Published OnlineFirst September 30, 2010; DOI: 10.1158/1078-0432.CCR-10-2138

Cancer Therapy: Clinical

Clinical Cancer Research

A Randomized Trial of *Ex vivo* CD40L Activation of a Dendritic Cell Vaccine in Colorectal Cancer Patients: Tumor-Specific Immune Responses Are Associated with Improved Survival

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Abstract

Purpose: To determine whether an autologous dendritic cell (DC) vaccine could induce antitumor immune responses in patients after resection of colorectal cancer metastases and whether these responses could be enhanced by activating DCs with CD40L.

Experimental Design: Twenty-six patients who had undergone resection of colorectal metastases were treated with intranodal injections of an autologous tumor lysate– and control protein [keyhole limpet hemocyanin (KLH)]–pulsed DC vaccine. Patients were randomized to receive DCs that had been either activated or not activated with CD40L. All patients were followed for a minimum of 5.5 years.

Results: Immunization induced an autologous tumor-specific T-cell proliferative or IFNγ enzyme-linked immunospot response in 15 of 24 assessable patients (63%) and a tumor-specific DTH response in 61%. Patients with evidence of a vaccine-induced, tumor-specific T-cell proliferative or IFNγ response 1 week after vaccination had a markedly better recurrence-free survival (RFS) at 5 years (63% versus 18%, $P = 0.037$) than nonresponders. In contrast, no association was observed between induction of KLH-specific immune responses and RFS. CD40L maturation induced CD86 and CD83 expression on DCs but had no effect on immune responses or RFS.

Conclusion: Adjuvant treatment of patients after resection of colorectal metastases with an autologous tumor lysate–pulsed, DC vaccine–induced, tumor-specific immune responses in a high proportion of patients. There was an association between induction of tumor-specific immune responses and RFS. Activation of this DC vaccine with CD40L did not lead to increased immune responses. *Clin Cancer Res; 16(22); 5548–56. ©2010 AACR.*

Of 147,000 patients who develop colorectal cancer in the United States each year, approximately 50,000 die of metastatic cancer (1). Many colorectal cancer patients develop metastases, especially to the liver, which are amenable to surgical resection. However, despite resection of metastases, 70% to 80% of these patients die from recurrent colorectal cancer (2, 3). Although recent studies have shown that postoperative (4) and perioperative (5) chemotherapy increases the recurrence-free survival (RFS) of patients after liver metastasis resection, over half the patients in both these studies recurred by 3 years after resection. New therapies are needed for treatment of these patients.

A tumor vaccine consisting of irradiated autologous tumor cells plus Bacillus Calmette-Guerin (BCG) has been used in phase III trials to treat patients after resection of primary colon cancer (6, 7). In these trials, although overall survival was not increased, the patients who developed an immune response to their autologous tumor manifested improved survival compared with those who did not, suggesting that treatment with more potent vaccines could improve survival. In a murine model, we found that a tumor lysate–pulsed dendritic cell (DC) vaccine generated an order of magnitude greater antitumor immunity than immunization with irradiated tumor cells plus the classic adjuvant, *Corynebacterium parvum* (8).

Although studies have shown that DC vaccines can induce immune responses in cancer patients, optimal parameters for immunization remain under investigation (9). Here, we based the route of immunization on our observations in murine models of enhanced antigen-specific T-cell proliferation and IFNγ production using intranodal

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doi: 10.1158/1078-0432.CCR-10-2138

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Translational Relevance

Several studies of the use of dendritic cell (DC) vaccines to attempt to treat measurable metastatic tumor deposits have been unsuccessful. There has been limited study of DC vaccines in the adjuvant setting after resection of metastatic tumors in general, and colorectal cancer in particular. We conducted a clinical trial in which patients were treated with a DC vaccine in the adjuvant setting after resection of metastatic disease. Observations in murine models in our lab were used to formulate one of the hypotheses we tested in the clinical trial and were used to determine the route of DC vaccination.

We show that an autologous tumor lysate–pulsed DC vaccine can induce immune responses against autologous tumor in a high proportion of patients. The clinical relevance of these immune responses was evaluated. We found an association between induction of an autologous tumor-specific immune response and improved recurrence-free survival.

injections when compared with subcutaneous or intravenous routes of immunization (10). A clinical study subsequently confirmed that an intranodal DC injection was more likely to induce peptide-specific T-cell responses than intradermal or intravenous injections (11).

In murine models, we also observed that signaling through CD40, a molecule on the surface of DCs, is required for multiple different vaccines to induce antitumor immunity (12). Engagement of CD40 by CD40L promotes DC cytokine production and costimulatory molecule expression, enabling DCs to effectively trigger T-cell activation and differentiation (13). In mice, activation of tumor lysate–pulsed DCs with CD40L leads to more potent induction of systemic immunity than DCs prepared without CD40L activation (14).

Therefore, we initiated a clinical trial whereby patients underwent resection of metastatic colorectal cancer and were then treated with intranodal injection of autologous tumor lysate–pulsed DCs. Patients were randomized to receive DCs treated with or without CD40L. We hypothesized that this vaccine would induce tumor-specific immune responses, and that these responses would be enhanced by CD40L activation.

Materials and Methods

This study was approved by the Dartmouth Committee for the Protection of Human Subjects and performed according to U.S. Food and Drug Administration Investigational New Drug 9554. Approximately 4 weeks after resection of all detectable metastatic colorectal cancer, patients signed their informed consent. Eligibility criteria included Karnofsky performance status >60%, recall delayed-type hypersensitivity (DTH) to either Candida or tetanus, and enough tumor cells to form the vaccine and do the immunoassays (>75 × 10^6 cells). Patients were excluded if they were receiving immunosuppressive drugs or had active autoimmune disease, HIV, or hepatitis B or C. A leukopheresis was scheduled 1 to 2 weeks after the patient consented. A computer tomography scan was performed just before pheresis to determine if residual measurable disease was present. Eligible patients were randomized in a blinded fashion into two groups: one had CD40L added to their DC culture, and the other did not. The data were unblinded following completion of all immunoassays. Patients received their first DC vaccination 8 days after pheresis. The second and third vaccinations were given 3 and 6 weeks after the first vaccination, respectively.

Preparation of tumor cells

Tumors were transferred from the Operating Room to Pathology, where under sterile conditions the margins of resection were evaluated and a sample was removed for histologic confirmation of adenocarcinoma. The tumor was then mechanically dissociated with a scalpel and filtered. The filtered cells were enumerated. Some were aliquoted for DTH testing, irradiated 10,000 cGy, and cryopreserved at 2 million cells/mL in human AB serum (Gemini BioProducts) containing 10% DMSO (Sigma). The remaining cells were combined with tumor chunks that did not go through the filter and were enzymatically digested using 4 mg/mL collagenase (Sigma) and 0.2 mg/mL DNase (Sigma). These cells were then washed, irradiated 10,000 cGy, and subjected to three freeze-thaw cycles in liquid nitrogen to form the tumor lysate.

Preparation of DC vaccines

Patients underwent leukopheresis using the Cobe Spectra Apheresis System in the Cellular Therapy Center of the Norris Cotton Cancer Center. The leukopheresis product was enriched for monocytes by counter flow centrifugation elutriation using a Beckman J6-MI elutriation centrifuge. Monocytes were cultured in Life Cell culture bags (Baxter Nexell Therapeutics) in AIMV media (Life Technologies) at an initial concentration of 2.5 × 10^6 cells/mL with 10 ng/mL recombinant human granulocyte macrophage colony-stimulating factor (rhuGM-CSF; Immunex) and 20 ng/mL recombinant human interleukin-4 (rhuIL-4; Schering Plough) added on days 0, 3, and 6 of culture. On culture day 6, autologous tumor lysate was added at a ratio of one tumor cell equivalent (TCE) to one DC. Keyhole limpet hemocyanin (KLH; Vacmune, Biosyn Corp.) was added at 25 μg/mL. On culture day 7, recombinant human CD40L (Amgen) was added at 1 μg/mL to the cultures of those patients who randomized to this treatment. On culture day 8, DCs were harvested and viable cells were enumerated and administered if they met the following release criteria: viability >70%, no organisms on gram stain, cultured samples with no bacterial growth, and endotoxin

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levels of <5 EU/kg. Five million DCs were injected in a volume of 0.5 mL into each of two inguinal lymph nodes under ultrasound guidance. The remainder of the DCs were cryopreserved in autologous serum/10% DMSO at 1×10^7 DCs/mL for the second and third vaccinations, which were performed 3 and 6 weeks after the initial vaccination in the same nodal basin.

Immunoassays

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood drawn from three different time points (before vaccine, 1 week after all vaccinations, and 3 months after all vaccinations) using Ficoll-Paque Plus (GE Healthcare). After separation, PBMCs were washed, then frozen in a 10% DMSO/fetal bovine serum (FBS; Lonza) solution, and stored at −140°C. PBMCs from all three time points were concurrently assayed.

Autologous DCs, cryopreserved after 6 days of culture, were thawed and washed in serum-free AIMV (Life Technologies) medium and then resuspended in AIMV supplemented with rhuGM-CSF at 500 IU/mL and rhuIL-4 at 20 ng/mL. DCs were incubated in six-well Ultra Low Adhesion plates (Costar) at 37°C/5% CO_2. After 24 hours of incubation, DCs were pulsed with antigens for 24 hours. The following antigens were used: KLH at 25 μg/mL and autologous tumor lysate at a ratio of one TCE to one DC. DCs were then harvested and washed in serum-free AIMV medium, then resuspended in conditioned medium, and used as stimulators. For peptide antigens, DCs were first activated with recombinant human CD40L (1 μg/mL) and then pulsed with peptides and B_2 microglobulin (Calbiochem) at 3 μg/mL for 4 hours at 37°C. The HLA-A2–restricted peptides Ep-Cam p263-271 (GLKAGVIAV), CEA p571-579 (YLSGANLNL), and Her-2/neu p654-662 (IISAVVGIL; Peptide Technologies Corp.) were used at 20 μg/mL.

Enzyme-linked immunospot assay

The enzyme-linked immunospot (ELISPOT) assay was performed on 96-well Immobilon-P acrylic polyvinylidene difluoride plates (Millipore), which were coated with 5 μg/mL mouse anti-human IFNγ antibody (1-DIK, Mabtech) and incubated at 4°C overnight. After thawing, PBMCs were washed and transferred to tissue culture–treated polystyrene flasks (Corning) for a 10-minute adherence depletion at 37°C. Cells were harvested from flasks, and then 3×10^5 cells were added to each well in triplicate. Antigen-pulsed DCs were added at 20,000 per well. Phytohemagglutin (Sigma) was used as a control at 20 μg/mL. After an 18-hour incubation, the plates were washed with PBS/0.05% Tween 20 (Sigma). The biotinylated detection antibody (IFNγ: Mab 7-B6-1 Biotin, Mabtech) was added at 1 μg/mL and incubated at 37°C for 2 hours. The plates were then washed, avidin-peroxidase complex (Vectastain ABC kit, Vector Laboratories) was added, and plates were incubated at room temperature for 1 hour. AEC substrate (3-amino-9-ethylcarbazole, Sigma) was added for 4 minutes, and the plates were washed and analyzed using the KS ELISPOT Axioplan 2 Imaging system (Carl Zeiss).

Restimulation ELISPOT assay

PBMCs were thawed, washed, and plated at 1×10^6 per well in 24-well plates. DCs were antigen loaded as previously described and added to wells at 1×10^5 per well. IL-12 (PeproTech) was added to each well at 1 ng/mL. On culture day 2, IL-2 (PeproTech) was added at 10 units/mL. Cells were incubated for an additional 5 days. On day 6, the cells were harvested and plated onto a precoated IFNγ ELISPOT plate (as described above) at a concentration of 3×10^4 per well. Antigen-pulsed DCs were added to these cells, and the ELISPOT plate was developed and analyzed as described above.

Dye dilution proliferation assay

The dye dilution proliferation assay (DDPA) was performed as previously described (15, 16). Briefly, PBMCs were thawed, washed, centrifuged, and resuspended in Diluent C (Sigma) at a concentration of 2×10^7/mL. An equal volume of Diluent C containing 4 μmol/L PKH-67 (Sigma) was added. Cells were incubated for 3 minutes in the dark at room temperature. PKH-67 was quenched by adding an equal volume of FBS for 1 minute. PBMCs were then washed, aliquoted at 1×10^6 cells/mL, and stimulated with medium or DCs at a ratio of 1:10 DC to PBMC. After 7 days of culture at 37°C and 5% CO_2, the cells were harvested, transferred to 12 × 75–mm tissue culture tubes (USA Scientific), and blocked with H-IgG (Sigma). Cells were stained with CD4 APC (Becton Dickinson) and CD8 PE (Becton Dickinson), incubated on ice for 30 minutes, and fixed with 1% paraformaldehyde. Cells were acquired on a Becton Dickinson FACSCalibur and analyzed with WinList and ModFit (Verity).

DTH testing

DTH tests were done by intradermal injection of 1 million mechanically dissociated, irradiated viable autologous tumor cells or 5 μg KLH. Tests were read 2 days later and were considered positive if >5 mm of induration were observed.

Statistics

For the DDPA and ELISPOT assays, all samples were run in triplicate to calculate the mean percentage of proliferating cells and spots (±SD). A response for a particular patient at one time point was considered positive if the mean value obtained as a result of a specific antigenic stimulus (e.g., tumor lysate–pulsed DCs) was significantly greater (by *t* test) than the mean value derived from the control stimulus (e.g., unpulsed DCs). Values reported are the response to the antigenic stimulus minus the response to the control stimulus.

The study was designed with a sample size of 24 patients to achieve a power of 80% to detect a difference in vaccine-induced immune responses in the DDPA assay between the CD40L and non-CD40L groups. The

Published OnlineFirst September 30, 2010; DOI: 10.1158/1078-0432.CCR-10-2138

Kaplan-Meier product limit method was used to estimate relapse-free survival and overall survival. The log-rank test was used to compare the survival of vaccine responders with nonresponders.

Results

Between December 2001 and December 2003, 26 patients were accrued to this trial. Six additional patients were assessed but were not enrolled due to the following reasons: tumor cell yield was too low (3) and patient declined enrollment (3). Twelve patients received DCs that were stimulated with CD40L; 14 patients received DCs not treated with CD40L. There was no statistically significant difference between the two groups (CD40L versus no CD40L) when variables of potential prognostic importance were considered (Table 1). The clinical risk score (CRS), a prognostic indicator based on a large database of patients who underwent hepatic resection (2), was similar for each group. When calculating the CRS, we excluded the two patients who had lung metastases resected to form the vaccine and the three patients who had previously had liver metastases resected, because such patients were not included in the database used to derive the CRS. All patients had all detectable tumor resected at the time of the operation with negative margins. All patients had a positive DTH test to Candida or tetanus after tumor resection. Two patients had new measurable metastases detected on the computer tomography scan done just before their vaccinations. The remainder of the patients had no measurable tumor and normal CEA levels at the time of initiation of adjuvant immunotherapy.

Addition of CD40L to the cultures changed the phenotype of the DCs. When DCs grown in the presence of CD40L were compared with DCs grown without CD40L, there was no change in expression of MHC class I, MHC class II, or the costimulatory molecule CD80. Ninety-eight percent of DCs expressed MHC class I, 90% expressed MHC class II, and 52% expressed CD80. However, the percentage of DCs expressing the costimulatory molecule CD86 were higher in the CD40L group (87% versus 68%, $P = 0.01$) and the mean fluorescence intensity (MFI) was higher in the CD40L group (390 versus 165, $P = 0.03$). In addition, the percentage of DCs expressing the maturation marker CD83 were higher in the CD40L group (30% versus 10%, $P = 0.01$) and the MFI was higher in the CD40L group (61 versus 29, $P = 0.007$).

No toxicity was observed in any of the patients. The two patients with measurable disease progressed at 6 months and died of colorectal cancer 11 months after vaccination. The 24 patients with no measurable disease treated with an adjuvant vaccine were followed for a minimum of 5.5 years. Eight are alive and disease-free, 4 are alive with recurrent disease, and 12 have died of colorectal cancer. The RFS of patients treated with adjuvant vaccine was 58% at 1 year, 42% at 2 years, and 38% at 5 years. The

Table 1. Patient characteristics

Characteristic	Randomization to		
	No CD40L	CD40L	Total
No. patients	14	12	26
Mean age, y (range)	64 (43-81)	57 (41-76)	61 (41-81)
No. males	10	10	20
Characteristics of the primary colorectal cancer			
No. with colon cancer	9	8	17 (65%)
No. with rectal cancer	5	4	9 (35%)
No. node positive	4	8	12 (46%)
Treatment before resecting the tumor used to form the vaccine			
Patients who had liver metastases resected	2	1	3 (11%)
Patients who had lung metastases resected	2	0	2 (8%)
Index metastases treated with chemotherapy	5	4	9 (35%)
Any chemotherapy	10	8	18 (69%)
Patient characteristics when metastasis resected to form the vaccine			
Liver metastasis	12	12	24 (92%)
Lung metastasis	2	0	2 (8%)
Also had extrahepatic, intra-abdominal metastases resected	1	2	3 (11%)
Mean no. metastases resected	1.4	1.7	1.6
No. patients with liver met >5 cm	4	4	8 (31%)
Mean disease-free interval (mo)	17	12	15
Patients with CEA level >200	0	0	0
Mean clinical risk score (2)	2.1	2.3	2.2

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Table 2. Dye dilution proliferation assay

Patient	Percentage of T cells proliferating in response to antigenic stimulus (CD4/CD8)					
	Tumor lysate			KLH		
	Pre-vax	1 wk Post-vax	3 mo Post-vax	Pre-vax	1 wk Post-vax	3 mo Post-vax
1	—	**2.0/2.0**	—	—	—	—
2	—	—	—	—	—	—
3	2.8/4.0	4.0/1.0	3.5/0.3	—	**1.3**/—	**1.3**/—
4	—	—	—	—	—/**1.8**	—
5	ND	ND	ND	ND	ND	ND
6	—	—	—	—	—	—
7	—/6.0	—/7.0	—/4.0	—	—	—
8	—/6.0	—/4.0	—/0.6	—/2.0	—	—
9	—	—/**2.1**	—	—	**4.0/6.2**	**3.9/1.1**
10	—	—	—	—	—	—
11	—	—	—	—	—	—
12	—	—	**3.4/1.7**	—	**1.1**/—	**3.4**/—
13	—	—	—	—	—	—
14	—	—	—	—	—	—
15	—	**0.7/0.9**	**0.7**/—	—	**2.1/6.1**	**2.8/3.3**
16	—	—	—	0.9/1.4	—	2.1/0.9
17	—	—	—	—	**2.3**/—	**3.5/1.2**
18	ND	ND	ND	ND	ND	ND
19	—	**2.3/2.7**	—/**0.9**	1.4/—	**7.0/3.9**	**9.0/10.0**
20	—	—	—	—	**3.3**/—	**7.8/0.9**
21	—/0.3	—	—	—	**0.7**/—	**1.4**/—
22	—	—	—	—	**1.0/0.7**	**0.8**/—
23	1.2/—	1.7/—	1.7/—	—	**4.3/1.0**	**2.5/1.7**
24	—	—	—/**0.3**	—	—	—
25	—	**1.3**/—	—	1.0/—	**8.0/2.6**	**5.0/1.4**
26	—	**0.4/0.4**	**0.6**/—	—	**0.9/0.3**	**0.7**/—

NOTE: Numbers represent the percentage of CD4 and CD8 T cells that proliferated in response to the antigenic stimulus (DCs pulsed with autologous tumor lysate or DCs pulsed with KLH) using the DDPA assay. "—" indicates that there was no proliferation significantly higher than that observed when unpulsed DCs were used as the stimulus. Values in bold are significantly higher than prevaccine values, indicating a response induced by the vaccine.
Abbreviation: ND, the assay was not done due to lack of cells.

overall survival was 96% at 1 year, 83% at 2 years, and 58% at 5 years.

Tumor-specific immunoassay results

Twenty-four patients had cells available for the DDPA. Detectable tumor-reactive CD4 or CD8 T cells were present before vaccination in five patients (Table 2). In four of five patients, that response continued to be detected at 1 week and at 3 months after vaccination, but the magnitude of response was not increased by vaccination. CD4/CD8 T cells that proliferated in response to tumor were first detected 1 week after vaccination in six patients. In these six patients, the mean percentage of tumor-reactive CD4 and CD8 T cells was 1.3% and 1.6%, respectively. A response continued to be detectable 3 months after vaccination in three of these six patients. Two patients were first noted to have a tumor-specific proliferative response 3 months after vaccination. Overall, immunization induced an autologous tumor-specific proliferative T-cell response in 33% (8 of 24) patients.

We evaluated PBMC cytokine secretion in response to tumor lysate–pulsed DCs in a primary ELISPOT immunoassay and after a single round of *in vitro* stimulation. In the primary ELISPOT assay, two patients had evidence of induction of tumor-specific IFNγ-secreting cells 1 week after vaccination. In the restimulated ELISPOT assay (Table 3), a tumor-specific IFNγ response was detectable in four patients before vaccination. In three of these four patients, the response was significantly increased 1 week after vaccination, and in five additional patients, a new tumor-specific IFNγ response was noted.

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Therefore, 33% (8 of 24) of patients had evidence of induction of a tumor-specific IFNγ response 1 week after vaccination. In five of these eight patients, this response was still detectable 3 months after vaccination. An additional two patients first manifested a tumor-specific IFNγ response 3 months after vaccination. Overall, immunization induced a tumor-specific IFNγ response in 42% (10 of 24) patients.

Table 3. Number of PBMCs secreting IFNγ in response to tumor and KLH

Patient	Tumor lysate			KLH		
	Pre-vax	1 wk Post-vax	3 mo Post-vax	Pre-vax	1 wk Post-vax	3 mo Post-vax
1	—	—	—	—	—	—
2	—	—	—	—	—	—
3	—	—	—	—	**102**	**64**
4	70	—	—	—	**59**	—
5	ND	ND	ND	ND	ND	ND
6	—	—	**43**	—	—	**13**
7	—	—	—	—	—	—
8	—	—	—	—	—	—
9	1091	**2010**	944	—	**17**	—
10	—	—	—	—	—	—
11	—	—	—	—	—	—
12	—	—	—	—	—	—
13	90	**163**	112	—	—	—
14	—	**65**	—	14	13	—
15	—	**46**	—	—	**20**	—
16	—	—	**42**	—	—	—
17	—	**41**	**55**	—	—	—
18	ND	ND	ND	ND	ND	ND
19	—	—	—	—	—	—
20	—	—	—	—	—	—
21	10	**136**	**136**	—	—	—
22	—	—	—	—	**5**	**4**
23	—	**38**	—	—	**20**	**42**
24	—	—	—	—	—	—
25	—	—	—	—	**24**	—
26	—	**282**	**188**	—	**10**	—

NOTE: Numbers represent the number of cells per 3×10^5 PBMCs that secreted IFNγ in response to the antigenic stimulus in an ELISPOT assay. To measure tumor-specific responses, PBMCs were exposed to autologous tumor lysate–loaded DCs and cultured for 1 wk before stimulus by DCs pulsed with autologous tumor lysate (the Restimulation ELISPOT assay described in Materials and Methods). KLH responses were measured without prior *in vitro* stimulation. "—" indicates that the number of spots was not significantly higher than that observed when unpulsed DCs were used as the stimulus. Values in bold are significantly higher than prevaccine values, indicating a response induced by the vaccine.

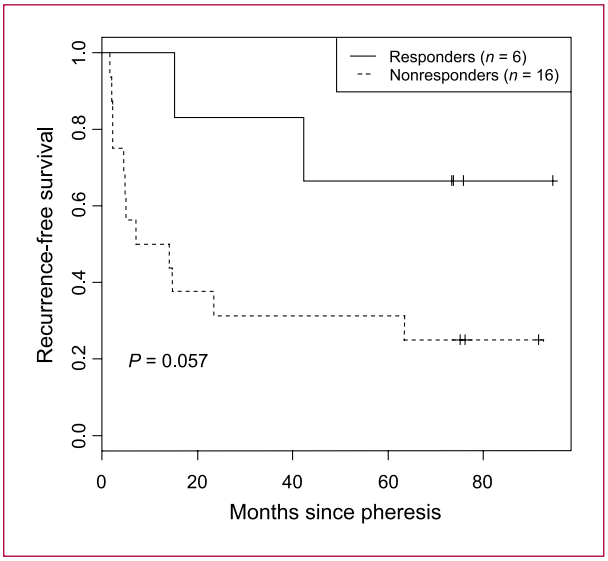

Fig. 1. Autologous tumor-specific proliferative response 1 wk after DC immunization is associated with an improved RFS (log-rank $P = 0.057$).

Two of 25 patients had a positive DTH reaction to autologous tumor before vaccination. After vaccination, 64% of patients (16 of 25) developed a positive DTH test to autologous tumor cells.

Association between tumor-specific immune responses and survival

Tumor-specific immune responses before vaccination were not associated with improved RFS. In contrast, the six patients who had evidence of a vaccine-induced antitumor proliferative response 1 week after vaccination had a markedly better RFS at 5 years (67% versus 31%, $P = 0.057$) than nonresponding patients (Fig. 1). There was a trend toward increased RFS at 5 years (63% versus 28%, P = not significant) in the group of patients who developed a tumor-specific IFNγ response 1 week after immunization. The eleven patients who developed a tumor-specific proliferative or IFNγ response 1 week after vaccination had a significantly greater RFS at 5 years (63% versus 18%, $P = 0.037$) than those who did not develop a response (Fig. 2).

There was no significant difference in RFS between the patients who had evidence of a vaccine-induced anti-KLH proliferative or IFNγ response and patients with no evidence of a response. Thus, it is the induction of an autologous tumor-specific immune response, rather than simply the ability to be immunized, that is associated with improved RFS.

There were no significant differences in the clinical characteristics of the 11 patients who developed tumor-specific proliferative or IFNγ immune responses 1 week after vaccination and the patients who did not. Similar numbers of patients in each group had prior surgery for liver metastases, chemotherapy before liver metastasis resection, or concomitant resection of extrahepatic

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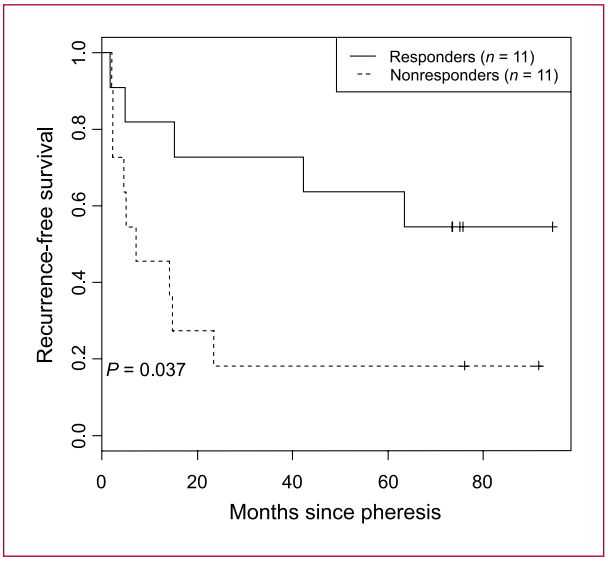

Fig. 2. Autologous tumor-specific proliferative or IFNγ ELISPOT response 1 wk after DC immunization is associated with an improved RFS (log-rank $P = 0.037$).

intra-abdominal metastases. There was no significant difference in the mean CRSs (2) of the immune responders compared with the nonresponders. Thus, the improved RFS observed in patients who developed a tumor-specific immune response cannot be explained by clinical prognostic factors.

There was no difference in the RFS of patients who developed a positive tumor-specific DTH response compared with patients who had a negative DTH test.

Three patients developed tumor-specific proliferative and IFNγ-secretory responses at 1 week after vaccination and had a positive tumor DTH test. Two of these patients are free of recurrence. The third patient developed a recurrence 3.5 years after vaccination, which we resected, and remains free of disease 7 years after vaccination.

Immune responses to KLH

Of 24 patients, 13 (54%) had KLH-specific proliferative responses induced by vaccination (Table 2). The mean percentage of KLH-reactive CD4 and CD8 T cells was 2.8 and 1.7, respectively. In all but one of these patients, KLH-reactive T cells continued to be detectable 3 months after vaccination. In the primary IFNγ ELISPOT assay, vaccination induced a KLH-specific IFNγ response in 38% (9 of 24) of patients (Table 3). In the restimulation IFNγ ELISPOT assay, 83% (20 of 24) of patients had evidence of a KLH-specific response 1 week after vaccination. The mean response was 473 spots/3 × 10^5 PBMCs. Sixty percent of patients developed a positive DTH response to KLH.

Effect of CD40L on immune responses

CD40L activation of DCs did not significantly affect the percentage of patients with positive antitumor or anti-KLH immune responses as measured by ELISPOT, DDPA, or DTH assay. Patients treated with CD40L matured DCs and those treated with DCs that were not exposed to CD40L had a similar RFS.

Antipeptide immune responses

Eight HLA-A2–positive patients had a sufficient number of cells for restimulation ELISPOT assay. We evaluated three peptides from colorectal cancer–associated proteins that had previously been shown to induce T-cell responses in patients with colorectal cancer (17). Before vaccination, CEA-, Her-2/neu–, and Ep-Cam–reactive cells

Table 4. Number of PBMCs secreting IFNγ in response to tumor peptides

Patient	CEA			Her-2/neu			Ep-Cam		
	Pre-vax	1 wk Post-vax	3 mo Post-vax	Pre-vax	1 wk Post-vax	3 mo Post-vax	Pre-vax	1 wk Post-vax	3 mo Post-vax
1	—	—	—	—	—	—	—	—	—
2	—	**201**	—	36	**96**	—	—	**120**	—
3	88	**159**	—	—	—	—	74	—	—
7	—	—	—	31	**200**	—	—	—	—
14	—	—	—	—	**125**	**59**	—	**161**	—
16	—	—	—	—	—	—	—	—	—
21	39	**115**	**302**	19	**201**	**196**	—	—	**183**
23	33	—	—	112	97	29	—	—	**72**

NOTE: Numbers represent the number of cells per 3 × 10^5 PBMCs that secreted IFNγ in response to the antigenic stimulus in an ELISPOT assay. To measure tumor peptide–specific responses, PBMCs were exposed to peptide-loaded DCs and cultured for 1 wk before stimulus by DCs pulsed with autologous peptide (the Restimulation ELISPOT assay described in Materials and Methods). "—" indicates that the number of spots was not significantly higher than that observed when unpulsed DCs were used as the stimulus. Values in bold are significantly higher than prevaccine values, indicating a response induced by the vaccine.

Published OnlineFirst September 30, 2010; DOI: 10.1158/1078-0432.CCR-10-2138

were detectable in three, four, and one patient, respectively (Table 4). An IFNγ-secreting antipeptide response was either induced or significantly increased by vaccination in three patients for the CEA peptide, and in four patients for the Her-2/neu and Ep-Cam peptides. Thus, the use of a whole-cell antigen source can induce peptide-specific immune responses.

Discussion

Our study is the first to evaluate the role of CD40L-induced maturation as a component of the DC manufacturing process in a prospectively randomized trial. At the time we began this trial, it was known that CD40 activation of murine and human DCs led to enhanced generation of CTLs by increasing DC expression of the costimulatory molecules CD80 and CD86 and by inducing IL-12 secretion (14, 18). It had also been showed that activating murine tumor lysate–pulsed DCs with CD40L led to more potent induction of systemic immunity than DCs prepared without CD40L activation (14). Therefore, we hypothesized that CD40L-activated human DCs would induce a more potent immune response than DCs grown without a specific maturation factor. However, treatment of DCs with CD40L did not increase tumor or KLH-specific immune responses in our patients. It is unlikely that this was due to inactive or inadequate amounts of CD40L, as we observed significant upregulation of CD86 and CD83 expression. One possible explanation for this finding is that inadequate Toll-like receptor signaling was present to optimize CD40-induced IL-12 expression in our vaccine. Subsequent to the initiation of our trial, it was shown that DC stimulation with the combination of CD40L and lipopolysaccharide increased IL-12 secretion several fold compared with CD40L alone (19, 20). We did not measure DC IL-12 secretion.

Others have attempted to induce antitumor responses in colorectal cancer patients with measurable metastatic tumors using DC vaccines. Chang et al. (21) treated two patients with an autologous tumor lysate–pulsed, intradermally injected DC vaccine and showed a tumor-specific response in one patient. Tamir et al. (22) observed tumor-specific responses in two of six patients treated with an autologous tumor lysate–pulsed DC vaccine. Mazzolini et al. (23) treated five patients with intratumoral injections of IL-12–transfected DCs and observed a transient decrease in CEA in one patient. In two studies using CEA peptide as the antigenic source, minimal antipeptide responses were observed, but in another study, 2 of 12 patients had tumor regression and induction of peptide-specific CD8 cells (24–26).

We focused our efforts in the adjuvant setting because of the paucity of data showing objective responses to vaccination in patients with measurable metastases (27, 28). In a review of 527 patients with measurable metastatic colorectal cancer treated on 32 studies with active specific cancer vaccination, the response rate was only 0.9% (27). Effective immunologic treatment of patients with measurable disease burdens has required adoptive transfer of large numbers of tumor-reactive T cells into lymphodepleted hosts, resulting in a high percentage of circulating antitumor T cells that persist over time (29). It is unclear, however, whether one would need to generate such a high proportion of antitumor T cells to eliminate or control micrometastatic tumor deposits that exist after resection of macrometastatic disease.

We treated patients with a DC vaccine in the adjuvant setting with no measurable disease after colorectal metastasis resection. These patients are an attractive group to attempt to immunize because they are immunocompetent (as we have now shown by recall antigen DTH testing) and approximately 70% will die from eventual tumor growth despite being treated with best current therapies. Only one previous study has reported adjuvant treatment of colorectal cancer patients with a DC vaccine after metastasis resection. In this study, 9 of 13 patients treated with a CEA mRNA-loaded DC vaccine relapsed at a median of 122 days, and few antipeptide responses were detected (30). Using a tumor lysate–pulsed, intranodally injected DC vaccine, we were able to induce an autologous tumor-specific T-cell proliferative or IFNγ-secretory response in 15 of 24 patients (63%), and a DTH response to autologous tumor cells in 14 of 23 patients (61%). In those patients who developed a vaccine-induced proliferative T-cell response 1 week after vaccination, the mean percentage of tumor-reactive CD4 and CD8 T cells was 1.3% and 1.6%, respectively. Also, 7 of the 11 patients (64%) who developed a tumor-specific proliferative or IFNγ response 1 week after vaccination had evidence of a persistent response 3 months after vaccination. Patients who developed a vaccine-induced tumor-specific response had a significantly better RFS at 5 years (63% versus 18%, $P = 0.037$) than the remainder of the patients. The similarity in the standard prognostic clinical characteristics of the tumor-specific immune responders and nonresponders increases the possibility that a causal relationship exists between DC vaccination and improved RFS. This study also suggests that a modest level of persistent antitumor T-cell reactivity may be sufficient to eliminate residual micrometastases or, at the least, keep them in a state of equilibrium with the immune system (31).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

1R29CA776612-01 and P20 RR16437 (R.J. Barth).
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Received 08/08/2010; revised 09/14/2010; accepted 09/19/2010; published OnlineFirst 09/30/2010.

Published OnlineFirst September 30, 2010; DOI: 10.1158/1078-0432.CCR-10-2138

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun M. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225–49.
2. Fong Y, Fortner J, Sun R, Brennan M, Blumgart M. Clinical score for predicting recurrence after hepatic resection for metastatic colorectal cancer: analysis of 1001 consecutive cases. Ann Surg 1999;230: 309–18.
3. Nordlinger B, Guiguet M, Vaillant JC, et al. Surgical resection of colorectal carcinoma metastases to the liver. Cancer 1996;77:1254–62.
4. Portier G, Elias D, Bouche O, et al. Multicenter randomized trial of adjuvant fluorouracil and folinic acid compared with surgery alone after resection of colorectal liver metastases: FFCD ACHBTH AURC 9002 trial. J Clin Oncol 2006;24:4976–82.
5. Nordlinger B, Sorbye H, Glimelius B, et al. Perioperative chemotherapy with FOLFOX4 and surgery vs surgery alone for resectable liver metastases from colorectal cancer: a randomised controlled trial. Lancet 2008;371:1007–16.
6. Vermorken JB, Claessen A, Tinteren H, et al. Active specific immunotherapy for stage II and stage III human colon cancer: a randomised trial. Lancet 1999;353:345–50.
7. Harris J, Ryan L, Hoover H, et al. Adjuvant active specific immunotherapy for stage II and III colon cancer with an autologous tumor cell vaccine: ECOG study E5283. J Clin Oncol 2000;18:148–57.
8. Lambert L, Gibson G, Maloney M, Barth R. Equipotent generation of protective anti-tumor immunity by various methods of dendritic cell loading with whole cell tumor antigens. J Immunother 2000;24:232–6.
9. Engell-Noerregaard L, Hansen T, Andersen M, Straten P, Svane I. Review of clinical studies on dendritic cell based vaccination of patients with malignant melanoma: assessment of correlation between clinical response and vaccine parameters. Cancer Immunol Immunother 2009;58:1–14.
10. Lambert L, Gibson G, Maloney M, Durell B, Noelle R, Barth R. Intranodal immunization with tumor lysate pulsed dendritic cells enhances protective anti-tumor immunity. Cancer Res 2001;61:641–6.
11. Bedrosian I, Mick R, Xu S, et al. Intranodal administration of peptide-pulsed mature dendritic cell vaccines results in superior $CD8^+$ T-cell function in melanoma patients. J Clin Oncol 2003;21:3826–35.
12. Mackey M, Gunn J, Ting P, et al. Protective immunity induced by tumor vaccines requires interaction between CD40 and its ligand CD154. Cancer Res 1997;57:2569–74.
13. Elgueta R, Benson M, de Vries V, Wasiuk A, Guo Y, Noelle R. Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunol Rev 2009;229:152–72.
14. Labeur MS, Roters B, Pers B, et al. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. J Immunol 1999;162:168–75.
15. Givan AL, Fisher J, Waugh M, Ernstoff M, Wallace P. A flow cytometric to estimate the precursor frequencies of cells proliferating in response to specific antigens. J Immunol Methods 1999;230:99–112.
16. Bercovici N, Givan A, Waugh M, et al. Multiparameter precursor analysis of T cell responses to antigen. J Immunol Methods 2003;276:5–17.
17. Nagorsen D, Keilholz U, Rivoltini L, et al. Natural T cell response against MHC class I epitopes of epithelial cell adhesion molecule, her-2/neu and CEA in patients with colorectal cancer. Cancer Res 2000;60:4850–4.
18. Cella M, Scheidegger D, Palmer-Lehman K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of IL-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med 1996;184:747–52.
19. Lapointe R, Toso J, Butts C, Young H, Hwu P. Human dendritic cells require multiple activation signals for the efficient generation of tumor antigen specific T lymphocytes. Eur J Immunol 2000;30: 3291–8.
20. Butterfield L, Gooding W, Whiteside T. Development of a potency assay for human dendritic cells: IL-12 p70 production. J Immunother 2008;31:89–100.
21. Chang A, Redman B, Whitfield J, et al. A phase I trial of tumor lysate pulsed dendritic cells in the treatment of advanced cancer. Clin Cancer Res 2002;8:1021–32.
22. Tamir A, Basaglia E, Kagahzian A, et al. Induction of tumor specific T cell responses by vaccination with tumor lysate loaded dendritic cells in colorectal cancer patients. Cancer Immunol Immunother 2007;56: 2003–16.
23. Mazzolini G, Alfaro C, Sangro B, et al. Intratumoral injection of dendritic cells engineered to secrete IL-12 by recombinant adenovirus in patients with metastatic gastrointestinal carcinomas. J Clin Oncol 2005;23:999–1010.
24. Morse MA, Deng Y, Coleman D, 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.
25. Itoh T, Ueda Y, Kawashima I, et al. Immunotherapy of solid cancer using dendritic cells pulsed with the HLA-A24-restricted peptide of carcinoembryonic antigen. Cancer Immunol Immunother 2002;51:99–106.
26. Fong L, Hou Y, Rivas A, et al. Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. Proc Natl Acad Sci U S A 2001;98:8809–14.
27. Nagorsen D, Thiel E. Clinical and immunological responses to active specific cancer vaccines in human colorectal cancer. Clin Cancer Res 2006;12:3064–9.
28. Rosenberg S, Yang J, Restifo N. Cancer immunotherapy: moving beyond current vaccines. Nat Med 2004;10:909–15.
29. Dudley M, Wunderlich J, Yang J, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J Clin Oncol 2005;23:2346–57.
30. Morse M, Nair S, Mosca P, et al. Immunotherapy with autologous, human dendritic cells transfected with carcinoembryonic antigen mRNA. Cancer Invest 2003;21:341–9.
31. Dunn G, Old L, Schreiber R. The three Es of cancer immunoediting. Ann Rev Immunol 2004;22:329–60.

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Clin Cancer Res 2010;16:5548-5556. Published OnlineFirst September 30, 2010.