Developing a Rational Tumor Vaccine Therapy for Renal Cell Carcinoma: Immune Yin and Yang

Marc S. Ernstoff,[1,2,3,11] Todd S. Crocenzi,[13] John D. Seigne,[3,6,7] Nancy A. Crosby,[1,2] Bernard F. Cole,[3,10,12] Jan L. Fisher,[1,2] Jill C. Uhlenhake,[1,2] Diane Mellinger,[1,2] Cathy Foster,[1,2] Conrad J. Farnham,[3] Kathleen Mackay,[3] Zbigniew M. Szczepiorkowski,[3,4,5,11] Susan M. Webber,[4,5] Alan R. Schned,[3,5] Robert D. Harris,[8] Richard J. Barth, Jr.,[3,7,11] John A. Heaney,[3,6,7] and Randolph J. Noelle[3,9,11,12]

Abstract In patients with progressive malignancy, the natural balance between proinflammatory (Yang) and inhibitory (regulatory or Yin) immune pathways is disrupted and favors cancer-specific immune suppression. Therapy with interleukin 2 (IL-2) can mobilize immune effector cells that recognize and destroy cancer. High-dose IL-2 is the only therapy that has consistently induced complete durable remissions in patients with metastatic renal cell carcinoma (RCC) but only in a few of them. The lack of benefit in most metastatic RCC patients is likely due to the ineffective manipulation of other immune circuits critical in regulating tumor cytotoxic pathways. The limited clinical activity of IL-2, RCC vaccines, and other immune therapies to date leads us to postulate that effective clinical treatment strategies will need to simultaneously enhance proinflammatory pathways and disrupt regulatory pathways. We present preliminary studies in RCC patients to highlight the complexity of the regulatory pathways and our approach to shifting the balance of proinflammatory and regulatory immune pathways using dendritic cell – tumor lysate vaccine followed by cytokine therapy.

Until recently, the most successful therapy for metastatic renal cell carcinoma (RCC) has been that of single-agent, high-dose interleukin 2 (IL-2; ref. 1). The addition of other proinflammatory agents, such as IFN-α, or adoptive cellular therapy with *ex vivo* expanded autologous effector cells, such as lymphokine-activated killer cells or tumor-infiltrating lymphocytes, to IL-2 has failed to significantly improve clinical outcome compared with IL-2 alone (1 – 5). Vaccine strategies have also had limited benefit for patients with metastatic RCC (6). Recently introduced "directed therapies," such as bevacizumab, sorafenib, sunitinib, and temsirolimus, have had an effect on the survival of metastatic RCC patients but rarely induce durable complete remissions (7 – 10).

Immunotherapy with high-dose IL-2, which mobilizes immune effector cells that recognize and destroy cancer, can induce complete durable remissions but only in few metastatic RCC patients. The reason why IL-2 therapy fails in most metastatic RCC patients may be explained, in part, by a persistent imbalance between proinflammatory/stimulatory (Yang) and regulatory (Yin) pathways (11 – 13).

The $CD8^+$ CTLs mediate tumor destruction and represent a common pathway for many immunotherapeutic approaches. $CD8^+$ tumor-specific T cells naturally exist in the lymphocyte memory compartment and can be identified in the peripheral blood of cancer patients (14, 15). Memory $CD8^+$ T lymphocytes consist of a heterogeneous mixture of two lineage groups of cells that evolve in parallel: central memory $CD8^+$ T cells, which recirculate through lymph nodes, and effector memory $CD8^+$ T cells, which are mostly found in peripheral tissues (16). Central memory and effector memory T-cell responses can be enhanced by dendritic cell (DC) – presenting tumor antigens (17). Thus, DC vaccination approaches may help to optimize antitumor CTL generation.

Tolerogenic pathways that involve regulatory cells are a mechanism of tumor-specific anergy (18 – 20). $CD4^+CD25^{+high}$ regulatory T (T_{reg}) cells function in a non – antigen-specific manner through T cell to T cell contact or other contact-dependent mechanisms or through expression of immunosuppressive cytokines. $CD8^+CD28^-$ suppressor T (T_s) cells inhibit immune responses via cell-to-cell interactions or using antigen-presenting cells as a bridge to the $CD4^+$ T helper cell in an antigen-specific, MHC-restricted manner (21 – 23). Under experimental conditions in murine models, tumor-DC vaccinations can overcome tolerance and enhance therapeutic outcome (24). IL-2 and IFN-α can also contribute in overcoming these regulatory pathways (25, 26). Other inhibitory loops include both natural killer T cells and a subpopulation of

Authors' Affiliations: [1]Medical Oncology Immunotherapy Group, [2]Section of Hematology/Oncology, [3]Norris Cotton Cancer Center, [4]Cell Therapy Center, [5]Department of Pathology, [6]Section of Urology, [7]Department of Surgery, [8]Department of Diagnostic Radiology, [9]Department of Microbiology and Immunology, [10]Section of Biostatistics and Epidemiology, Department of Family and Community Medicine, and [11]Immunotherapy Program, Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire; [12]Dartmouth Medical School, Hanover, New Hampshire; and [13]Earle A. Chiles Cancer Research Center, Portland, Oregon
Received 8/17/06; revised 11/10/06; accepted 11/16/06.
Grant support: NIH grant RO1 CA5648 and Berlex, Inc.
Presented at the Second Cambridge Conference on Innovations and Challenges in Renal Cancer, March 24-25, 2006, Cambridge, Massachusetts.
Requests for reprints: Marc S. Ernstoff, Dartmouth-Hitchcock Medical Center, Lebanon, NH 03756. Phone: 603-650-5534; Fax: 603-650-7791; E-mail: Marc.S.Ernstoff@Hitchcock.org.

doi:10.1158/1078-0432.CCR-06-2064

inhibitory DCs (27, 28). The contribution of each of these regulatory compartments to tumor tolerance in the cancer patient is currently unknown (29, 30).

The lack of ability of cancer patients' CTLs to mount a complete response against tumor (anergy) may also be due to the loss of the T-cell receptor ζ chain (TCRζ), defective signaling downstream from TCRζ, and/or activation of inhibitory pathways by regulatory cells (31, 32). Some of these defects can be corrected by subsequent cytokine signals, including IL-2 (33). Agents that exploit proinflammatory activities that both enhance CTL function and disrupt regulatory pathways may synergistically act in causing immune-mediated tumor destruction. We hypothesize that immunotherapy using mature DC vaccine administered intranodally, IL-2, and IFN-α designed to shift the balance between immune stimulatory and inhibitory (regulatory) pathways will enhance therapeutic benefit.

DCs are the most potent antigen-presenting cells and are able to activate proinflammatory tumor-specific immune pathways as well as disrupt regulatory pathways (13). Immature DCs sample and process antigens and then migrate to T cell–rich areas in lymphoid organs (13, 34). In lymphoid organs, mature DCs, which express costimulatory molecules, present antigen and stimulate antigen-specific T cells to proliferate. In the tumor-bearing host, *in vivo* DC maturation and function seem inhibited (35–37). Treatment of tumor-bearing mice with an *ex vivo* generated DC vaccine reduces T-cell tolerance and enhances antitumor immunity (24, 38). Although the optimal route of DC administration is not defined, DC cross-priming of T lymphocytes requires cell-to-cell contact, suggesting that direct administration of mature DCs to lymph nodes would be advantageous. This is supported by mouse studies that confirm enhanced protective systemic immunity by intranodally administered DC-tumor vaccines, as well as preliminary data in human studies using intranodal vaccinations, which suggest that this route is superior in eliciting CD8 T-cell responses (39, 40). The source and type of antigens that are best suited for exploiting DC biology to initiate immunity remain unclear; defined tumor peptides, autologous tumor lysate, apoptotic tumor cell bodies, DC-tumor cell fusion, and genetically engineered DCs to express antigen have all been used with some evidence of immune stimulation (41–44). The uses of therapeutic tumor vaccines for metastatic RCC in the past have shown a low level of clinical activity (6). The poor clinical performance, in humans, of therapeutic cancer vaccines is due to the lack of ability of the vaccines, when given alone, to effectively influence proinflammatory and regulatory pathways needed to augment and maintain CTL response. Alternatively, antigen-bearing DCs may not have been appropriately matured *ex vivo* with inflammatory signals to effectively stimulate protective antitumor immune responses. This suggests the need for postvaccination signaling as immune manipulation. Combining therapeutic vaccines with single-agent proinflammatory cytokines, such as IL-2, or with inhibiting regulatory pathways with anti–CTL antigen-4 (CTLA-4) antibody does not seem to improve clinical outcomes, further suggesting the need for multitargeted combinations (45). The approach we have used differs from those previously reported by adopting an intranodal DC vaccination, followed immediately by combining IL-2 and IFN-α cytokine therapies.

Regulatory Pathways and T_s Cells

Over the past few years, disruption of the $CD4^+$ T_{reg} function through the blocking of CTLA-4 has been a clinical focus of immunotherapy. It is not clear whether anti–CTLA-4 antibody blockade directly works on the T_{reg} cells or by blocking negative signals to effector T cells. CTLA-4 molecule is constitutively expressed on $CD4^+$ T_{reg} cells and competes with CD28 (a T-cell stimulatory receptor) to bind to B7.1/B7.2 on antigen-presenting cells (45). When CTLA-4 is disabled, it is believed that CTLs are released from inhibition and the regulatory effects of T_{reg} cells are diminished. Disabling CTLA-4 can be achieved by a blocking antibody (anti–CTLA-4; ref. 46). Preliminary clinical trials of blocking anti–CTLA-4 antibodies in melanoma and RCC patients suggest that this approach has a small therapeutic effect (47, 48). In one study, the addition of IL-2 to anti–CTLA-4 antibody therapy in melanoma patients failed to show additional clinical activity over IL-2 or anti–CTLA-4 antibody therapy alone, suggesting an overlap in the mechanisms of action of these agents on $CD4^+$ T_{reg} cells or the presence of other regulatory pathways (49).

To determine the presence of other regulatory cell populations in cancer patients, we studied peripheral blood mononuclear cells from healthy donors and advanced RCC patients for the presence of T_s cells. T-cell subpopulations were identified by multicolor flow cytometry using anti-CD8, anti-CD28, anti-CD4, and anti-CD25 antibodies (Coulter/Immunotech, Marseille, Cedex, France). Phenotypical differences in the proportion of T-cell subsets between healthy donors and RCC patients were analyzed using an unpaired *t* test. T_s cell function was measured by a $[^3H]$thymidine $CD4^+$ T-cell proliferation assay using irradiated KG-1 cells, which act as an antigen-presenting cell bridge (21).

Blood samples from 24 healthy donors and 29 RCC patients were analyzed. The percentage of $CD8^+CD28^-$ T_s cells was significantly higher in RCC patients (46.2%) than in healthy donors (39%; $P = 0.044$). The percentage of T_{reg} cells ($CD4^+CD25^{+bright}$) was also significantly higher in RCC patients (38.3% versus 21.3%; $P = 0.0006$).

RCC patients' T_s cells consistently suppressed autologous $CD4^+$ cell proliferation. The percentage of reduction in proliferation ranged from 31% to 47%. Irradiation of T_s cells before cell culture did not affect their ability to suppress CD4 proliferation. Low concentrations of IL-2 (50 IU/mL) were able to reverse the suppressive effects of T_s cells on $CD4^+$ cell proliferation (Fig. 1).

Thus, we have shown a significantly higher percentage of functioning T_s cells in RCC patients. Their contribution to tumor tolerance is still unclear, but they may play a significant role along with the other regulatory cell populations. Recent data suggest that DCs made tolerant by $CD8^+$ T_s cells have reduced expression of costimulatory molecules (21). Thus, one approach to overcome $CD8^+$ T_s cell suppression would be to induce costimulatory molecules by maturing DCs *ex vivo* before clinical use.

Therapeutic Strategies Using a Yin-Yang Approach for Metastatic RCC

By exploiting the proinflammatory properties (Yang) of the DC vaccine, IL-2, and IFN-α, and the ability of IL-2 and mature

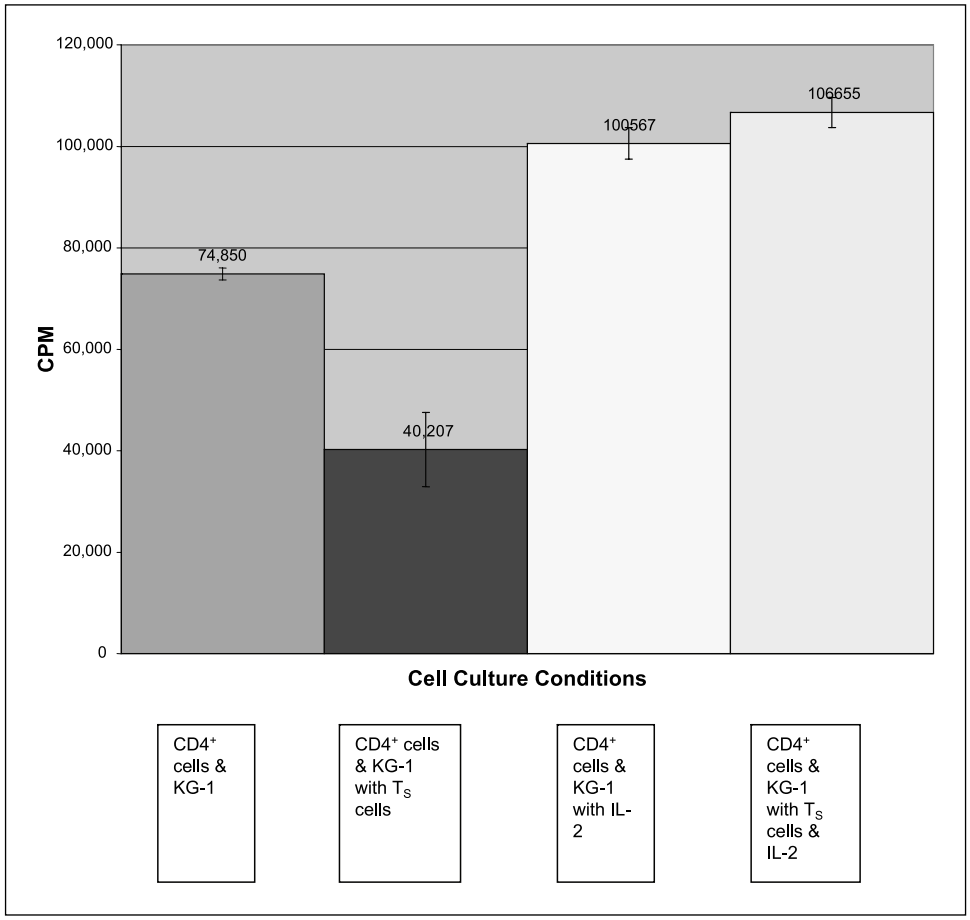

Fig. 1. Example in a RCC patient of inhibition of CD4 cell proliferation by T_s cells using a [^{3}H]thymidine assay. Functional assay using a purified $CD4^+$ cell stimulated with irradiated KG-1 cells ($CD4^+$ cell/KG-1, 1:0.5) and pulsed with [^{3}H]thymidine as the base culture condition. The addition of T_s cells ($CD4^+$ cell/T_s cell/KG-1, 1:1:0.5) inhibits $CD4^+$ cell proliferation (% suppression, −46%). In the presence of IL-2, $CD4^+$ cell proliferation is enhanced. No significant reduction in proliferation is observed with the addition of T_s cells when cultured in the presence of IL-2. CPM, cell count per minute.

DCs to disrupt regulatory pathways (Yin), we hoped to improve clinical therapy for metastatic RCC patients. We have analyzed the first 13 metastatic RCC patients (11 men and 2 women) treated with DC vaccine, IL-2, and IFN-α. Patient demographics are summarized in Table 1.

Patients who met the eligibility criteria received two induction cycles of IL-2/IFN-α2a given on days 1 and 14 and three maintenance cycles at 28-day intervals (Fig. 2; ref. 50). An intranodal matured DC vaccine was given the day before each cycle. An 18×10^6 IU/m^2 dose of IL-2 (Chiron, Inc., Emeryville, CA) was administered i.v. as 24-h continuous infusion for 5 days per cycle. IFN-α2a (Hoffmann-La Roche, Inc., Nutley, NJ) was given every other day, for three doses, by s.c. injection; at the start of each cycle, a dose of 6 MIU was used. Continuous infusion of IL-2 and/or IFN-α2a were interrupted for serious toxicity, and two dose reductions of IL-2 to 75% and 37.5% were allowed with subsequent cycles of therapy. Toxic effects that required further interruptions of therapy resulted in patients discontinuing IL-2 therapy and continuing IFN-α2a and vaccine therapies.

Patients were characterized for prognosis based on the criteria described by Motzer et al. (51). Clinical response, defined by using the Response Evaluation Criteria in Solid Tumors from the National Cancer Institute, was assessed by computed tomography of the chest, abdomen, and pelvis and bone scan before therapy, at the completion of the second induction cycle, between the second and third maintenance cycle, and at the completion of the study (52). Subsequent follow-ups for responding and stable patients consisted of the same imaging methods every 3 months thereafter or as clinically indicated. Survival was evaluated using the Kaplan-Meier method.

Tumor lysate–loaded, mature DCs were obtained from leukapheresis products under IND 11162. DCs were generated and matured over 9 days *ex vivo* in Lifecell tissue culture bags with serum-free AIM-V medium, 500 IU/mL of granulocyte-macrophage colony-stimulating factor (Berlex, Inc., Richmond, CA), 20 ng/mL of IL-4 (R&D Systems, Inc., Minneapolis, MN),

Table 1. Patient characteristics

Characteristics	No. patients (*N* = 13)
Sex	
Male	11
Female	2
Age, y (median, 61.8 y)	
40-49	1
50-59	3
60-69	6
70-79	3
Karnofsky performance status, %	
80	7
90	6
Motzer risk group (no. risk factors)	
Favorable risk (0)	6
Intermediate risk (1-2)	7
Poor risk (>2)	0

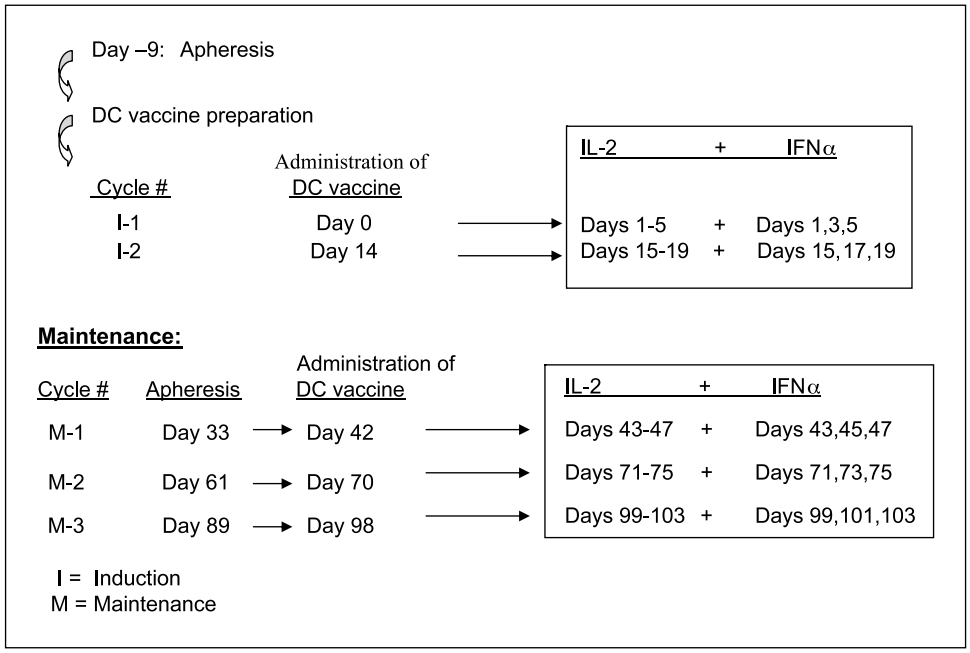

Fig. 2. Treatment schema.

autologous tumor lysate (1-3 tumor cell equivalents per DC added on day 5), and 50 ng/mL of tumor necrosis factor α (added on day 6; R&D Systems). These matured DCs typically express high levels of costimulatory molecules (CD80, CD86, MHC classes I and II) and up-regulate CD83 expression. DCs were harvested on day 9, and a total of 1×10^7 DCs in 1 mL of Ringer's lactate were injected into two inguinal lymph nodes under ultrasound guidance. A portion of these DCs was frozen in 90% autologous serum and 10% DMSO and thawed for vaccine 2. Subsequent DC vaccines (vaccines 3, 4, and 5) were generated from monocyte precursors obtained from freshly prepared pheresis products. Requirements for release of the final DC preparation include >70% viability, negative sterility Gram stain, and an acceptable low endotoxin level.

To assess the effect of the therapy on immunologic function, we assessed tumor-specific CD4 and CD8 precursor levels using a dye dilution proliferation assay (46, 47). TCR function was assessed via a redirected cytotoxicity assay (53, 54). The dye dilution proliferation assay has a sensitivity of 10^{-5} and differentiates the renal cell–specific $CD4^+$ and $CD8^+$ T-cell populations.

Determination of the clinical response rate was the primary objective of the study. Six (46%) of the 19 patients achieved an objective partial or complete clinical response to treatment (Table 2). The two complete responders remain free of disease at >17 and 11 months of follow-up, respectively. Six patients had favorable Motzer criteria, and seven patients were in the intermediate risk group. Clinical responses were seen in both visceral (lung, liver, and adrenal) and lymph node–based disease sites. Responses were even observed in sites of bone involvement. Ten of thirteen patients remain alive from 19+ to 33+ months.

Table 2. Clinical responses

Patient no.	Metastatic disease sites	Response	Response duration (mo)	Survival (mo)
1	Lung, adrenal	SD	15	18+
2	Lung	PR	9	17+
3	LN (mediastinal/RP/pelvic)	SD	9	13
4	Lung, LN (mediastinal/para-aortic)	CR	15+	17+
5	Lung	PD	NA	13+
6	Bone	PD	NA	13
7	Lung, adrenal, LN (mediastinal)	PR	8	11+
8	Lung, bone, LN (mediastinal)	CR	10+	11+
9	Lung, liver, LN (RP)	PR	7	9+
10	Bone, LN (RP/para-aortic)	SD	4	5+
11	Lung, LN (RP)	PR*	2	4+
12	Lung, LN (mediastinal)	SD	4+	4+
13	Lung	SD	2	4+

Abbreviations: LN, lymph nodes; NA, not applicable; RP, retroperitoneal; SD, stable disease; PR, partial response; CR, complete response; +, ongoing response and/or survival.
*Resected to no evidence of disease status.

Table 3. Adverse events (grade 3 or 4) during treatment

Adverse events	No. patients (%)
Electrolyte deficiency	12 (92)
Hypotension	11 (85)
Rash/desquamation/pruritis	11 (85)
Neurological symptoms	6 (46)
Diarrhea	4 (31)
Infection	4* (31)
Hypoxia	3 (31)
Alkaline phosphatase elevation	3 (23)
Creatinine elevation	3 (23)
Anuria/oliguria	2 (15)
Nausea or vomiting	2 (15)
Thrombocytopenia	2 (15)
Mucositis	1 (8)
Bilirubin elevation	1 (8)
Cardiac symptoms	1† (8)

*Includes two patients with *Clostridium difficile* colitis and two patients with central venous catheter-associated bacteremia.
†One patient developed a transient incomplete right bundle branch block and a self-limited cardiomyopathy.

Table 3 lists the grade 3 or 4 adverse events observed during the treatment with IL-2, IFN-α2a, and DC vaccine, all of which were expected. We were particularly interested in the development of high-grade, autoimmune-like toxicity, which has been suggested to correlate with clinical responses. No grade 3 or 4 adverse events directly related to vaccine occurred. No treatment-related deaths occurred. Eighty-five percent of patients developed significant dermatitis, which is probably an autoimmune-like reaction. One patient who achieved a complete response developed glomerulonephritis, cardiomyopathy, and lung radiographic changes suggestive of an interstitial pneumonitis after three vaccine doses and four cycles (two induction and two maintenance) of IL-2 and IFN-α2a. An endomyocardial biopsy specimen was nondiagnostic, and renal biopsy was deferred. All of these manifestations normalized at ~3 to 4 months after the completion of the therapy.

Tumor-specific T-cell precursor frequency (Fig. 3) includes a summary of $CD4^+$ T-cell precursor frequencies as measured based on IFN-γ production and proliferation in response to autologous, tumor lysate–loaded DCs. Included are the four patients who received at least three vaccine doses and had blood samples obtained at the designated time points for analysis for which data are currently available. All four patients showed a relatively small increase in tumor-specific $CD4^+$ T-cell precursor frequencies at a minimum of one time point after induction or maintenance phases of treatment compared with pretreatment baseline values. Three of these patients experienced a clinical partial response. No measurable increases in $CD8^+$/IFN-γ^+ precursors have been detected to date.

The effect of treatment on the $CD8^+$ TCRζ pathway was determined via lytic activity in a redirected cytotoxicity assay, which uses an anti-CD3 antibody that engages the TCRζ (CD3) on the effector cell and the Fc receptor on the target P815 cell (Fig. 4; ref. 54). This assay allows a functional assessment of the TCRζ signal transduction pathway. A measurable increase in the $CD8^+$ T-cell lytic activity after treatment was observed in three of six patients, suggesting enhanced signaling through TCRζ and reversal of signaling pathway defects. The other three patients had relatively stable posttreatment activity compared with pretreatment levels. Those patients with a detectable increase in lytic activity during or after their course of therapy included two patients with a partial response and one patient with a stable disease. The one complete responder of this group of six patients had a stable lytic activity.

Discussion

Our broader understanding of the complexity of immune inflammatory (Yang) and regulatory (Yin) pathways in cancer patients now offers new paradigms that will affect immunotherapy strategies for patients. Our observation of functional $CD8^+$ T_s cells in RCC patients and the limited clinical benefit of disruption of the CD4 T_{reg} cell–CTLA-4 pathway suggests that multiple regulatory pathways are likely to be operative in cancer patients. Induction of proinflammatory pathways or down-regulation of immune tolerance alone is frequently insufficient to produce antitumor responses in most cancer patients. We hypothesize that the generation of the appropriate balance of immunologic Yin and Yang may well be the key to enhancing therapeutic benefit. DCs, IL-2, and IFN-α play roles in both enhancing inflammatory responses and down-regulating or limiting peripheral tolerance mediated by T_{reg} and T_s pathways (22, 56). Preliminary data from our trial of autologous mature DC-tumor vaccination followed by IL-2 and IFN-α2a therapy provides support for combination immunotherapy, addressing both proinflammatory and regulatory pathways.

In our study, the administration of autologous, tumor lysate–loaded, mature DC vaccine, coupled with continuous infusion of IL-2 and IFN-α2a, resulted in a clinical objective response in 6 of 13 patients with metastatic RCC, twice the response rates historically seen with high-dose IL-2 or with continuous infusion of IL-2 with IFN-α (1, 2, 50) and better than the response rates reported to RCC tumor vaccines,

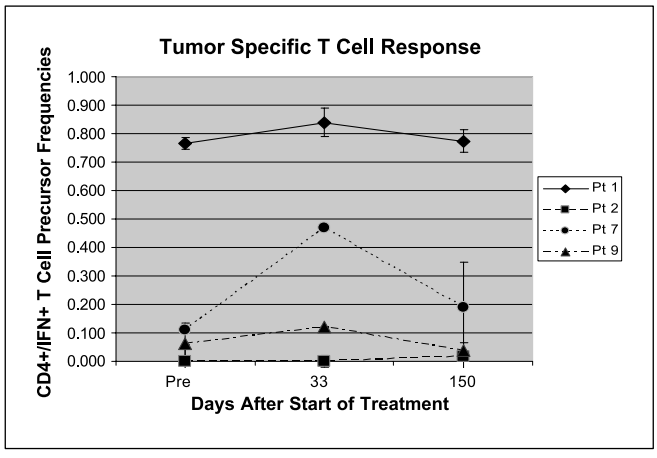

Fig. 3. To date, patients (*Pt*) 1, 2, 7, and 9 have had full time course of assessed immune variables. Antigen-specific $CD4^+$ T-cell precursor frequencies are determined by IFN-γ production of PKH67-labeled lymphocytes in culture with tumor lysate – loaded, autologous DCs in a dye dilution proliferation assay. Points, actual frequency values done in replicate assays; bars, SD. Precursor frequency values were measured 9 d before treatment (*Pre*), 33 d after start of treatment (completion of induction treatment cycles), and at 150 d (after completion of maintenance treatment cycles).

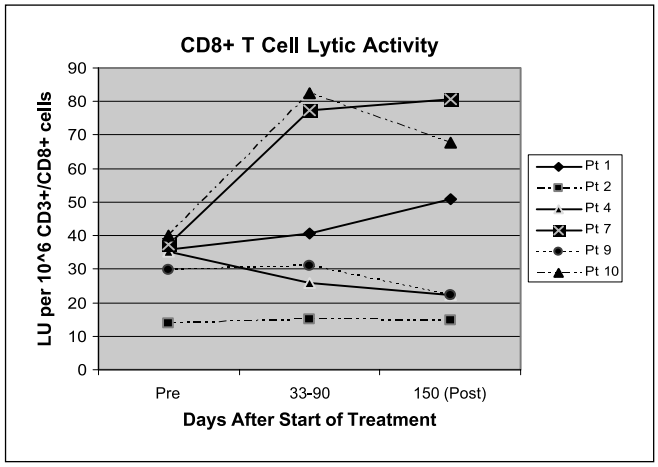

Fig. 4. To date, patients 1, 2, 4, 7, 9, and 10 have had full time course of assessed immune variables. $CD8^+$ T-cell lytic activity is determined through a redirected cytotoxicity assay that identifies $CD8^+$ cells by flow cytometry and CTL specificity using P815 cell line as the target cell. Quantitation of lytic activity is expressed in lytic units (*LU*) per 10^6 $CD3^+CD8^+$ cells based on 30% target cell lysis. Values are shown for the six patients who received at least three DC vaccine doses and for whom blood samples were obtained for the three time points of interest. Pretreatment samples were obtained 9 d before the start of therapy. Another blood sample was obtained between 33 and 90 d from start of therapy (following treatment induction cycle 2 or before second or third maintenance cycles). The last blood sample was obtained at 150 d (*Post*), ~60 d from the last cycle of therapy.

including DC vaccines. We would attribute the improvement of the objective response rate observed in our study, if confirmed, to the addition of the DC vaccine component in our regimen. Optimization of this therapy using the Yin-Yang hypothesis would ultimately need to be tested in larger randomized studies.

The limitations of DC vaccination strategies for human cancers include DC biological complexity, the immunologic complexity of the host, and the production of homogeneous GMP grade product. DC function can be classified into initiating a T helper 1 cellular immune response, initiating a T helper 2 humoral immune response, or inhibiting immune responses via the induction of the T_{reg} function (57). Each of these functions has been associated with different DC phenotypes, although it seems that, in humans, these functional phenotypes are somewhat plastic. Thus, the best DC precursor to use for initiating *in vitro* cultures and the conditions that would provide the most effective T helper 1 skewing are unclear. The complexity of the host immune environment may differ between patients with the same cancer and between patients with different cancers, which may limit the general value of DC vaccination. Developing production methods that can provide high-quality, GMP-grade DC products which are homogeneous in their maturational state for a large number of patients is also a challenge. This may ultimately limit the application of DCs to a larger population of patients.

The IL-2 dose of the Negrier regimen is still quite substantial, and we observed toxicity with our regimen as expected of a high-dose IL-2–containing regimen (50). Serious adverse events were transient and were managed with standard supportive measures consistent with published guidelines and in a center experienced in administering IL-2 (58). Of note, we observed higher rates of grade 3 or 4 hypotension than that reported by Negrier et al. (50). Interestingly, we also observed a significantly higher rate of grade 3 or 4 skin manifestations, including skin peeling, erythema, and pruritus, which have been considered to be autoimmune-like effects and correlated to better clinical outcomes in other immunotherapy studies (47, 48). This, too, would support our hypothesis that combining DC vaccine with induction of inflammatory pathways and inhibition of regulatory pathways is crucial to improving clinical outcomes. We suspected a unifying autoimmune process in the one patient who developed a cardiomyopathy, glomerulonephritis, and interstitial infiltrates on his lung radiograph after three complete cycles of therapy. This patient went on to achieve a complete response, along with spontaneous resolution of the apparent treatment-related toxic effects.

We previously reported that $CD8^+$ CTL activity in advanced RCC patients is impaired but can be restored with IL-2 *in vitro* (33). In our current study, we observed a posttreatment increase in $CD8^+$ CTL activity in three of the six patients tested, although this did not necessarily correlate with the clinical responses. The lack of a consistent increase in CTL activity, particularly in patients with clinical responses, may reflect the individual heterogeneity in a functional TCRζ signaling apparatus in peripheral blood lymphocytes (11, 12, 21, 32). To isolate a more tumor-specific immune response, we used a dye dilution proliferation assay to measure IFN-γ–producing $CD4^+$ or $CD8^+$ T cells in response to the culture with tumor lysate–loaded, autologous DCs (53, 54). Thus far, four patients who were tested exhibited an increase in at least one time point in antigen-specific $CD4^+$ T-cell precursor frequency in response to treatment.

Our encouraging preliminary results raise the possibility of enhancing the objective response rate and, particularly, the durable clinical responses with therapy that takes advantage of enhancing inflammatory and limiting regulatory pathways.

Open Discussion

Dr. Atkins: What is the end point of your renal cancer vaccine trial?

Dr. Ernstoff: This is a classic two-stage, phase 2 trial design, employing response as a primary end point. We've already seen six responders in the first cohort of patients, which exceeds our criteria for the first stage, and thus anticipate accruing our planned 33 patients.

Dr. Vieweg: What kind of T-cell subsets do you induce during vaccination? Do you get T-cell memory? Do you induce CD4 or CD8 T-cell responses?

Dr. Ernstoff: We are still in the process of analyzing data for specific T-cell subsets. We are employing a panel using seven-color flow cytometry to determine the induction of different CD8 and CD4 T-memory and T-effector cells. The tumor-specific proliferation assay that we employ uses autologous DCs loaded with lysed tumor cells or unloaded DCs as stimulators and peripheral blood lymphocytes as responding cells. By use of the dye dilution method and subtraction of the fraction of T cells proliferating in response to unloaded DCs, we can determine tumor-specific T-cell responses in peripheral blood lymphocytes. We are still in the process of completing this assessment in all patients, but preliminary data suggest induction of tumor-specific CD4 response to treatment.

Dr. Vieweg: Are your DCs migratory?

Dr. Ernstoff: We haven't looked at the migratory nature of the DC population that we used to treat patients. We have looked at both DC phenotype and cross-presentation, so we know that there is up-regulation of CD83 and other costimulatory molecules and that the DCs are able to cross-present antigen *in vitro*.

Dr. Atkins: Do the DCs need to migrate if you are injecting them into the nodes?

Dr. Vieweg: Other studies suggest that (CD40L) activated DCs that are not migratory can cause tolerance.

Dr. Atkins: Is there potentially a selection bias in your study that comes from detecting recurrence early in these patients or is the patient population enrolled in this study mostly the typical population referred in with metastatic disease?

Dr. Ernstoff: Our referral pattern is such that these patients are representative of the everyday metastatic renal cell patient. Approximately half of the patients have metastatic disease at presentation and have a nephrectomy as standard of care. The others are frequently followed up in the community and sent to us only when their disease recurs.

Dr. Sosman: How durable is your treatment response?

Dr. Ernstoff: The two complete responders continue to be in complete response. The longest response is now about 18 or 19 months. Of the four partial response patients, two continue with partial response with no further therapy, probably about 10 or 11 months out, and two have progressed about that time and gone on to antivascular endothelial growth factor therapy.

References

1. Tretter C, Savage Paul D, Muss Hyman B, Ernstoff MS. Interferon-α and -β: clinical applications renal cell cancer. 3rd ed. In: Rosenberg SA, editor. Principles and practice of the biologic therapy of cancer. Baltimore: Lippincott Williams & Wilkins; 2001. p. 252–65.
2. Atkins MB, Sparano J, Fisher RI, et al. Randomized phase II trial of high-dose interleukin-2 either alone or in combination with interferon α-2b in advanced renal cell carcinoma. J Clin Oncol 1993;11:661–70.
3. Atzpodien J, Kirchner H, Jonas U, et al. Interleukin-2- and interferon α-2a-based immunochemotherapy in advanced renal cell carcinoma: a prospectively randomized trial of the German Cooperative Renal Carcinoma Chemoimmunotherapy Group (DGCIN). J Clin Oncol 2004;22:1188–94.
4. Negrier S, PhilipT, Stoter G, et al. Interleukin-2 with or without LAK cells in metastatic renal cell carcinoma: a report of a European multicentre study. Eur J Cancer Clin Oncol 1989;25:S21–8.
5. Goedegebuure PS, Douville LM, Li H, et al. Adoptive immunotherapy with tumor-infiltrating lymphocytes and interleukin-2 in patients with metastatic malignant melanoma and renal cell carcinoma: a pilot study. J Clin Oncol 1995;13:1939–49.
6. Schwaab T, Heaney J, Schned AR, et al. A randomized phase II trial comparing two sequence combinations of autologous vaccine and human recombinant interferon γ and human recombinant interferon α-2B therapy in patients with metastatic renal cell carcinoma: clinical outcome and analysis of immunological parameters. J Urol 2000;163:1322–7.
7. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med 2003;349:427–34.
8. Motzer RJ, Michaelson MD, Redman BG, et al. Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. J Clin Oncol 2006; 24:16–24.
9. Ahmad T, Eisen T. Kinase inhibition with BAY 43-9006 in renal cell carcinoma. Clin Cancer Res 2004; 10:6388S–92S.
10. Atkins MB, Hidalgo M, Stadler WM, et al. Randomized phase II study of multiple dose levels of CCI-779, a novel mammalian target of rapamycin kinase inhibitor, in patients with advanced refractory renal cell carcinoma. J Clin Oncol 2004;22:909–18.
11. Whiteside TL. Signaling defects in T lymphocytes of patients with malignancy. Cancer Immunol Immunother 1999;48:346–52.
12. Uzzo RG, Rayman P, Kolenko V, et al. Renal cell carcinoma-derived gangliosides suppress nuclear factor-κB activation in T cells. J Clin Invest 1999;104: 769–76.
13. Banchereau J, Steinman RM. Dendritic cells and control of immunity. Nature 1998;392:245–52.
14. Finke JH, Rayman P, Edinger M, et al. Characterization of a human renal cell carcinoma specific cytotoxic CD8+ T cell line. J Immunother 1992;11:1–11.
15. Jantzer P, Schendel DJ. Human renal cell carcinoma antigen-specific CTLs: antigen-driven selection and long-term persistence *in vivo*. Cancer Res 1998;58: 3078–86.
16. Lauvau G, Vijh S, Kong P, et al. Priming of memory but not effector CD8 T cells by a killed bacterial vaccine. Science 2001;294:1735–9.
17. Wiethe C, Dittmar K, Doan T, Lindenmaier W, Tindle R. Provision of 4-1BB ligand enhances effector and memory CTL responses generated by immunization with dendritic cells expressing a human tumor-associated antigen. J Immunol 2003;170:2912–22.
18. Javia LR, Rosenberg SA. $CD4^+CD25^+$ suppressor lymphocytes in the circulation of patients immunized against melanoma antigens. J Immunother 2003;26: 85–93.
19. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. *Ex vivo* isolation and characterization of CD4(+)CD25(+) T-cells with regulatory properties from human blood. J Exp Med 2003;193:1303–10.
20. Cortesini R, LeMaoult J, Ciubotariu R, Cortesini NS. $CD8^+CD28^-$ T suppressor cells and the induction of antigen-specific, antigen-presenting cell-mediated suppression of Th reactivity. Immunol Rev 2001;182: 201–6.
21. Chang CC, Ciubotariu R, Manavalan JS, et al. Tolerization of dendritic cells by Ts cells: the crucial role of inhibitory receptors ILT3 and ILT4. Nat Immunol 2002;3:237–43.
22. Feinberg MB, Silvestri G. Ts cells and immune tolerance induction: a regulatory renaissance? Nat Immunol 2002;3:215–7.
23. Cortesini R, LeMaoult J, Ciubotariu R, Cortesini NS. $CD8^+CD28^-$ T suppressor cells and the induction of antigen-specific, antigen-presenting cell-mediated suppression of Th reactivity. Immunol Rev 2001;182: 201–6.
24. Fields RC, Shimizu K, Mule JJ. Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses *in vitro* and *in vivo*. Proc Natl Acad Sci U S A 1998;95:9482–94.
25. Anderson PO, Sundstedt A, Yazici Z, et al. IL-2 overcomes the unresponsiveness but fails to reverse the regulatory function of antigen-induced T regulatory cells. J Immunol 2005;174:310–9.
26. Knoefel B, Nuske K, Steiner T, et al. Renal cell carcinomas produce IL-6, IL-10, IL-11, and TGF-β1 in primary cultures and modulate T lymphocyte blast transformation. J Interferon Cytokine Res 1997;17: 95–102.
27. Terabe M, Swann, J, Ambrosino E, et al. A nonclassical non-Vα14Jα18 CD1d-restricted (type II) NKT cell is sufficient for down regulation of tumor immunosurveillance. J Exp Med 2005;202:1627–33.
28. Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. Nat Rev Immunol 2004;4:762–74.
29. Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. Clin Cancer Res 2003;9:606–12.
30. Jarnicki AG, Lysaght J, Todryk S, Mills KH. Suppression of antitumor immunity by IL-10 and TGF-beta-producing T cells infiltrating the growing tumor: influence of tumor environment on the induction of CD4+ and CD8+ regulatory T cells. J Immunol 2006;177:896–904.
31. Cardi G, Heaney JA, Schned AR, Phillips DM, Branda M, Ernstoff MS. T-cell receptor ζ chain expression on tumor infiltrating lymphocytes from renal cell carcinoma. Cancer Res 1997;57:3517–9.
32. Finke JH, Zea AH, Stanley J, et al. Loss of T-cell receptor ζ chain and p56lck in T-cells infiltrating human renal cell carcinoma. Cancer Res 1993;53: 5613–6.
33. Crocenzi TS, Tretter CPG, Schwaab T, et al. Impaired cytolytic activity in peripheral blood t cells from renal cell carcinoma patients. Clin Immunol 2005;117:6–11.
34. Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immun 1991;9: 271–96.
35. Gabrilovitch DI, Corak J, Ciernik IF, et al. Decreased antigen presentation by dendritic cells in patients with breast cancer. Clin Cancer Res 1997;3:483–90.
36. Troy AJ, Summers KL, Davidson PJT, Atkinson CH, Hart DNJ. Minimal recruitment and activation of dendritic cells within renal cell carcinoma. Clin Cancer Res 1998;4:585–93.
37. Schwaab T, Schned AR, Heaney JA, et al. *In vivo* description of dendritic cells in human renal cell carcinoma. J Urol 1999;162:567–73.
38. Lou Y, Wang G, Lizée G, et al. Dendritic cells strongly boost the antitumor activity of adoptively transferred T cells *in vivo*. Cancer Res 2004;64:6783–90.
39. Lambert LA, Gibson GR, Maloney M, Durell B, Noelle RJ, Barth RJ, Jr. Intranodal immunization with tumor lysate-pulsed dendritic cells enhances protective antitumor immunity. Cancer Res 2001;61: 641–6.
40. Czerniecki BJ, Carter C, Rivoltini L, et al. Calcium ionophore-treated peripheral blood monocytes and dendritic cells rapidly display characteristics of activated dendritic cells. J Immunol 1997;159:3823–37.
41. Bedrosian I, Mick R, Xu S, et al. Intranodial administration of peptide-pulsed mature dendritic cell vaccines results in superior $CD8^+$ T-cell function in melanoma patients. J Clin Oncol 2003;21:3826–35.
42. Mackey MF, Gunn JR, Maliszewsky C, Kikutani H, Noelle RJ, Barth RJ, Jr. Dendritic cells require maturation via CD40 to generate protective antitumor immunity. J Immunol 1998;161:2094–8.
43. Morse MA, Zhou LJ, Tedder TF, Lyerly HK, Smith C. Generation of dendritic cells *in vitro* from peripheral

blood mononuclear cells with granulocyte/macrophage colony stimulating factor, interleukin-4 and tumor necrosis factor α for use in cancer immunotherapy. Ann Surg 1997;226:6–16.
44. Gibson SJ, Lindh JM, Riter TR, et al. Plasmacytoid dendritic cells produce cytokines and mature in response to the TLR7 agonists, imiquimod and resiquimod. Cell Immunol 2002;218:74–86.
45. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. Nat Rev Immunol 2001; 1:220–8.
46. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science 1996;271:1734–6.
47. Attia P, Phan GQ, Maker AJ, et al. Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti- cytotoxic T-lymphocyte antigen-4. J Clin Oncol 2005;23: 6043–53.
48. Blansfield JA, Beck KE, Tran K, et al. Cytotoxic T-lymphocyte-associated antigen-4 blockage can induce autoimmune hypophysitis in patients with metastatic melanoma and renal cancer. J Immunother 2005;28:593–8.
49. Maker AV, Phan GQ, Attia P, et al. Tumor regression and autoimmunity in patients treated with cytotoxic T lymphocyte-associated antigen 4 blockade and interleukin 2: a phase I/II study. [see comment]. [Clinical trial, phase I. Clinical trial, phase II. Journal article] Ann Surg Oncol 2005;12:1005–16.
50. Negrier S, Escudier B, Lasset C, et al. Recombinant human interleukin-2, recombinant human interferon α-2a, or both in metastatic renal-cell carcinoma. Groupe Francais d'Immunotherapie. N Engl J Med 1998;338:1272–8.
51. Motzer RJ, Bacik J, Mazumdar M. Prognostic factors for survival of patients with stage IV renal cell carcinoma: Memorial Sloan-Kettering Cancer Center experience. Clin Cancer Res 2004;10:6302S–3S.
52. Trillet-Lenoir V, Freyer G, Kaemmerlen P, et al. Assessment of tumour response to chemotherapy for metastatic colorectal cancer: accuracy of the RECIST criteria. Br J Radiol 2002;75:903–8.
53. Bercovici N, Givan AL, Waugh MG, et al. Multiparameter precursor analysis of T-cell responses to antigen. J Immunol Methods 2003;276:5–17.
54. Givan AL, Fisher JL, Waugh M, Ernstoff MS, Wallace PK. A flow cytometric method to estimate the precursor frequencies of cells proliferating in response to specific antigens. J Immunol Methods 1999;230:99–112. Erratum in: J Immunol Methods 2000;237:207.
55. White HD, Crassi KM, Givan AL, et al. $CD3^+CD8^+$ CTL activity within the human female reproductive tract: influence of stage of the menstrual cycle and menopause. J Immunol 1997;158:3017–27.
56. Spiotto MT, Fu Y, Schreiber H. Tumor immunity meets autoimmunity: antigen levels and dendritic. Curr Opin Immunol 2003;15:725–30.
57. Kapsenberg ML. Dendritic-cell control of pathogen-driven T-cell polarization. Nat Rev Immunol 2003;3: 984–93.
58. Dutcher J, Atkins MB, Margolin K, et al.; Cytokine Working Group. Kidney cancer: the Cytokine Working Group experience (1986–2001): part II. management of IL-2 toxicity and studies with other cytokines. Med Oncol 2001;18:209–19.