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REVIEW

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Clinical applications of dendritic cell vaccination in the treatment of cancer

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Abstract Dendritic cell (DC) immunotherapy has shown significant promise in animal studies as a potential treatment for cancer. Its application in the clinic depends on the results of human trials. Here, we review the published clinical trials of cancer immunotherapy using exogenously antigen-exposed DCs. We begin with a short review of general properties and considerations in the design of such vaccines. We then review trials by disease type. Despite great efforts on the part of individual investigative groups, most trials to date have not yielded data from which firm conclusions can be drawn. The reasons for this include nonstandard DC preparation and vaccination protocols, use of different antigen preparations, variable means of immune assessment, and nonrigorous criteria for defining clinical response. While extensive animal studies have been conducted using DCs, optimal parameters in humans remain to be established. Unanswered questions include optimal cell dose, use of mature versus immature DCs for vaccination, optimal antigen preparation, optimal route, and optimal means of assessing immune response. It is critical that these questions be answered, as DC therapy is labor- and resource-intensive. Cooperation is needed on the part of the many investigators in the field to address these issues. If such cooperation is not forthcoming, the critical studies that will be required to make DC therapy a clinically and commercially viable enterprise will not take place, and this therapy, so promising in preclinical studies, will not be able to compete with the many other new approaches to cancer therapy presently in development. Trials published in print through June 2003 are included. We exclude single case reports, except where relevant, and trials with so many variables as to prevent interpretation about DC therapy effects.

Keywords Dendritic cell · Human clinical trial · Vaccination · Immunotherapy · Cancer

Abbreviations *CEA* carcinoembryonic antigen · *CTL* cytotoxic T lymphocyte · *DC* dendritic cell · *DTH* delayed-type hypersensitivity · *EpCAM* epithelial cell adhesion molecule · *FCS* fetal calf serum · *Flt3L* Flt3 ligand · *G-CSF* granulocyte colony stimulating factor · *GM-CSF* granulocyte-macrophage colony stimulating factor · *HBsAg* hepatitis B surface antigen · *HLA* human leukocyte antigen · *HPV* human papillomavirus · *IFN* interferon · *Ig* immunoglobulin · *IL* interleukin · *KLH* keyhole limpet hemocyanin · *MHC* major histocompatibility complex · *MUC1* mucin gene antigen · *PAP* prostatic acid phosphatase · *PBMC* peripheral blood mononuclear cell · *PEG* polyethylene glycol · *PSA* prostate-specific antigen · *PSMA* prostate-specific membrane antigen · *PTH* parathyroid hormone · *RT-PCR* reverse transcription polymerase chain reaction · *TNF-α* tumor necrosis factor α

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Introduction

Immunotherapy of cancer is a field of intense research, especially the induction of active immunity with either therapeutic or adjuvant intent. The existence of tumor-specific antigens which can be the target of an immune response is well established [57]. The explosion of cancer genomics promises an even greater array of potential tumor-specific antigen targets. A variety of putative tumor vaccines are in clinical trials [13, 27, 55]. A variety of immunization preparations and technologies have been employed, including whole tumor cell vaccines, tumor lysate vaccines, specific tumor antigens, tumor peptides, heat shock proteins, DNA vaccines, dexosomes, and DC-based vaccines.

DCs are the professional antigen-presenting cells of the immune system [4, 33]. Indeed, they are likely the dominant antigen-presenting cells in vivo. Animal studies indicate that DCs can be manipulated to allow very effective antigen presentation. This can allow the generation of immune responses against antigens which might not otherwise be immunogenic. In vaccination against tumors, such a capacity would clearly be very desirable.

Production and properties of DC vaccines

The generation and administration of DC-based vaccines involves a number of discrete steps. Each step imposes potential variables, leading to a multitude of potential protocols for employment of this technology. DC precursors must first be obtained from the host. Although blood or bone marrow may serve as the initial source, peripheral blood mononuclear cells (PBMCs) from leukapheresis are the most common origin. Cytokine priming prior to harvest may be used to increase the yield of precursor cells. Both Flt3 ligand (Flt3L) and granulocyte colony stimulating factor (G-CSF) have been used for this purpose [14, 25]. The PBMCs may themselves be used as precursors, or $CD34^+$ cells may be selected and used as precursors [78]. Stift and coworkers [73] have used a method based on magnetic bead selection to obtain a population enriched with $CD14^+$ cells to serve as precursor cells.

After obtaining precursors in adequate quantity, the cells are grown, to expand and differentiate the precursors. Most often, the precursor cells will be initially grown in the presence of supplemental granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4). This induces the ''immature'' DC phenotype. The immature cells may be exposed to antigen and administered, or they may be subjected to a further differentiation step, to generate the ''mature'' DC phenotype. There are multiple ways to accomplish the second differentiation step. The most common protocol supplements the medium with tumor necrosis factor α (TNF-α), prostaglandin E2, IL-1β, and IL-6. This combination is not used universally; there are a number of other protocols used, most being variations on this theme. The process of generation typically takes 5 days to generate immature DCs, and another 2 days to generate mature DCs. The different phenotypes are distinguishable by in vitro assays of biological activity and by surface marker phenotype. Flow cytometry is the method most often used to confirm the identity of cells to be used in vaccination protocols.

The question of whether mature or immature DCs are to be used in a vaccination protocol appears to be an important one. This is based primarily on the results of animal studies [42]. Immature DCs, while being better at antigen uptake and processing, may be less effective at immunization than mature DCs. Indeed, immature DCs have even been thought to have a tolerizing activity. Obviously such a property is undesirable in a vaccine. This distinction in the biological activities of mature and immature DCs has led to various sequences of antigen administration in relation to cell maturity, with antigen most often, though not always, being administered in the context of the immature phenotype. The cells are then subjected to the maturation step, and administered as mature DCs.

Antigens and other reagents introduced into DCs

Many different antigens have been investigated as possible DC immunogens in vaccination against cancer. These may be classified as defined and undefined preparations, depending on whether or not the exact composition of the preparation is known. Defined antigen preparations include specific protein antigens, specific cDNAs encoding target antigens, and peptides representing human leukocyte antigen (HLA)–specific epitopes from target antigens. Murine prostatic acid phosphatase (PAP) was used to generate an immune response in humans against human PAP [16]. Parathyroid hormone (PTH) has been used to generate responses against parathyroid carcinoma [63]. The cDNA for the mucin gene tumor antigen (MUC1), introduced into DCs by liposomal transfection, was used to generate responses against MUC1-expressing tumors [54]. Peptides representing the HLA-specific epitopes of known tumor antigens have been used in many studies (for examples, see [14, 48]).

The use of undefined antigen preparations has some advantages over the use of defined antigen preparations. Undefined preparations include whole tumor cells, tumor cell lysates, eluates of HLA peptides from surface of tumor cells, and tumor cell mRNA. The primary advantage lies in targeting a wide variety of potential tumor antigens. Targeting a cancer from multiple directions is more likely to be effective in controlling a tumor than relying on a single target, as the opportunities to escape immune surveillance by modulation of antigen expression are more limited. Unfortunately, it is more difficult to monitor the quality or intensity of the consequent immune response when using an undefined preparation, as many of our monitoring technologies rely on defined targets to generate data. Furthermore, antigens which may be important in the response may not be present in adequate concentrations in an undefined preparation to elicit a response reliably. A further complication is the source of tumor antigen preparation. One would presume that autologous tumor would possess specific antigens corresponding most closely with a given patient's tumor. However, it may be impractical to obtain autologous tumor. In such cases, preparations from other persons' tumors of the same type might be used. These may not have the right pattern of antigen expression. Though technologies such as microarray assessment may eventually provide some information in this regard, there is no simple way to determine if one

person's tumor matches another's closely enough to elicit an acceptable response. Given these constraints, most studies with undefined preparations have relied on autologous tumor.

Whole tumor cells have been fused with DCs by polyethylene glycol (PEG)- and electrical-based fusion protocols [32, 34, 35, 39, 49]. These have been conducted while incorporating a treatment step, typically irradiation, to prevent subsequent growth of tumor cell in the patient. Lysates of autologous tumor cells, prepared by freeze-thaw cycling and hypotonic methods, have also been used [20, 22, 50, 64, 73]. An eluate of major histocompatibility complex (MHC) class I peptides from a glioma was used as antigen in one study [86]. Tumor cell mRNA has been used as antigen by a number of workers [19, 46, 56].

Pharmacologic considerations

Like any new drug therapy, there are a number of practical pharmacologic considerations which still must be answered with regard to DC vaccination in cancer. Many routes of vaccination have been utilized, but there is limited information regarding the optimal route. Fong and coworkers [16, 17] found that intravenous, intranodal, and intradermal routes appear to be equally effective, although they noted differences in the quality of the response induced by different routes. In contrast, work in melanoma has suggested that the intravenous route may not work well [53]. This has not been determined in a controlled fashion, however. Other workers have elicited detectable responses by the subcutaneous route.

Optimal cell dose has not been determined. As will be apparent from the studies reviewed herein, no dose-limiting toxicities have been reliably identified. It seems that maximal cell dose is limited, at present, by the ability to culture the cells in large numbers. At least one study suggested that there may be better immunologic effects with higher cell doses [71].

There is no standard schedule for DC vaccine administration. Almost every group uses its own schedule. Optimal vaccination schedules, the utility of maintenance vaccinations, and the use of revaccination after failure have not been addressed.

A number of potential adjuvants have been proposed for DC vaccines. The most widely promoted has been IL-2 [73]. At least one group felt that it did not help in the context of DC vaccination against renal carcinoma [50]. GM-CSF has been proposed as an adjuvant, but one group found that it was also ineffective in this role [70]. Okada and coworkers [49] proposed the cointroduction of IL-4-secreting autologous fibroblasts as an adjuvant along with DC vaccine. Work in mice with keyhole limpet hemocyanin (KLH) suggested that it had an adjuvant-like effect in DC vaccination against an experimental lymphoma [76]. In vitro studies in human cells supported such an effect [52]. This effect has not been proven in human DC vaccination [82], although a number of groups have used the observation to justify inclusion of KLH in experimental protocols (see, for example [3, 18, 20, 50, 56, 77]).

Toxicity

Remarkably, DC vaccination has shown essentially no significant toxicity in the cancer trials in which it has been employed. Local reactions predominate. One of the major theoretical concerns regarding DC vaccines concerned the possible induction of autoimmunity. This is especially important when one considers the use of DC vaccination in cancer, where the immunogens are likely to be tumor-specific molecules, though not absolutely so. This is theoretically less of a problem in defined antigen preparations than in undefined preparations. These worries have not been borne out in preliminary studies.

A number of groups have assessed for the development of autoantibodies after DC vaccination. In a number of studies, autoantibodies were detectable after vaccination, but were not clinically significant [18, 29, 47, 73, 82]. There can be reactions to components of the cell preparation. In one case, immunoglobulin (Ig) E was generated in response to fetal calf serum (FCS) in the DC preparation [29]. This interfered with the use of delayed-type hypersensitivity (DTH) as an immune response marker.

DC vaccines in specific malignancies

Over about the last decade, there have been a variety of early clinical studies of DC vaccines. As noted above, there are many variables in the generation of such vaccines. These vaccines have undergone preliminary testing in patients with a variety of tumors, using a variety of vaccination approaches. We will review the clinical studies that have been conducted to date, according to tumor type.

Malignant melanoma

Melanoma is a disease strongly subject to immunomodulation. Present immunotherapeutic strategies employed in this disease include nonspecific immunostimulation with IL-2 and interferon (IFN), and a variety of vaccine-based approaches intended to prevent recurrence in individuals at high risk of recurrent disease or to treat patients with stage IV disease [9, 30, 31]. With the importance of immune mechanisms in its natural history, it is thus not surprising that melanoma has been of intense interest to DC vaccine researchers.

One of the earliest studies was that of Nestle and coworkers [47]. PBMCs, cultured with IL-4 and GM-CSF, were pulsed with either HLA-specific peptides from

the melanoma antigens tyrosinase, Melan-A/MART-1, gp100, MAGE-1 and MAGE-3, or autologous tumor cell lysate prepared by the freeze-thaw technique. These were given to stage IV melanoma patients. Patients received 10^6 antigen-loaded DCs intranodally on a weekly basis for 4 weeks, followed by a fifth vaccination 2 weeks later, and then monthly vaccinations as long as clinical response continued. No significant, treatment-limiting toxicities were noted. Antigen-specific DTH responsiveness was demonstrated in all of the 16 patients enrolled. Antigen-specific cytotoxic T lymphocytes (CTL) could also be demonstrated in 11 patients. Five patients responded clinically, with two complete and three partial responses. Complete responses were for greater than 15 months, and occurred in one patient with skin metastases and another with metastases in the skin and lungs. Partial responses were of 3–12 months duration and were seen in patients with disease in the skin, pancreas, and bone (Table 1).

Panelli and coworkers [53] assessed an intravenous route for DC administration. PBMCs were treated with IL-4 and GM-CSF and exposed to HLA-A0201-specific epitopes from MART-1 and gp100. These were administered intravenously to HLA-A0201-positive patients with stage IV melanoma four times at 3-week intervals. Two groups of patients received escalating doses of 6×10^7 and 2×10^8 DCs per vaccination. Of ten patients treated, two receiving the higher dose of cells also received IL-2 at vaccination. No toxicity attributable to DC vaccination was observed. Unfortunately, this protocol resulted in antigen-specific CTL activity in only one of five patients tested. A temporary clinical response was seen in one of seven evaluable patients, with subcutaneous and lung metastases. Due to the apparent poor specific immune response and clinical efficacy of this protocol, this study was stopped early. While there are many variables in this study, the results suggest that intravenous administration might not be an optimal route.

Table 1 Clinical trials of DCs in malignant melanoma

Tumor(s)	Subjects[a]	DC maturation[b]	Antigen	Route(s)[c]	Tumor immunity[d]	Clinical responses[e]	Ref.
Melanoma	24	Immature	HLA-specific peptides or autologous tumor lysate	IN	16/16	5/16 (31%); 2 CR, 3 PR	[47]
Melanoma	10	Immature	HLA-specific peptides	IV	1/5	1/7 (14%); 1 PR	[53]
Melanoma	16	Immature	HLA-specific peptides	IV	5/16	1/16 (6%); 1 CR	[36]
Melanoma	14	Mature	HLA-specific peptides	IV	5/14	1/14 (7%); 1 PR	[41]
Melanoma	11	Mature	HLA-specific peptides	SQ, ID, IV	8/11	6/11 (55%)	[74]
Melanoma	8	Mature	HLA-specific peptides	SQ, IV	8/8	0/8 (0%)	[68]
Melanoma	24	Mature	HLA-specific peptides	SQ	Most[f]	1/16 (6%); 1 CR	[69]
Melanoma	11	Mature and immature	HLA-specific peptides	IN	N/A	3/8 (37%); 3 PR	[29]
Melanoma	18	Mature	HLA-specific peptides	SQ	16/18	7/17 (41%); 3 CR, 4 PR	[5]
Melanoma	2	Mature	HLA-specific peptides	SQ	2/2	0/2	[1]
Melanoma	17	Immature	Tumor cell/DC fusion	SQ	?[g]	1/17 (6%); 1 PR	[34]
Melanoma (12), NSCLC (2), colorectal (1), head/neck (2), sarcoma (2), bladder (1), adrenocortical (1), glucagonoma (1)	22	Immature	HLA-specific peptides	IV, SQ	12/20	2/16 (12%);[h] 1 CR1 PR	[82]
Melanoma (11), colorectal (2), neublastoma (1)	14	Immature	Autologous tumor lysate	ID	7/11	1/14 (7%)	[11]
Melanoma	22	Immature	Autologous tumor peptide eulate	ID	6/19	3/19 (16%); 1 CR, 2 PR	[72]
Melanoma	19	Mature	DC/tumor cell coculture	ID	?[i]	6/17 (35%); 3 CR, 3 PR	[51]

[a]Total number of subjects initially enrolled in the given study. May include persons who were unable to complete the study or were not evaluable

[b]DC maturation state at time of vaccination

[c]Routes of vaccination were *ID* intradermal, *IV* intravenous, *SQ* subcutaneous, *IN* intranodal

[d]Patients in whom tumor-specific immunity of any kind was elicited by vaccination. Total number of patients tested may differ from number enrolled

[e]Clinical response rates may differ from total number enrolled due to persons not completing vaccination protocol. *CR* complete response, *PR* partial response

[f]Tumor-specific immune responses were detected in most patients, but the specific number cannot be determined from the data presented [69]

[g]Data not sufficient to determine number of tumor-specific immune responses [34]

[h]Clinical responses assessed only in those with measurable disease at study entry [82]

[i]Data not sufficient to determine number of tumor-specific immunes [51]

Other workers also explored use of the intravenous route [36]. Plastic-adherent PBMCs were exposed to IL-4 and GM-CSF, producing immature DCs. These were pulsed with HLA-specific peptides from tyrosinase, gp100, and/or MART-1. These peptides were modified by single amino acid substitution to increase the strength of HLA-binding. Sixteen patients with stage IV melanoma in a variety of sites, including the viscera, lungs, bone, brain, and skin, were treated. Each cycle lasted 1 month and consisted of an initial infusion of fresh, antigen-pulsed DCs, followed 2 weeks later by an infusion of frozen DCs. The frozen cells were pulsed with antigen at the same time as the initial batch, and thereafter frozen. DCs were administered in progressively greater doses, starting at 10^7 antigen-treated cells and increasing to 10^8 cells. One patient with lung nodules had a complete response, lasting 12 months. Only 5 of the 16 patients developed specific immune responses, as detected by an assay for IFN-γ production in response to gp100 or tyrosinase. These results are difficult to interpret, as there are so many variables among the different patients. However, a lack of detectable immune responses in this study correlates with rapid disease progression and death, with 10 of the 11 nonresponders progressing. This study again suggests that the intravenous route may be suboptimal. Alternatively, this may reflect the use of immature DCs for vaccination. The results do support the concept that lack of an immune response, as assessed by in vitro methods, indicates a poor prognosis.

The melanoma studies noted above use a DC-generation protocol which would not produce mature DCs. Mackensen and colleagues [41] used a protocol which would do so. Fourteen patients were treated with G-CSF to mobilize $CD34^+$ cells in the peripheral blood. These were harvested by leukapheresis and cultured with IL-3 and IL-6 for 7 days, followed by IL-4 and GM-CSF for 3 weeks. TNF-α was added to the medium for 24 h to achieve final maturation. The cells were pulsed with a mixture of HLA-specific peptides from either the MAGE-1 and MAGE-3 melanoma antigens or Melan-A, gp100, and tyrosinase. Expression of the different melanoma antigens was assessed by reverse transcription polymerase chain reaction (RT-PCR) in biopsy samples from each individual patient. Patients received four intravenous vaccinations of 5×10^6 to 5×10^7 DCs at 2-week intervals. Side effects were minimal and included fever and local reactions in a minority of participants. One patient developed vitiligo. Results were otherwise disappointing. Specific immune responses were noted in only 5 of 14 patients, and an objective partial clinical response was seen in only one patient. This consisted of complete regression of a single large subcutaneous metastasis, though another lesion was unchanged. The results of this study, which used mature DCs, suggest that the lack of efficacy in this and in the studies noted above may not be due to use of immature DCs, but rather to the intravenous route of administration [36, 53].

Thurner and coworkers [74] initially obtained DCs from PBMCs treated with GM-CSF and IL-4. The cells were matured by treatment with medium conditioned by growth of autologous monocytes for 24 h, supplemented in some cases with TNF-α. The cells were pulsed with an HLA-A1-specific peptide from MAGE-3. Eleven melanoma patients received five vaccinations at 2-week intervals, the first three being subcutaneous and intradermal injection of 3×10^6 DCs and the last two being intravenous injection of 6×10^6 and 12×10^6 DCs. Minimal toxicities occurred, including local reactions, low-grade fever, and localized lymphadenopathy. Increased antigen-specific CTLs were detected in eight patients. Interestingly, intravenous vaccination seemed to result in a decrease in CTL levels, a finding consistent with the results of others [41, 52]. Six of 11 patients showed regression of individual metastases. These were in the lung, liver, subcutaneous tissues, and lymph nodes. It is not possible from the data presented to draw conclusions regarding overall extent of response in individual patients. In two patients, lesion regression correlated with development of localized erythema and $CD8^+$ lymphocyte infiltration. Nonregressing lesions in two patients did not show MAGE-3 antigen expression and did not display $CD8^+$ lymphocyte infiltration. As all patients had MAGE-3 antigen expression at enrollment, this result suggests selection for tumor cells lacking MAGE-3 expression. To avoid this problem, any putative vaccine should incorporate components from as diverse a selection of target antigens as possible.

This group next conducted a study to determine if the results of the prior study were applicable in a different haplotype [68]. Using a similar protocol, eight HLA-$A2.1^+$ melanoma patients with advanced metastatic disease received DCs pulsed with an HLA-specific MAGE-3 peptide. All eight developed specific $CD8^+$ cells. In contrast to the previous studies, the intravenous vaccinations in this study had a less noticeable negative impact on the development of specific CD8+ lymphocytes. Of the eight, one patient with disease confined to the lymph nodes was stable, though the duration of this stability was not noted.

Subsequently, these workers showed that a type 1 helper T-cell response against melanoma antigens could also be generated by DC vaccination [69]. DCs were prepared by exposure of the plastic-adherent fraction of PBMCs to medium with autologous serum, IL-4 and GM-CSF, followed by a mixture of TNF-α, IL-1β, IL-6, and prostaglandin E2. The matured cells were exposed to cocktails of HLA-specific melanoma antigen peptides, derived from the MAGE-1, MAGE-3, MAGE-4, MAGE-10, tyrosinase, Melan-A, and gp100 antigens. Four million DCs pulsed with each antigen were administered subcutaneously in the vicinity of lymph nodes. The total cell dose was a multiple of this base dose and the total number of antigens administered in each case. This varied with different HLA haplotypes. Sixteen of 24 enrolled patients with advanced stage IV melanoma received the full course of five vaccinations,

given every 2 weeks. While the exact number of responders is not clear from the report, most patients generated tumor-specific Th1 responses, with increased levels of antigen-specific IFN-γ- and IL-2-producing cells. Clones of these cells from some patients were even capable of lysing target cells expressing the native MAGE-3 target antigen. Clinical responses were assessed 1 month after completion of the full vaccination series. Only one patient, who had pancreatic and pulmonary metastases, exhibited an objective complete clinical response. No dose-limiting toxicity occurred. Eight enrolled patients were not able to complete the vaccination series due to death or progressive disease, suggesting a problem with patient selection. This approach to vaccination, using a cocktail of peptides, mature DCs, and a prolonged administration protocol in the vicinity of lymph nodes, appears to be effective in generating immunologic responses against melanoma antigens.

Finally, this group also conducted an important study to determine whether mature or immature DCs were better for inducing specific immunity [29]. Eleven patients with advanced melanoma were enrolled, of whom eight were evaluable at the end of the vaccination protocol. The adherent fraction of PBMCs from each patient was grown in the presence of GM-CSF and IL-4. Cells intended to be used as mature DCs were grown in a commercial serum-free medium. Cells intended to be used as immature cells were grown in conventional medium supplemented with FCS. Cells to be used as mature DCs were then supplemented with IL-1β, IL-6, TNF-α, and prostaglandin E2. Mature and immature DCs from each patient were then each exposed to a different, HLA-specific melanoma peptide from Melan-A/Mart-1, MAGE-1, or tyrosinase, and to a different control antigen (tetanus toxoid or tuberculin). Patients received intranodal vaccinations with each DC cell population at different sites. Six vaccinations were given, at 2-week intervals.

In vitro antigen-specific responses were clearly more potent against antigens presented in the context of mature DCs. This was the case both for the HLA-specific melanoma peptides and for control antigens. This was reflected in proliferation assays, increased expansion of specific $CD8^+$ cells, and increased frequencies of antigen-specific CTLs. One might wonder why the authors chose to culture mature DCs in a serum-free medium and immature DCs in serum-supplemented medium. However, it is hard to accept that this difference explains the difference in the immune responses induced by the two different means of preparing DCs for vaccination.

Clinical responses were a secondary endpoint in this study. Patients' disease status was assessed 2 weeks after the third vaccination and 2 weeks after the sixth. Two patients, one with liver and lung metastases and another with lymph node, skin, and subcutaneous disease, were stable at time of assessment. Three patients were judged to have partial responses and three had progressive disease. The limited interval between completion of treatment and assessment prevents one from drawing firm clinical conclusions based on this study.

Several recent studies in melanoma have demonstrated that in vitro assays of immune reactivity after DC vaccination predict clinical outcome. One group vaccinated patients with metastatic melanoma, with DCs exposed to a mixture of HLA-specific peptides [5]. These peptides were derived from Melan-A/MART-1, gp100, tyrosinase, and MAGE-3 melanoma antigens. $CD34^+$ cells were obtained from the patients by administration of G-CSF for 5 days, followed by leukapheresis. These were grown in serum-free medium supplemented with GM-CSF, TNF-α, and Flt3L. Flt3L has been shown to be capable of inducing a mature DC phenotype [14]. DCs were administered subcutaneously every 2 weeks over 6 weeks for a total of four injections. Escalating doses of antigen-loaded DCs, from 10^5 to 10^6 cells, were given at each injection, and three injections occurred at each administration.

Of 18 patients enrolled, 16 developed detectable specific immune responses to the target antigens. Clinical response was assessed during the study and at 4 weeks after the vaccination course. Of 17 evaluable patients, three complete responders and four partial responders were identified. Complete responses were only seen in patients with low initial disease burdens.

In vitro immune responses correlated with clinical response. Six of seven patients responding to less than three antigens showed disease progression. Only one of ten patients responding to three or more antigens experienced disease progression. Two patients who did not respond to any antigen showed rapid progression. As a corollary, this observation suggests that targeting a variety of antigens would be more effective than targeting individual antigens.

A case report supports this observation [1]. Two patients with widely metastatic melanoma progressing despite conventional therapy were treated. Mature DCs were generated from each patient and exposed to a cocktail of HLA-specific peptides from the MAGE-3, gp100, and MART-1 melanoma antigens. Approximately $2x10^7$ to $3x10^7$ antigen-loaded DCs were administered subcutaneously every 2 weeks. The patients also received IL-2 and temozolomide. The primary endpoints in the study were the assessment of specific immunologic responses. Disease stability correlated with development of cell-mediated immunity. Furthermore, disease progression occurred as antigen-specific immunity waned after vaccination. This admittedly small report supports the contention that immunity generated by DC-based vaccination can control melanoma.

In contrast to the previously noted studies, one report used fusion of tumor cells with DCs to generate a vaccine [34]. Seventeen patients with advanced melanoma refractory to conventional therapy were enrolled. Fresh tumor specimens were obtained either from easily accessible biopsy specimens or during medically indicated surgery. Cells were isolated from these specimens and frozen.

To generate DCs, monocytes were isolated from peripheral blood by either density-gradient centrifugation and plastic-adherence, or leukapheresis, density-gradient centrifugation, and countercurrent elutriation [2]. The cells were then grown for 5–7 days in the presence of IL-4 and GM-CSF to induce the immature phenotype. Approximately 10^7 immature DCs were then fused with an equal number of irradiated autologous tumor cells by PEG-mediated fusion. While fusion efficiency was not determined in each individual case, control experiments indicated fusion efficiencies of 2–15%. The mixture containing the fused cells was used for vaccination. No attempt was made to isolate the tumor-DC fusions from the other cell types (unfused cells, DC-DC fusions, tumor-tumor fusions) in the mixture.

The vaccine was administered subcutaneously in the vicinity of inguinal nodes. This was repeated monthly. Vaccinations continued until either disease progression occurred or the supply of tumor cells was exhausted. No significant adverse events or dose-limiting toxicities occurred.

The 17 patients enrolled were heavily pretreated for their disease. They had metastatic disease in a variety of sites, including subcutaneous, nodal, lung, liver, spleen, bone, brain, and adrenal gland. Clinical results were limited, and included a partial response in one patient, lasting 6 months. Interestingly, this patient also developed some loss of hair pigmentation, similar to vitiligo. One patient had a mixed response and another had stable disease for 6 months. All remaining patients had progressive disease over 1–3 months or were not evaluable due to rapid disease progression.

The authors also attempted to analyze immune responses to the vaccination by DTH and IFN-γ ELISpot assay. No DTH reactivity against irradiated autologous tumor cells was appreciated in any patient. ELISpot assay could be conducted in only five patients, due to inadequate cell yields in the others. The results obtained were essentially uninterruptible.

This study demonstrates the safety of this approach. In one patient, an apparent significant clinical response was observed. This approach to therapy may have difficulty achieving very much in the face of very advanced disease, from which all the patients enrolled in the study suffered. The study also suffers from lack of a detailed analysis of immune responses generated by the vaccine. Such information about immunogenicity would have been useful, even if no clinical responses were observed.

One group has examined the immunogenicity of DC-based vaccination with HLA-specific MAGE peptides in vaccination against a variety of tumor types, with an emphasis on melanoma [82]. Twenty-two patients were enrolled in the study and were evaluable. While 12 patients had melanoma, other tumor types were represented, including non–small cell lung cancer (2), colorectal cancer (1), head and neck cancer (2), sarcoma (2), bladder cancer (1), adrenocortical cancer (1), and glucagonoma (1). The tumors of all patients expressed MAGE-A1 or MAGE-A3 tumor antigens, and all patients had to possess at least one of four specific HLA types. The primary endpoint of the study was immunogenicity. Enrolled patients received the vaccine either as immunotherapy of established disease or as adjuvant therapy for disease at high risk of relapse.

To produce DCs, PBMCs were harvested by leukapheresis and the adherent cell fraction was grown for 7 days in serum-free medium in the presence of GM-CSF and IL-4, yielding the immature DCs. The immature DCs were then exposed for 2 h to between one and three HLA-specific peptides from MAGE-1 or MAGE-3, according to a given patient's haplotype and tumor antigen expression. Ten patients received this vaccine. In a second part of the study, the cells of 16 patients, including 4 from the first phase were also exposed to KLH as a control and potential "helper" antigen. DCs were generated anew for each vaccination. In the first phase of the study, each DC preparation was divided into aliquots, one of which was given intravenously and the remaining (3–5) were injected subcutaneously in the vicinity of lymph nodes. In the second phase, incorporating KLH, the patients received only subcutaneous vaccinations. Three vaccinations were given at 3-week intervals, and a fourth vaccination was given 6 weeks later. In responsive cases, subsequent vaccinations were given every 2 months for a year and then every 6 months for 2 years.

Vaccinations were well tolerated, with no dose-limiting toxicities. Two patients with preexisting antinuclear antibodies experienced an increase in titer during vaccination. This reversed after immunization, and was not associated with clinical findings of autoimmunity.

Immune responses elicited by this vaccination protocol were the primary endpoint of the study. In the first part of the study, no control antigen was included. In this phase, immune responses were monitored by measuring the frequency of peptide-specific, IFN-γ-producing cells after vaccination by cytokine flow cytometry (CFC) and ELISpot assays. Six of eight (75%) evaluable patients produced a detectable response to a target peptide. These assays correlated so well that only CFC was used to monitor responses in the second phase, in which KLH was also administered. Only 6 of 16 (38%) tested responded to a target peptide. If the four patients from the initial phase of the study are excluded, the response rate increases to 50%. It is possible that the initial vaccination series interfered with the generation of immune responses in the second phase of the study.

Clinical responses were a secondary endpoint in this study, and it is hard to draw any firm conclusions based on the results. Melanoma patients comprised over half of the study participants. Sixteen patients had measurable disease. One patient with melanoma of the axilla seems to have had a complete response, although this is not entirely clear from the report, and must be inferred. Another patient had a partial response, with disappearance of lung metastases. No firm conclusions can be drawn regarding clinical effect: the tumors and stage of

disease are so different among the enrollees as to be noncomparable.

This vaccination protocol appears to be immunogenic, at least in a significant fraction of the vaccinees. Addition of KLH does not appear to markedly increase the immunogenicity of the preparation. This result argues against the use of KLH as a "helper" antigen. Other variables in the study interfere with the conclusions that can be drawn. Nonuniformity in the study, specifically eliminating the intravenous route of administration, and including persons from the first phase in the second phase, makes it more tenuous to compare results of the different phases. The authors also used immature DCs and a relatively short duration of peptide antigen-pulsing, which may have further compromised their results. The preparations do appear to be safe and well tolerated.

Another group conducted a phase I study of autologous tumor lysates as antigen in DCs in a patient population consisting mostly of melanoma patients [11]. The study was intended to assess the effects of increasing DC doses, and enrolled patients with stage IV malignancies. Twenty-four patients were enrolled in the study, but only 14 completed the planned vaccination protocol, suggesting a problem with patient selection. Of the 14 patients who were considered evaluable, 11 had melanoma, 2 had colorectal carcinoma, and 1 had neuroblastoma. The other ten patients included one with colorectal carcinoma, who progressed prior to any study intervention. The other nine included three with breast cancer and six with melanoma. These all progressed during the treatment. All patients were immunocompetent prior to vaccination, as demonstrated by DTH positivity to at least one of four recall antigens.

Vaccine was prepared from PBMCs obtained by leukapheresis. Aliquots of PBMCs were frozen to prepare subsequent vaccine doses, except for the first dose, which was prepared from fresh PBMCs. The plastic-adherent fraction of PBMCs was grown in serum-free medium in the presence of GM-CSF and IL-4 for 6 days. The cells were then exposed separately for 18 h to either an autologous tumor lysate, produced by freeze-thaw cycling and irradiation, or to KLH. A dose of 10^6, 10^7, or 10^8 cells was given, with half of the dose being tumor-exposed and half being KLH-exposed. Vaccine was administered intradermally in the vicinity of uninvolved lymph nodes. Patients received three vaccinations at 2-week intervals, at which time re-staging studies were conducted. If there was no disease progression, patients received another course of vaccinations.

Primary endpoints in this phase I study were safety and tolerability. No dose-limiting toxicities occurred at any dose. Several patients experienced minor local reactions at the injections site. A panel of autoimmune antibodies was assessed in a subset of the patients. One patient developed a positive anti-nuclear-antibody titer after vaccination, which was not associated with clinical signs. Another patient with melanoma developed vitiligo, although not associated with disease regression.

Immune responses to the vaccine were assessed in a number of ways. Unfortunately, data from all patients could not be generated. Data regarding KLH proliferative responses prior to and after vaccination were available from 11 of 14 patients, 10 of whom displayed an increase in KLH-specific reactivity after vaccination. Thus, the vaccine was immunogenic, at least against the control antigen.

The assays to assess for tumor-specific immunity were even less complete. Tumor-specific DTH, ELISpot, and PBMC proliferation assays were conducted, although their application appears to have been haphazard. While not explicitly noted, 7 of 11 patients tested were positive in at least one of these assays for tumor-specific immunity. Clinically, one patient with melanoma had a limited partial response, though the report does not detail this response.

This study has some serious limitations. First, use of immature DCs may have limited their effectiveness. Second, and perhaps more critically, while the goal of a phase I study is not to determine clinical response, a well-designed study can produce information regarding clinical responses to guide later studies. That nine patients succumbed to their disease during treatment indicates either that there was a problem with patient selection, or that the treatment was not especially effective. Even among the 14 evaluable patients, only one partial response is reported. Furthermore, better planning might have yielded more complete immunologic data than was actually obtained. Conducting fewer immune assays, but actually performing them on all patients, would have been a better approach. Such data could have been valuable to assess whether there is a DC dose-response effect on the immune parameters. The authors attempt to draw conclusions about this, but they are restricted by the data they obtained. Better planning might have yielded firmer conclusions.

Another study looked at the response to DCs exposed to autologous, tumor-associated peptides [72]. This was designed as a phase I/II study, with endpoints being both cell dose tolerance and melanoma-specific responsiveness. Twenty-two patients with stage IV melanoma were enrolled. To prepare vaccine, PBMCs were obtained from either whole blood or leukapheresis product. The adherent cell fraction was then cultured for 5–7 days in the presence of GM-CSF and IL-4, generating the immature DCs. The cells were used fresh for vaccine production or frozen for later use. Antigen was an acid eluate of peptides from autologous tumor specimens, which was prepared from a fresh surgical specimen and then lyophilized. For the first three patients, DCs were exposed to the peptide elute and β_2-microglobulin. β_2-Microglobulin was added as an adjuvant [59]. In the remaining 19 patients, hepatitis B surface antigen (HBsAg) was added as a control antigen. Patients received 1×10^6 to 10×10^6 antigen-exposed DCs

intradermally every 2 weeks with the goal of reaching eight vaccinations.

The initial goals in the study were dose-ranging and assessing tolerability. No dose-limiting effects were noted at any cell dose. Several patients developed vitiligo and others experienced transient flu-like symptoms. One patient developed an inflammatory arthritis in response to intraarticular melanoma deposits. Once the dosing safety was established in the first 13 patients, the remaining patients received a fixed dose of 0.1×10^6 DCs/kg. Such a weight-based dosing scheme is common in oncology studies, but this is one of the few studies incorporating this approach to dosing.

The authors were able to subdivide their patients into two groups: those who developed immune responses against HBsAg and those who did not. These groups appear to differ in their overall immunocompetence. Among the nine responders, five developed detectable cellular responses against autologous melanoma peptides, as assessed by DTH or ELISpot for peptide-specific, IFN-γ-secreting cells in peripheral blood. This group also included all of the patients with responses, including one complete response in a patient with subcutaneous disease, and two partial responses in patients with visceral disease. In contrast, only one of the ten nonresponders to HBsAg developed a detectable immune response to the melanoma peptides. All of these patients progressed.

This study is well designed, compared to many trials. It adheres to a phase I/II standard, with close clinical and immune monitoring, and has well-defined criteria for response. All patients are accounted for. The results are thus more reliable than many studies in this field. Use of β_2-microglobulin in the DC-sensitization protocol is unusual; the published report does not comment on this. The clinical responses to the vaccine used are clearly very limited, and probably do not justify a phase III trial. It is unclear whether use of mature, rather than immature, DCs would change the results. The results highlight an important point in vaccine studies: one needs to assess the general immunocompetence of one's study subjects, as this will greatly impact their ability to respond to any immune intervention.

A very recently reported phase I/II trial of DC therapy in melanoma obtained some apparent complete responses [51]. The study enrolled 19 patients with stage IV melanoma. None had brain metastases. Pretreatment varied, from no treatment at all to very aggressive, multimodality therapy. Extent of disease also varied, from disease limited to the lymph nodes to extensive visceral disease.

The vaccine preparation protocol was somewhat unusual. Tumor was obtained from fresh surgical specimens. A single cell suspension was produced from the specimen, the cells were irradiated, and then cryopreserved. PBMCs were isolated from either whole blood or leukapheresis product. After purification of mononuclear cells by density-gradient centrifugation, the plastic-adherent fraction was cultured for 6 days in the presence of autologous serum, GM-CSF, and IL-4. The resulting immature DCs were cryopreserved for later use. Three days prior to vaccination, DCs were thawed and cocultured with the irradiated autologous tumor cells. After 6 hours, autologous monocyte conditioned medium was added to the cultures to induce maturation to the mature DC phenotype. After 2.5 days in culture, the cells were washed and administered to the study patients.

Patients received the vaccine intradermally at one of two dose levels: low dose (average 0.9×10^6 cells) and high dose (average 5×10^6 cells). The "priming" regimen involved administration of a dose of vaccine every 2 weeks for a total of six doses. Thereafter, vaccine was administered at a dose of 10^6 cells in the same manner every 6 weeks in patients with stable or responding disease.

The vaccine was well tolerated at both low and high dose, without significant side effects or dose-limiting toxicities. In two patients, vaccine could not be prepared due to inadequate tumor cell yield. Thus, only 17 of the original 19 enrollees received any vaccine at all. Ten patients received low-dose vaccination and seven received high-dose vaccination. One patient withdrew prior to completion of priming for personal reasons, developing progressive disease thereafter. Four patients, at both low and high vaccine doses, developed disease progression while receiving priming.

Of the remaining 12 patients, 3 ($3/17 = 18\%$) had complete responses. One patient had disease limited to the lung, with a single 1.4-cm lung nodule which resolved with treatment. This patient remained in remission at greater than 30 months after treatment. Another patient, who presented with disease limited to the mesenteric lymph nodes and was noted to have a single metastasis in the skull during treatment, resolved at both of these sites during treatment and remained in remission at 44 months. A third patient, who presented with more extensive visceral disease, remained in remission at greater than 55 months. A number of characteristics of these patients are notable. All had relatively low-volume disease, with the largest resolving lesion being a 4-cm mediastinal lymph node. They were relatively young (34, 24, and 39 years, respectively), and all had not been subject to extensive pretreatment. In contrast, the other patients had larger volume disease, were older (average age 50 years), and had received more extensive pretreatment, including seven who had already received chemo- and/or immunotherapy. In addition to patients with complete responses, the authors note three patients who had partial responses in disease of the skin and lymph nodes. These patients subsequently progressed.

The report does not present much analysis of the immune responses generated, other than the results of DTH testing. These data are not complete, although magnitude of DTH response does not appear to correlate with clinical response. The authors also assess levels of the S-100B serum tumor marker in patients prior to treatment. Low levels of this marker correlate with

complete and partial responses, and longer survival. These data agree with that noted clinically, that the patients with complete responses had lower volume disease.

This study is one of the few DC vaccination studies to achieve, in most respects, the goals of a phase I/II study. Dose of DCs appears not to be a significant parameter. The DC vaccine is well tolerated and safe. A number of complete responses were observed, and these appear to have been durable. The frequency of these responses must remain in perspective, especially in the context of a phase I/II trial. Initial studies of dacarbazine therapy reported similar response rates [28, 83]. This suggests that the control arm of a phase III trial might receive conventional dacarbazine therapy, with a possible cross-over design to allow those failing one therapy to receive the other. The authors have identified a number of factors which need to be addressed in further studies. Specifically, they need to control for disease volume in allocating patients to different arms of a controlled trial. The S-100B serum antigen level may provide a quantitative means of doing this. Immune monitoring, at least as reported, does not identify a useful assay, though it does indicate that DTH testing appears to be virtually useless for this purpose. A more reliable means of correlating immune response with clinical response would be a worthwhile contribution to both the study of vaccination against melanoma and to the study of DC vaccination generally. Despite some problems, this study's results justify a phase III trial.

With perhaps the exception of the last study cited, the problems with the above-cited melanoma trials are numerous and preclude firm conclusions. These include small numbers of patients, incompletely described clinical staging, treatment of patients with advanced refractory disease, inadequate description and documentation of responses, variable vaccine preparation and administration methods, and short follow-up periods. None of the studies were large or uniform enough for valid statistical analysis. One can only conclude that DC therapy induced some antitumor immune responses, the vaccine preparations described appear to be safe, and there are some apparent remissions. There is no firm data on survival. Remissions in melanoma often do not translate into prolonged survival. Thus, despite the great deal of effort expended in these studies, these data are of little value and call for larger, more uniform, controlled trials.

Hematologic malignancies

Multiple myeloma

Multiple myeloma is a compelling target for immune intervention. It is characterized by the production of a unique antigenic target produced by the malignant cells, the paraprotein. Levels of the paraprotein in the peripheral blood provide a ready means of following response to treatment. Myeloma has indeed been the subject of a number of studies using DC-based vaccination (Table 2).

Wen and coworkers [84] reported a 43-year-old male with advanced, heavily pretreated myeloma. PBMCs were obtained by leukapheresis, and plastic-adherent cells were cultured with GM-CSF and IL-4 for 7 days. The cells were pulsed on days 1 and 6 with purified paraprotein and KLH. The patient received three vaccinations intravenously with 5×10^6, 30×10^6, and 45×10^6 antigen-exposed DCs. T- and B-cell-specific responses developed against both the control antigen and the paraprotein. The patient experienced a decrease in serum paraprotein levels, but the disease subsequently progressed. Interestingly, the paraprotein-specific T-cell responses were still detectable 2 months after subsequent high-dose melphelan therapy. Thus, the immunity generated by DC vaccination exhibits some durability, even in the face of potent immunosuppression. This study confirms that DC-based vaccination in

Table 2 Clinical trials of DC immunotherapy in hematologic malignancies. *NHL* non-Hodgkin's lymphoma

Tumor(s)	Subjects[a]	DC maturation[b]	Antigen	Route(s)[c]	Tumor immunity[d]	Clinical responses[e]	Ref.
Myeloma	1	Immature	Paraprotein	IV	1/1	0/1	[84]
Myeloma	2	Immature	Paraprotein	IV	2/2	0/2	[12]
Myeloma	6	Immature	Paraprotein	IV	4/5	0/5	[37]
Myeloma	11	Mature	Paraprotein peptides and $F(ab')_2$	SQ	2/10	1/11 (9%); 1 PR	[78]
Myeloma	5	Mature	Paraprotein	SQ, IV	5/5	1/5 (20%); 1 PR	[85]
NHL	4	Mature	Idiotypic antibody	IV	4/4	2/4 (50%); 2 CR	[24]
NHL	31	Mature	Idiotypic antibody +/− KLH conjugation[f]	SQ, IV	18/31	9/31 (29%); 9 CR	[77]

[a]Total number of subjects initially enrolled in the given study. May include persons who were unable to complete the study or were not evaluable

[b]DC maturation state at time of vaccination

[c]Route of vaccination: *IV* intravenous, *SQ* subcutaneous

[d]Patients in whom tumor-specific immunity of any kind was elicited by vaccination. Total number of patients tested may differ from number enrolled

[e]Clinical response rates may differ from total number enrolled due to persons not completing vaccination protocol. *CR*, complete response, *PR* partial response

[f]Idiotypic, tumor-specific antibody was administered either chemically conjugated to KLH or along with free KLH [77]

human myeloma can generate specific immune responses.

Another group demonstrated similar results using a slightly different vaccination protocol [12]. PBMCs were obtained from the blood of two myeloma patients, both of whom had been extensively pretreated. Adherent cells were cultured in the presence of GM-CSF and IL-4, yielding immature DCs. At the initiation of culture and 24 h prior to harvest, the cells were pulsed with purified paraprotein and KLH. This was done twice, with half of each harvest being administered intravenously to the patient, and the remainder being frozen for a second vaccination. Patients received a total of four vaccinations, given every 2 weeks. Each vaccination consisted of 4×10^6 to 40×10^6 cells. At each vaccination, the patients also received 300 μg of GM-CSF subcutaneously as an adjuvant. One patient experienced mild, flu-like symptoms for 24 h after each booster vaccination. The vaccinations were otherwise well tolerated. Treatment elicited cell-mediated immunity against both the paraprotein and KLH. Increased antigen-specific T-cell proliferation in response to stimulation with both of these antigens was observed. Paraprotein-specific, IFN-γ production was also detectable. Furthermore, humoral immune responses occurred against both antigens. Clinically, the rate of rise of paraprotein level in one patient decreased during the period of vaccination, although the absolute level continued to rise. Levels of paraprotein in the other patient, which were stable at study initiation, remained stable. Again, this study presents evidence for immune responses in this disease with DC vaccination, but no evidence of significant clinical effect.

Lim and Bailey-Wood [37] conducted a study with six patients. Three had been extensively pretreated, while the others were untreated. PBMCs were obtained by leukapheresis, and adherent cells were cultured for 7 days in the presence of GM-CSF and IL-4. Purified paraprotein and KLH were added to the cultures on days 1 and 6. The antigen-exposed DCs then were harvested and divided into three aliquots, two of which were frozen for later administration. The remaining aliquot was immediately administered intravenously. The patients received two additional doses at 2-week intervals. Depending on culture yield, patients received 3.5×10^6 to 89×10^6 cells per vaccination. Of the six patients, five completed the vaccination series.

All five evaluable patients developed proliferative responses and humoral responses to KLH. Four of the five patients developed proliferative responses to the paraprotein. The fifth patient had a high baseline, prevaccination proliferative response. Three of the five patients developed paraprotein-specific IgM and four of the five developed paraprotein-specific IgG. Interestingly, the single patient who failed to develop increased proliferative responses against the paraprotein also did not develop a humoral response. This patient experienced the most rapid disease progression.

One previously untreated patient experienced a minor response, with paraprotein levels decreasing from about 20 g/l prevaccination to about 16 g/l postvaccination. This response persisted for at least 13 months. Paraprotein levels in two patients remained stable during the protocol, and for at least 8 months thereafter. The other two patients progressed 2 and 5 months after completion of the study.

Like the two reports noted above, this study also shows that DC-based vaccination can elicit detectable immune responses in myeloma patients and may have some clinical benefit. The study suffers from being a case series, without adequate controls. The conclusions that can be drawn are limited, especially in light of the limited clinical effects observed.

Another study was conducted in 11 patients with heavily pretreated, stage II–III myeloma [78]. DCs were generated from $CD34^+$ peripheral blood cells, obtained by leukapheresis. Patients received G-CSF for 5 days prior to leukapheresis to mobilize the stem cells. The $CD34^+$ cells were purified in a two-step process using magnetic cell sorting and a $CD34^+$-specific column. The cells were grown for 10–12 days in medium supplemented with human serum, TNF-α, and GM-CSF. The DCs produced in this manner were capable of stimulating antigen-specific T cells in vitro. Eighteen hours prior to vaccination, the DCs were exposed to paraprotein-derived peptides and $F(ab')_2$ fragments as the antigen. Vaccinations consisted of 1×10^6 to 20×10^6 cells administered subcutaneously. Nine patients received one dose of antigen-exposed DCs and three booster doses every 2 weeks afterward of 100-μg GM-CSF and 100-μg paraprotein-derived peptides. Two patients received four vaccinations with only paraprotein-exposed DCs.

Three of ten patients developed a humoral response to the paraprotein. One of the two patients receiving four DC vaccinations was excluded from the analysis. Paraprotein-specific cellular responses were detectable in both of the patients receiving four DC vaccinations. None of the eight patient vaccinated once with antigen-exposed DCs and three times with antigen and GM-CSF showed a detectable cellular response.

One of the 11 patients studied had a partial response to the treatment, as indicated by a reduction in bone marrow plasma cells from 8–10% to 4%. Otherwise, all treated patients experienced disease progression. This would appear to correlate with the weak immune responses generated.

Like the other studies, this is essentially a case series, with no control group. The already limited numbers of patients were further subdivided into two different experimental treatment groups. One might hypothesize that using four rounds of vaccination with antigen-exposed DCs provokes better cellular responses and weaker humoral immune responses than the protocol using one course of DC-based vaccination followed by three rounds of antigen-GM-CSF treatment. Unfortunately, the numbers of patients do not allow firm

conclusions. One can only infer that the latter treatment is not an effective vaccination approach against myeloma.

Yi and coworkers [85] studied five patients, all with myeloma in stable partial remission after high-dose chemotherapy. Adherent PBMCs, obtained by leukapheresis, were cultured for 3 days in the presence of FCS, GM-CSF, and IL-4. At day 3, the medium was supplemented with twice the concentration of cytokines. On day 7, the cells were exposed to purified paraprotein and grown for 24 h in the presence of IL-1β and TNF-α, to induce DC maturation. On day 8, the cells were harvested and used for vaccination. Each patient received a total of at least 20×10^6 cells injected subcutaneously at six sites every 2 weeks for three vaccinations. Remaining cells were injected intravenously or intravenously and subcutaneously. As an adjuvant, IL-2 was given subcutaneously for 5 days after each vaccination.

The primary endpoint was the immune response generated by vaccination. Paraprotein-specific proliferative responses were seen in two of five subjects. Vaccination induced or augmented the number of paraprotein-specific, IFN-γ-secreting cells in four of five subjects. These cells correlate with Th1-type activity. In contrast, no increase in paraprotein-specific, IL-4-secreting cells, which correlate with Th2-type activity, occurred. All five patients also demonstrated an increase in the frequency of paraprotein-specific B cells. This vaccination protocol is able to induce paraprotein-specific cellular and humoral immune responses. One patient experienced a 50% drop in paraprotein levels. Three had stable disease and one experienced disease progression. Interestingly, the patient with disease progression failed to generate a significant increase in paraprotein-specific, IFN-γ-secreting cells.

The same concluding comments regarding the limitations of melanoma studies apply equally to the reviewed myeloma studies. In the future, we hope that more standard phase I and II trials will be conducted. In this way, the true value of this approach can be evaluated more critically.

Non-Hodgkin's lymphoma

Hsu and coworkers [24] first reported the results of a pilot study of four patients in low-grade B-cell lymphoma. Three had measurable disease and one had apparent mesenteric disease and atypical cells in the bone marrow. All had been extensively pretreated for their disease with conventional chemotherapy.

As antigen, these authors used the idiotypic protein of the patients' particular lymphoma, obtained by hybridoma capture, as previously described by the same group [10, 23]. That the Ig produced by the hybridoma was the same as that of the lymphoma was confirmed by comparison of the hybridoma's heavy chain variable region DNA sequence with that of the corresponding lymphoma. The resulting antibody was purified by affinity chromatography. KLH was used as a control antigen.

PBMCs were obtained by leukapheresis and density-gradient centrifugation. These were divided and cultured in the presence of a low concentration (2 μg/ml) of either tumor-specific Ig or KLH. After 24 h, lymphocytes were removed from the preparations by further density-gradient centrifugation. The resulting DCs were then cultured for 14–18 h with higher concentrations of the specific antigen (50 μg/ml). This led to maturation of the DCs without the addition of exogenous cytokines. The DCs were then combined and used for immunization. Patients received one vaccination cycle per month for 3 months, followed by another vaccination cycle after 5–6 months. Each cycle consisted of an intravenous injection of 2×10^6 to 32×10^6 antigen-exposed DCs, determined by the yield in the preparation procedure. Two weeks later, the patients received a booster vaccination consisting of the idiotypic antibody and KLH in saline, each injected subcutaneously at different sites.

Specific antibodies and a specific cellular proliferative response were detected against KLH in all four subjects. Cellular proliferative responses against the idiotypic antibody were also detectable in the four subjects. No IgG anti-idiotypic IgG was detected, but IgM anti-idiotypic IgM was present in one subject. Anti-idiotypic CTLs were detectable in one patient.

Clinical responses to the vaccination were noted at the time of the original report, and later updated [77]. One patient remained in clinical remission for 44 months after treatment, but then relapsed. Another developed progressive disease after the initial series of three vaccinations, underwent rescue vaccination with idiotypic antibody conjugated to KLH. This patient's disease stabilized, allowing the fourth DC vaccination to be given at 6 months. The patient remained stable for 83 months, but then relapsed. A third patient developed progressive disease at 10 months. The fourth patient in the series underwent a sensitive molecular analysis of the blood and bone marrow, using PCR directed at the variable heavy chain specific for the patient's tumor. The lymphoma-specific DNA sequence was present in both blood and bone marrow prior to, but not after, vaccination. This patient remained without evidence of disease at least 79 months after vaccination.

The later report of this group describes the results obtained in an additional 31 patients [77]. Unfortunately, the authors introduced variations on the treatment protocol, making it difficult to evaluate the results rigorously. The authors treated a further six patients who had relapsed or had residual disease after initial conventional treatment. Of the six, four developed anti-idiotypic proliferative responses. One of the six developed anti-idiotypic IgM. All six developed humoral and cellular responses to KLH, demonstrating immunocompetence. Five patients had progressive disease within 1 year of beginning treatment. One had a complete clinical response, developing progressive disease 57 months later, while another developed progressive disease within 1 year. The other four developed progressive disease within 12 months of vaccination.

In summary, of the first ten patients vaccinated under this protocol, one had a complete clinical and molecular remission and remained in remission 79 months after vaccination. Two had complete responses, but later relapsed. One had a partial response. Six patients developed progressive disease within 1 year.

The authors studied a further 25 patients in their first remission after initial chemotherapy. They sought to prevent recurrence or progression of disease after initial chemotherapy. Twelve patients received DCs exposed to idiotypic antibodies or KLH. The remaining 13 patients received a DC vaccine pulsed with idiotypic Ig conjugated to KLH. Patients received an average of 0.7×10^6 to 12×10^6 DCs per vaccination. Two developed disease progression after receiving the initial series of three vaccinations. All remaining patients developed cellular and proliferative immune responses against KLH. Eleven patients remained in the first group, which received DCs pulsed with the individual antigens. Five developed cellular responses, while one developed anti-idiotypic IgM. Thus, 6 of 11 (55%) patients developed anti-idiotypic responses. Of the 11, five showed no evidence of disease at 43–56 months, two were stable at 46 and 51 months, and three patients had progressed at 25–51 months.

In the second group, which received DCs pulsed with the idiotype-KLH conjugate, 4 of 12 developed cellular immune responses. Six developed humoral responses. Overall, nine (75%) had a detectable immune response to the tumor-specific idiotype. Three patients had no evidence of disease at 24–45 months after vaccination, five were stable at 23–39 months, and four progressed at 16–28 months.

It is very difficult to interpret the results of this study, because of the large number of uncontrolled variables. First, some patients had recurrent disease and others had disease in first remission. Patients in the later group also received different DC vaccines, with the target idiotype either native or conjugated to KLH. The use of non-DC booster vaccinations makes it difficult to attribute all of the effects to the DC treatments specifically. There was no control group. The most useful analysis is in one of the first three patients, in whom a complete molecular remission was documented. It is unfortunate that the authors did not, in the follow-up article of 2002, confirm this effect in a larger, comparable patient group. Based on these results, one can conclude that DC therapy can induce cellular and humoral immune responses in low-grade lymphoma and that clinical remissions can be induced or maintained. A rigorous pursuit of this lead in the form of controlled clinical trials seems warranted.

Genitourinary malignancies

Renal and bladder carcinoma

One group has conducted a number of trials in renal cell carcinoma, a tumor which is responsive to immune interventions [21, 58]. To produce DCs, they obtained PBMCs by density-gradient centrifugation from peripheral blood. The plastic-adherent fraction was cultured for 5 days in medium supplemented with autologous serum, GM-CSF, and IL-4. On day 5, the cells were exposed to a tumor cell lysate and KLH for 1 h, after which fresh medium, containing TNF-α and prostaglandin E2, was added; the cells were cultured for another 24 h prior to use in vaccination. This yielded mature DCs, with high levels of cell surface HLA and associated molecules (Table 3).

Tumor cell lysate was produced from tumor cells cultured from nephrectomy specimens. Lysis was accomplished by treatment with a hypotonic solution; the lysates were then irradiated and frozen. As a control, normal kidney tissue was obtained from noninvolved portions of the nephrectomy specimens and prepared in parallel. Four patients with metastatic renal carcinoma were enrolled. They received three monthly intravenous infusions of DCs, beginning 4 weeks after nephrectomy. The patients received 5×10^6 to 10×10^6 antigen-exposed DCs per infusion. Fever was the only notable side effect.

One patient developed a partial clinical response to the vaccination. He exhibited partial regression in the size of all five measurable lung metastases. Two patients had stable disease and one experienced progression. Immunity in the responding patient was compared with that of the other three. The responding patient appeared to be more immunocompetent than the other patients, as evidenced by his development of a strong DTH response against KLH after a single vaccination. The other patients did not develop such a response. The responding patient also developed a proliferative response against tumor antigen after a single vaccination. The two patients with stable disease developed this only after several vaccinations; the patient with progressive disease hardly developed such a response at all. These data suggest that the inherent immunocompetence of patients is a key determinant in their response to this particular modality.

One unusual way of generating DCs for vaccination is the use of fused tumor cell/DC hybrids. This has been reported in the context of human renal cell carcinoma in 17 patients with metastatic renal cell carcinoma. The related paper has been marred by subsequent accusation of fraud [6] and, although the charges were not substantiated, the authors admitted sloppiness in the work and the paper was retracted [35].

A phase I/II study of DC vaccination was undertaken in 15 patients with progressive metastatic renal cancer [43]. To produce DCs, PBMCs were obtained from the buffy coats of peripheral blood by density-gradient centrifugation. The plastic-adherent cell fraction was cultured in medium supplemented with autologous serum, GM-CSF and IL-4. On the 5th day of culture, TNF-α was added. The cells were harvested on day 7 for vaccination. Antigen was added to the culture wells on days 1–4, prior to the maturation step.

Table 3 Clinical trials of dendritic cell immunotherapy in genitourinary malignancies

Tumor(s)	Subjects[a]	DC maturation[b]	Antigen	Route(s)[c]	Tumor immunity[d]	Clinical responses[e]	Ref.
Renal cell	4	Mature	Tumor cell lysate	IV	4/4[f]	1/4 (25%); 1 PR	[21, 58]
Renal cell	17	Mature	Tumor/DC fusion	SQ	11/17	7/17	[35][g]
Renal cell	11	Mature	Tumor cell lysate	IN, SQ	2/9	1/11 (9%); 1 PR	[43]
Renal cell	3	Immature	Tumor cell lysate	ID	2/3	0/3	[3]
Renal cell	12	Immature	Tumor cell lysate	ID	0/12	0/12	[50]
Bladder Cancer	4	Immature	HLA-specific peptide	SQ	N/A	3/4 (75%); 1 CR, 2 PR	[48]
Prostate	19	Immature	HLA-specific peptides	IV	?[h]	5/19 (26%); 5 PR	[44, 79]
Prostate	33	Immature	HLA-specific peptides	IV	N/D[i]	8/25 (32%); 2 CR, 6 PR	[80]
Prostate	37	Immature	HLA-specific peptides	IV	N/D[i]	12/37 (32%); 1 CR, 11 PR	[45, 81]
Prostate	31	Mature	PAP-GM-CSF	IV	20/31	3/31 (10%)	[71]
Prostate	13	Mature	PAP-GM-CSF	IV, SQ[j]	12/12	0/12	[8]
Prostate	21	Mature	Murine PAP	IV, IN, ID	11/21	0/21	[16, 17]
Prostate	16	Immature	PSA mRNA	IV, ID	9/9	1/7 (14%); 1 PR	[19]
Ovarian (6), uterine sarcoma (2)	8	Mature	Tumor cell lysate	IC	2/6	0/6	[20]
Cervical	1	Mature	EBV E7 protein[k]	SQ	1/1	0/1	[61, 62]

[a]Total number of subjects initially enrolled in the given study. May include persons who were unable to complete the study or were not evaluable

[b]DC maturation state at time of vaccination

[c]Routes of vaccination: *IV* intravenous, *SQ* subcutaneous, *IN* intranodal, *IC* intracutaneous, *ID* intradermal

[d]Patients in whom tumor-specific immunity of any kind was elicited by vaccination. Total number of patients tested may differ from number enrolled

[e]Clinical response rates may differ from total number enrolled due to persons not completing vaccination protocol. *CR* complete response, *PR* partial response

[f]While immunity was elicited in all patients, its intensity varied [21, 58]

[g]Validity of results later called into question [6, 35]

[h]Not able to be determined from the data [44, 79]

[i]Not determined [45, 80, 81]

[j]DC vaccination was given intravenously. Only booster doses with naked fusion protein were given subcutaneously [8]

[k]Epstein-Barr virus E7 protein [61, 62]

Tumor antigen was produced by freeze-thaw lysis of primary cell cultures of the patients' tumors. In nine cases, the DCs were pulsed with only tumor lysate. Two patients received DCs pulsed with both tumor lysate and KLH. Four control patients received DCs which had not been pulsed with tumor lysate. One was pulsed with only KLH and the remaining three received DCs that were not exposed to any antigen. Two patients from the group receiving tumor lysate–pulsed DCs and one patient from the unpulsed group also received treatment with a modified chemobiotherapy regimen. This appears to have occurred after the end of the experimental protocol. The reason for this treatment is not explained, although one would presume this was because of disease progression.

Patients received 0.91×10^6 to 14.4×10^6 DCs per vaccination. Two vaccinations were administered 1 week apart and then further booster vaccinations were administered every 2 weeks to a total of five vaccinations. Vaccinations were administered intranodally or, in the alternative, subcutaneously. There is no information provided as to the relative numbers of injections given by each route or the means of assuring that injections were intranodal, if given by that route. No adverse events were reported in association with the vaccination.

All patients received DTH testing against common recall antigens. Of the 11 patients who received DCs pulsed with tumor lysate, 6 exhibited an increase in such reactivity. Four of these 11 were not evaluable. None of the patients receiving DCs unpulsed with tumor lysate showed any such increase in reactivity. DTH testing was also conducted using the specific antigens individual patients received. Of the 11 patients receiving tumor lysate, 9 were evaluable in this regard, but only 2 showed even minimal reactivity by this assay after vaccination. Three of four patients receiving KLH-pulsed DCs were also assessed for DTH reactivity against this antigen. Two developed a detectable response after vaccination.

One patient, who received DCs pulsed with tumor lysate alone, experienced a partial response to the treatment. Seven had stable disease and the remainder had disease progression. Interestingly, two patients in the stable disease group received DCs without tumor lysate pulsing. The results of this study are of limited use. The experimental design is confusing. The immunologic assessment is limited. One can only conclude that this treatment approach, while apparently safe, is not immunogenic in this circumstance and does not lead to significant clinical effect.

A case series with three patients with metastatic renal cell carcinoma was reported from Japan [3]. DCs were generated from the adherent fraction of PBMCs by growth for 5 days in the presence of GM-CSF and IL-4. On days 6–7, the cultures were exposed to an autologous tumor lysate and KLH, as an ''immunomodulator'' and

positive control antigen. Patients received intradermal injections of the cells four times at 2-week intervals. The authors reported no associated toxicity.

Immunogenicity was assessed by DTH testing. All three developed DTH responses to KLH after vaccination. Two of the three patients developed DTH reactivity against autologous tumor lysate. One patient had stable disease, and two patients progressed despite therapy.

While it is hard to draw conclusions from a study of three patients, it is interesting to compare this study to studies noted above, which employed similar vaccination strategies [21, 58]. Vaccine preparation methods were similar, but the Japanese study did not include a DC maturation step: they did not add TNF-α or prostaglandin E2 at the end of the DC culture period. Nevertheless, the Japanese study was able to demonstrate development of immune responses against positive control antigen in all three patients and against tumor antigen in at least two. This was despite their reliance on DTH testing, a relatively crude and insensitive assessment tool. This leads one to question the necessity of such a maturation step in DC preparation.

Another group enrolled 12 patients with metastatic renal carcinoma in a trial of DC therapy [50]. Only nine of the patients had a positive DTH reaction to any of a panel of recall antigens. To obtain DCs, PBMCs were first isolated by density-gradient centrifugation. The plastic-adherent fraction was cultured for 7 days in medium supplemented with autologous serum, GM-CSF, and IL-4. Immature DC phenotype was confirmed at the end of this culture period by flow cytometry. Cells were prepared freshly at each vaccination. Interestingly, the phenotype of the cells changed during succeeding isolations as regards the fraction of $CD14^+$ cells. This may have been related to the use of IL-2 as an adjuvant agent, as noted below.

The antigen used for vaccination was a tumor cell lysate. Tumor cells were grown in culture from nephrectomy specimens. These were then subjected to lysis by freeze-thaw cycling and frozen for later use. DC cultures were exposed to the antigen preparation for about 3–4 h on day 7, and then injected. The cultures of six patients were also pulsed with KLH on days 4–7. After pulsing with lysate, all generated DCs were injected intradermally three times every other week. Total cell numbers were an average of 9.1×10^6 cells for the first vaccination, increasing to 14.6×10^6 cells for the third vaccination. The increased cell numbers were attributed to increased cell yields. After each vaccination, the patients received subcutaneous IL-2 for 5 days. Patients experienced some mild local effects and mild reactions typical of IL-2.

As noted above, DTH testing revealed that three of the study participants had no reaction to a panel of recall antigens prior to treatment. None of the enrolled patients developed a reaction to tumor lysate. DTH testing results against KLH are not mentioned in the report. Proliferative responses of PBMCs were evaluated. A moderate increase in proliferation against KLH was observed in several of the patients treated with KLH-exposed DCs. No increases in reactivity against tumor lysate or *Candida albicans*, a negative control, were observed. No humoral immune responses against KLH or tumor lysate were elicited.

The clinical results of this trial were essentially negative. Eight patients had stable disease for 3 to greater than 18 months. The remaining four displayed disease progression. No control group was available.

Inclusion of IL-2 treatment makes it impossible to distinguish an IL-2 effect from a DC effect. Further, the duration of DC antigen exposure was quite brief. Most studies utilizing immature DCs expose the cells to antigen for about 2 days, starting about day 5 of culture. It is interesting to note that some immune responses were generated against KLH, which was introduced into cell culture on days 4–7.

One study has explored the use of DC vaccination in metastatic bladder cancer in four patients [48]. The tumors of all four patients were proven by RT-PCR to express the cancer antigen MAGE-3. The antigen was an HLA-A24-specific nonapeptide epitope of MAGE-3. All four patients were HLA-A24-positive.

To isolate DCs, PBMCs were isolated from whole blood by density-gradient centrifugation and the adherent fraction was cultured in medium supplemented with human serum, IL-4, and GM-CSF for 7 days. The DCs were exposed to the MAGE-3-peptide "just before each vaccination," but it is unclear from the paper for what duration and when this occurred. A total of 10^7 to 10^8 antigen-pulsed DCs were administered subcutaneously every 2 weeks. Three patients received 6 vaccinations, while one received 18 vaccinations. All vaccinations were well tolerated.

No attempt to assess the induction of specific immune responses was reported. Of the four patients, one had a complete response. Unfortunately, this patient died of sepsis associated with a bowel perforation. The authors do not comment in detail about this event, although they imply that this was not likely to be related to DC vaccination. A pathologic complete remission in this patient was documented at postmortem examination, with the patient's nodal disease being completely absent. Two other patients had partial responses, while one patient died from progressive disease.

Prostate cancer

The most extensively developed approach to DC therapy in prostate cancer uses autologous DCs pulsed with one of two HLA-A0201-specific peptides from prostate-specific membrane antigen (PSMA) [44, 79]. In the phase I study, 51 patients with advanced, hormone-refractory prostate cancer enrolled, of whom 30 were positive for HLA-A2. PBMCs were isolated by density-gradient centrifugation. Plastic-adherent cells were cultured for 4–6 days in medium supplemented with GM-CSF and IL-4, and then exposed to one of the two HLA-specific peptides for 2 h.

Patients received four to five intravenous vaccinations every 6–8 weeks. Patients were divided into five

groups. One received 1×10^7 to 20×10^7 DCs that had not been exposed to antigen. Two groups received 0.2–20 μg of either of the two HLA-specific peptides without DCs. The final two experimental groups received 2×10^7 DCs exposed to one of the two HLA-specific PSMA peptides. Of the 51 patients, 19 received DCs pulsed with HLA-specific peptide: 9 with the PSM-P1 peptide. Twelve received unpulsed DCs at various concentrations; 20 received the naked peptides absent DCs.

At study entry, DTH reactivity against three recall antigens was evaluated. Only 25% reacted to all three antigens, with 63% reacting to none or to only one antigen, indicating impaired cell-mediated immunity. One would expect an immune-based intervention to be less effective in this population.

Toxicity was moderate. Mild to moderate hypotension, without reflex tachycardia, occurred in 24 of the subjects. The incidence was highest after the first infusion, and declined thereafter. Experiencing this effect after the one infusion did not predict the effect during later infusions.

Groups were subdivided into those with high initial prostate-specific antigen (PSA) values (> 10 ng/ml) and low initial PSA values (0–10 ng/ml). In the group receiving DCs pulsed with PSM-P1, those with high initial PSA showed a modest increase in PSA, while those with low PSA showed no significant change. Patients receiving DCs pulsed with PSM-P2 showed no significant change in PSA over the course of the study. In contrast, PSA increased modestly in the groups receiving either peptide alone or DCs alone, irrespective of initial PSA value. Based on National Prostate Cancer Project and PSA criteria, the authors identified seven partial responders. Two received peptide alone, four received DCs pulsed with PSM-P1 and one received DCs pulsed with PSM-P2.

Immunologic monitoring revealed modestly increased cellular proliferative activity only in the group receiving DCs exposed to PSM-P2. As a group, this became significantly elevated after the second DC infusion. Data presented are limited, and do not allow conclusions regarding number of responders in in vitro assays of immunity.

This group next conducted a phase II study. Part of the study consisted of 33 patients from the initial phase I study who wished to enroll in the phase II study [80]. The vaccination protocol was similar. DCs were pulsed with a tenfold higher concentration of either the PSM-P1 or PSM-P2 peptides for 2 h. Furthermore, the DCs were exposed to both peptides at the same time. Vaccinations were administered six times at 6-week intervals. On average, 6.4×10^6 DCs were administered with each intravenous infusion. Seventeen of the patients enrolled also received a 7-day course of subcutaneous injections with GM-CSF, to evaluate the adjuvant effect of this agent. Neither an effect on clinical response to DC vaccination nor an improvement in DTH reactivity was attributable to GM-CSF treatment [70]. Nine patients exhibited a partial response. No impact could be attributed to haplotype. Six of the nine partial responders were HLA-A2-positive, while 17 of 24 patients with progressive or stable disease were HLA-A2-positive. This was somewhat unexpected, as the vaccine was targeted toward this haplotype. Four of the nine partially responding patients were also responders in the phase I study.

Thirty-three vaccine-na patients were also enrolled in this phase II study [45, 80, 81]. Twenty-five received at least one infusion of the vaccine, and were evaluable. All had hormone-refractory prostate cancer and 14 were HLA-A2-positive. Partial response was defined as either satisfaction of National Prostate Cancer Project response criteria coupled with a 50% or greater decrease in PSA, or significant improvement on Prostascint scan. Two patients experienced complete responses, with disappearance of all evidence of prostate cancer. Six experienced a partial response, as defined above. Overall response rate was 32% (8/25). Median duration of response was 144 days.

The authors also conducted a phase II study in patients who developed local recurrences after initial therapy [81]. Thirty-seven patients participated. Treatment regimen and criteria for response were the same as in the group with metastatic disease. One complete and 11 partial responders were identified, for an overall response rate of 30% (11/37). Median duration of response was 184 days. Responders in both the metastatic disease and local recurrence groups displayed no significant change in PSA values when comparing poststudy to pre-study values. In contrast, the nonresponders exhibited 404% and 100% increases, respectively.

The authors evaluated immune status of the study patients by DTH to recall antigens and by IFN-γ secretion by PBMCs after anti-CD3 stimulation [40]. No statistically significant differences among the different patient response groups were evident.

DC pulsing has also been studied with a fusion protein antigen, consisting of PAP linked to GM-CSF and designated PAP-GM-CSF [71]. This study was conducted as a phase I/II trial. Twelve patients were initially enrolled to receive escalating doses of antigen-pulsed DCs to determine toxicity. An additional 19 patients were then enrolled to receive vaccinations at the highest tolerated dose. Patients were eligible if they had histologically confirmed prostate cancer, had failed antiandrogen therapy, had castrate levels of serum testosterone, and had a life expectancy of at least 3 months.

PBMCs were obtained by leukapheresis. DC precursors were obtained by a sequential, two-step purification process, using density-gradient centrifugation. The purified DCs were grown in medium without serum or supplemental cytokines for 40 h in the presence of PAP-GM-CSF. Mature DC phenotype was confirmed by cell surface marker analysis. In five patients during the initial phase of the study, one or two vaccine doses were prepared in which half of the harvested DCs were pulsed with the PAP-GM-CSF target antigen and half were

pulsed with KLH to provide a positive control. Patients received a fixed dose of vaccine intravenously at 0, 4, and 8 weeks. In patients with stable or responding disease, an additional vaccination was given at 24 weeks. The vaccinations were tolerated at all doses tested. Fever occurred during 15% of the infusions. Five patients experienced mild urinary complaints, including obstructive voiding symptoms, incontinence, urgency, and nocturia.

All patients developed T-cell proliferative responses against PAP-GM-CSF. On the basis of cytokine secretion profiles, the authors further characterized the responses as Th1-type. Interestingly, 15 patients (57%) had preexisting responses to GM-CSF, with 3 of these patients having been previously exposed to GM-CSF. Twenty patients developed T-cell proliferation in response to PAP, while 11 did not. The median time to disease progression was 34 weeks in the former group, while it was only 12 weeks in the later group, suggesting that a response against PAP is important in control of the disease. The authors also demonstrated that the five patients who received DCs primed with KLH developed a specific T-cell response against this antigen, while nine that were not exposed to this antigen did not. Thus, the response induced against PAP-GM-CSF was not a result of nonspecific immunostimulation. Sixteen (52%) patients developed antibodies against PAP. Ten patients had preexisting antibodies against GM-CSF, which increased to 25 (80%) patients after treatment.

The authors did not precisely define their clinical endpoints. They do note that "objective disease progression" was an endpoint for study participation. They note that a decline in PSA of greater than 50% of pretreatment values is generally viewed as significant. In three of their patients, such a decline was observed. The 12 patients in the phase I portion of the trial had median time to disease progression of 12 weeks, compared with 29 weeks for the 19 patients in the phase II portion of the trial. Phase I patients were more heavily pretreated and had higher median PSA values than those in the phase II portion of the trial. These factors may explain the difference in time-to-progression.

This study accomplished the goals of a phase I study. Specifically, the maximal tolerable dose of DC preparation was assessed, and not exceeded. The authors also showed that measurable specific immune responses against targeted antigens could be elicited, and that possession of a cell-mediated response against PAP had some correlation with time-to-progression. Clinical responses to the vaccine were limited. Interpretation of the clinical results was hampered by lack of precise definitions of clinical response.

Another group used the same antigen and DC preparation protocol in a slightly different approach to vaccination [8]. Specifically, a prime-boost approach was used, with priming by antigen-exposed DCs and boosting with the naked protein antigen. Thirteen patients with hormone-refractory prostate carcinoma were eligible. DCs were isolated, prepared, and exposed to the PAP-GM-CSF construct. Participants received two doses of intravenous DCs at 1-month intervals. Patients then received three subcutaneous booster doses of PAP-GM-CSF protein itself. Eight received all three vaccinations. Adverse events consisted of fever and chills associated with the infusions of DCs, myalgias and fatigue several days after DC infusion, and mild local reactions to the subcutaneous boost injections. All were less then grade II and self-limited.

Twelve patients completed the DC vaccination phase of the protocol. Three of these could not be assessed for proliferative responses due to high background levels. In the remaining nine, all developed specific proliferative responses against both GM-CSF and PAP. Specific proliferation did not markedly increase after administration of the naked antigen, indicating that these cellular responses were due to the DC treatments.

Only 2 of 11 patients developed humoral responses to the test antigen after DC vaccination alone. After subcutaneous vaccination with the PAP-GM-CSF construct, all patients developed antibodies. Interestingly, this was primarily attributable to humoral responses against GM-CSF. Responses against PAP were only detected in five patients, and these were of considerably lower titer than the response against GM-CSF.

Clinical response data were limited. One patient experienced rapid progression. Three experienced greater than 50% declines in PSA values. One of these three, however, developed progressive disease coincident with this decline. Three patients, including two of those that experienced significant PSA declines, also experienced drops in serum PAP. No radiographically detectable clinical benefit was appreciated.

This study, like that of Small and coworkers [71], was a phase I study. Its main purpose was to evaluate the tolerability and safety of the proposed treatment. In this respect, the study was successful. It also demonstrated the immunologic effects of the treatment. Specifically, even with a prime-boost approach, T-cell responses against the test antigen are primarily due to the DC treatment. The boost phase appears to induce humoral responses. The humoral response is primarily directed at the GM-CSF component of the fusion protein, as opposed to the PAP component. One anticipates that it would be the response against PAP that would result in clinical activity. Clinical effects were disappointing, with only four patients experiencing a decrease in serum PSA and/or PAP. Unfortunately, the authors did not define precisely the parameters for clinical regression, progression, or stability, making evaluation of clinical responses ambiguous.

Twenty-one men with biopsy-confirmed prostate cancer enrolled in a study using a xenoantigen-based approach [16, 17]. The DC sensitizing antigen was recombinant murine PAP (mPAP), which shares 81% amino acid homology with human PAP (hPAP). Seven of these patients had hormone-refractory disease.

DCs were isolated by leukapheresis from unmobilized peripheral blood. Enrichment of the DCs was by

density-gradient centrifugation [24]. The cells were then incubated for 24 h with mPAP at 2 μg/ml and 10% human serum. No additional cytokines were used. DCs were then further purified by metrizamide density-gradient centrifugation, and cultured overnight in medium with 50 μg/ml of mPAP. The cells were washed, resuspended in 5% autologous serum, and administered. The investigators administered the two monthly vaccinations to each patient by three different routes: intravenous, intranodal, and intradermal. One third of the study group received the vaccine by each route. The authors noted no differences in immunologic parameters or clinical responses attributable to route of administration. Mean cell dose was 11.2×10^6 cell per vaccination.

All toxicities attributable to vaccine administration were mild and self-limited. They consisted of grade II fevers and rigors in two patients, grade I erythema at the injection site in one patient, and a transiently swollen and painful draining lymph node in one patient. Before and after vaccination, the authors also assessed for the presence of antinuclear and rheumatoid factor antibodies. Five patients developed antinuclear antibodies and one developed rheumatoid factor. No clinical changes attributable to these antibodies were observed.

Proliferative responses against the test antigen were detected in all patients. Eleven developed specific T-cell proliferation in response to hPAP, indicating that tolerance to this self-antigen had been broken by the mPAP. Antibody responses elicited against mPAP and hPAP were less impressive. Four patients developed antibodies against both. Four others developed low titer antibodies against mPAP, and two developed low titer antibodies against hPAP.

The ''Materials and methods'' section of the report clearly defines clinical parameters for disease progression, stability, and response. Progression was a PSA rising to greater than 50% above baseline or the development of new lesions on imaging procedures. Regression was a greater than 50% reduction in measurable disease. Stability met neither of these criteria. Six of the 21 patients were classified as having disease stability. The remainder experienced disease progression. Notably, all six with disease stability developed T-cell responses against hPAP, in contrast to only five of the 15 with progression. Development of a cellular response against PAP can therefore be associated with a clinical response. This must be confirmed in a larger controlled trial, with disease response as primary endpoint. The immunologic results of the study nevertheless support its theoretical underpinnings: DC vaccination in humans with a xenoantigen homolog of a human antigen can break tolerance to the native antigen.

It is also possible to use mRNA encoding human PSA for DC vaccination [19]. Sixteen patients were enrolled in a phase I study. All had metastatic prostate cancer and serum PSA greater than or equal to 4.0 ng/dl. They all had adequate performance status, life expectancy greater than 6 months, adequate end organ function, and had not received active therapy, other than hormonal therapy, within 6 weeks of entry.

DCs were isolated from leukapheresis-derived PBMCs. These were cultured, and the ''semi-adherent cell fraction'' used for further DC generation. These were then cultured for 7 days in serum-free medium supplemented with IL-4 and GM-CSF. The cells were transfected with PSA mRNA by coincubation for 45 min. Vaccination was given intravenously at three escalating doses of 1×10^7, 3×10^7, and 5×10^7 mRNA-exposed cells. A dose of 10^7 cells was also given intradermally at each vaccination. Three vaccinations were given to each patient at 2-week intervals.

Of 16 patients originally enrolled, 2 withdrew due to rapid disease progression, and 1 developed sepsis unrelated to the vaccine trial. Vaccine administration was well tolerated at all doses: all toxicities were self-limited and grade I. These consisted of mild flu-like symptoms in four patients and mild injection-site reactions. One patient developed transient elevation in antinuclear and rheumatoid factor antibodies, but these returned to baseline after vaccination.

PBMCs of nine vaccinated patients, with three from each dose group, were analyzed before and after vaccination for the frequency of IFN-γ-secreting T-cells. In all tested patients, there was a marked increase in the frequency of these cells. There was no such change detected for a control antigen kallikrein, which shares significant amino acid homology with PSA.

Chromium release assays used autologous DCs transfected with mRNA for PSA or a control antigen as targets. T cells were stimulated with the mRNA-loaded DCs and then were used in the assay. In nine patients, all developed increased levels of specific lysis after vaccination. No postvaccination increase in specific lysis was observed with targets loaded with control antigen. Specific lysis was suppressed with an anti-CD8 antibody. The vaccination protocol thus elicited PSA-specific, $CD8^+$ CTLs.

PSA levels were used as a surrogate clinical endpoint. Seven patients were considered evaluable. One had an absolute decrease in PSA. The remaining six had increases in their PSA. However, when analyzed with regard to serum PSA slope velocities, five of these six had a decrease in slope velocity after vaccination. The sixth patient's slope velocity was unchanged. It is possible that the change in slope velocity of serially measured PSA values was decreased due to the formation of PSA-specific antibodies. The authors do not provide information regarding the humoral responses to their vaccination protocol. Measurement of serum PAP, either as absolute values or as slope velocities, might have avoided this criticism.

The authors used RT-PCR to assess vaccine effects on the levels of circulating tumor cells in three patients. Ten healthy male volunteers were used to determine a normal threshold level of PSA or epithelial cell adhesion molecule (EpCAM), another marker associated with prostate cancer. Prior to treatment, two patients had

elevated peripheral blood PSA copy numbers and one had elevated EpCAM copy number. All three demonstrated suppression of the elevated marker into the normal range while receiving vaccination. After vaccination, copy number values returned to pretreatment levels rapidly. These data support an antitumor effect for the vaccination protocol. They also indicate that vaccine treatment may have to be continuous to maintain antitumor effects.

In summary, these studies of DC therapy in prostate cancer confirm the induction of cell-mediated immunity to antigens associated with prostate cancer (PSA, PAP) and provide some evidence of antitumor activity. These are only surrogate markers for treatment efficacy in this disease. Measurable tumor regression, prolongation of time-to-progression, and prolonged survival will be the critical measures of efficacy that can only be confirmed in controlled clinical trials. As the optimal approach to DC therapy in this cancer has not yet been established, it may be some time before it is possible to even contemplate such trials.

Female genital tract cancers

A phase I study of DC vaccination was conducted in six patients with advanced ovarian carcinoma and two patients with advanced uterine sarcoma, using a previously reported method to produce DCs [20, 75]. PBMCs were isolated by density-gradient centrifugation. Adherent cells were cultured for 6–7 days in serum-free medium supplemented with GM-CSF and IL-4. At harvest, the nonadherent cell fraction was pulsed with either an autologous tumor cell lysate, derived from material obtained at surgery, or with KLH, along with GM-CSF, IL-4, and TNF-α. Cells were harvested on day 7 for leukapheresis-derived cells and on day 10 for those derived from fresh peripheral blood.

Patients received vaccination intracutaneously in the vicinity of the axillary nodes. Four patients received vaccinations at 10-day intervals and three received them at 4-week intervals. One patient received 14 vaccinations at 10-day intervals and nine at 4-week intervals. Vaccinations were continued until disease progression occurred, although one patient elected to continue vaccinations after surgical management of her progressive disease. Cell doses were quite variable, ranging from 1×10^6 to 90×10^6 cells per vaccination. Treatments were well tolerated in all subjects, with no toxic effects greater than grade II, including self-limited skin reactions at vaccination sites in six patients and mild fatigue and low-grade fever in two patients.

DTH testing against a panel of recall and test antigens was conducted before and after vaccination in six of the eight patients. Two were excluded due to rapid disease progression. Interestingly, the two excluded patients had the weakest DTH responses to a battery of test antigens administered prevaccination. All of the other six developed a response to the control antigen KLH. However, only one of the six developed a measurable DTH response to autologous tumor lysate. T-cell proliferative responses to vaccination were also assessed. Again, none of the patients had preexisting responses to the test antigens. Six of the eight patients developed responses to KLH, with the two nonresponders being those who experienced rapid disease progression and who had the weakest DTH responses prevaccination. Only two patients developed specific proliferative responses to autologous tumor lysate, one of whom was the individual who developed a DTH response to tumor lysate. ELISpot assays showed that two patients developed expansion of IFN-γ-secreting T cells after vaccination. Both of these patients were those who also developed specific proliferative responses to both KLH and to tumor lysate. One of these two also developed DTH responses to tumor lysate. This later patient also developed a detectable and durable increase in the levels of tumor lysate–specific, IFN-γ-secreting cells in the ELISpot assay. The authors have demonstrated that their vaccine is capable of inducing immune responses detectable in vitro. Responses to tumor lysate were seemingly more difficult to induce than those against KLH. Persons with poor responses in initial DTH testing did not develop responses.

Both patients who failed to develop detectable in vitro responses displayed rapid disease progression. Both patients with uterine sarcoma progressed rapidly, one of whom was one of the patients noted who failed to develop in vitro immune responses. Four of the remaining five patients remained progression-free for 11–25 weeks. The final patient was the one who developed significant responses to both KLH and tumor lysate in vitro. She remained progression-free for 45 weeks. After progression, she underwent surgical resection of her progressive disease and then received six further vaccinations, with 23 additional weeks prior to subsequent progression.

This study confirmed that the treatment was tolerable and established a toxicity profile. This vaccination protocol was capable of inducing responses to test antigens, including responses against autologous tumor lysate in at least two participants. Although there were no clinical responses, the patient who developed the strongest in vitro immune responses to tumor antigens also appeared to have some degree of disease stability. Unfortunately, the study suffers from the same deficiencies as other pilot and phase I and II studies described above.

Cervical cancer provides an interesting potential target for DC therapy, as an infectious agent—specifically human papillomavirus (HPV)—has been implicated in the disease's etiology. In much the same way that one might target an infectious disease, one can target viral proteins associated with oncologic transformation. DCs primed with the HPV antigen E7 are able to elicit specific $CD4^+$ and $CD8^+$ immune responses in vitro against tumor cells [61]. This observation led to a human trial in a 52-year-old patient, who had been heavily pretreated for local HPV-type-18-associated cervical

carcinoma and developed lung metastases 3 years later [62]. She received therapy with subcutaneous, mature DCs, primed with the type-specific E7 protein. She received five injections of $3x10^6$ to $5x10^6$ cells about every 2 weeks. After three of the five initial vaccinations, she also received autologous T cells stimulated in vitro with HPV-type-18-primed DCs. Furthermore, she received low-dose IL-2 intravenously. It is not clear whether this was just with autologous T cell infusion or was with each vaccination. She received an additional nine vaccinations every 1 to 2 months, apparently consisting only of DCs. The treatments were well tolerated, except for local symptoms at injection sites and flu-like symptoms associated with IL-2 infusion. The authors were able to demonstrate a DTH-response to the E7 antigen and to autologous tumor cells, although no control was administered. The patient's disease remained stable for 20 months. The authors attribute the clinical benefit to DC therapy, although this patient received so many different treatments that it is not reasonable to reach such a conclusion.

Three ovarian cancer patients were included in another DC study, which focused primarily on breast cancer [7]. The results are discussed below.

Gastrointestinal malignancies

Colorectal carcinoma

One group explored the use of DCs pulsed with total tumor RNA from colorectal cancer [56]. Fifteen patients with metastatic colorectal cancer were enrolled. To produce DCs, PBMCs were isolated from the buffy coat of whole blood, and the plastic-adherent cells were cultured for seven days in the presence of IL-4 and GM-CSF. The DCs were pulsed with total tumor RNA and KLH. Half of the pulsed cells were injected intravenously and half were cryopreserved for a booster vaccination. Patients received $0.4x10^6$ to $2x10^6$ antigen-exposed DCs per vaccination. Patients underwent two collections, with each providing material for two vaccinations (Table 4).

Vaccinations were well tolerated. Two patients developed mild, self-limited rigors and malaise after booster vaccinations. Three of the 15 patients died of progressive disease prior to completion of the planned four-dose course. Eleven patients developed DTH reactions to KLH. The authors did not assess the development of specific antitumor responses, but focused on changes in levels of serum carcinoembryonic antigen (CEA). Serum CEA levels fell in seven patients, and the rate of rise was reduced in two patients. No clinical responses were observed.

This study demonstrated the safety and tolerability of the authors' vaccination protocol. It also showed that a majority of patients developed DTH responses to a control antigen. A more rigorous analysis of the vaccine's clinical effects would have been worthwhile. An attempt at defining induction of a specific antitumor immune response, in particular against CEA, would also have been a worthwhile addition.

Another study examined patients with metastatic or recurrent cancer with rising CEA [15]. Twelve patients were enrolled, with either colorectal cancer or non–small cell lung cancer. The authors do not define the relative numbers of each type of patient. Due to the nature of the antigen used, all patients were required to be HLA-A0201$^+$. This study actually addresses three major issues: the ability of pretreatment with Flt3L to improve the yield of DC precursors from PBMCs, safety and tolerability of increasing DC doses, and the ability of DCs to elicit an immune response against a specific target peptide. Pretreatment with Flt3L increased the yield of DCs approximately 60-fold. This increase could only be partially explained by an increase in the yield of PBMCs, which only increased threefold after the treatment.

Table 4 Clinical trials of DC immunotherapy in gastrointestinal malignancies

Tumor(s)[a]	Subjects[b]	DC maturation[c]	Antigen	Route(s)[d]	Tumor immunity[e]	Clinical responses[f]	Refs.
Colorectal	15	Immature	Tumor RNA	IV	N/D[g]	0/15	[56]
Colorectal, NSCLC	12	Mature	HLA-specific peptide	IV	7/12	2/12 (17%); 2 CR	[15]
Colorectal (7), NSCLC (2), gastric (1)	10	Immature	HLA-specific peptide	SQ, ID	2/10	0/10	[25]
Gastric (6), esophageal (3), colorectal (3)	12	Immature	HLA-specific peptide	IV	5/9	0/12	[60]
HCC (8), CCC (2)	10	Immature	Tumor lysate	IN	N/D[g]	0/10	[26]

[a]Tumors: *NSCLC* non–small cell lung cancer, *HCC* hepatocellular carcinoma, *CCC* cholangiocarcinoma
[b]Total number of subjects initially enrolled in the given study. May include persons who were unable to complete the study or were not evaluable
[c]DC maturation state at time of vaccination
[d]Routes of vaccination: *IV* intravenous, *SQ* subcutaneous, *IN* intranodal, *ID* intradermal
[e]Patients in whom tumor-specific immunity of any kind was elicited by vaccination. Total number of patients tested may differ from number enrolled
[f]Clinical response rates may differ from total number enrolled due to persons not completing vaccination protocol. *CR* complete response
[g]not determined

After leukapheresis, PBMCs were further purified by density-gradient centrifugation. They were then initially cultured for 24 h with a low concentration of KLH and 10% human serum. DCs were then purified by metrizamide gradient centrifugation, and cultured overnight with a higher concentration of KLH and the test antigen 610D. This is a nonapeptide derived from an HLA-A0201-specific peptide of CEA. A substitution of aspartate for asparagine results in increased stimulation of CTLs in vitro. Interestingly, this substitution does not alter the ability of these lymphocytes to recognize and lyse tumor cells displaying authentic CEA. After exposure to antigen, the matured cells were resuspended in autologous serum for vaccination. Patients received two intravenous injections, separated by 1 month, of progressively increasing doses of antigen-exposed DCs, up to a maximum dose attainable. This was less than 10^{10} cells. No dose greater than 10^7 was attainable without patient pretreatment with Flt3L. Seven of 12 patients developed mild self-limited rigors and fever. Five developed mild diarrhea, which began 2–6 days after vaccination and lasted for 2–3 days. No information was provided regarding the relation of these side effects to total cell dose.

Vaccinations elicited specific immune responses to both KLH and CEA. PBMCs displayed KLH-specific proliferation after a single vaccination in all participants. CTL activity specific for the 610D peptide was detectable in 7 of 12 patients. As predicted, these CTLs also recognized and lysed target cells presenting the native, HLA-A0201-specific nonapeptide from CEA. In 5 of the 12 patients, tetramer analysis revealed that vaccination led to more than 1% of peripheral $CD8^+$ T cells being specific for 610D. They had a similar percentage of peripheral $CD8^+$ cells able to recognize the native, unmodified CEA peptide. That the same population of T cells was recognizing both antigens was confirmed by blocking the specific native peptide tetramer-binding with a preparation containing the 610D peptide. No humoral response against CEA was detected.

Two of 12 patients had complete clinical responses. One patient with metastatic colon cancer had a drop in CEA below the limits of detection and remained disease-free for at least 10 months. Another patient with colon cancer had resolution of her pulmonary metastases, but recurred in the abdomen after 10 months. One mixed response and two cases of stable disease (stabilization of both CEA and measurable disease) were observed, with stability lasting between 4 and 6 months. There was a statistically significant correlation between clinical response and both the percentage of specific $CD8^+$ cells on tetramer assay and the level of $CD8^+$ expansion over baseline.

This study demonstrates a number of important observations. First, Flt3L is a useful adjunct to increase DC yield, and these cells appear to be fully functional. Second, DCs seem to be tolerable and safe even at the highest doses attainable. Third, a measurable immune response can be elicited against a specific antigen, and the magnitude of the response appears to have some bearing on clinical response. The authors do not address whether higher DC doses yield better immune responses, which would be important in considering whether Flt3L pretreatment is even necessary.

Itoh and coworkers [25] used a similar vaccination strategy. As antigen, they used a nonapeptide from CEA, designated CEA652, which is specific for binding to HLA-A24. This HLA antigen is very common in Japan, where the study was conducted. Ten patients with metastatic cancer were enrolled. Seven had colorectal cancer, two had lung cancer, and one had gastric cancer. All were HLA-A24-positive and all had elevated CEA levels. PBMCs were harvested from peripheral blood by leukapheresis after 5 days of priming with G-CSF. PBMCs were purified from the leukapheresis product by density-gradient centrifugation. They were stored in liquid nitrogen until needed to prepare vaccine. Stored cells were thawed and cultured in medium supplemented with human serum for 2 h. Plastic-adherent cells were cultured for 7 days in this medium, further supplemented with GM-CSF and IL-4. The cells were then exposed to the CEA652 peptide for 2 h, and used for vaccination.

Patients received a total of ten vaccinations administered every two weeks. A total of 2.7×10^7 to 16×10^7 DCs were administered over the course of treatment. Vaccinations were given in the inguinal region subcutaneously and intradermally. The first three study patients, all with colorectal cancer, received DC vaccinations alone. The subsequent seven patients received adjuvant IFN-α and TNF-α on days 2 and 5 of each 14-day vaccination cycle.

The authors indicate that use of G-CSF pretreatment increased the ultimate yield of DCs about sixfold, though they do not present data to support this conclusion. The treatments were well tolerated. Two patients experienced transient local reactions at the injection site after the third vaccination. One developed progressive liver function test abnormalities and another developed mild anemia, both of which were attributed to rapid disease progression. After addition of adjuvant cytokines to the regimen, no reactions were noted.

DTH testing was conducted at study initiation and after each vaccination, using DCs with or without the CEA652 peptide. Purified protein derivative was used as positive control. Detailed results of the testing, in particular the reaction to the positive control, are not provided in the paper. Two patients who both received adjuvant cytokines developed positive DTH responses after the seventh or eighth vaccinations. In these two patients, the specific CTL response against the CEA652 peptide was assessed. One of the two demonstrated such a response.

Two patients, one with lung cancer and another with colorectal cancer, had disease stabilization for 6 and 9 months, associated with decreased and stable CEA levels, respectively. These patients both developed posi-

Table 5 Clinical trials of DC immunotherapy in miscellaneous malignancies and non-tumor-specific approaches

Tumor(s)[a]	Subjects[b]	DC maturation[c]	Antigen	Route(s)[d]	Tumor immunity[e]	Clinical responses[f]	Refs.
Astrocytoma(2), glioblastoma(7)	9	Immature	Tumor cell surface-associated peptides	ID	4/5	?[g]	[86]
Malignant glioma	8	Mature	DC/tumor cell fusion	ID	6/6	0/8[h]	[32]
Nasopharyngeal	16	Mature	HLA-specific peptide	IN	9/16	2/16 (12%); 2 PR	[38]
NSCLC	1	Immature	RNA for CEA	IV, ID	1/1	0/1	[46]
Breast (7), ovarian (3)	10	Mature	MUC1 or HER-2/*neu* HLA-specific peptide	SQ	5/10	1/10 (10%); 1 PR	[7]
Parathyroid	1	Mature	Tumor lysate, PTH	SQ	1/1	1/1; 1 PR	[63, 64]
Neuroendocrine pancreas	1	Mature	Tumor lysate	SQ	1/1	1/1; 1 PR	[64, 65]
Medullary thyroid	7	Mature	Calcitonin, HLA-specific CEA peptide	IC	7/7	1/7 (14%); 1 PR	[66, 67]
Breast (7), pancreas (2), papilla of Vater (1)	10	Immature	MUC1-expressing plasmid	IN	5/10	0/10	[54]
Pancreas (9), medullary thyroid (4), cholangiocarcinoma (4), hepatocellular (2), adrenocortical (1)	20	Mature	Tumor cell lysate	IN	18/20	?3/20[i] (15%)?3 PR	73]
Neuroblastoma (3), primitive neuroectodermal (2), Ewing's (1), sarcoma (6), renal cell (1), Wilms (1)	15	Immature	Tumor cell lysate	ID	3/6	1/15 (7%); 1 PR	[18]

[a]Tumor: *NSCLC* non–small cell lung cancer
[b]Total number of subjects initially enrolled in the given study. May include persons who were unable to complete the study or were not evaluable
[c]DC maturation state at time of vaccination
[d]Routes of vaccination: *IV* intravenous, *SQ* subcutaneous, *IN* intranodal, *ID* intradermal, *IC* intracutaneous
[e]Patients in whom tumor-specific immunity of any kind was elicited by vaccination. Total number of patients tested may differ from number enrolled
[f]Clinical response rates may differ from total number enrolled due to persons not completing vaccination protocol. *CR* complete response, *PR* partial response
[g]Improved survival versus historical controls [86]
[h]Based on objective radiologic criteria [32]
[i]Not stated explicitly by authors. Inferred from data presented [73]

tive DTH responses to the vaccinating peptide. A third patient with lung cancer had stabilization of CEA levels, but progressive disease on imaging studies. All three of these patients received adjuvant cytokine therapy, in addition to the DC vaccinations.

The conclusions which can be drawn from this study are limited by the protocol used. G-CSF may be a useful adjunct for increasing DC yields in leukapheresis. The specific vaccine and adjuvant cytokine treatments are apparently well tolerated. The limited immunologic testing indicates that the vaccine, even with adjuvant treatment, is not especially immunogenic. Furthermore, the clinical responses were limited. Whether the responses were due to the DC vaccine or to the adjuvant cytokines is unclear.

Yet another group employed a peptide-based approach, using a peptide from the MAGE-3 antigen [60]. MAGE-3 is normally thought to be expressed on melanoma, but it is also expressed in a significant number of gastrointestinal tumors, including esophageal, gastric, hepatocellular, and colorectal carcinomas. Twelve patients were enrolled, including six with gastric cancer, three with esophageal cancer, and three with colon cancer. All patients were either HLA-$A2^+$ or HLA-$A24^+$. These are the two most common HLA haplotypes in Japan, where the study was conducted.

DCs were produced from peripheral blood cells collected by leukapheresis. PBMCs were purified by density-gradient centrifugation and plastic adherence. The adherent fraction was cultured for 7 days in the presence of autologous serum, GM-CSF, and IL-4, generating immature DCs. The DCs were then pulsed with either of two HLA-specific MAGE-3 peptides, depending on the given patient's HLA haplotype.

Patients received vaccinations intravenously every 3 weeks, to a total of four vaccinations. The first vaccination consisted of 10^7 cells, with the remaining vaccinations using 3×10^7 cells. No significant or dose-limiting toxicities were observed. Three patients died from progressive disease prior to receiving all planned vaccinations.

A number of immunologic studies were conducted comparing results prior to vaccination with those after all four planned vaccinations. Eight patients were evaluable for development of peptide-specific CTL precursors in the blood, with the ninth patient yielding inadequate cell numbers at leukapheresis for this purpose. Four of the eight had a significant increase in the

frequency of CTL precursors. The authors also assessed DTH responses to the immunizing peptide before and after vaccination. Three of eight patients assessed had a positive reaction.

Clinical responses were evaluated by monitoring levels of peripheral tumor markers and changes in target lesion size in imaging studies. Three patients died due to progressive disease prior to finishing the vaccination protocol. Two of these demonstrated increases in tumor marker level prior to death, although one showed a moderate decrease. Of the remaining nine patients, three experienced mixed responses. All three patients also had a decrease in their serum tumor marker levels. Two of these patients had esophageal cancer and one had colon cancer. The patient with colon cancer had a marked decrease in the size of a chest wall mass, the results of which are presented in the paper. This patient also developed both a DTH reaction to the immunizing peptide. Of the remaining six patients, all had progressive disease on imaging. Three had increases in tumor marker levels and three had decreases.

This study demonstrates the somewhat monotonous tolerability and relative lack of toxicity of the DC vaccination approach. The immunologic results are mixed and difficult to interpret. At the least, this vaccination approach appears able to elicit some kind of detectable immune response in five of nine evaluable patients. The significance of the elicited responses is open to question. Clinical responses are also difficult to interpret. The authors attempt to introduce parameters other than strict imaging criteria. The three mixed responses noted on imaging, including an impressive decrease in chest mass size presented in the paper, would be a reasonable response in any phase I study. Changes in serum tumor markers, which might be expected to be relatively objective response measures, do not correlate well with clinical outcome. Inclusion of data regarding time-to-progression and survival would have been a useful addition to the study.

Hepatocellular carcinoma and cholangiocarcinoma

Liver cancer is a major worldwide health problem, especially in Asia. One pilot study has been recently published from Japan, examining the role for DC therapy in this disease [26]. Ten patients were enrolled, eight with hepatocellular carcinoma and two with cholangiocarcinoma. The group was heavily pretreated. Samples of tumor cell were obtained by needle biopsies and single cell suspensions were generated from the samples. These were subjected to freeze-thaw cycling and filtration to generate a tumor lysate for vaccination and immune monitoring.

To generate DCs, PBMCs were isolated from peripheral blood or leukapheresis product by density-gradient centrifugation. The adherent cell fraction was cultured in the presence of autologous serum, GM-CSF, and IL-4. On day 6, the cells were exposed to the tumor lysate for 12 h. Then, TNF-α and KLH were added, and the cells were cultured until day 9. Flow cytometric analysis prior to vaccination revealed that the cells had the immature DC phenotype, despite an attempted maturation step.

All patients received at least four weekly intranodal vaccinations with 1×10^6 to 10×10^6 DCs. Thereafter, stable or responding patients were to receive monthly booster vaccinations. All vaccinations were well tolerated. The authors report no adverse events, including no development of autoimmunity.

Immune responses were evaluated by the development of DTH reactions to KLH. Seven of the ten vaccinees developed a positive response. Development of such a response did not correlate with disease regression or stability. One patient had a mixed response, with partial regression of a single liver lesion. Biopsy of this lesion after regression showed no detectable tumor cells. The report indicates that six people had stable disease, though there is no indication of its duration. Three patients had progressive disease during the study. In eight patients, serum tumor marker levels were assessable. Two patients, both with ''stable disease,'' had moderate decreases in tumor marker levels.

This study has a number of deficiencies. The method for preparation of DCs did not yield the anticipated mature DC phenotype, although other studies have used immature DCs. The vaccine appears to have been immunogenic in the majority of patients, at least as regards KLH. Inclusion of a prevaccination assessment of immunocompetence, such as DTH testing against recall antigens, would have distinguished whether the lack of response was due to the vaccine or to immunodeficiency on the part of the nonresponders. Assessment of tumor-specific immunity would have been desirable, but may have been limited by available tumor sample. In vitro assessments, such as proliferation assays, may have allowed such an assessment while conserving precious tumor lysate samples. Perhaps the most significant deficiency in the study is the clinical assessment. In particular, lack of data regarding duration of disease stability is a major omission. The study did accomplish its primary goal, specifically establishing the safety of the vaccine preparation. It also highlighted some problems which must be addressed prior to any further studies based on these results.

Other malignancies and non-tumor-specific vaccination approaches

Brain tumors

Nine patients were enrolled in a phase I study of DC vaccination in glioma [86]. Two had anaplastic astrocytoma and seven had glioblastoma multiforme. All underwent surgical resection of their tumors, followed by adjuvant radiation therapy. All patients were off steroid therapy at the time of vaccination. Tumor cell–associated surface peptides served as antigen. To isolate these peptides, resected glioma cells were cultured until

an adequate number of cells were available. Surface peptides, in particular those associated with surface MHC class I molecules, were derived by acid elution (Table 5).

To prepare DCs, PBMCs were obtained from peripheral blood by density-gradient centrifugation, cultured for 2 h, and the adherent cell fraction retained. They were then cultured for 7 days in medium supplemented with FCS, IL-4, and GM-CSF. For antigen loading, adherent DCs were briefly exposed to an acid buffer to strip peptides associated with surface MHC molecules. The cells were then incubated overnight in medium supplemented with autologous serum and 50 μg/ml of the tumor peptide eluate. Patients received intradermal vaccinations with 10^6 cells three times at 2-week intervals. Vaccinations were given every 2 weeks. Other than brief, self-limited fever in one patient and lymphadenopathy that persisted for 2 months in another, the vaccinations were well tolerated.

Seven patients were tested for peptide-specific CTLs at various times relative to vaccination. It is unclear from the report why two of the nine enrolled patients were omitted from this part of the analysis. Two of the tested patients possessed CTL activity prior to vaccination. Of the remaining five, four developed specific CTL activity with vaccination. This activity was persistent, lasting, where detectable at all, until at least 3 months after vaccination. As further observation of the cellular responses induced by vaccination, intratumoral lymphocyte infiltration was present in four patients undergoing reoperation for recurrent tumor. Two patients displayed $CD8^+$ CTL and $CD45RO^+$ memory T-cell infiltration. These were not present in prevaccination tumor specimens. No B- or NK-cell infiltration was detectable, indicating that the infiltrating cells were not present nonspecifically. Tumors from reoperation in four unvaccinated control patients did not display such tumor-infiltrating T lymphocytes.

Forty-two unvaccinated patients served as historical controls. They were all treated at the same institution by the same surgeons within 2 years of study initiation. All had glioblastoma multiforme and were compared with the seven study patients with this disease. Both groups had similar clinical and demographic characteristics. Median survival was 455 days in the vaccinated group and 257 days in the control group. The authors did not attempt to draw statistical conclusions, due to the small test group size and the possibility of selection bias. This study demonstrates the safety of this vaccination approach. It also provides evidence regarding its apparent ability to induce immune responses in vaccine recipients, in a region of the body thought to be less amenable to immune interventions. Appropriately, the authors relate the possible survival benefit, but note that these results require confirmation in more rigorous studies.

Another approach to vaccination against gliomas involves fusion of DCs with the tumor cells [32]. Eight patients with malignant gliomas progressing despite conventional therapy were enrolled. All had cells from a surgical specimen growing in culture. PBMCs were isolated from peripheral blood by density-gradient centrifugation and plastic adherence. The adherent cell fraction was cultured for 7 days in the presence of autologous serum, IL-4, GM-CSF, and TNF-α, producing mature DCs. To generate the vaccine, DCs were admixed with irradiated autologous tumor cells and fused using a PEG-based protocol. The cells were then placed in the DC culture medium noted above, for 48 h. Fusion efficiency was estimated at 9% to 35%.

Patients were vaccinated intradermally at a site close to cervical lymph nodes. They received treatments every 3 weeks. It was intended to administer three to seven vaccinations, although two patients deteriorated prior to the third vaccination. No significant adverse effects or dose-limiting toxicities from the vaccinations occurred.

In the six evaluable patients, clinical responses to the treatment were judged from both a clinical evaluation and from radiologic findings. The clinical evaluation component of the study appears somewhat subjective. Eight weeks after vaccination, two patients were judged to be partial responders and the remaining four were reported with stable disease. The authors do not present time-to-progression data, although one patient remained clinically stable at 3 months after vaccination. By objective radiologic criteria, one mixed response was observed, in a patient judged to be "stable" clinically at 8 weeks.

Production of IFN-γ by PBMCs in response to vaccination was assessed in the six evaluable patients. PBMCs obtained before or after vaccination were mixed with either autologous tumor cells or an allogeneic glioma cell line U87MG. All patients displayed increased levels of production of IFN-γ in response to both cell types after vaccination.

This study demonstrates an apparent immunologic effect of the treatment, specifically an apparent increase in the IFN-γ secretion of PBMCs on exposure to autologous and allogeneic glioma cells. It would have been useful to include another, unrelated cell type, such as autologous fibroblasts, to show the specificity of the response. The data presented show that the vaccine is safe, satisfying the most basic goal of a phase I study. The primary weakness lies with the clinical evaluation. The authors use a relatively subjective clinical evaluation approach as their primary means of evaluation. Based on this, they claim at least two partial responses from six evaluable patients. The radiologic evidence indicates only one mixed response to treatment, in a patient judged clinically to be "stable." A single mixed response might not be such an unreasonable expectation in a phase I study of a disease with such a grave prognosis, especially in patients failing conventional therapy. When faced with such divergence, one must give greater weight to the objective radiologic data.

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma is a significant clinical problem in Asia, and one study of DC therapy for this disease originated there [38]. Sixteen patients were enrolled, all of whom had a local recurrence of this disease or developed metastases. All had at least one measurable target lesion and were positive for at least one of the following HLA alleles, which are common in the study population: A1101, A2402, or B40011.

To produce DC vaccine, PBMCs were obtained by leukapheresis and cryopreserved for later use. The plastic-adherent cell fraction was cultured for 7 days in the presence of FCS, GM-CSF, and IL-4. TNF-α was then added for an additional 3 days of culture. Mature DC phenotype was confirmed by flow cytometry, at which time the cells were pulsed for 6–8 h with KLH and one of three, HLA-specific peptides from LMP2, an Epstein-Barr virus protein expressed on the surface of nasopharyngeal carcinoma cells. Choice of peptide depended on a given patient's HLA haplotype. The cells were then washed and injected intranodally. Patients received four weekly injections. Cell dose is not noted, but apparently represents the entire yield of a DC preparation starting with $1x10^8$ to $2x10^8$ PBMCs.

Patients tolerated the immunizations well, with self-limited local reactions, low-grade fever, and flu-like symptoms as the only reported adverse effects. Overall immunocompetence was assessed by DTH testing for KLH reactivity after completion of the vaccination series. Four patients were negative in this regard, all of whom were negative by in vitro assays for peptide-specific immune responses and exhibited disease progression.

Three assays were conducted to assess for peptide-specific responses: ELISpot for peptide-specific, IFN-γ-secreting peripheral T cells; cytokine flow cytometry (CFC) to assess for peptide-specific, IFN-γ-secreting peripheral $CD8^+$ cells; and cytotoxicity assays for peptide-specific CTLs. ELISpot and CFC assays correlated perfectly, with 9 of the remaining 12 KLH-reactive patients being positive in both assays. Only five of these nine patients had detectable levels of specific CTLs. This may reflect an inherently lower sensitivity of CTL assays, as compared to the others. The ELISpot was conducted longitudinally. This revealed a peak in activity at about 2 weeks after vaccination, which persisted for about 3 months, declining to baseline at 6 months. The remaining three KLH-reactive patients were negative for any tumor-specific reactivity in these tests.

Clinical results were limited. One patient with a single vertebral metastasis had a partial response, which improved the patient's symptoms and decreased intake of opiate pain medication. This persisted for at least 1 year. Another patient with tumor in the lungs and mediastinal lymph nodes had a partial response lasting about 10 months, but then experienced disease progression.

The vaccine preparation in this study appears well tolerated. Information regarding the cell dose administered would have been useful. Further, prevaccination DTH testing for general immunocompetence would have been desirable to exclude those not likely to respond to the vaccine. Further immune testing in more advanced studies could be limited to ELISpot testing, as these results correlate well with CFC testing. CTL testing is less sensitive, and, in light of its greater labor intensity, does not really add enough to warrant its continued execution. It is surprising that no plan for continued immunization was included for those responding to the initial vaccination series. The ELISpot data, showing a waning of immunity 2 months after discontinuation of immunization, suggests that such a strategy might be effective in responders. Overall, this is a relatively well-conducted study, the results of which probably justify a phase II study, with some moderate alterations in the protocol.

Non-small cell lung cancer

One patient with metastatic non–small cell lung cancer was vaccinated initially with DCs transfected with CEA RNA, and 6 months later with DCs exposed to total tumor RNA [46]. DCs were obtained from PBMCs by density-gradient centrifugation and grown in serum-free medium supplemented with GM-CSF and IL-4. Lipid transfection was used to insert RNA of interest into the cells. The patient received four monthly immunizations with 3×10^6 intravenous and 1×10^6 intradermal RNA-exposed DCs. Neither adverse events associated with the immunization, nor apparent clinical benefit was observed. They do not comment on this patient's CEA levels prior to or during treatment.

PBMCs were obtained from this patient prior to any immunization, after immunization with DCs transfected with CEA RNA, and after immunization with DCs transfected with total tumor RNA. They were used in CTL assays for CEA-specific lytic activity. After the first round of immunization with DCs containing CEA RNA, specific CTL activity was detected in chromium release assays, even without in vitro restimulation. This was further enhanced after vaccination with DCs transfected with total tumor cell RNA. One cannot distinguish whether enhancement of this effect was due to the second round of vaccination or would have happened spontaneously. The latter might have happened, for example, by exposure to CEA from the patient's own tumor. Testing PBMCs for CTL activity after CEA RNA-DC immunization, but just prior to total RNA-DC vaccination, would help distinguish these possibilities.

At least two studies looking at DC-based vaccination with CEA have included some lung cancer patients [15, 25]. These are discussed in detail above under "Colorectal carcinoma."

Breast cancer

Only one study is published examining DC therapy in breast cancer [7]. The authors enrolled seven breast cancer and three ovarian cancer patients. All were HLA-$A2^+$, and their tumors expressed either HER-2/*neu* or MUC1 antigens. All were heavily pretreated, had measurable disease, and reacted adequately on DTH-testing of common recall antigens. The last requirement excluded those with inadequate overall immune function.

PBMCs were isolated by density-gradient centrifugation from whole blood. The adherent cell fraction was grown in serum-free medium supplemented with IL-4, GM-CSF, and TNF-α for 7 days. Mature DC phenotype was confirmed by flow cytometry. The cells were pulsed for 2 h with each of two HLA-A2-specific peptides from either HER-2/*neu* or MUC1. The selected peptide depended on the pattern of antigen expression in a given patient's tumor. Patients with tumors expressing both antigens were vaccinated with only the HER-2/*neu*-derived peptides. Patients received subcutaneous injections of 2×10^6 to 17×10^6 cells in the vicinity of the inguinal lymph nodes. These were administered every 14 days for three vaccinations, followed by vaccination every 28 days if tumor was stable or regressing. The authors report no significant side effects attributable to vaccination.

To assess the generation of a specific immune response, the authors examined IFN-γ production by peripheral $CD8^+$ T lymphocytes in response to stimulation with the vaccinating peptides. This was conducted using two-color flow cytometry. Three of four patients vaccinated with MUC1 peptides and two of six patients vaccinated with HER-2/*neu* peptides were positive in this assay after three vaccinations. The same patients also reacted against their respective vaccinating antigens in an in vitro cytotoxicity assay.

One breast cancer patient, who was noted to develop responses against MUC1 in the immune assessment, demonstrated regression of almost all her systemic disease on staging 6 months after trial initiation. This was associated with a drop in CA-125 and CA-15.3 tumor antigen levels. Notably, she developed central nervous system disease at the time of this restaging, suggesting that the treatment was not efficacious in the central nervous system. Interpretation of the results is confounded by the patient's undergoing oophrectomy shortly after the second vaccination. The authors argue that the patient was chemically oophrectomized prior to this, and the surgery was less likely to be an explanation for her clinical response than was the vaccination. One ovarian cancer patient had stable disease for at least 8 months, and another had a short period of about 8 weeks with stable disease. The authors do not report clinical responses in any of the other patients.

One German group looked at DC vaccination against mucin using transfection of MUC1 cDNA [54]. Seven of ten patients enrolled had breast cancer. This is discussed in more detail below under "Non-tumor-specific approaches."

Endocrine tumors

One group has investigated the use of DC therapy in the treatment of endocrine tumors. Their first study was a case series enrolling two patients, one with metastatic parathyroid carcinoma and another with a neuroendocrine tumor of the pancreas [63, 64, 65]. Tumor was obtained at surgery and subjected to lysis. To obtain DCs, PBMCs were isolated from peripheral blood by density-gradient centrifugation, placed in culture for 2 h and the adherent cells retained. They were cultured for 6 days in medium supplemented with FCS, GM-CSF, and IL-4. On day 7, the medium was replaced with medium supplemented with FCS, GM-CSF, TNF-α, and one of three antigens: PTH, tumor cell lysate, or KLH. After 4 h, the cells were used for immunization.

The patients received four weekly subcutaneous vaccinations of 5×10^6 cells, followed by monthly booster immunizations. The parathyroid carcinoma patient had previously received a vaccination series with tumor lysate–treated DCs [63]. She developed specific in vitro proliferative responses and DTH responses after vaccination, but progressed clinically. This led the authors to change the immunogen to DCs pulsed with PTH [64]. The patient with neuroendocrine pancreas tumor received tumor lysate–treated DCs. Both patients also received KLH-treated DCs. KLH served as both a control antigen and as a "CD4+ helper antigen." How these were administered is not clear, though they seem to have been given during the first four weekly immunizations at sites different from those receiving tumor antigen–exposed DCs. Both patients tolerated the immunizations well.

Both patients developed DTH reactions against their particular immunogens after several treatments. The parathyroid carcinoma patient had been previously noted to have KLH-specific DTH reactions from her prior vaccination series. A fourfold decrease in the serum PTH, a tumor marker in this disease, occurred after treatment. The neuroendocrine pancreas tumor patient also developed specific immune responses with treatment. DTH reactivity was detected against both KLH-exposed and tumor lysate–exposed DCs. However, the authors noted that some of this reactivity could have been attributable to FCS. Strong proliferative responses to both tumor lysate and KLH persisted after 1 year of vaccination treatment. Ovalbumin served as a negative control in these assays, and the proliferative response against it did not change with vaccination. From a clinical standpoint, this patient showed disappearance of a serum tumor marker (PTH) and partial regression of a measurable liver metastasis. He was clinically stable 20 months after initiation of treatment.

This group also reported a pilot study of DC vaccination in seven patients with advanced medullary

thyroid carcinoma [66, 67]. DCs were produced as above. On day 7 of culture, the DCs were exposed to calcitonin and an HLA-A2-specific nonapeptide from CEA, except in one patient who was HLA-A2-negative. The patients received 1x10 to $5x10^6$ antigen-exposed DCs intracutaneously weekly for 1 month, followed by booster immunizations every 4–8 weeks. During the first two weekly treatments, KLH was also administered, as "a $CD4^+$ helper antigen." This appears to have been given along with the DCs. One patient experienced self-limited fever with immunization, but the treatments were otherwise without adverse event.

DTH responses to CEA-peptide and calcitonin-pulsed DCs were present in all seven patients by the fifth immunization. The authors do not describe the controls in this testing, and thus do not exclude the possibility that this reactivity could be due to nonspecific reactions, such as to components of the culture media. All patients appeared to develop significant levels of specific T-cell proliferation against the CEA-peptide and calcitonin after vaccination.

One patient experienced a partial response, as evidenced by regression of liver and pulmonary masses and a significant decrease in serum tumor marker levels. Two patients had mixed responses and four patients had stable disease biochemically and morphologically. This study demonstrates an interesting potential use for DC vaccination in a tumor which is resistant to treatment, but any further conclusions from either this study or the case report of this group noted above are prevented by inconsistent experimental design and varying antigen and vaccine preparation protocols.

Non-tumor-specific approaches

A number of studies have been conducted using DC therapy as a general treatment for cancers. Pecher and coworkers [54] exploited the observation that disparate tumor types express the MUC1 antigen. Ten patients, all with metastatic tumors confirmed to express MUC1, were enrolled. Most were extensively pretreated. Seven patients had breast cancer, two had pancreatic cancer, and one had cancer of the papilla of Vater.

To produce DCs, PBMCs were isolated from peripheral blood by sequential density-gradient centrifugation. The plastic-adherent cell fraction was grown for 5 days in medium supplemented with human serum, GM-CSF, and IL-4. On day 5, the DCs were transfected by liposomes with plasmids expressing the MUC1 antigen under the control of the cytomegalovirus immediate-early promoter. These were grown for another day in essentially the same medium, at which point a glycosylation inhibitor was added. The glycosylation inhibitor allows better exposure of mucin peptides on the cell surface for better $CD8^+$-lymphocyte recognition. The cells were incubated for another day and then frozen. Quality assessment included surface marker analysis by flow cytometry to confirm DC phenotype and flow cytometry to confirm mucin expression as a means to determine transfection efficiency. All preparations had the expected DC surface phenotype. Transfection efficiency was 2–53%. Patients received 10^6 transfected cells injected intranodally under ultrasound guidance to the inguinal nodes. Vaccinations were given every 3 weeks, with the goal of achieving three vaccinations. Two patients died of disease progression prior to the third vaccination. No side effects were reported.

DTH testing and assessment of PBMCs for IFN-γ secretion in response to vaccine were used to assess immunogenicity. Nine of the ten patients had no DTH reactivity to the vaccine prior to vaccination. Two of these nine patients developed reactivity after vaccination. In nine patients, it was possible to assess the frequency of mucin-specific $CD8^+$ cells by examining IFN-γ secretion in response to antigenic stimulation. The antigen used was a synthetic peptide derived from mucin. CTLs can recognize this repeat in an HLA-independent manner. Four of the patients developed two- to tenfold increases in the frequency of these cells. Two patients had decreased frequencies. Three patients showed no change. Of note, one of the patients developing a positive DTH response to vaccine did not show an increase in the frequency of circulating mucin-specific $CD8^+$ cells. Therefore, effector cells for the DTH response recognize different mucin epitopes than the one used for CTL assessment.

The immunologic assessment indicated that the vaccine treatment was of limited immunogenicity. The clinical results were poor. One patient had at least a 3 month period of disease stability. The remainder experienced disease progression despite the treatment. Lack of immunogenicity was a significant problem. One obvious weakness in this study is the great variability in transfection rate. A more reliable method of transfection, such as electroporation, might be worth considering. One cannot conclude that this approach does not work. Rather, one can only conclude that the vaccine was not immunogenic as applied here. Nevertheless, attacking tumors immunologically in a non-organ-of-origin manner is attractive.

Another study sought to apply this approach using tumor lysate in stage IV carcinomas of poor prognosis [73]. Twenty patients were enrolled. Nine had pancreatic adenocarcinoma, four had medullary thyroid carcinoma, four had cholangiocarcinoma, two had hepatocellular carcinoma, and one had adrenocortical carcinoma.

A lysate prepared from resected tumor served as antigen. After resection and maceration, tumor lysate was prepared by sequential freeze-thaw cycling. To produce DCs, PBMCs were first isolated from whole blood by density-gradient centrifugation. Then, the $CD14^+$ cell fraction was isolated by adherence of cells coated with anti-CD14 antibodies to magnetic microbeads. $CD14^+$ cells were cultured for 5 days in medium supplemented with GM-CSF and IL-4. On day 5, the cells were pulsed with tumor cell lysate and incubated until day 7 in medium supplemented with

GM-CSF and TNF-α. This induced the mature DC phenotype.

Although not explicitly stated in the paper, patients appear to have received $1x10^7$ to $2x10^7$ DCs per vaccination. These were administered into the inguinal lymph nodes under ultrasound guidance. Patients received between four and ten vaccinations at 3-week intervals. Criteria for halting vaccination are not clearly delineated in the report, although it appears to have been due to disease progression in most cases. Starting on the day of vaccination during each cycle, patients also received subcutaneous IL-2 for 12 days as an adjuvant treatment.

Toxicity appears to have been minimal. Some patients had self-limited fever; one patient developed a self-limited rash after some of the vaccinations. Development of autoantibodies in the patients was carefully assessed. Patients were assessed for the presence of antibodies against a variety of known self-antigens. Sixteen patients were evaluable. Autoantibodies were detectable in four patients prior to initiation of therapy and in seven patients during therapy. No clinical symptoms attributable to these antibodies were observed.

The primary means for monitoring immunogenicity in this study was DTH, using mature DCs either unpulsed or pulsed with tumor cell lysate. Eighteen of 20 vaccinated patients were positive by this assay after four vaccinations. Results of the control injection of unpulsed DCs are not reported. Strong immunogenicity of the vaccine was thus demonstrated.

Clinical results were poorly presented. While definition of disease progression was defined, no clear definition of disease response was defined. No patient had a complete response. Possible partial responses occurred in three patients, but this is not stated explicitly. There are possible decreases in the levels of serum tumor markers in four of eight patients with tumors expressing such markers.

This study presented evidence of a DC vaccine with good immunogenicity. There was minimal toxicity. Of note is a detailed evaluation of possible generation of autoantibodies, which, although occurring in coincidence with vaccination, does not appear to be clinically significant. The inclusion of IL-2 treatment confounds conclusions regarding immunogenicity. It would be interesting to know if this is necessary to induce specific responses in such a high proportion of test subjects. Clinical responses were minimal, though this might reflect the advanced nature of the tumor types included in the study and the generally poor prognosis that these particular tumors have. In this regard, it is noteworthy that survival of only four patients exceeded 12 months and only half of the patients survived longer than 6 months. A more rigorous clinical evaluation would have been welcome.

One study examined the use of DC vaccination in pediatric patients with advanced solid tumors [18]. Fifteen patients were enrolled in the study, with neuroblastoma, sarcoma and renal tumors. All had recurred despite extensive pretreatment. As antigen, fresh tumor cells were subjected to freeze-thaw cycling and irradiation. They were then frozen for later use. To produce DCs, PBMCs were obtained by leukapheresis of peripheral blood. These were purified by density-gradient centrifugation and plastic-adherence. The adherent cell fraction was cultured in serum-free medium for 6 days in the presence of GM-CSF and IL-4. A total of 10^7 cells were then pulsed for 18 h with either tumor lysate equivalent to 10^7 tumor cells or with KLH. The DCs pulsed with the two antigens were then mixed. A total of 10^6–10^7 mixed cells were administered intradermally every 2 weeks for three vaccinations. Ten of the 15 enrolled patients completed all three vaccinations.

No significant or dose-limiting toxicities were noted. The authors assessed patients for the development of autoimmune antibodies after completing vaccination. One patient developed a positive anti-dsDNA titer, while another developed a positive anti-nuclear-antibody titer. Both later returned to baseline and neither was associated with clinical symptoms.

Immune responses were assessed against both KLH and tumor lysate. Against KLH, seven of nine evaluable patient developed significant increases in specific proliferation after vaccination. Proliferation against tumor lysate was not evaluated. In an ELISpot assay for the presence of IFN-γ-secreting cells in the peripheral blood, six of ten patients had an increase in KLH-specific, IFN-γ-secreting cells. Six patients could be assessed for tumor-specific, IFN-γ-secreting cells. Three developed an increase in such cells. In one patient with neuroblastoma, the authors evaluated the specificity of the ELISpot response. This patient had an increase in the number of secreting cells against an HLA-matched neuroblastoma cell lysate, but not against an unrelated tumor. Given the greater simplicity of proliferation assays, it would have been desirable if PBMC proliferative responses against tumor lysate had also been assessed.

Only ten of the patients were clinically evaluable at the end of the study. The authors do not comment on the five patients who dropped out. If this was due to disease progression, it suggests a problem with patient selection in the study. Of the remaining ten patients, one patient had a partial response. This patient had a fibrosarcoma with lung and thoracic spine recurrences. The authors note a very significant response in this patient. Five patients exhibited disease stability.

The paper focuses on the immunologic characterization of the response to vaccination, as well as the tolerability of their vaccination procedure. In these regards, their vaccination protocol appears to elicit detectable responses in a majority of those tested and to be safe and well tolerated. Furthermore, this study demonstrates that a DC vaccination protocol is practicable in a pediatric population, including children as young as 3 years old. The clinical results are reasonable for a phase I study, although the apparent loss of five patients, due presumably to progressive disease, suggests a problem with patient selection criteria. Use of a step to

mature the DCs prior to vaccination might have led to a better immunologic response. Nevertheless, the study appears promising and warrants further follow-up, in the context of a phase II study.

Discussion

The field of DC therapy has generated exciting data confirming that specific immune responses against tumor can be generated by a wide variety of DC vaccination approaches. Further, DC-based vaccines have shown negligible toxicity at all doses of cells achievable. However, most of the studies to date have demonstrated only limited clinical responses. There is also no firm data regarding remission duration and survival.

As this review illustrates, deficiencies in the trials to date lay in the wide variability in methods used at all steps in the vaccination process. In detail, these include (1) different DC sources; (2) different precursor cell mobilization methods; (3) different DC culture methods and different cytokine mixtures to induce their development; (4) different antigens; (5) different durations, concentrations, and other parameters in the antigen exposure process, including relative DC maturity at time of antigen exposure; (6) different DC maturation states at the time of vaccination; (7) different routes, schedules, and cell doses for immunization; (8) different adjuvant therapies; (9) use of more than one type of vaccine or other treatment in a given trial; (10) nonstandardized means of assessing induced immune responses; (11) utilization of nonrigorous or ill-defined criteria to assess response; and (12) incomplete description of clinical responses. In addition, DC therapy has not, with few exceptions, been approached in a manner approximating the systematic, sequential, phase I, II, and III trials, as used widely in other cancer therapies. Without a systematic effort to define the optimal preparation, dose, route, schedule, and duration of treatment, in a statistically evaluable number of patients, it is unlikely that DC therapy for cancer will achieve regulatory approval and wide-spread use.

Based on the trials reviewed, we can suggest what might be a worthwhile approach to a phase I/II DC trial in a generic tumor. Prevaccination immune testing for general immunocompetence needs to be conducted to exclude subjects incapable of responding to vaccination. This is most easily accomplished by DTH testing against common recall antigens. Each person enrolled needs to have objectively measurable lesions. PBMCs may be isolated by leukapheresis from peripheral blood, possibly after stimulation with G-CSF to increase yield. The adherent cell fraction is isolated, and grown in the presence of IL-4 and GM-CSF to generate the immature DCs. The immature cells are exposed to a lysate of autologous tumor cells and KLH as a control antigen. The cells are then grown 2 more days in medium supplemented with IL-1β, TNF-α, IL-6, and prostaglandin E2, to generate the mature DCs. The cells are injected subcutaneously in the vicinity of lymph nodes, every 2 weeks for three vaccinations. At the end of that time, assessment is made of immune responses against both the KLH control and the tumor lysate. This should be done by a simple, easily interpretable assay, such as a proliferation assay of PBMCs in response to antigen. Clinical response is assessed by reference to measurable target lesions, measured by reproducible, objective means, such as computed tomography. Determination of disease status after vaccination would be made according to standard clinical trials criteria. Vaccination might continue as long as there is no disease progression. Duration of response or stable disease, time-to-progression, and survival would be mandatory components of the study.

Such a trial would have a number of distinct advantages. The methods for obtaining and generating DCs have been used by a variety of workers, and been found to be effective in generating DCs. Unnecessary variables and steps are not introduced. While an undefined antigen preparation is utilized, this is most likely to sensitize a patient to the broadest range of antigens present in his or her own tumor. A standard control antigen is included to assess the patient's immunocompetence and the immunogenicity of the vaccine preparation as a whole. Immune assessment, specifically by a lymphocyte proliferation assay, is intended to answer whether the preparation generates a cellular immune response; this should be in the cheapest, simplest, most objective, and most reproducible way possible. More complicated immune analyses can be deferred to later studies, once an effective preparation is identified. Clinical response criteria must be well defined and objective.

It is possible that the deficiencies in DC therapy trials derive from the belief that DC therapy is somehow special and different from other therapies. We contend that this is not the case: the well-established phase I–III process used to develop conventional therapeutics can and should be applied in this field. There is one aspect of this therapy which differs from more conventional pharmacologic therapies: DC therapy is very labor and resource intensive. Each patient receives personalized treatment. This makes it difficult for any one center to conduct large trials. One concludes that the most efficient way to conduct the needed research may be through a cooperative group- or corporate-type model, with a central facility to process tumor specimens and generate DCs. Such groups would be able to determine the important questions to be answered. Participation by many centers would then make it possible to answer these questions efficiently. It would determine if the DC technology can be disseminated and whether it can be standardized, and it would generate data in a sufficient number of patients to produce definitive and statistically valid conclusions. Such an approach would address one of the major deficiencies of the present approach to this technology: many groups are working on different tumors using their own treatment protocols without any coordination with other groups.

For potential success, this field desperately needs the establishment of standards throughout the entire process. These would include the determination of optimal protocols for precursor collection and processing, DC generation, DC maturation, DC antigen pulsing, DC storage, and all of the standard aspects of clinical trials (dose, route, schedule, duration of treatment, and follow-up). After proper phase I and II trials, which have yet to be done in conformance with the standards of normal drug development practice, controlled phase III trials with survival as the primary endpoint will have to be undertaken. This must involve the pharmaceutical and biotechnology industries. Such studies will have to be conceived based on the data from well-conducted phase II clinical trials, indicating that there is sufficient promise for these therapies to be effective and commercially viable. Technology must also be developed to allow the development of cost-effective production methods according to Good Manufacturing Practice standards. It is only by this route that DC therapy, which shows such promise in animal models, has any hope of ultimately leading to treatments which can be marketed and can effectively compete with other new and promising cancer therapeutics now in the pipeline.

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