.
13. Wingren AG, Parra E, Varga M, et al. T cell activation pathways: B7, LFA-3, and ICAM-1 shape unique T cell profiles. Crit Rev Immunol 1995;15:235–53.
14. Hodge JW, Sabzevari H, Yafal AG, Gritz L, Lorenz MG, Schlom J. A triad of costimulatory molecules synergize to amplify T-cell activation. Cancer Res 1999;59:5800–7.
15. Zaremba S, Barzaga E, Zhu M, et al. Identification of an enhancer agonist cytotoxic T lymphocyte peptide from human carcinoembryonic antigen. Cancer Res 1997;57:4570–7.
16. Marshall J, Odogwu L, Hwang J, et al. A phase I study of sequential vaccinations with fowlpox-CEA(6D)-TRICOM (B7.1/ICAM-1/LFA-3) alone, and sequentially with vaccinia-CEA(6D)-TRICOM and GM-CSF in patients with CEA-expressing malignancies [abstract 662]. Proc Am Soc Clin Oncol 2003;22:165.
17. Hodge JW, Rad AN, Grosenbach DW, et al. Enhanced activation of T cells by dendritic cells engineered to hyperexpress a triad of costimulatory molecules. J Natl Cancer Inst 2000;92:1228–39.
18. Zhu M, Terasawa H, Gulley J, et al. Enhanced activation of human T cells via avipox vector-mediated hyperexpression of a triad of costimulatory molecules in human dendritic cells. Cancer Res 2001;61:3725–34.
19. Tsang KY, Zhu M, Even J, Gulley J, Arlen P, Schlom J. The infection of human dendritic cells with recombinant avipox vectors expressing a costimulatory molecule transgene (CD80) to enhance the activation of antigen-specific cytolytic T cells. Cancer Res 2001;61: 7568–76.
20. Keilholz U, Weber J, Finke JH, et al. Immunologic monitoring of cancer vaccine therapy: results of a workshop sponsored by the Society for Biological Therapy. J Immunother 2003;25:97–138.
21. Ridgway D. The first 1000 dendritic cell vaccines. Cancer Invest 2003;21:873–86.
22. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med 2001;193:233–8.
23. Dhodapkar MV, Steinman RM, Sapp M, et al. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. J Clin Invest 1999;104:173–80.
24. Hanada K, Yewdell JW, Yang JC. Immune recognition of a human renal cancer antigen through post-translational protein splicing. Nature 2004; 427:252–6.

Clinical Cancer Research

Phase I Study of Immunization with Dendritic Cells Modified with Fowlpox Encoding Carcinoembryonic Antigen and Costimulatory Molecules

Michael A. Morse, Timothy M. Clay, Amy C. Hobeika, et al.

Updated version	Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/11/8/3017

Cited articles	This article cites 23 articles, 12 of which you can access for free at: http://clincancerres.aacrjournals.org/content/11/8/3017.full#ref-list-1
Citing articles	This article has been cited by 11 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/11/8/3017.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/11/8/3017. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.