Cancer Therapy: Clinical

Clinical Benefit of Allogeneic Melanoma Cell Lysate–Pulsed Autologous Dendritic Cell Vaccine in MAGE-Positive Colorectal Cancer Patients

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Abstract

Purpose: We evaluated the clinical benefit of an allogeneic melanoma cell lysate (MCL)–pulsed autologous dendritic cell (DC) vaccine in advanced colorectal cancer patients expressing at least one of six MAGE-A antigens overexpressed by the cell line source of the lysate.

Experimental Design: DCs were cultured from peripheral blood mononuclear cells (PBMC), pulsed with the allogeneic MCL, and matured using cytokines that achieved high CD83- and CCR7-expressing DCs. Each patient received up to 10 intradermal vaccinations ($3\text{-}5 \times 10^6$ cells per dose) at biweekly intervals.

Results: Twenty patients received a total of 161 vaccinations. Treatment was well tolerated and quality of life measurements did not vary much across time. One patient experienced partial response [5%; 95% confidence interval (CI), 1-24%] and seven achieved stable disease (35%; 95% CI, 18-57%), one of whom also achieved late tumor regression, yielding a clinical benefit response rate of 40% (95% CI, 22-61%). Although overall median progression-free survival was 2.4 months (95% CI, 1.9-4.1 months), five patients (25%) experienced prolonged progression-free survival (>6 months), two of whom (10%) remain progression-free for >27 and >37 months, respectively. This result is particularly meaningful as all patients had progressive disease before treatment. Overall, DC vaccination was associated with a serial decline in regulatory T cells. Using an antibody array, we characterized plasma protein profiles in responding patients that may correlate with vaccine activity and report a prevaccination protein signature distinguishing responders from nonresponders.

Conclusion: This phase II vaccine study using mature, MCL-pulsed DCs has shown promising results and warrants further evaluation in a prospective randomized setting. (Clin Cancer Res 2009;15(24):7726–36)

The efficacy of therapeutic cancer vaccines in humans has in general been poor (1–3). An overall objective response rate of only 3.3% was observed in 1,306 vaccine treatments of cancer patients with advanced disease (3). This lack of success might reflect poor immunogenicity of the adopted tumor-associated antigens (TAA) as well as downmodulation of tumor-associated antigenicity in advanced tumors (4). Local and systemic suppression mediated by the growing tumor and its environment, including activation and stimulation of regulatory T lymphocytes (Treg), tolerogenic dendritic cells (DC), myeloid-derived suppressor cells, and angiogenic factors, can also counteract vaccine-based immunotherapy (5). In clinical trials that have used autologous DC-based vaccinations, differentiated and immunogenic DCs generated *ex vivo* from peripheral blood monocytes were pulsed or transfected with a variety of TAA. This approach might more effectively expose the immune system of the patient to immunogenic TAA, and because the DCs are already differentiated, may overcome the tolerogenic tumor environment in the patient. This view is supported by theoretical (4) as well as practical considerations. Preclinical studies have shown the efficacy of DC-based vaccination in murine colorectal cancer models (6). In humans, an overall response rate of 9% in a total of 626 patients treated with DC-based vaccination has been achieved to date (1).

Translational Relevance

We present an allogeneic melanoma cell lysate–pulsed autologous dendritic cell (DC) vaccine clinical study in MAGE-enriched advanced colorectal cancer patients, using DCs generated by a proprietary DC production method that achieved highly mature DC phenotype with potentially better lymph node trafficking. Our result showed a 40% clinical benefit rate with some durable responses and even late tumor shrinkage. This DC vaccine was associated with reduction in the immunosuppressive regulatory T-cell population, which might have contributed to enhancing antitumor immunity. We adopted a novel antibody array system, with validation by ELISA, to analyze the plasma protein profiles of the patients. We identified a prevaccination signature that may potentially predict response to DC vaccination. We also identified potential biomarkers of response following DC vaccination. Such a translational protein signature analysis may be useful for enrichment of patients for optimizing cancer vaccine efficacy and for further understanding the antitumor immune response.

Although only a limited percentage of vaccinated DC actually traffic to the T-cell sites of regional draining lymph nodes, high immune activating potential is still achievable. The degree of DC maturation is believed to be critical to effective priming of antigen-specific T cells and hence potentially produce a more successful outcome for cancer vaccination. Immature DCs are known to populate within the cancer stroma and draining lymph nodes that contributes toward creating an immunosuppressive milieu (7). Strategies to generate functionally mature DCs with improved lymph node homing capabilities and which can downregulate Tregs may contribute to improving DC cancer vaccine efficacy (7–9).

In the present single arm phase II clinical trial, we used a newly developed cancer vaccine (DanDrit Biotech A/S, DDB). The vaccine consists of autologous DCs generated by a proprietary DC production method comprising a cocktail of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and prostaglandin E to create a consistently mature, uniform, and highly CCR7- and CD83-expressing DC phenotype (10). DCs were pulsed with DDB's proprietary allogenic melanoma cell (DDM-1) lysate (MCL) rich in cancer/testis antigens. The tumors of the target population, comprising colorectal cancer patients with advanced disease, were prescreened for expression of cancer/testis antigens and matched to those antigens present in the MCL.

Thus, in our study, intradermal DC vaccination was administered to patients whose colon cancer cells expressed at least one of six MAGE-A cancer/testis antigens present in the MCL. MAGE gene expression in colorectal cancer is reported at 42.1% in Asians (11). This MCL also contains other recently identified antigens (Supplementary Table S1), potentially maximizing antitumor immunity in this MAGE-enriched patient population. There are also established evidence that specific T lymphocyte subsets in the colorectal cancer microenvironment may determine prognosis and clinical outcome, which may po-

tentially allow the modulation of T cells by cancer immunotherapeutic approaches (12, 13).

Our objectives were to determine clinical activity, immune responses, and tolerability of the DC vaccine in progressing metastatic colorectal cancer patients. We also studied the effects of our DC vaccine on Treg *in vivo*.

Although established immune monitoring, such as with CTL and ELISPOT assays, have shown some correlation with clinical efficacy to cancer vaccines (5), this has not universally been consistent across numerous clinical cancer vaccine studies. Therefore, we used a novel antibody array that simultaneously detects 507 plasma proteins to identify potential predictive markers of response to DC vaccination and also potential biomarkers of a putative immune response following DC vaccine.

Materials and Methods

Study design. This prospective, open-labeled phase II study was approved by the Institutional Review Board of the National Cancer Centre Singapore. Consenting patients underwent tumor biopsy to determine *MAGE-A* gene expression. Only patients whose tumors expressed at least one of six *MAGE-A* genes (*A1, A3, A4, A6, A10,* and *A12*) were enrolled. Upon accrual, each patient was scheduled to undergo venesection for generation of DC vaccine. Each patient received up to 10 intradermal vaccinations with the autologous DC-based vaccines of 3 to 5 × 10^6 cells each at biweekly intervals. If required, additional venesection (s) were done during treatment to generate enough vaccines as specified by the clinical protocol.

The primary study end point included clinical benefit response (CBR) rate [complete response, partial response (PR), or stable disease (SD) of at least 4 wk as defined by the Response Evaluation Criteria in Solid Tumors (RECIST) criteria and time to tumor progression], whereas secondary end points included progression-free survival (PFS), overall survival (OS), quality of life (QoL) analysis, and delayed-type hypersensitivity (DTH) reactivity. The sample size was calculated based on the assumption that the cancer vaccine would be worth a further confirmatory phase III study if the CBR was at least 16% against a no further interest rate of 2%. The trial was modeled after A'Hern's single stage phase II design, and with a type I error of 5% and power of 80%, a total of 20 patients (including a 10% dropout rate) would be required (14). If at least two CBR were observed, the trial was then deemed worthy of further study.

Patient eligibility. Patients with histologically proven *stage IV* colorectal cancer, who had progressed during or following palliative chemotherapy and biological therapy, or who refused chemotherapy were eligible. Patients between ages 25 and 80 y, with expected survival time of over 4 mo, and a performance status of ≤2 were required to have normal cardiopulmonary function, hemoglobin of >8.0 g/dl, platelet of >100,000/mm³, WBC of >3,000/mm³, adequate liver function [total bilirubin <34 μmol/l, aspartate aminotransferase and alanine aminotransferase of <3 × upper limit of normal, or aspartate aminotransferase and alanine aminotransferase of <5 × upper limit of normal (in patients with liver metastases], and serum creatinine of <140 μmol/L. Exclusion criteria included uncontrolled infection, central nervous system involvement, HIV, chronic Hepatitis B or C infections, and participation in another clinical trial <2 mo before entry. Patients were required to stop chemotherapy, radiotherapy, biological therapy, corticosteroids, or any other immunosuppressive agents at least 2 mo before study entry.

Detection of MAGE-A antigen expression. Tumor core biopsies were obtained from patients. Expression of *MAGE-A* was studied by reverse transcription-PCR. Briefly, RNA was extracted using RNeasy Mini kit (Qiagen) and converted to cDNA using Superscript II Reverse Transcriptase (Invitrogen). *MAGE-A* expression and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase were analyzed using specific primers (Supplementary Table S2).

Isolation of peripheral blood mononuclear cells from peripheral blood. For generation of DC vaccines, patients underwent 1 to 4 venesections of 250 to 350 mL of whole blood. Research bloods were drawn serially, at each vaccination time point and at months 5, 6, 7, and 12. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation. The PBMCs were either cryopreserved or used immediately for DC vaccine production (see next section) or Treg isolation and detection (see Molecular Treg analysis section below). The resulting plasma was stored in aliquots at -80°C.

Preparation and administration of DC vaccine. Vaccine production was done under strict Good Manufacturing Practice conditions. Immature DCs were generated from PBMCs by a 5-d culture and then pulsed with allogeneic cell lysate prepared from patented melanoma cell line DDM-1 as previously described (15). A cytokine cocktail described by Jonuleit et al. (10) was added on day 6 to initiate DC maturation. On day 8, nonadherent cells were harvested. An aliquot of 3 to 5×10^6 cells was used for immediate vaccination and remaining cells were cryopreserved in 3 to 5×10^6 cell aliquots. Quality control of DCs consisted of sterility tests, phenotyping, and viability testing by trypan blue staining. Each vaccine was prepared as described previously and administered intradermally on the inner thigh (15).

Response and toxicity evaluation. Serial computed tomography (CT) scans were done at baseline, after second, fourth, and sixth vaccinations, and on months 6, 7, 8, and 12, and then on physicians' judgment. CBR was determined after the second vaccination. Duration of response was measured from documentation of response to date of disease progression. Time to progression was measured from time of registration to the date of CT scan showing disease progression. Overall survival was measured from time of registration to death. Toxicity was monitored and graded according to the National Cancer Institute Common Toxicity Criteria (version 3.0).

DTH test. DTH tests were done with tumor lysate–pulsed autologous DCs and unpulsed DC controls, 0.5×10^6 cells each, at the fifth and tenth vaccination. Skin induration diameters were measured 48 h later. Positive reaction was defined *arbitrarily* as ≥5 mm induration on the lysate-pulsed DC arm and ≥3 mm larger than the control DC arm.

Immunophenotyping. DC vaccines were phenotyped by gating cells of high size and granularity in the forward/sideward scatter plot and using anti-human antibodies: CD14-FITC (Dako, Denmark), CD1a-PE, CD83-PE, and HLA-DP,DQ,DR-FITC (BD), and CD86-PE and CCR7-FITC (R&D systems). DC vaccine purity was determined by assessing the percentage of CD83$^+$HLA-DP,DQ,DR$^+$ cells. CD4$^+$CD25^{Hi} and CD4$^+$Foxp3$^+$ Treg were assessed using anti-human CD4-FITC and CD25-PE antibodies (BD). CD4$^+$CD25^{Hi}CD127$^{-/Lo}$Foxp3$^+$ Treg were assessed using anti-human CD4-APC, CD25-FITC, and CD127-Cy7 antibodies (BD). Negative controls were fluorochrome-conjugated isotype-matched irrelevant antibodies (Dako). Intracellular staining for Foxp3 was carried out using PE Anti-Human Foxp3 Staining Set and rat IgG2a-PE isotype control antibody (Ebioscience) according to manufacturer's instructions.

Molecular Treg analysis. Tregs were isolated using the CD4$^+$CD25$^+$ Regulatory T-Cell Isolation kit and MACS Separation Columns according to manufacturer's instructions (Miltenyi Biotec). Briefly, CD4+ cells were isolated from PBMCs by negative selection, and then incubated with anti-CD25 microbeads. The CD4$^+$CD25$^+$ fractions were then positively selected over magnetized columns.

RNA from CD4$^+$CD25$^+$ T cells was isolated using RNeasy kit (Qiagen) and first-strand cDNA was generated by Superscript III Reverse Transcriptase (Invitrogen,) according to manufacturers' recommendations. Primers for GITR and housekeeping gene β-actin were designed using Primer Express Software version 3.0 (Applied Biosystems). Primers for Foxp3 were described previously (Supplementary Table S2; ref. 16). Gene expression was analyzed by quantitative PCR using SYBR Green PCR Master Mix and ABI 7500 Fast Real-time PCR System (Applied Biosystems). Actin was used for normalization for each sample. Relative fold change of target cDNA was determined using $\Delta\Delta Ct$ method.

Plasma protein analysis. Proteins in patients' prevaccine and postvaccine plasma samples were profiled using RayBio L-Series 507 Biotin Label–based Antibody Array system (RayBiotech). Fifty-six plasma samples from 18 patients, collected at prevaccination and at months 2 and 4 postvaccination, were analyzed. Normalization was done using the signal of internal controls on each protein array chip. Quality control was done with the removal of proteins detected below the raw signal intensity of 50, which is twice the maximum intensity of the negative control probes. Significance testing was done by *t* test and fold change was cut off at 1.5. To validate data from the L Series 507 array analysis, selected proteins from prevaccine and postvaccine signatures were quantified using multiplex sandwich ELISA arrays (Custom Quantibody Array, Raybiotech, Inc.). Plasma IL-20 levels were validated by ELISA using a matched antibody pair and recombinant human IL-20 (R&D Systems) according to the manufacturer's instructions. All samples were run in triplicates and the analysis was repeated at least once for every sample. The lower limit of detection for each marker was determined based on the average raw data of two sets of standard curves and from the average of four negative controls and their SD (by calculating average + 2× SD).

Statistical analysis. The 95% confidence interval (CI) of proportion of CBR was estimated based on Wilson method (17). Survival curves were estimated by Kaplan-Meier method and the difference between curves was tested by log-rank test statistics. QoL data were analyzed based on the guidelines for assessing QoL in European Organization for Research and Treatment of Cancer clinical trials. No imputation was carried out for missing values. Difference of QoL between baseline and each cycle was done by paired *t* test. Exploratory analyses of changes in Treg variables between responders and nonresponders were done using the Wilcoxon rank sum test. These analyses were done in SAS 9.2 and $P \leq 0.05$ was considered significant. For the plasma protein data from antibody array analysis, unpaired *t* test was performed. Change in CD4$^+$CD25^{Hi}CD127$^{-/Lo}$Foxp3$^+$ with time was analyzed using multi-level model in MLwin 2.02. The differences in serum protein levels between responders and nonresponders at specific time points (prevaccination, month 2, and month 4) were analyzed by the Wilcoxon rank sum test and *t* test. Statistical analyses for Quantibody Array and ELISA results were done in STATA 9.1 and $P \leq 0.05$ was considered significant.

Results

Patients. Between October 2005 and November 2006, 29 patients were screened and 26 patients underwent tumor core biopsy for *MAGE-A* gene detection. Twenty-five patients expressed at least one *MAGE* gene and 20 patients (9 males and 11 females) were eventually accrued. Their median age was 64 years (range, 33-77 years). Four of the 20 patients had received prior radiotherapy and 18 patients received prior palliative chemotherapy, with a median of two chemotherapy regimens (range, 0-5) and 2.5 chemotherapy agents (range, 0-5) used. All patients were Asians and had documented progression of colorectal cancer on CT tomogram before accrual (Table 1).

The most common *MAGE-A* gene expressed among the 26 patients screened was *MAGE-A4* (69.2%), followed by *MAGE-A12* (57.7%), *MAGE-A1* (53.8%), *MAGE-A3* (34.6%), *MAGE-A10* (19.2%), and *MAGE-A6* (15.4%). Among the 20 accrued patients, this ranking remained consistent (Supplementary Table S3). Seven of the 20 expressed 1 *MAGE-A* gene (35%), 5 expressed 2 genes (25%) and 8 expressed 3 or more genes (40%).

Vaccine administration. A total of 161 vaccines were administered and 12 patients completed the full 10 cycles of

vaccinations (mean, 4.17×10^6 cells per vaccine). The median dose per patient was 38.57×10^6 cells (range, $12.81\text{-}47.75 \times 10^6$ cells).

Toxicity and QoL analysis. Treatment was overall well tolerated. Most patients experienced mild grade 1 or grade 2 toxicities (Supplementary Table S4). There were only three nonhematologic adverse events that were possibly related to treatment and were all grade 1 severity: arthralgia, itch over thighs, and skin rash. QoL measurement using global health score was 68.3 at baseline and did not vary much across time.

Tumor response. One patient experienced PR (5%; 95% CI, 1-24%) and seven achieved SD (35%; 95% CI, 18-57%; Table 1) leading to a CBR rate of 40% (95% CI, 22-61%).

Survival. The median OS was 7.4 months (95% CI, 4.5-17.5 months; Fig. 1A). Two patients were still alive at the time of analysis. An exploratory analysis showed that patients with PR and SD had significantly better survival (median, 18.1 months) compared with those with progressive disease (median, 6.2 months; $P = 0.007$; Fig. 1B).

Although the median time to tumor progression was short at 2.4 months (95% CI, 1.9-4.1 months), five patients experienced a prolonged PFS of >6 months (Fig. 1C), and two of them (reviewed below) continued to be progression-free at time of analysis (PFS >27 and >37 months).

Patient 1, a 72-year-old Chinese female, was accrued in January 2006 with disease progression in her bulky, histologically confirmed intra-abdominal lymph nodes. She achieved disease stabilization following 10 DC vaccinations. At month 16 of follow-up, she experienced a late tumor shrinkage of 21%

(not amounting to PR by unidimensional RECIST criteria; Fig. 2A). Her latest CT scan in April 2008 (month 27) continued to show response. She was unwilling for a repeat CT scan in her 2009 follow up but remained well with Eastern Cooperative Oncology Group of 0.

Patient 9 is a 62-year-old female with poorly differentiated rectal carcinoma to the lungs, lymph nodes, and pelvis whose best response to prior capecitabine and oxaliplatin was SD. She achieved a PR of 79 days (2.5 months) after DC vaccination. At month 32, her CT scan continued to show tumor regression (Fig. 2B), and she remains alive and in PR at latest follow-up in March 2009 (month 37).

Lysate-specific response detected by DTH. DTH was done in 15 of 20 patients at fifth vaccination. Positive reactions were seen in eight patients with mean induration diameter of 19.8 mm (median, 16.5 mm; range, 5-45 mm). DTH was repeated in 12 patients at the 10th vaccination. Positive reactions were seen in nine patients, four of whom were tested negative previously. The mean induration diameter was 13.1 mm (median, 10.0 mm; range, 5-25 mm; Supplementary Fig. S1). Thus, a significant higher number of patients were DTH positive than DTH negative at the 10th vaccination ($P = 0.044$, Fisher's Exact test). The apparent increase in number of DTH-positive patients from the 5th (8 of 15) to the 10th vaccination (9 of 12) time point was not significant ($P = 0.68$). Correlative analysis found no correlation between clinical responses (SD/PR, $n = 8$) and DTH responses [frequencies or scores (mm)].

DCs generated expressed matured phenotype. Immunophenotyping of DC vaccines generated confirmed a mature phenotype

Table 1. Patients' characteristics

ID	Age (y)/sex	PS	Site of disease	No. of chemo-regimens	Disease at accrual	No. of vaccinations	BOR	Time to tumor response (mo)	Duration of response (mo)	TTP (mo)	Survival time (mo)
1	72/F	1	LN	1	PD	10	SD	2.7	> 25.0*	> 27.7*	39.7[†]
2	67/F	1	Lung	0	PD	10	SD	2.9	4.2	7.1	35.6
3	53/F	2	Lung, LN, pelvic, bone	4	PD	10	SD	1.7	5.2	6.9	6.9
4	43/F	1	Lung, adrenal, LN	4	PD	3	PD	—	—	2.6	5.9
5	54/M	1	Liver, lung, ascites, LN	3	PD	3	ND[‡]	—	—	> 3.8[‡]	3.8[‡]
6	76/M	0	Liver, peritoneum, pelvic, lung, LN, serosa	3	PD	10	SD	1.8	2.4	4.1	7.6
7	33/F	1	Bone	2	PD	9	PD	—	—	2.0	6.5
8	75/F	0	Lung	0	PD	10	PD	—	—	1.9	13.1
9	62/F	1	LN, lung and pelvic	3	PD	10	PR	2.5	> 35.4*	> 37.9*	37.9[†]
10	73/M	0	Liver, LN	2	PD	10	PD	—	—	2.1	19.6
11	64/M	0	Liver	1	PD	10	PD	—	—	2.1	6.4
12	57/M	1	Lung, liver, LN	2	PD	10	PD	—	—	2.3	7.5
13	65/F	1	LN, pleural, lung, liver	5	PD	5	PD	—	—	1.6	2.9
14	49/M	1	Lung, liver, peritoneum	5	PD	8	SD	2.3	1.2	3.5	7.2
15	72/M	0	LN, pleural, liver, lung	2	PD	4	PD	—	—	1.8	3.2
16	77/M	1	Liver, bone, lung	4	PD	10	SD	1.6	1.9	3.5	13.0
17	75/F	0	Lung, liver	1	PD	10	PD	—	—	1.9	17.5
18	54/F	0	LN, lung	1	PD	10	SD	1.8	4.9	6.7	23.2
19	75/M	0	Lung, liver,	2	PD	3	PD	—	—	2.0	2.9
20	41/F	1	Lung, skin, LN, bone	5	PD	6	PD	—	—	1.9	4.5

Abbreviations: PS, performance status according to Eastern Cooperative Oncology Group; BOR, best overall response; TTP, time to tumor progression; LN, lymph node; F, female; M, male; SD, SD of at least 4 wk; PD, progressive disease; ND, CT scan not done.
*Indicates that the patient has not progressed at the time of analysis.
[†]Patients who are alive at the time of analysis have their survival time censored at the time of last follow-up.
[‡]Patient withdrawn due to poor performance status; survival time was censored at last date in the study.
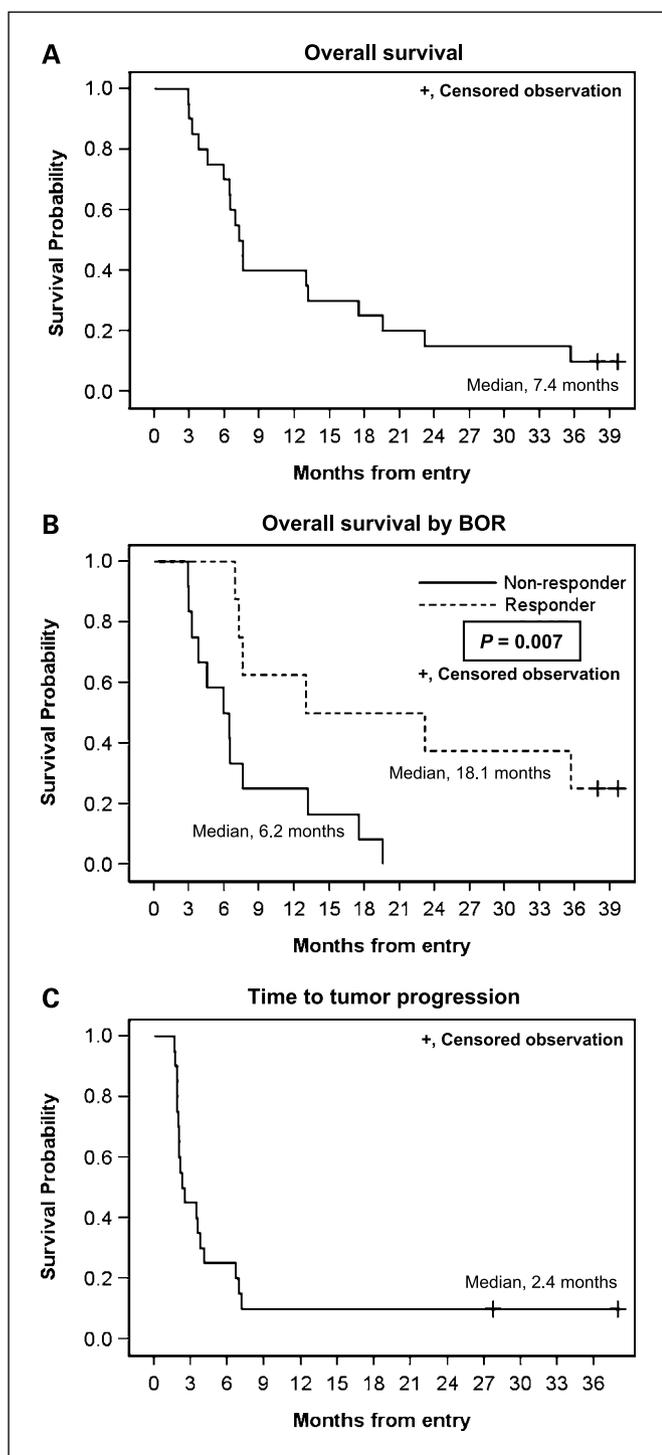
Fig. 1. Kaplan-Meier estimates. *A,* OS. *B,* OS by best overall response (*BOR*). Responder, PR and SD of at least 4 wk; Nonresponder, progressive disease and nonevaluable. *C,* time to tumor progression.

with median percentage of cells expressing HLA-Class II at 99.79%, CD83 at 82.07%, CD86 at 99.76%, CCR7 at 60.24%, CD1a at 38.52%, and low monocyte marker CD14 at 17.08% (Supplementary Fig. S2). The median purity of DCs generated (CD83⁺HLA-DP,DQ,DR⁺) was 80.65% [interquartile range (IQR), 51.14-93.61%].

DC vaccination downregulates peripheral Treg. Downregulation of circulating $CD4^+Foxp3^+$ and $CD4^+CD25^{Hi}$ Treg cells was observed in all 13 evaluable patients by month 2 postvaccination. Analysis for Treg was not available for the other seven as one refused research blood draw and the other six progressed rapidly. The average decrease was significant (Fig. 3A and B). More recently, $CD127^{-/Lo}$ has been shown as an additional marker to define Treg cells (18, 19). We analyzed for $CD4^+CD25^{Hi}CD127^{-/Lo}Foxp3^+$ cells in 12 patients, comprising both responders and nonresponders, for whom sufficient PBMC samples were available. Decrease in this Treg population was seen in 10 patients, and Treg increase was seen in only patients 8 and 11, both nonresponders. Overall, there was significant downregulation of $CD4^+CD25^{Hi}CD127^{-/Lo}Foxp3^+$ T cells between prevaccination and month 4 postvaccination (Fig. 3C). Also, quantitative PCR analysis revealed a downward trend in the expression of Treg markers Foxp3 and GITR from specifically $CD4^+CD25^+$ T cells following DC vaccination (Fig. 3D). We observed no correlation when comparing magnitude of Treg frequency changes with objective responses.

Differential plasma protein profiles identified in vaccinated patients. Using RayBio L-Series 507 Antibody Array that simultaneously detects changes in the levels of 507 proteins, we profiled the plasma proteins of prevaccination and postvaccination (months 2 and 4) samples. We identified 58 and 47 proteins that were significantly modulated at months 2 and 4, respectively, compared with prevaccination. Of these, 28 showed persistent upregulation as they were increased at both postvaccination time points (Supplementary Table S5). We further characterized protein profiles in responders to identify biomarkers potentially correlative with vaccine efficacy. Seven proteins were found significantly modulated at month 2 and five at month 4 postvaccination compared with the prevaccination profile. Two of these (CCL26 and IL-20) were persistently upregulated at both postvaccination time points (Table 2).

By comparing the baseline prevaccination profiles of patients who achieved CBR to DC vaccination to the profiles of nonresponders, we identified distinct patterns that may be used to predict vaccine efficacy. Twenty-eight proteins were found significantly modulated in the plasma of responders compared with nonresponders, of which 26 were upregulated and 2 were downregulated. Several of these proteins have been associated with chemotactic activities, immunomodulation, cell adhesion, and tumor suppression (Table 3).

Selected proteins from the prevaccine signature, including I-TAC (CXCL11), L-selectin (CD62L), monocyte chemotactic protein-1 (MCP-1), and soluble gp130 (sgp130), were quantitated using a multiplex sandwich ELISA system to validate data from the large-scale antibody array analysis. Consistent with the antibody array results, plasma samples from vaccine responders had higher levels of these four proteins compared with the nonresponders (Fig. 4A). Only the mean sgp130 level in responders (113.51 ng/mL) versus nonresponders (76.09 ng/mL) was found to be statistically significant (t test, $P = 0.046$).

IL-20 levels in plasma were quantitated by ELISA to validate our findings from the antibody array analysis and to further evaluate IL-20 trends in responders versus nonresponders. The difference between these two groups was evident even at prevaccination (Fig. 4B), with only 2 of the 10 nonresponders

showing detectable IL-20 in their plasma (limit of detection, 0.4 ng/mL), whereas a much higher proportion of the responders, 5 of 8, had detectable IL-20 in their plasma. After DC vaccination, plasma IL-20 levels decreased in the two nonresponders but increased in four of the five responders. The median concentrations in responders versus nonresponders were 2.4 versus 0 ng/mL at prevaccination, 2.1 versus 0 ng/mL at month 2, and 4.8 versus 0 ng/mL at month 4. In the nonresponders, the IQR of IL-20 concentration at all three time points were 0 to 0 ng/mL. As such, the rare IL-20–positive results in nonresponders were all considered as outliers in the statistical analysis. In addition, mean plasma IL-20 levels were consistently higher in responders compared with nonresponders at prevaccination (6.9 versus 1.79 ng/mL, $P = 0.07$), month 2 (8.17 versus 1.20 ng/mL, $P = 0.03$), and month 4 (8.85 versus 1.79 ng/mL, $P = 0.06$).

Discussion

Our DC vaccine trial used mature autologous DCs pulsed with an allogeneic MAGE-containing MCL. The melanoma cell line, DDM-1, was generated from a patient selected for high levels of CTL-mediated cytotoxicity against autologous tumor and against other HLA class I–matched MAGE-positive tumors. The patient had long postsurgical disease-free interval of several years. Our DC preparations showed overall high expression of CCR7 (>60%) and CD83 (>82%). MAGE-A–expressing metastatic colorectal cancer patients with prior progressive disease achieved a competitive CBR of 40%. Although patients with single metastatic sites in either lung or nodal regions tended to have more durable responses (patients 1, 2, and 9), SD was also attained in patients with bulky multiple metastases (patient 6). Five patients notably remained progression-free for over 6 months and two patients with significant tumor burden (patients 1 and 9) are still progression-free for over 27 and 37 months, respectively. We recognize that adopting the primary end point of CBR using RECIST criteria has limitations. Nevertheless, we did evaluate PFS and OS as secondary end points, which may better reflect true vaccine efficacy (20). We focused our attention on the PFS curve, whose long tail suggests that there is likely a subgroup of patients who may benefit disproportionately from vaccine therapy.

Fig. 2. *A,* patient 1 CT scans. This patient was assessed as having SD by RECIST criteria despite tumor shrinkage. *B,* CT scans of patient 9 who has PR. The right upper lobe mass decreased in size and the right upper paramediastinal mass (*arrows*) underwent cavitary necrosis.
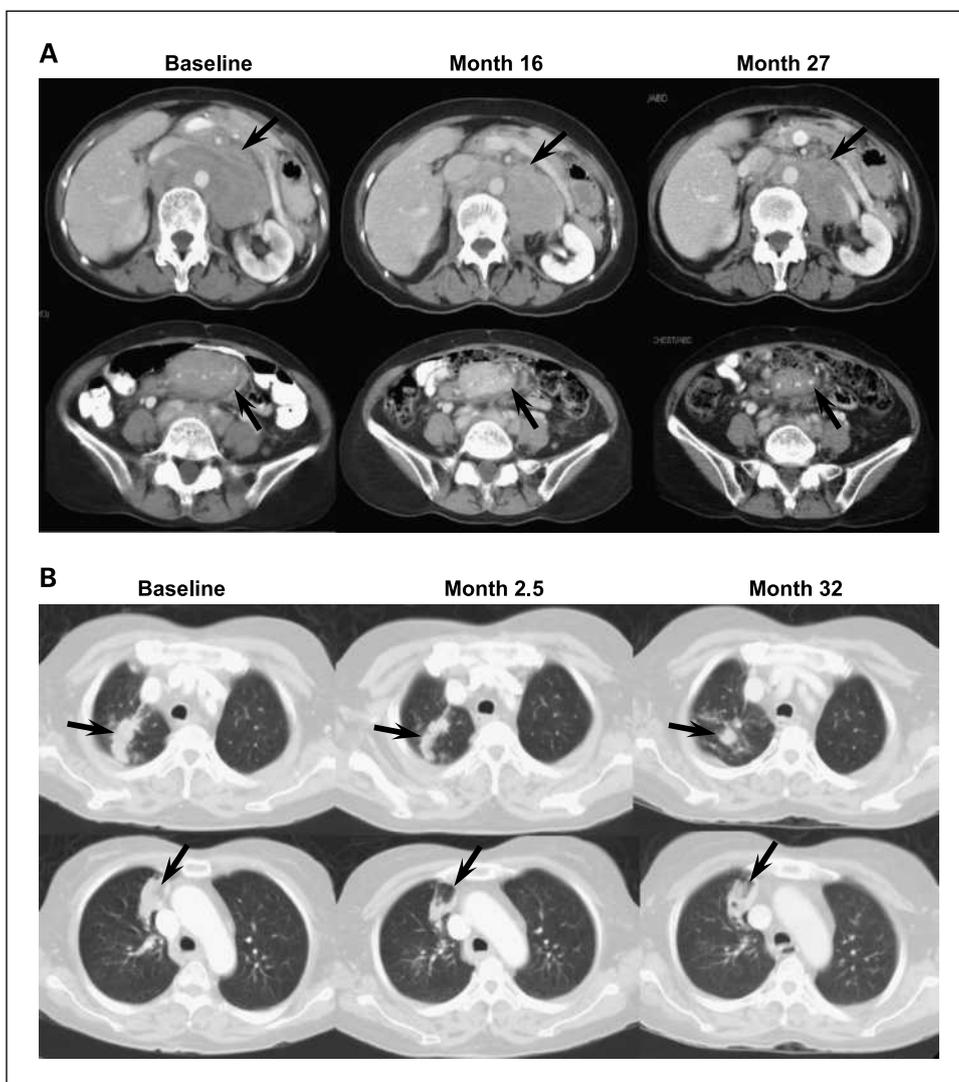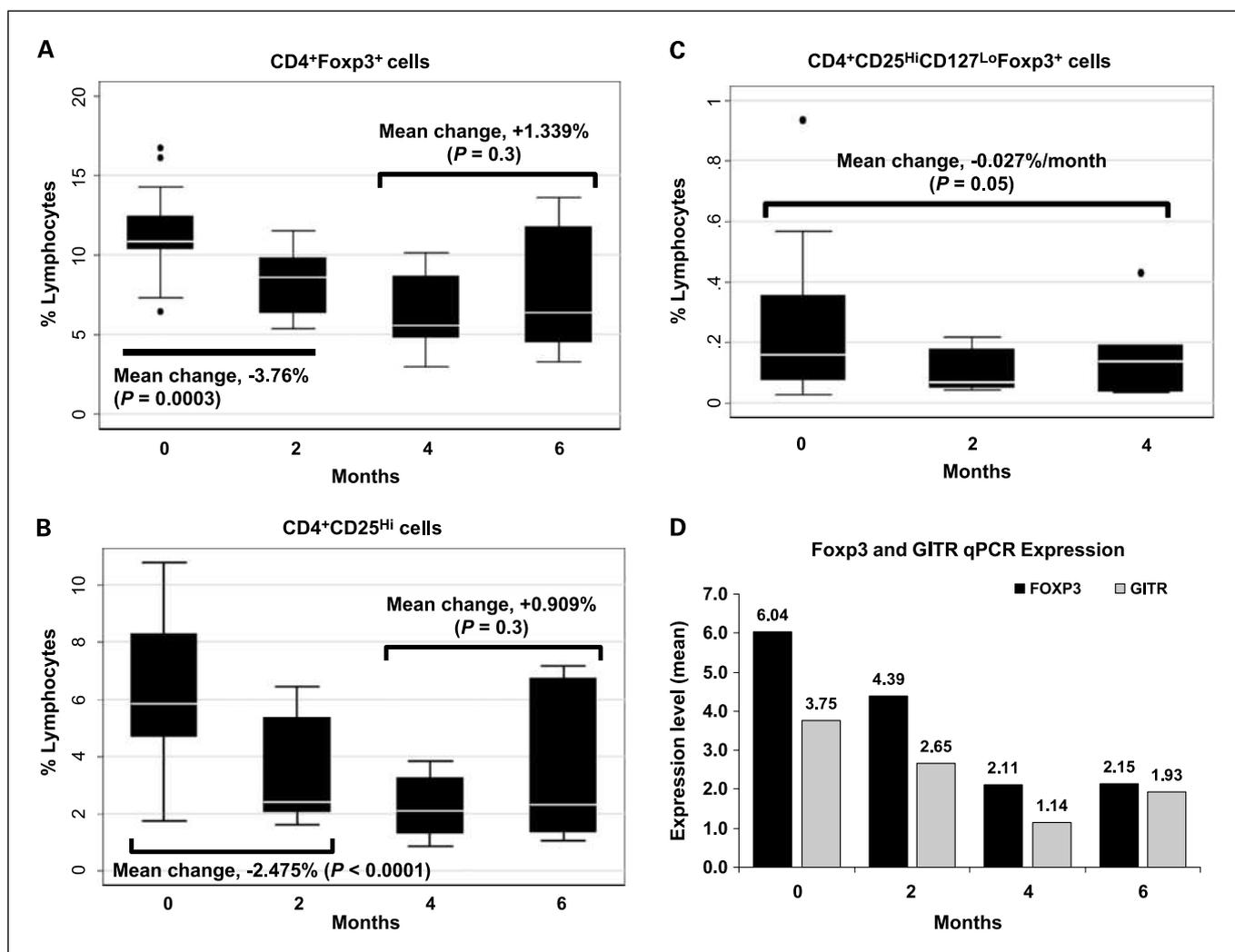
Fig. 3. Modulation of peripheral regulatory T cells. Box plots summarizing Treg (*% lymphocytes*) analysis by flow cytometry at each time point. *A*, CD4+Foxp3+, (*B*) CD4+CD25^{Hi}, and (*C*) CD4+CD25^{Hi}CD127$^{-/Lo}$Foxp3+ cells were significantly downregulated postvaccination. *D*, quantitative change in Foxp3 and GITR mRNA levels in CD4+CD25+ cells showing a downward trend (1 mo = 4 wk).

Cancer vaccine trials are under way combining vaccine with chemotherapy and/or radiotherapy.[5] Chemotherapy and radiotherapy may act synergistically with vaccines through mechanisms that include increasing the range and dose of antigens for presentation, improving T-cell traffic into the tumor, enhancing CTL killing through upregulation of death receptors or Fas ligand, and also augmenting cellular danger signals (21–23).

A meta-analysis of 32 cancer vaccine clinical studies in patients with advanced colorectal cancer reported a CBR in 11.2% of patients and an overall response rate (complete response and PR) of 0.9%. The defined clinical benefit rate (complete response, PR, SD, or mixed response) was observed in 17% (12 of 70) of colorectal cancer patients who received DC vaccines, including one pulsed with MAGE-3 peptide (2, 24). A recent Danish trial using a allogeneic melanoma lysate–pulsed DC vaccine similar to ours achieved 4 SD among 20 advanced colorectal cancer patients (25). However, their DC production

method was different, and used only TNF-α as DC maturation stimulus, and their colon cancer patients were not selected for MAGE-A antigen expression, whereas the DC production method adopted in this study had been further optimized to generate cells of more mature phenotype and higher proportion with lymph node–homing capability when formally analyzed against the DC quality of the Danish study.[6]

In addition to the six MAGE-A antigens, the MCL used in our study also contains other TAAs. Therefore, DTH test was adopted to assess lysate-specific response following DC vaccination. Significant lysate-specific DTH reactivity occurred in most patients only after 10 vaccines, indicating that increasing and repeated vaccination frequency may be important in activating lysate-specific T-cell immunity (20).

In many solid tumor microenvironments characterized by an immunosuppressive milieu, the immature myeloid DC is a key player in generating Treg and inducing T-cell deletional

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[5] ClinicalTrials.gov (http://www.clinicaltrials.gov/).

[6] Unpublished data.

Table 2. Significantly modulated plasma proteins (*P* < 0.05) in responders, comparing month 2 and month 4 postvaccination profiles with prevaccination profiles

Name of protein	Fold change*	*P*
Month 2		
Eotaxin-3/CCL26	2.40	0.004
Fas/TNFRSF6	2.26	0.028
IFN-γ	1.92	0.032
IL-17	1.72	0.027
IL-20	2.32	0.044
MIG	2.05	0.026
TNF-β	2.00	0.030
Month 4		
Eotaxin-3/CCL26	1.92	0.026
IL-15 R α	1.82	0.018
IL-17B	4.03	0.000
IL-20	2.70	0.043
TMEFF1/Tomoregulin-1	-3.25	0.041

*A positive value indicates an increase and a negative value indicates a reduction relative to prevaccination.

tolerance that contributes toward tumor progression (7). Interestingly, in one study, Foxp3$^+$ Treg density in the normal colonic mucosa prognosticated for a significantly poorer survival in a large series of colon cancer patients, whereas intratumoral Foxp3$^+$ Treg prognosticated for a favorable survival (13).

Recent cancer vaccine studies showed paradoxically either an increase or decrease in Treg in human patients following vaccination (26, 27). Our analyses for CD4$^+$CD25^{Hi}, CD4$^+$Foxp3$^+$, and CD4$^+$CD25^{Hi}CD127$^{-/Lo}$Foxp3$^+$ Treg at pre- and post-DC vaccination, supported by quantitative PCR results for Foxp3 and GITR transcriptome expression in isolated CD4$^+$CD25$^+$ T cells, showed that Treg levels in the present study decreased significantly following DC vaccination. The CD4$^+$CD25^{Hi}CD127$^{-/Lo}$Foxp3$^+$ Treg levels showed an increase in only two patients, who had rapid progression. However, we did not observe a clear correlation between DTH or CBR with Treg reduction. In contrast, a recent clinical study showed Treg reduction to a specific *in vivo* antimelanoma immune response in melanoma patients receiving an allogeneic cell lysate–pulsed autologous DC vaccination (28). Although the actual mechanism of Treg reduction postvaccination remains unclear, it may result from mature DC-T lymphocyte interactions in the lymphohematopoietic compartment (29). This observed effect has important implications for tumor immunotherapy, although conclusive benefits of Treg reduction following cancer vaccination will need to be derived from randomized clinical trials.

The novel antibody array analysis revealed a significant number of proteins increased in prevaccination profiles of responders relative to that of nonresponders. The CXC chemokine, IFN-γ–inducible T-cell α chemoattractant (I-TAC), is a chemotactic agent for CXCR3-expressing effector T cells, promoting transendothelial migration during an IFN-γ dominant immune response (30). The chemokine MCP-1 is a chemoattractant for monocytes where in a breast cancer vaccine study, high serum MCP-1 correlated with more favorable clinical outcome (31). L-selectin (CD62L) is expressed by most leukocytes and is rapidly shed upon activation (32), suggesting that lower plasma

L-selectin levels may indicate an impairment in immune activation. sgp130 is a soluble circulating receptor of IL-6 that antagonizes IL-6 biological activity (33). IL-6 is known to play an important role in cancer progression (34). Therefore, a higher level of sgp130 may reflect slower tumor growth kinetics. Overall, the data suggests a baseline profile more favorable to developing an effective antitumor immune response and that opposes tumor growth in the responders. Validation by ELISA confirmed the trends detected by the array. However, because of small sample size, only sgp130 upregulation in the responders was statistically significant. These results will need to be further validated in a larger patient cohort.

In the responders only, IFN-γ, IL-17, Fas, IL-20, TNF-β, CCL26 (eotaxin-3), and MIG (CXCL9) were significantly elevated by midvaccination. These molecules might be significant in antitumor immunity by direct cytocidal mechanisms or through attracting activated T and natural killer cells (35–42). IL-17 is a key cytokine for Th17 cells, known to be inversely related to Treg, and has been shown to inhibit tumor growth (36). IL-20 has been shown as an inhibitor for cyclooxygenase-2 expression, whereas CCL26 is a chemokine that activates eosinophils (39, 41). MIG is a chemokine associated with T-cell infiltration in cancers and promotes tumor necrosis (42). In a recent study in colorectal cancer, MIG (CXCL9) was found to be coexpressed with Guanylate binding protein-1, a chemokine

Table 3. Differentially expressed plasma proteins (*P* < 0.05) between prevaccination profiles of responders and nonresponders

Name of protein	Fold change*	*P*
CXCL14/BRAK	2.92	0.022
CXCR2/IL-8 RB	2.42	0.029
Decorin	2.28	0.022
Dtk	2.41	0.041
ErbB4	4.03	0.025
Erythropoietin	3.78	0.013
FGF-9	2.93	0.029
FGF-13 1B	2.32	0.020
Follistatin	4.88	0.041
Follistatin-like 1	3.03	0.017
IFN-β	2.55	0.046
IL-1 sRII	3.73	0.034
IL-4 R	-3.04	0.016
IL-15 R α	2.69	0.038
Inhibin B	6.10	0.022
Insulin R	6.67	0.037
I-TAC/CXCL11	2.26	0.023
Kremen-2	2.28	0.035
LIGHT/TNFSF14	2.65	0.049
L-Selectin (CD62L)	6.51	0.011
Lymphotactin/XCL1	4.77	0.008
Lymphotoxin β/TNFSF3	3.69	0.046
MCP-1	3.39	0.025
MMP-16/MT3-MMP	-3.09	0.026
sgp130	2.14	0.017
TFPI	2.76	0.022
Thrombospondin-2	2.21	0.043
TMEFF1/Tomoregulin-1	2.59	0.008

*A positive value indicates an upregulation and a negative value indicates a downregulation in responders relative to nonresponders.
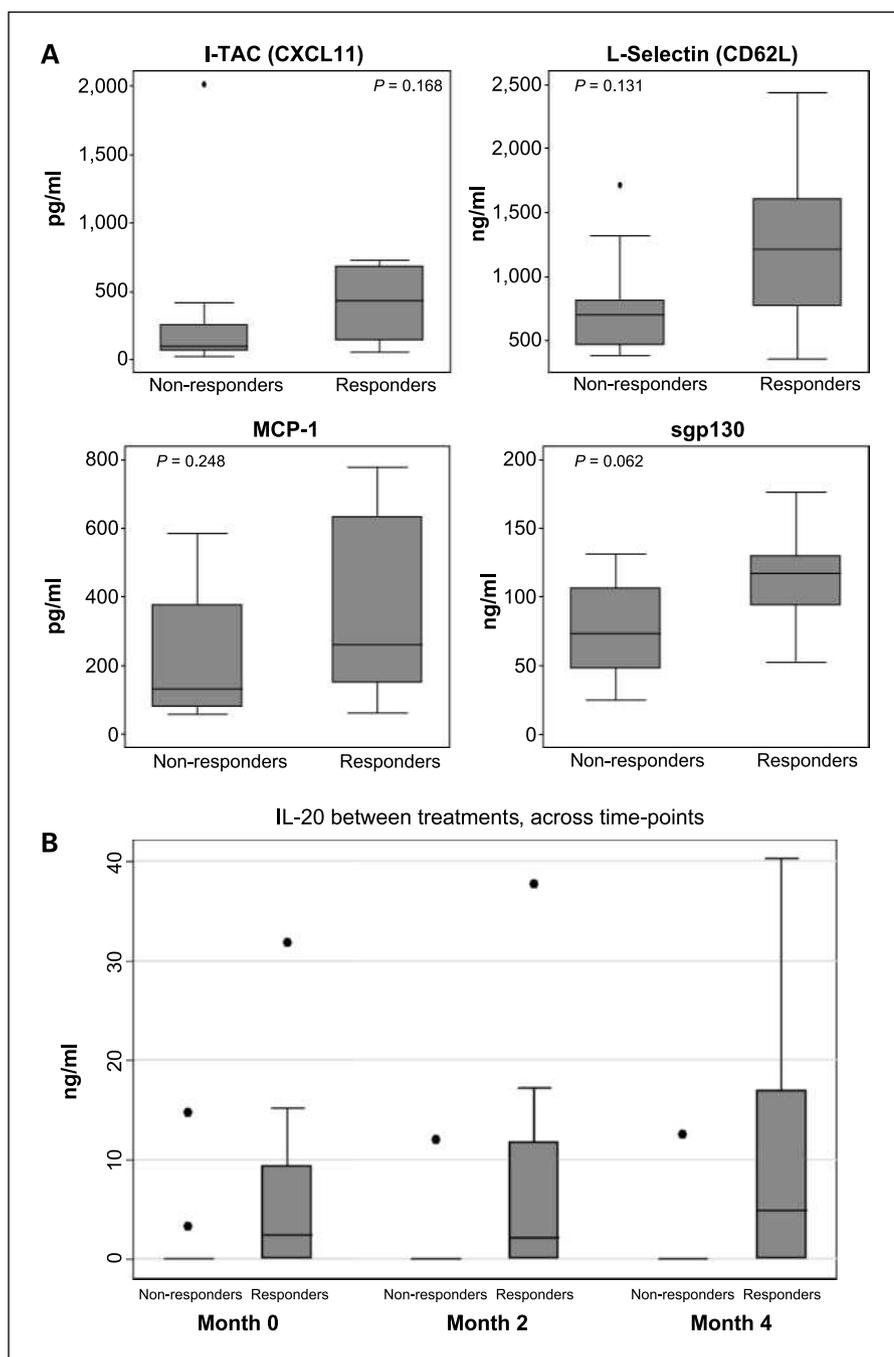
Fig. 4. Validation of plasma protein expression levels by ELISA. *A,* prevaccination plasma I-TAC, L-selectin, MCP-1, and sgp130 concentrations in responders and nonresponders. Quantitation by multiplex sandwich ELISA analysis (Quantibody Array). *B,* plasma IL-20 concentration in vaccinated patients. Quantitation by ELISA showed increasing IL-20 trend in responders following vaccination. Responders also had consistently higher levels of IL-20 compared with nonresponders (6.8-fold higher at month 2, *P* = 0.03). In the absence of outliers (MCP-1 and sgp130), the cutoff points were the maximum and minimum values of the range. In the presence of outliers (I-TAC, L-selectin, and IL-20), the cutoff points were 1.5 × IQR above 3rd quartile (upper limit) and 1.5 × IQR below 1st quartile (lower limit).

that plays an important role in a Th-1 angiostatic immune reaction, and that may be a biomarker of favorable outcome in colorectal cancer (43). At later vaccinations, the cytokine protein profile of these clinical responders still showed sustained upregulation of IL-20 and CCL26, but now also showed a significant upregulation of IL-17B, a critical inducer of TNF-α (44).

IL-20, a member of the IL-10 family of cytokines, is a proinflammatory and prokeratinocyte proliferation cytokine (45). Maturing DCs, and to a lesser extent matured DCs, are major source for IL-20 and may promote innate immunity (46). IL-20 enhances the proliferation of anti-CD28 antibody–induced T cells (47) and inhibits cyclooxygenase-2 expression, which has been implicated in the immunosuppressive function of reg-

ulatory T cells on effector cells (39, 48). IL-20 also showed paradoxically both proangiogenic and antiangiogenic effect in separate studies (39, 49). Still, it is also plausible that the IL-20 increase in DC vaccine responders and decrease in nonresponders may be an epiphenomenon of a nonspecific immune or inflammation-mediated event than a potential biomarker of a specific and functional vaccine-mediated immune response.

As of 2006, there were a total of eight clinical DC vaccination studies in patients with metastatic colon cancer, all with peptide-pulsed DC (2, 24). The clinical activity of this present DC-based vaccine might reflect its polytopic nature, its allogenic adjuvant-like components, the quality of the DC preparation (i.e., high uniform expression of CD83, CD86, HLA class II, and CCR7),

the intradermal route of vaccine injection securing optimal lymph drainage to regional lymph nodes, the presence of MAGE expression in both patients and vaccine, and the increased frequency of delivery (ten injections). In support of the vaccine activity presented here, we and others (50) have shown that lysate and peptide-pulsed DCs induce HLA-restricted helper and cytotoxic T-cell responses *in vitro* against a variety of HLA-matched MAGE expression tumor cell lines.

In conclusion, 8 of 20 end-stage CRC patients with progressive disease vaccinated with autologous DC pulsed with an allogenic MAGE containing tumor lysate showed CBR and durable disease control in a few patients. In the course of DC vaccination, Treg levels declined and vaccine-specific DTH levels increased. We believe that this is the first time a comprehensive cytokine protein array analysis is used in a cancer clinical trial. The plasma protein profiling revealed a prevaccination protein signature in DC vaccine responders, including molecules known to contribute to antitumor immunity and tumor growth kinetics—a signature potentially of use in future clinical trials for enrichment of patients to maximize therapeutic efficacy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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