Active Immunotherapy with Flt3-Ligand Mobilized Peripheral Blood Dendritic Cells Loaded With Carcinoembryonic Antigen Peptide in Patients with Metastatic Malignancies

Michael A. Morse, MD*
Timothy M. Clay, PhD†
Amy C. Hobeika, PhD†
Stephen Chui, MD∗
Paul J. Mosca, MD, PhD‡
Dania Caron, MD‡
H. Kim Lyerly, MD†§

*Department of Medicine, †Department of Surgery, Duke University Medical Center, Durham, North Carolina
‡Amgen Corporation, Seattle, Washington
§Departments of Immunology and Pathology, Duke University Medical Center, Durham, North Carolina

KEY WORDS: cancer vaccine, mobilization, immune assays.

ABSTRACT
Background: Dendritic cells (DC) loaded with tumor antigens induce immune responses in some cancer patients. However, the most commonly used method for obtaining clinical grade DC requires in vitro generation over 7 days in media containing cytokine, increasing the complexity and cost of the cellular vaccine product. The cytokine Flt3-ligand (FL) increases peripheral blood DC numbers in humans permitting an alternative approach to obtaining an adequate number of clinical grade DCs. We sought to evaluate the safety and immunogenicity of immunizations with FL-mobilized DC loaded with the tumor antigen CEA.

Methods: Patients with CEA-expressing cancers received FL 20 μg/kg/d SQ x 10 d and were then leukapheresed to obtain peripheral blood mononuclear cells enriched for DC (FL-DC). After maturation overnight, FL-DC were coadministered (1 x 10⁷ cells) as a combined ID and SQ injection with a class I peptide fragment of CEA, hepatitis B core antigen, tetanus toxoid or KLH protein every 3 weeks for four immunizations.

Results: We found that FL and FL-DC immunizations were well tolerated. Following FL, the CD11c+CD14− DC
population within the PBMC increased (1.5 ± 1.4% to 8.5 ± 4.2%). After overnight maturation, 10 to 20% of these DC expressed the maturation marker CD80. Antigen-specific DTH reactivity was observed at injection sites in all but 1 patient. Antigen specific immune responses were detected in the peripheral blood of 4 of 6 patients by intracellular cytokine staining, ELISPOT, proliferation, or tetramer analysis. Among the 6 patients who completed the immunizations, there was 1 with stable disease and 5 with progressive disease. Immunization with mobilized dendritic cells loaded with tumor antigens appears feasible and induces low levels of immune response.

**INTRODUCTION**

In recent years, the search for clinically effective cancer vaccines has centered heavily on delivering tumor antigens along with dendritic cells (DC) or with adjuvants that attract dendritic cells to the injection site. Dendritic cell-based vaccines have demonstrated promising results in a variety of tumors, but require further optimization to improve their efficacy. Currently, clinical grade DC are generated over 5 to 14 days from peripheral blood mononuclear cells (PBMC) or CD34+ hematopoietic progenitors in media supplemented with cytokines such as GM-CSF, IL-4 or IL-13, and TNF-alpha. These methods, although yielding adequate numbers of DC, are expensive, labor intensive, and involve many steps during which serious errors or contamination could occur. Furthermore, the cells produced are different in a number of ways from blood DC (including their requirement for additional cytokines in order to mature). Because of the challenges in generating DC vaccines, procedures that optimize numbers of clinical grade DC are desirable. Mobilization of DC into the peripheral blood is one method to increase the numbers of DC available for vaccine development.

Flt3L, a cytokine that stimulates hematopoietic stem cells, has more recently been found to mobilize monocytes and DC into the peripheral blood of mice and humans 10 to 40 fold. In addition, Flt3L may stimulate the immune response to systemically administered antigens. We hypothesized that Flt3L would mobilize sufficient numbers of DC into the peripheral blood to exploit in a vaccination strategy.

The choice of an antigen for a vaccination strategy is complex. Antigens may be delivered to DC as peptides, proteins, DNA or RNA, with viral vectors, or tumor lysates or whole tumor cells. We have chosen as an index antigen, the HLA A*0201-restricted epitope of carcinoembryonic antigen (CEA) (CEA<sub>405-413</sub>) called CAP-1, because CEA is broadly expressed in a variety of malignancies and because this epitope is recognized by cytolytic T lymphocytes (CTL). We hypothesized that immunization of cancer patients with FL-DC loaded with CAP-1 would be both safe and feasible, and would induce CAP-1 specific immune responses. While we were performing our study, Fong and colleagues reported the safety of intravenous administration of Flt3-L-mobilized DC loaded with a heteroclytic variant of CAP-1 called CAP-1(6D). This paper reports upon our study focusing on intradermal administration of FL-DC.

**METHODS**

**Patients**

Patients were recruited from the medical and surgical oncology clinics of Duke University Medical Center and provided signed informed consent approved by the Duke University Medical Center Institutional Review Board before enrollment. They were required to be immune type HLA...
A*0201, have a histologically confirmed metastatic cancer expressing CEA as defined by immunohistochemical analysis, and have adequate hematologic (WBC >4.0), renal (Cr <2.0), and hepatic function (Bilirubin <1.5). Patients were excluded if they had had chemotherapy, radiation therapy, or immunotherapy within the prior 4 weeks, 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. The study was intended to enroll up to 18 patients, but during its conduct, FL became unavailable due to a pending business merger and therefore, the study was terminated at 6 patients.

**Patient Treatment and DC Vaccine Production**

A preliminary 2-hour leukapheresis was performed to obtain mononuclear cells for immunologic analysis. Patients then self-administered FL (20 µg/kg/d, max 1500 µg/d) subcutaneously for 10 days to mobilize DC into peripheral blood (FL-DC), followed by a second leukapheresis (2-4 hours, processing 7-12 liters of blood). The leukapheresis product was separated by density gradient centrifugation over Ficoll in a Cell Separator (Cobe BCT, Inc., Lakewood, Colo) to obtain peripheral blood mononuclear cells (PBMC) containing the FL-DC.

In the first 2 patients, the FL-DC were incubated overnight in serum free media to induce spontaneous maturation, then mixed with 20 µg/mL CAP-1 peptide (YLSGANLNL) (produced by Enzyme Systems Products, Livermore, CA) for 2 to 4 hours, were washed, and cryopreserved in autologous plasma/10% DMSO. These patients later received CAP-1 peptide loaded FL-DC, 100 x 10^6 total cells IV and 1 x 10^6 total cells ID every 3 weeks for 4 injections.

In the next 4 patients, the FL-DC (1 x 10^7 total cells) also incubated overnight to induce spontaneous maturation, then mixed with CAP-1 500 µg and administered as 1 x 10^6 cells (in a volume of 100 µL) intradermally and 9 x 10^6 cells (in a volume of 900 µL) subcutaneously every 3 weeks for 4 immunizations. Controls consisted of FL-DC mixed with hepatitis B core peptide (HBc) (FLPSDFPPSV) (Enzyme Systems Products (500 µg), Tetanus toxoid (TT) (4 Lf), and/or KLH (100 µg).

**Skin Testing (DTH)**

At least one of: Candida (1:1000), Mumps (undiluted), Trichophyton (1:1000), tetanus toxoid (0.1 mL of 1.6 Lf/mL), and CAP-1 peptide (4 µg), HBc peptide (4 µg), and KLH protein (10 µg) were injected intradermally prior to the FL and after all the immunizations. The diameter of erythema and induration was measured 48 hours afterwards. Similarly, the diameter of erythema and induration was measured at the FL-DC injection sites.

**Immunologic Assays**

Fresh PBMC were analyzed before and after FL, 1 week following the first injection, before the second and third injections, and after the last injection of FL-DC in the following assays:

**Proliferation Assay**

PBMC were incubated with CAP-1, HBc peptide, TT, or KLH at various dilutions for 96 hours after which proliferation was measured by ³H-thymidine incorporation. For each sample, PBMC were cultured at 1 x 10^5 cells per well of a 96 well plate in the presence of tetanus toxoid, preservative-free recombinant hepatitis B surface antigen (HBsAg; kind gift from Merck Research Laboratories, West Point, Pa), phytohemagglutinin (PHA; Sigma Chemicals, St. Louis, Mo) or medium alone for 4
days. The following antigens were assayed in triplicate in a total volume of 200 µL: no antigen; CAP-1 at 0.04, 0.2 or 1 µg/mL, HBe at 0.04, 0.2 or 1 µg/mL; TT (preservative-free; Connaught Laboratories Willowdale, Ontario, Canada) at 1, 5, or 25 µg/mL; KLH at 1 or 5 µg/mL, and PHA at 5 µg/mL. After 4 days, 3H-thymidine (1 µCi; NEN Life Science Products, Boston, Mass) was added to each well for 18 to 20 hours and the DNA was harvested onto filter paper using a TOMTEC automated cell harvester (Wallac, Turku, Finland). 3H-thymidine incorporation was determined using a MicroBeta Plus Scintillation counter (Wallac). Data are presented as mean stimulation index (S.I.), which was determined by subtracting the mean cpm of media alone wells from cpm of wells incubated with antigen divided by the mean cpm of media alone.

**Intracellular Cytokine Staining**

Ficolled PBMC (2.4 x 10^6 PBMC in 2 mL media) were stimulated with 1 µg/mL CAP-1, TT at 1, 5, or 25 µg/mL; or KLH, 5 µg/mL pokeweed mitogen (Sigma Chemicals, St. Louis, Mo) or media alone for 4 hours at 37°C in the presence of 1 µg CD28 (BD Biosciences, San Jose, Calif) per 10^6 PBMC. After 1 hour, brefeldin A (5 µg/mL; Sigma) was added, and the incubation was continued for another three hours. The stimulated cells were washed with 1% BSA/PBS, fixed with 1% paraformaldehyde, and permeabilized using FACS Permeabilizing Solution (BD Biosciences). Permeabilized cells were then stained with anti-IFNαFITC, anti-CD69-PE, anti-CD8-PerCP, and anti-CD4-APC for 30 minutes at room temperature. Cells were gated by forward and side scatter for lymphocytes and positively for CD4 or CD8. The percentage of CD4+ or CD8+ lymphocytes that were IFNα double positive was determined by gating on lymphocytes by forward and side scatter and then on CD4+ or CD8+ cells.

**ELISPOT Assay**

Multiscreen-HA 96-well plates (Millipore, Bedford, Mass) were coated overnight at 4°C with 100 µL/well of 10 µg/mL mouse anti-human IFNαMab (diaPharma Group, Inc., West Chester, Ohio) in Dulbecco’s Phosphate Buffered Saline [PBS] (Life Technologies, Gaithersburg, Md). The plates were washed 3 times for 5 minutes each with 150 µL DPBS/well and blocked with 200 µL/well of RPMI-1640, 10% HuABS, 25 mM Hepes, 100 U/mL Penicillin, and 100 µg/mL Streptomycin, and 2mM Glutamine for 1 hour at 37°C in 5% CO₂. PBMC were stimulated with CAP-1, TT, KLH, Pokeweed Mitogen (PWM), or Media in replicates of 6. PBMC were plated at 100,000, 50,000, or 25,000 PBMC/well with either Cap-1, 1 µg/mL, TT, 5 µg/mL; KLH, 5 µg/mL, PWM 2 µg/mL, or media in a total volume of 200 µL/well for 18 to 20 hours at 37°C in 5% CO₂.

The plates were washed with 0.05% Tween/DPBS using the Tecan 96PW plate washer (Tecan, Research Triangle Park, NC). 100 µL of mouse anti-human IFNαMabbiotinylated Mab (diaPharma Group, Inc., West Chester, Ohio) at 1 µg/mL in DPBS were added to each well and the plates incubated for 2 hours at 37°C, 5% CO₂. Vectastain ABC Peroxidase (Vector Labs, Inc., Burlingame, Calif) was added at 100 µL/well for 1 hour at room temperature after washing. 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 ([AEC] Sigma, St. Louis, Mo) reconstituted in an acetate buffer for 4 minutes at room temperature in the dark. Color development was stopped with deionized water.

Basins were removed and the mem-
branes dried overnight in the dark. Membranes were removed using sealing tape (Millipore, Bedford, Mass) and the number of spots per well was determined by color “footprint” on the well membrane indicating the presence of an IFNγ secreting cell. Membranes were read by Zellnet Consulting, Inc. using the KS ELISPOT Automated Reader System with the KS ELISPOT 4.2 Software (Carl Zeiss, Inc., Thornwood, NY).

**Table 1. Patient Characteristics**

<table>
<thead>
<tr>
<th>Patients (n=6)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>Prior chemotherapy</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Primary site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Colorectal</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Metastases sites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Peritoneum</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Performance status</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

**MHC-peptide Tetramer Binding Analysis**

Peptide-MHC tetramer staining was performed on fresh PBMCs using 10⁶ cells per individual tetramer analysis. Cells were washed using 1% BSA/PBS and analyzed by multiparameter flow cytometry. Approximately 25,000 gated events were collected.

Total number of tetramer positive cells per 10⁶ PBMC was determined by gating on lymphocytes by forward and side scatter, positively for CD3 cells and negatively for CD4, 14, and 19 to calculate total number of CD8+/tetramer positive T cells.

**CTL Assay**

In selected patients, we attempted to detect the induction of cytotoxic CEA specific T cells (CTL) in peripheral blood. The mononuclear cells were stimulated to expand the CTL as previously described. Briefly, autologous PBMC were mixed with autologous DC loaded with KLH, TT, CAP-1, or HBC peptide at a stimulator to responder ratio of 1:10 in RPMI-10% FCS (Life Technologies) containing IL-7 (10 ng/mL). After 3 days, IL-2 (20 IU/mL) (Genzyme,
Cambridge, Mass) was added to the cultures. After incubation for 7 to 12 days, the CD8+ lymphocytes were selected using CD8 microbeads (Miltenyi Biotech, Sunnyvale, Calif) as per manufacturers protocol. The CD8+ T cells were tested for CEA-specific cytotoxic activity. Targets were autologous DC loaded with KLH, TT, or CAP-1. A standard europium release CTL assay was performed. Spontaneous release was less than 25% of total release by detergent in all assays. Standard errors of the means of triplicate cultures was less than 5%. Percent specific cytotoxicity was calculated as follows: [(experimental release - spontaneous release)/(total release - spontaneous release)] X 100.

RESULTS
Patient Characteristics and Tolerance of Treatment
The characteristics of the 6 patients are listed in Table 1. All patients had failed prior chemotherapy, but nonetheless retained an excellent performance status at study entry. Four of the six patients had colon cancer metastatic to liver sites; a single breast cancer and a lung cancer patient, respectively, were also enrolled into this study. The FL injections were very well tolerated with mild injection sites reactions infrequently reported and no myalgias, arthralgias, or fevers. The FL-DC immunizations were very well tolerated with one episode of mild hypertension following IV administration and no skin injection site ulceration. One patient experienced a large, tender and erythematous FL-DC-TT injection site associated with fever.

Vaccine Characteristics
The mobilized PBMC were analyzed to determine the percentage of FL-DC and their expression of maturation markers. FL-DC were characterized using FACS analysis by their size (high forward scatter) and expression of CD11c and lack of expression of CD14 (Figures 1A, and B). We have previously observed that these cells also lack lymphocyte lineage markers (CD3, CD19, and CD56). Across all six patients, the CD11c+/CD14- DC represented 8.5 ± 4.2% of the PBMC. After overnight culture in serum free medium, spontaneous maturation occurred in 10 to 88% of the FL-DC based on expression of the maturation markers CD80 and CD83. The costimulatory molecule CD86 was also upregulated on the majority of the FL-DC and the mean fluorescence intensity of HLA-DR also increased.

Clinical Results
Among the 6 patients who completed the immunizations, there was 1 with stable disease for approximately three months and 5 with progressive disease. The patient with stable disease had metastatic breast cancer and at the end of the study had no change in mediastinal lymphadenopathy and pulmonary nodules, but on repeat CT scan 3 months after receiving the intravenous DC, had experienced progression of disease.

Clinical Immunologic Results
We evaluated DTH reactivity at the FL-DC intradermal injection sites after each injection. As demonstrated in Figure 2 for a representative patient, DTH reactivity was variable and repeated injections did not clearly augment DTH response following the first injection. DTH responses to FL-DC across three patients increased two- to eight-fold after completing the immunization course, although no increase in reactivity was seen in the remaining three patients. We also performed DTH tests on the peptides and protein themselves and recall antigens (mumps, candida) before and after Flt3L and after all 4 immunizations. We did not observe any increase in baseline reactivity to any of the recall antigens, nor did we observe
Figure 1 A, B. FACS analysis of FLT3L mobilized peripheral blood DC (FL-DC) before and after overnight maturation. The mobilized PBMC were stained with CD11c, CD14 and either CD80, CD83, CD86, or HLA-DR and at least 10,000 cells were analyzed by FACS. A gate (R1) was placed around the large (high FSC), complex (high SSC) cells and the cells were analyzed as CD11c+CD14- (dendritic cells) (R2) and CD11c+CD14+ (monocytes and DC precursors) (R3). A representative of the 6 patients is depicted.
significant reactivity against CAP-1 following the immunizations in those who were tested (data not shown). Interestingly, one patient had an increase in reactivity (6 mm erythema, 3 mm induration) to CAP-1 and another had an increase in KLH (10 mm erythema) following the FL and prior to any of the immunizations.
**CEA-specific T Cell Results**

We performed an extensive immunologic analysis of the PBMC before and after FL and the immunizations in the four patients that received intradermal injections of DC. In Figure 3, the results for the proliferative response are presented. In some patients the proliferative activity decreased following FL and then rebounded. The proliferative response specific for tetanus and KLH increased initially, but was variable after subsequent immunizations. There was minimal CAP-1-specific proliferation. Figure 4 depicts the results for intracellular cytokine production using flow cytometry for a selected patient. An increase in CD4+ T cells secreting interferon-γ in response to the FL-DC-tetanus toxoid and FL-DC-KLH, but not DC-CAP-1, immunizations was observed. There was no increase in the number of CD8+ T cells secreting interferon-γ in response to the immunization. In Figure 5, we present the results for an ELISpot analysis to detect the number of interferon-γ-secreting cells in response to the various immunogens in those patients who received intradermal injections of DC. The number of cytokine-producing PBMC in response to tetanus was variable while the number responding to KLH increased. Small increases in reactivity to CAP-1 were also detected in 2 of 4 patients. In selected patients, we attempted to analyze the
immune response specific for CEA by evaluating the ability of lymphocytes obtained before and after immunization to lyse CEA expressing target cells. As demonstrated in Figure 6, PBMC obtained before and after the immunizations were stimulated once with antigen and then tested for the ability to lyse antigen-loaded DC target. There appeared to be an effect of FL alone since cytolytic activity of the PBMC was greater following FL (LPB). The cytolytic activity appeared to be augmented further by the tetanus and CAP-1 (LPC), but not KLH in this patient. There was greater cytotoxic T cell activity following the immunizations. Finally, we evaluated the percentage of T cells with receptors that recognized CAP-1 using CAP-1-tetramers. In one patient, an increase in the percentage of CAP-1 tetramer positive cells was observed (Figure 7). The remainder had no increase in tetramer-specific T cells. No tetramers were available to test the Tetanus or KLH responses.

DISCUSSION
Specific immune responses to tumor antigens may be improved by the administration of autologous antigen presenting cells that present and stimulate immune effector elements against target epitopes. With the recognition that dendritic cells can strongly stimulate antigen-specific T cell responses, there has been considerable interest in DC-based anti-tumor vaccines. However, the optimal source and manufacturing technique of dendritic cells for vaccine use is unknown. Since the peripheral blood mononuclear cell compartment contains a small number of circulating DC that might be able to activate immune responses when administered with antigens, we hypothesized that a strategy that increased the number of DC might increase the efficacy of vaccine immunotherapy.

Our prior experience indicated that FL could increase DC number by 16- to 40-fold in peripheral blood. On the basis of this observation, in this study we investigated whether a cellular product enriched for DC by FL mobilization was safe and feasible as an immunotherapy. Indeed, we found that FL-mobilized DC
The Journal of Applied Research

Intracellular IFN-γ CD4 Response

**Figure 4.** Intracellular cytokine staining detected by FACS analysis for PBMC (CD4+) stimulated by antigen at various time points. The percentage of activated (CD69+), interferon gamma secreting CD4+ T cells recognizing the various antigens is plotted for the different time points before and after FL and following each immunization for a representative patient.

Loaded with CAP-1 peptide was feasible to produce and well-tolerated when administered to patients with metastatic cancers. We did not see clinical activity in this small series, but our study concluded pre-maturely due to the further unavailability of FL. Our paper is only the second to report the use of Flt3L mobilized DC as a vaccine platform in humans. Previously, Fong et al.\(^6\) reported on the safety of administering Flt3L-mobilized DC and described immunologic and clinical responses to vaccination. The clinical responses detected in this previous 12-patient study could be attributed to the use of an altered heteroclytic CAP-1(6D) peptide with increased antigenic recognition by T cells, or perhaps to the occurrence of random spontaneous events.

The detailed immunologic analysis unique to our study permitted assessment of immune responses at serial time points throughout the course of immunization. Not only was immune responsiveness to CAP-1 and recall antigens different between individual patients, but the proportion and magnitude of response varied according to the immune assay performed. Interferon-gamma ELISpot detected increases in T cell activation to CAP-1 (2/4 patients),
KLH (4/4 patients) and TT (4/4 patients) throughout the course of vaccination, whereas CAP-1 tetramer staining showed that only 1 of 6 patients exhibited an increase in tetramer-specific T cells. Proliferative responses to immunization, intracellular cytokine staining, and cytolytic activities against the CAP-1 target antigen and recall antigens were variable when multiple time points were compared throughout the course of treatment. Interestingly, most patients developed immune responses early, which did not increase with further immunizations. These data not only highlight distinctions between immune monitoring assays, but also underscores the importance of measuring both the magnitude and persistence of induced immune responses in immunotherapy trials.

Selection procedures to enrich for dendritic cells were not performed due in part to regulatory issues concerning the use of monoclonal antibodies to deplete bystander cells. Furthermore, our own experience of using monoclonal antibodies in vitro to deplete leuka-pheresed PBMC of CD3+, CD19+, and CD14+ cells resulted in a median 1 log decrease in DC available for injection (data not shown). Additionally, depletion of these populations might remove CD11c+CD14+ cells with the capacity to differentiate into dendritic cells during the maturation period or upon re-injection, further reducing the effective therapeutic DC population.

**Figure 5.** Interferon-gamma ELISPOT assay for PBMC obtained at various time points. The number of cells per 100,000 PBMC secreting interferon-gamma in response to the antigens (TT, KLH, and CAP-1) before and after FL and following each immunization for all 4 patients who received the intradermal administrations of FL-DC.
Figure 6. Cytotoxic T cell assay for PBMC obtained before Flt3L (LPA), after Flt3L (LPB), and after the immunizations (LPC). PBMC obtained at the various time points were stimulated once in vitro with DC loaded with KLH, tetanus toxoid, CAP-1, or Hbc peptide and were then tested for their ability to lyse targets labeled with europium and expressing their respective antigens.

We also began a pilot study of immunization with FL mobilized PBMC loaded with HER2/neu ICD. However, this trial was similarly stopped after enrolling two patients due to the unavailability of FL. Of note, in one patient vaccinated with FL-DC-HER2/neu ICD we observed activation of both ICD and tetanus toxoid specific immune responses following vaccination.

It is not known whether vaccination with larger numbers of DC would have generated specific immune responses of greater magnitude. The first two subjects enrolled in this study received a higher dose of intravenous DC compared to subsequent patients. However, given the small overall number of patients in this trial, we cannot assess whether a significant immunologic response could be generated by administering more DC.

It is possible that more consistent maturation and activation of the DC could have generated greater immune responses. In the manufacture of the cellular product in this study, overnight culture caused a certain extent of “maturation” of the DC in all specimens. However, the range of DC maturation after overnight culture remained wide. As well, these DC were not fully activated due to their lack of IL-12 secretion (data not shown); further activation would require cytokine treatment with CD40L and interferon gamma. No obvious correlation was observed between the degree of CD80, CD86, or HLA DR expression and induced immune responses. Even so, a matured
DC product might result in greater immune responsiveness.

Not unexpectedly, immune responses against the tetanus recall antigen and the immunogenic protein KLH were greater than those against the tumor antigen CEA. This likely occurred since CEA is a self antigen whereas the other targets represent foreign immunogens. Additionally, there is no antigen-specific T cell help following immunization due to the MHC Class I restriction of CAP-1 peptide. Conversely, the full-length tetanus and KLH proteins encode MHC Class II epitopes that lead to T cell help for induced immune responses. We hypothesize that loading the DC with a tumor protein would lead to greater tumor antigen-specific immune responses.

It should be noted that FL itself has diverse immunologic effects, which may have contributed to the results observed after administration of this vaccine. As we have observed previously, FL can cause a non-specific increase in DTH skin reactivity. We also noted that cytolytic activity against KLH increased in the one patient evaluated following FL administration, perhaps reflecting upon the effects by FL on NK cells.

In summary, a vaccine based on FL-
mobilized DC appears feasible to administer. We hypothesize that further modifications including potent maturation strategies, selection procedures to improve product purity, and the inclusion of class II help might improve the potency of this vaccine. Furthermore, analysis of immune responses at several time points during a vaccination strategy has importance in determining the kinetics of immune responses.

DISCLOSURES
This study was supported by R01-CA75472-01, and P01-CA78673-01. It also utilized the Duke University Medical Center General Clinical Research Unit funded by NIH grant M01RR00030. M.A.M. is a recipient of an American Society of Clinical Oncology Career Development Award and supported by NIH grant M01RR00030, the Wendy Will Case Fund, and the C. Douglas McFadyen Fund.

ACKNOWLEDGMENTS
The authors wish to thank Kirsten Colling, Manar Ghanayem, Rhonda Williams, Michelle St. Peter, Eva Fisher, and Rian Cumming for technical assistance; and Shubi Khan for management of the patients.

REFERENCES

15. Tsang KY, Zaremba S, Nieroda CA, Zhu MZ, Hamilton JM, Schom J. Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vac-
