

# Immunological and Clinical Responses in Metastatic Renal Cancer Patients Vaccinated with Tumor RNA-transfected Dendritic Cells<sup>1</sup>

Zhen Su,<sup>2</sup> Jens Dannull,<sup>2</sup> Axel Heiser, Donna Yancey, Scott Pruitt, John Madden, Doris Coleman, Donna Niedzwiecki, Eli Gilboa, and Johannes Vieweg<sup>3</sup>

Cancer Immunotherapy Program, Division of Urology, Department of Surgery [Z. S., J. D., A. H., D. Y., D. C., J. V.], Center for Cellular and Genetic Therapies, Department of Surgery [S. P., E. G.], Department of Pathology [J. M.], and Department of Biostatistics [D. N.], Duke University Medical Center, Durham, North Carolina 27710

## ABSTRACT

Autologous dendritic cells transfected with total renal tumor RNA have been shown to be potent stimulators of CTLs and antitumor immunity *in vitro*. A Phase I trial was conducted to evaluate this strategy for feasibility, safety, and efficacy to induce tumor-specific T-cell responses in subjects with metastatic renal cell carcinoma. Renal tumor RNA-transfected dendritic cells were administered to 10 evaluable study patients with no evidence of dose-limiting toxicity or vaccine-related adverse effects including autoimmunity. In six of seven evaluable subjects, expansion of tumor-specific T cells was detected after immunization. The vaccine-induced T-cell reactivities were directed against a broad set of renal tumor-associated antigens, including telomerase reverse transcriptase, G250, and oncofetal antigen, but not against self-antigens expressed by normal renal tissues. Although most patients underwent secondary therapies after vaccination, tumor-related mortality of the study subjects was unexpectedly low with only 3 of 10 patients dying from disease after a mean follow-up of 19.8 months. These data provide a scientific rationale for continued clinical investigation of this polyvalent vaccine strategy in the treatment of metastatic renal cell carcinoma and, potentially, other cancers.

## INTRODUCTION

Metastatic RCC<sup>4</sup> remains a therapeutic challenge because of its demonstrated resistance to conventional means of therapy (1). Although the administration of recombinant cytokines has become an accepted standard treatment for patients with metastatic RCC, overall response rates have remained unsatisfactory and lack consistency. Therefore, continued investigation in developing clinically effective and nontoxic strategies to eliminate metastatic disease remains of paramount importance.

As an emerging, alternative therapeutic option for patients with metastatic RCC, DC-based vaccines are currently under active clinical investigation. DCs, the most potent professional presenting cells, play a pivotal role in the induction of CD8<sup>+</sup> and CD4<sup>+</sup> T-cell responses *in vivo*. Therefore, vaccination with antigen-loaded DCs has evolved into an attractive approach to induce cellular, potentially therapeutic, immune responses in cancer patients. Accordingly, several recent clinical trials using antigen-loaded DCs have shown promise and sporadic clinical responses in metastatic RCC and other tumor systems (2, 3).

One key aspect that deserves further exploration is the identification of the optimal strategy for antigen delivery to DCs. Antigen loading can be accomplished either by coculture of DCs with HLA-class I- or -II-restricted antigenic peptides, proteins, apoptotic cell bodies, exosomes, tumor lysates, irradiated whole cell preparations or by genetic modification, each of which greatly influences the pathway and efficacy of T-cell activation (4).

Over the last years, we have investigated a novel, effective, and broadly applicable strategy to induce T-cell responses in cancer patients by using autologous DCs transfected with RNA-encoded antigens. Specifically, we have shown that monocyte-derived DCs cultured from the PBMCs of RCC patients and transfected with total RCC RNA are highly effective for stimulating polyclonal and tumor-specific T-cell responses *in vitro* (5). Moreover, we demonstrated that tumor RNA can be amplified by PCR, thereby providing large amounts of tumor antigens for DC transfection (6). Finally, a recently completed clinical trial using PSA RNA-transfected DCs has demonstrated immunological activity, an impact on surrogate clinical markers, and a lack of toxicity (7). On the basis of this experimental background, we have performed a Phase I clinical trial in which 10 patients with metastatic RCC received immunizations with RCC RNA-transfected DCs. The main objective of this trial was (a) to determine the safety of this approach and (b) to define its feasibility with respect to vaccine generation. Moreover, we were interested in characterizing the vaccine-induced immune and clinical responses in the treated patients.

Here, we show that the administration of RCC RNA-transfected DCs is not only safe and feasible but also capable of stimulating the expansion of tumor-specific, polyclonal T cells in the immunized patients. The vaccine-induced T-cell responses were directed against a broad set of antigens including hTERT, G250, and OFA, but not against normal cellular antigens expressed by autologous normal renal tissue. Although most patients elected to undergo secondary therapies after the completion of DC therapy, disease-specific mortality was unexpectedly low, with only 3 of 10 patients dying from disease after a mean follow-up of 19.8 months.

The demonstration of vaccine safety and of the induction of a potentially therapeutic and tumor-specific immune response in the majority of patients provides a foundation for ensuing studies that more directly address the question of clinical benefit using further optimized DC vaccines in patients with metastatic RCC.

## PATIENTS AND METHODS

**Vaccine Preparation.** Cell production was performed in a dedicated cell processing suite at Duke University Medical Center using standardized protocols developed according to good manufacturing procedure (GMP) guidelines. For DC culture, we used previously described techniques (7) and implemented modifications that allowed the processing of cellular material using defined serum-free media and supplements. Briefly, a concentrated leukocyte fraction was generated through a 2-h restricted peripheral blood leukapheresis that processed 6–8 liters of blood with each collection. The leukapheresis product was further separated by density gradient centrifugation over polysucrose/sodium diatrizoate (HISTOPAQUE; Sigma Diagnostics, St.

Received 12/18/02; accepted 2/28/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported in part by NIH Grant RO1-CA-89102 and by the National Center for Research Resources, NIH Grant MO1-RR-30, General Clinical Research Centers Program, NIH.

<sup>2</sup> Z. S. and J. D. contributed equally to this work.

<sup>3</sup> To whom requests for reprints should be addressed, at Duke University Medical Center, MSRB, Suite 466, Box 2626, Research Drive, Durham, NC 27710. Phone: (919) 684-9949; Fax: (919) 681-7414; E-mail: viewe001@mc.duke.edu.

<sup>4</sup> The abbreviations used are: RCC, renal cell carcinoma; DC, dendritic cell; ELISPOT, enzyme-linked immunospot assay; GFP, green fluorescent protein; IL, interleukin; OFA, oncofetal antigen; PBMC, peripheral blood mononuclear cell; RE, renal epithelium; SSCP, single-stranded conformational polymorphism; TAA, tumor-associated antigen; hTERT, human telomerase reverse transcriptase; TIL, tumor-infiltrating lymphocyte; GM-CSF, granulocyte macrophage colony-stimulating factor; RT-PCR, reverse transcription-PCR; TCR, T-cell receptor; PSA, prostate-specific antigen.

Louis, MO) and cells were resuspended in serum-free AIM-V medium (Invitrogen, Carlsbad, CA). PBMCs were incubated in a humidified incubator for 2 h at 37°C to allow plastic adherence. The adherent cell fraction was used for DC culture by incubation in serum-free AIM-V medium supplemented with recombinant human IL-4 (500 units/ml) and recombinant human GM-CSF (800 units/ml; R&D Systems, Minneapolis, MN). After 7 days of culture, cells were harvested and phenotypically characterized to ensure that they met the typical phenotype of immature DCs: CD3<sup>-</sup>, CD14<sup>-</sup>, CD56<sup>-</sup>, CD19<sup>-</sup>, MHC I<sup>+</sup>, MHC II<sup>+</sup>, CD40<sup>low</sup>, CD86<sup>low</sup>, CD80<sup>low</sup>, and CD83<sup>low</sup>. DC preparations fulfilling these phenotypic criteria were used for subsequent RNA transfection.

**RNA Generation from Human Tissues.** PBMCs, renal tumor tissue, and nonmalignant control tissue were collected after informed consent from human subjects treated on protocols approved by the Institutional Review Board at our institution. All of the primary tumor material was histologically classified as clear-cell carcinoma by an experienced pathologist (J. M.). Tissue samples were transported from the operating suite to the laboratory at room temperature in RNA preservation solution (RNAlater; Ambion, Austin, TX). Tissues were homogenized, and total cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's protocol. To attest to their intactness, all of the RNA preparations were subjected to electrophoresis in 1.2% agarose gel under denaturing conditions with clear visualization of intact 18S and 28S ribosomal bands after ethidium bromide staining. In addition, RT-PCR for  $\beta$ -actin was performed with successful amplification of the gene product visualized as a 838-bp fragment on agarose gel (data not shown). Control RNA was extracted from autologous benign RE and PBMCs.

In one patient, autologous renal tumor cells were generated from short-term primary cultures. Cells were minced with opposing scalpels and the resulting tissue fragments were cultured in RPMI 1640 supplemented with 10% FCS using a humidified incubator. The epithelial nature of these cells was confirmed by flow cytometry. Early-passage cells were used as targets in CTL assays as shown in Fig. 3.

**DC Transfection.** In contrast to a prior trial (7) in which cryopreserved and reconstituted RNA-transfected DC were administered, in this trial, RNA transfection was carried out on the day of administration using cryopreserved and reconstituted DCs. Cryopreserved DCs were washed twice in PBS, counted, and spun at  $300 \times g$  for 10 min. Subsequently, DCs were resuspended at a concentration of  $1 \times 10^7$  cells/ml in AIM-V medium and were cocultured for 60 min with 50  $\mu$ g/ml RNA in a humidified incubator at 37°C/5% CO<sub>2</sub>. After transfection with "naked" renal tumor RNA, cells were washed twice in PBS, resuspended in normal saline solution, and administered to patients.

**Analysis of Expression of TAAs in Tissues by RT-PCR.** Total RNA (2  $\mu$ g) were reverse-transcribed using the GeneAmp Gold RNA PCR Core kit (PE Biosystems, Foster City, CA) according to the manual provided by the manufacturer. One-tenth of the RT reaction was then subjected to 30 cycles of amplification using the following cycling parameters: 1 min at 95°C, 1 min at 60°C, 1 min at 72°C, and a final extension for 10 min at 72°C. Oligodeoxynucleotide primers used in our assays were: OFA<sub>sense</sub>, 5'-ATGTCCG-GAGCCCTTGATGTCC-3'; OFA<sub>antisense</sub>, 5'-TTAAGACCAGTCAGT-CAGTGGTTGC-3'; RUAS2<sub>sense</sub>, 5'-CTTCAGGAAGACTTCGAAGC-3'; RUAS2<sub>antisense</sub>, 5'-AAACTTGAGCGTCTACTCCG-3'; G250<sub>sense</sub>, 5'-GG-GACAAAGAAGGGGATGAC-3'; G250<sub>antisense</sub>, 5'-AAAGGCGGTGA-GGTGAA-3' (8); hTERT<sub>sense</sub>, 5'-CGGAAGAGTGTCTGGAGCAA-3'; hTERT<sub>antisense</sub>, 5'-GGATGAAGCGGAGTCTGGA-3' (9);  $\beta$ -actin<sub>sense</sub>, 5'-ATCTGGCACCACCTTCTACAATGAGCTGCG-3';  $\beta$ -actin<sub>antisense</sub>, 5'-CGTCATACTCTGCTTGTGATCCACATCTGC-3'. PCR reactions were subjected to electrophoresis on 1.5% agarose gels, and PCR products were visualized by ethidium bromide staining.

**Patient Eligibility.** Patients with a diagnosis of a metastatic RCC Stage IV (pT<sub>4</sub>N<sub>x</sub>M<sub>x</sub>) after nephrectomy were eligible for this study with the following restrictions: Eastern Cooperative Oncology Group performance status  $\leq 2$ , estimated life expectancy  $> 6$  months, WBC  $\geq 3000$  mm<sup>3</sup>, platelets  $\geq 100,000$ /mm<sup>3</sup>, serum creatinine  $< 2.5$  mg/dl, and bilirubin  $< 2.0$  mg/dl. Patients had to have recovered from all of the toxicities related to any prior therapy and not to have received chemotherapy, radiation therapy, or immunotherapy for at least 6 weeks before entry into the trial. Excluded from the study were patients with: central nervous system metastases; a history of autoimmune disease; serious intercurrent chronic or acute illnesses; concurrent second malignancy other than nonmelanoma skin cancer; or controlled superficial bladder cancer. Also excluded were patients on immunosuppressive agents.

**Dose and Immunization Schedule.** Renal tumor RNA-transfected DCs were administered at three proposed, escalating dose levels with the highest dose to be tested corresponding to the largest number of DCs that could be generated from the PBMCs of healthy volunteers isolated by a routine leukapheresis. Dose escalation was performed through an i.v. route using 3 vaccination cycles with  $1 \times 10^7$  (low dose),  $3 \times 10^7$  (medium dose), or  $5 \times 10^7$  (high dose) cells applied at study weeks 0, 2, and 4. To optimize vaccination, a concomitant dose of  $1 \times 10^7$  cells was given intradermally at each vaccination cycle. Patients were asked to undergo a second leukapheresis 2 weeks after the last dose to obtain sufficient numbers of cells for immunological monitoring.

**ELISPOT.** For ELISPOT analysis, T cells were isolated from pre- and posttherapy PBMCs (2 weeks after the final dose) by negative depletion (Pan T Cell Isolation kit; Miltenyi Biotec Inc., Auburn, CA). After blocking wells with complete RPMI medium,  $1 \times 10^5$  T cells and  $1 \times 10^4$  RNA-transfected DCs in 100  $\mu$ l of RPMI medium supplemented with 10% fetal calf serum were added to each well of flat-bottomed 96-well nitrocellulose plates (Multiscreen-IP; Millipore, Bedford, MA) precoated with 2  $\mu$ g/ml IFN- $\gamma$  capture antibody (Endogen, Woburn, MA). Plates were incubated for 20 h at 37°C, and after washing, biotinylated IFN- $\gamma$  detection antibody (0.2  $\mu$ g/ml; Endogen) was added to each well. Wells were incubated for an additional 2 h at room temperature, then were incubated with streptavidin-alkaline phosphatase (1  $\mu$ g/ml; Sigma, St. Louis, MO) and developed with substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). After washing, spots were counted using an automated Zeiss KS Elispot Compact reader (Carl Zeiss Inc., Minneapolis, MN).

**Cytolytic Assays.** Target cells ( $1 \times 10^6$ ) were labeled with 100  $\mu$ Ci of Na<sub>2</sub>[<sup>51</sup>Cr]O<sub>4</sub> (NEN, Boston, MA) in 200  $\mu$ l of complete RPMI for 1 h at 37°C in 5% CO<sub>2</sub>. After three washes, <sup>51</sup>Cr-labeled target cells ( $5 \times 10^3$ ) were incubated in 200  $\mu$ l of complete RPMI for 4 h at 37°C in 5% CO<sub>2</sub> at different E:T ratios. Then, 50  $\mu$ l of supernatant were harvested, and the release of <sup>51</sup>Cr was measured with a scintillation counter. Counts from triplicate wells were averaged, and the percentage of specific lysis was calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

**SSCP Analysis of TCR  $\beta$  Chain.** TCR  $\beta$  chain SSCP analysis was performed according to the method of Andrews *et al.* (10). Briefly, lymphocyte total RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase. The resulting cDNA was then used as the template for multiplexed PCR reactions using paired V- $\beta$  family-specific primers and a 6-carboxy-2',4,4',7,7'-hexachloro-fluorescein-labeled  $\beta$ -chain constant region primer, according to the method of Yassai *et al.* (11), but with 40 cycles of amplification. PCR products were then denatured in formamide and resolved by nondenaturing gel electrophoresis. Gels were then analyzed using an automated FMBioII fluorescence scanner (Hitachi, Brisbane, CA).

**Statistical Methods.** The time to disease-specific death or to last follow-up was calculated for 10 evaluable patients from the time of the first DC injection. The cumulative rates of deaths were calculated using the method of Kaplan and Meier.

## RESULTS

**Patient and Tumor Characteristics.** The group of patients enrolled in this study was made up of 15 individuals with metastatic RCC; the detailed patient characteristics are shown in Table 1. Immunotherapy with RNA-transfected DCs was offered to all of the patients who fulfilled the study eligibility criteria; no further selection was performed before study entry. Of the 15 patients, only 10 were fully evaluable for analysis because 5 patients could not receive the three proposed vaccination cycles and required premature withdrawal from the study because of rapid and symptomatic tumor progression after surgery or during the vaccination phase. As per study protocol, patients underwent standard radical nephrectomy before enrollment into the trial, and histologically confirmed renal tumor tissues were

Table 1 Characteristics of patients enrolled including status before vaccination (Vac) and response to vaccination

Pt. ID <sup>a</sup> no.	Age	Sex	ECOG	Metastases (study entry)	Assigned dose <sup>b</sup>	Actual dose	Time intervals <sup>c</sup> (in months)							T-cell frequencies (ELISPOT)		
							Lead time to Vac	Nx to Vac	F/U after Vac	F/U after DX	DX to death	Vac to additional therapy	Adjunctive therapy <sup>d</sup>	Status	Pre Vac	Post Vac
JB-01	55	M	1	BN/LN	Low	Low	2.7	2.3	26.2	28.9	N/A	0.7	Surgery	AWD	8	81
VC-02	40	M	1	PN/LN	Low	Low	3.1	3.1	8	11.1	12.1	3.9	IL-2	DOD	12	25
AE-03	50	M	1	PN	Low	Low	19.8	4.6	24.2	44	N/A	5.3	IL-2/XRT	AWD	2	32
PH-05	65	M	1	LN	Med	Med	5.1	3.5	23.6	28.7	N/A	7.3	IL-2	AWD	13	12
CW-06	73	M	0	PN	Med	Low	4.1	2.7	8.9	13	17.4	2.5	XRT	DOD		
FH-07	41	M	1	LN	Med	Med	4.4	4.5	22	26.5	N/A	N/A	None	AWD	11	89
RD-08	59	M	1	PN	Med	Low	3.5	2.3	2	5.5	7.3	N/A	None	DOD		
MS-10	74	F	1	PN	Med	Low	4.3	1.8	13.6	17.9	N/A	11.7	IL-2	AWD	13	86
SC-12	83	M	1	PN	Med	Low	2.6	1.9	2.5	5	N/A	2.5	IL-2	AWD		
CW-14	50	M	1	PN/BN	Med	Low	3	2.2	9.5	12.4	N/A	7.6	IL-2/IFN- $\alpha$ /5FU	AWD	4	52

<sup>a</sup> Pt. ID, patient identification; ECOG, Eastern Cooperative Oncology Group performance status; BN, bony metastases; LN, lymph node metastases; PN, pulmonary nodule metastases; N/A, not applicable; AWD, alive with disease; DOD, dead of disease.

<sup>b</sup> Assigned/Actual Dose: Low,  $1 \times 10^7$  cells i.v.,  $1 \times 10^7$  cells i.d.  $\times 3$  cycles; Med, medium,  $3 \times 10^7$  cells i.v.,  $1 \times 10^7$  cells i.d.  $\times 3$  cycles.

<sup>c</sup> Time intervals: Lead time to Vac, time between diagnosis and first DC Vac; Nx (nephrectomy) to Vac, time between nephrectomy and first DC Vac; F/U (follow-up) after Vac, time interval between Vac and last clinical/radiological follow-up; F/U after DX (diagnosis of disease), interval between diagnosis of metastatic disease and last clinical follow-up; DX to death, interval from first diagnosis of metastatic disease to death; Vac to additional therapy, interval from last Vac to adjunct therapy.

<sup>d</sup> Adjunct therapy: Surgery, metastasectomy of bony lesion; IL-2, high-dose IL-2 therapy; XRT, radiation therapy to metastatic lesions; None, no additional therapy; IFN- $\alpha$ , intravenous IFN- $\alpha$  administration; 5-FU, 5-fluorouracil therapy.

used for RNA extraction. All of the tissue samples were characterized by a pathologist (J. M.) as clear cell carcinoma and were further analyzed for gene expression of renal TAAs using RT-PCR. As shown in Fig. 1, only the TAA G250 (membrane antigen/carbonic anhydrase 9; Ref. 12), OFA (laminin receptor; Ref. 14), and hTERT were consistently present or overexpressed in all of the tumor samples, whereas the described TAA RUSAS2 (13) and WT1 mRNAs (14) did not exhibit a tumor-specific expression pattern (data not shown). Consistent with other reports, G250 and hTERT were the only TAAs that could be detected in all of the renal tumors but not in the corresponding benign renal tissues, whereas OFA demonstrated high expression in both tissue types examined at the RNA level. Unfortunately, RT PCR-based detection of amplified OFA transcripts does not account for potential posttranscriptional or posttranslational modifications of the laminin receptor suggested to be impaired in renal tumor tissues (15). This question can only be addressed once appropriate OFA-specific antibodies become available.

**Vaccine Characteristics.** Monocyte-derived DCs were generated from PBMCs cultured in serum-free medium supplemented with the cytokines IL-4 and GM-CSF and transfected with RNA in the absence of transfection agent, as described previously (16; 7). As demonstrated in prior studies, transfection of immature DCs with RCC RNA resulted in a modest increase (5–10%) of the cell surface markers CD83 and CD86, suggesting that RNA loading induced partial DC

maturation (7, 17, 18). Accordingly, RNA-transfected DCs induced proliferation of allogeneic T cells and stimulated tumor-specific T-cell responses *in vitro*, attesting to their potent antigen-presenting function. To assure vaccine consistency and safe administration, flow-cytometric analysis was performed on cryopreserved and reconstituted DC aliquots to determine DC phenotype and to document the absence of bacterial, fungal, *Mycoplasma*, or endotoxin contamination.

**Toxicity Profile and Feasibility of Vaccine Generation.** The trial design used in this study was a traditional three-tiered dose escalation scheme, in which RCC RNA-transfected DCs were administered i.v. at three dose levels over three treatment cycles, each performed 2 weeks apart ( $1 \times 10^7$  DCs, low dose;  $3 \times 10^7$  DCs, medium dose;  $5 \times 10^7$  DCs, high dose). In addition, a concomitant dose of  $1 \times 10^7$  intradermally (i.d.) injected DCs was given among all of the dose levels and at each treatment cycle. Among the 10 evaluable patients receiving the three treatment cycles, sufficient cells to allow treatment could be generated in 10 patients at a low dose level ( $1 \times 10^7$  i.v.;  $1 \times 10^7$  i.d.) and in 2 patients at a medium dose level ( $3 \times 10^7$  i.v.;  $1 \times 10^7$  i.d.). According to the protocol design, the low dose level was, therefore, considered the feasible dose level.

Vaccinations were generally well tolerated. No major (>grade I) toxicity including autoimmune toxicity was encountered in any subject receiving RCC RNA-transfected DCs, which suggests favorable toxicity profiles of the vaccine. A total of five subjects experienced grade I injection site reactions consisting of erythema lasting 48–72 h. There were no treatment-related hematological, hepatic, renal, or neurological toxicities. Three adverse events (anemia in one and dyspnea in two subjects) were noted and reported to regulatory agencies but were considered as unrelated to the treatment and were interpreted as tumor-related symptoms. Cumulatively, these data suggest low toxicity profiles of RCC RNA-transfected DCs. They also demonstrate the feasibility of generating reproducible numbers of RCC RNA-transfected DCs from patients using clinically compatible and safe cell manufacturing procedures.

**Immunological Response to Vaccine Therapy.** To determine whether vaccination with RCC RNA-transfected DCs is capable of stimulating tumor-specific T-cell responses in the treated patients, we first analyzed and compared directly the numbers of tumor-specific T cells from pre- and posttherapy PBMC samples using ELISPOT analyses. For this trial, we have developed a modified IFN- $\gamma$  spot-forming assay, in which RNA-transfected DCs were used for *in*

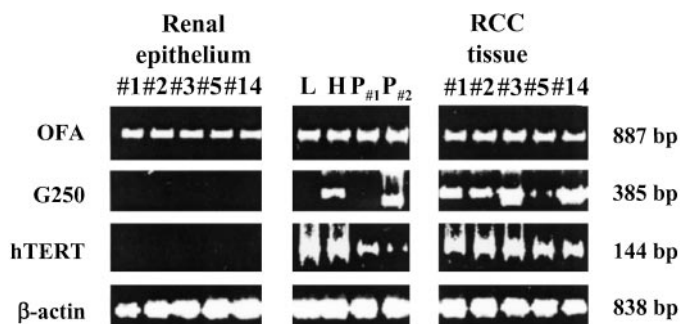


Fig. 1. Expression of TAAs by tumor and benign renal tissues. Analysis of TAA gene expression using RT-PCR. cDNA, generated from histologically confirmed renal tumor and the corresponding benign tissues, was amplified by 30 cycles of PCR and subjected to electrophoresis on 1.5% agarose gels. PCR products were visualized by ethidium bromide staining. As controls, RNA species extracted from HeLa cells (H; G250<sup>positive</sup>, hTERT<sup>positive</sup>), from LNCaP cells (L; G250<sup>negative</sup>, hTERT<sup>positive</sup>), and from the PBMCs of patients 1 and 2 (P<sub>#1</sub> and P<sub>#2</sub>) were used.



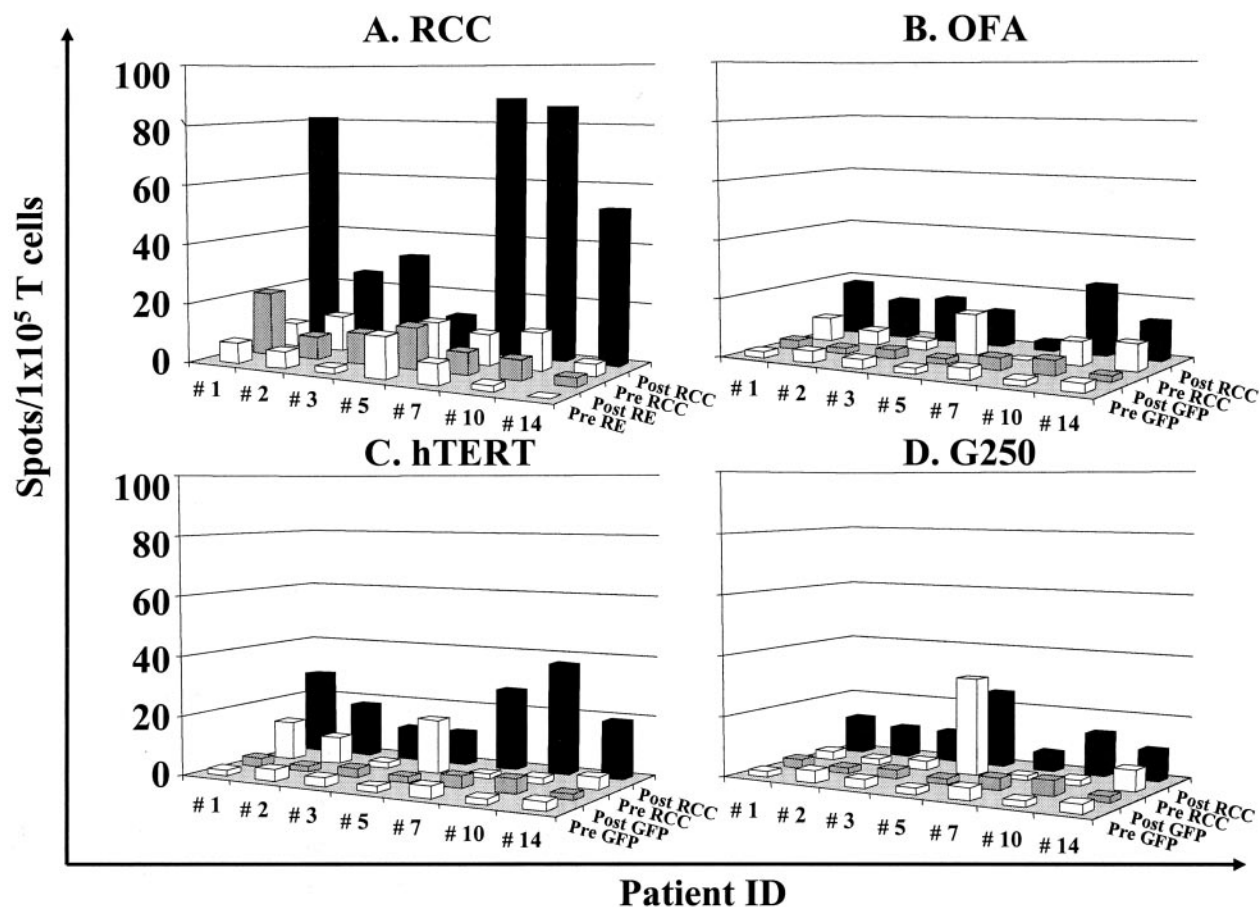


Fig. 2. Increase of tumor-specific T cells after vaccination with renal tumor RNA-transfected DCs. T cells isolated from pre- and postvaccination peripheral blood samples were incubated overnight with RNA-loaded DCs (targets) as described in the text. After short-term antigenic stimulation, T cells were plated onto IFN- $\gamma$  capture antibody-coated nitrocellulose plates. Visible spots were enumerated using an automated ELISPOT reader. As control targets, RE or GFP RNA-loaded DCs were used.

*in vitro* antigenic stimulation. We have shown previously (5, 7, 16) that RNA-transfected DCs express antigens facilitating their use as antigen-specific cellular targets in cytolytic assays as well as in ELISPOT analyses (19).

Specifically, aliquots of PBMCs obtained at baseline and at 2 weeks after the third vaccination (study week 6) were thawed, T cells were isolated by negative depletion and were cultured overnight with DCs that had been transfected with RCC RNA or RNA extracted from autologous benign renal tissue (RE; Fig. 2, panel A). As controls, unloaded DCs (data not shown), GFP RNA-transfected DCs, or RE RNA-transfected DCs were used as stimulators. Prior experiments had shown that overnight culture of  $1 \times 10^5$  T cells seeded per well with RNA-transfected DCs generates optimal results with respect to the detection efficacy of single spot-forming T lymphocytes (data not shown). As shown in Fig. 2, panel A, the numbers of tumor-specific IFN- $\gamma$  secreting cells was expectedly low or undetectable when T cells isolated from pretherapy PBMC samples were analyzed, whereas after vaccination, six of seven patients exhibited measurable expansion of tumor-specific T cells.

The observed increases in the numbers of detectable, tumor-specific T cells after vaccination is notable in view of the fact that analysis was performed directly from peripheral blood cells without the need for repeated *in vitro* restimulations. In stark contrast, no reactivity was observed against unloaded DCs or DCs transfected with GFP RNA in any PBMC sample obtained from study subjects before or after vaccination. Interestingly, when RE RNA-transfected DCs were used for antigenic challenge, IFN- $\gamma$  secretion by activated T cells increased consistently above background levels in all patients, albeit at very low

frequencies (Fig. 2, panel A), suggesting that minor cross-reactivity of the vaccine-induced T cells against antigens present on normal renal tissues may exist. On the other hand, in all patients treated neither deterioration of renal function (based on serum creatinine or urea levels) nor increases of autoimmune parameters such as rheumatoid factor, antinuclear- or antimitochondrial antibodies were observed during and after therapy.

Next, we sought to determine whether the tumor-specific T cells also recognize epitopes expressed by OFA, hTERT, and G250, as shown in Fig. 2. DCs transfected with G250 RNA, hTERT RNA, or OFA RNA were used as stimulators to detect potential vaccine-induced T-cell reactivities against each antigen, respectively. The data shown in Fig. 2, panels B–D, suggest that small increases in T-cell frequencies against hTERT, OFA, and G250 can be achieved after vaccination with renal tumor RNA-transfected DCs.

Although there was significant patient-to-patient variability in the magnitude of the vaccine-induced T-cell responses directed against hTERT, OFA, and G250, these antigen-specific T-cell responses were consistently inferior when compared with the corresponding overall tumor-specific response (as determined by the use of renal tumor RNA-transfected DCs) suggesting that reactivities against these individual antigens comprise a subcomponent of the polyclonal T-cell response.

To analyze and compare effector function between pre- and post-therapy T cells, we next determined whether the vaccine-induced and tumor-specific CTLs are functionally intact by analyzing their efficacy to kill autologous tumor targets in cytolytic assays. Here we performed cytolytic assays in patient 1, who exhibited an immuno-

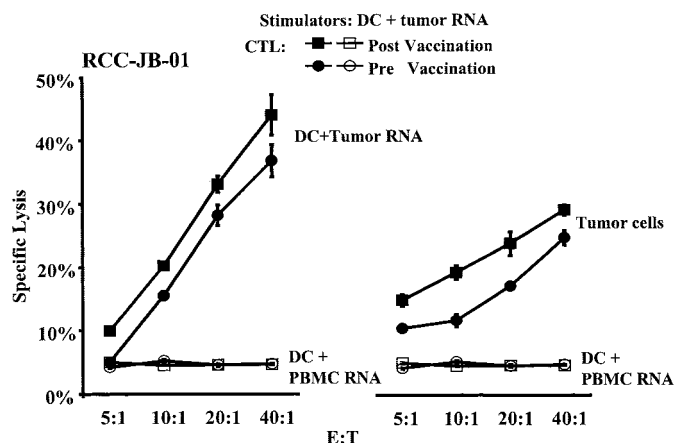


Fig. 3. Lytic activity of vaccine-induced CTLs. PBMCs derived from a renal cancer patient (patient 1) who received three vaccinations with  $1 \times 10^7$  renal tumor RNA-transfected DCs per cycle were restimulated twice to generate CTLs. We compared the efficacy of pretherapy and posttherapy CTLs to recognize and lyse renal tumor RNA-transfected DCs (*left panel*) and cultured autologous tumor cells used as targets in cytolytic assays (*right panel*).

logical response as determined by ELISPOT. Pre- and posttherapy PBMC samples were stimulated twice with autologous RCC RNA-transfected DCs to generate CTLs. After the two stimulation cycles, lytic activities of both of the T-cell preparations against tumor and control targets were analyzed. Because we were able to establish an autologous tumor cell line from patient JB-01, we used: (a) autologous tumor cells and (b) RCC RNA-transfected DCs as targets in cytolytic assays. DCs, transfected with RNA extracted from autologous PBMCs obtained before or after vaccination served as control targets. As demonstrated in Fig. 3, *left panel*, CTLs stimulated from posttherapy PBMCs were more effective in recognizing and lysing RCC RNA-transfected DC targets than were CTLs that were stimulated from pretherapy PBMC samples. Interestingly, we observed similar results when autologous tumor cells were used as targets (Fig. 3, *right panel*), except that the lytic levels at each E:T ratio were inferior. In both experiments, the tumor-specific CTLs did not recognize control targets in the form of nontransfected DCs or DCs transfected with PBMC RNA. At first glance, the differences in target lysis appear small. However, it needs to be pointed out that CTL assays are by no means quantitative. Therefore, even small but consistent differences in target cell lysis can be meaningful.

In summary, the data shown in Figs. 2 and 3 provide evidence that vaccination with RCC RNA-transfected DCs can stimulate the *in vivo* expansion of previously undetectable polyclonal tumor-specific T cells, which are capable of specifically recognizing and lysing tumor cells.

**Clonality of the Vaccine-induced Immune Response.** To provide further evidence for the presence of TAA-specific T cells as part of the polyclonal response induced by RCC RNA-transfected DCs, vaccine-induced T-cell clonality was analyzed by detection of predominant TCR  $\beta$  chains within T-cell populations via SSCP. More specifically, the diversity of TCR structure was determined by PCR-based detection of differences in the CDR3 region nucleotide sequence at the TCRV $\beta$ -N-D $\beta$ -N-J $\beta$  junction, which arise from somatic recombination of V (variable), D (diversity), J (joining), and C (constant) genes and introduction or deletion of template-independent N-nucleotides during ontogeny of T cells.

DCs transfected with (a) RCC RNA, (b) hTERT RNA, (c) OFA RNA, (d) G250 RNA, or (e) GFP RNA (control) were generated and used to stimulate CTLs from PBMCs. RNA extracted from these CTLs was reverse transcribed and further analyzed for individual

T-cell receptor usage using PCR primers specific for all 25 TCR V $\beta$  families in combination with a primer complimentary to C $\beta$  and subjected to SSCP analysis. RNA was isolated from autologous RCC tissue (hereafter referred to as TILs), standardized for CD8 expression via real-time PCR, and included in this analysis.

As shown in Fig. 4, we detected unique banding patterns for CTLs stimulated with RCC RNA-loaded DCs in V $\beta$ 11, V $\beta$ 22, and V $\beta$ 19 that were found neither in TILs nor in any other CTL preparations examined (exemplified in Fig. 4, *arrow 1*). This may suggest the expansion of CTLs recognizing patient-unique, TAA-specific epitopes or the generation of T-cell reactivity against shared antigens. Interestingly, we observed a simultaneous activation of hTERT-specific TCR V $\beta$ 11 CTLs, in addition to CTLs that can be found as a component of the TIL population (Fig. 4, *arrow 2*), by stimulation of PBMCs with both hTERT RNA-loaded DCs and RCC RNA-loaded DCs, suggesting the stimulation of hTERT-specific T-cell reactivities by RCC RNA-transfected DCs. Furthermore, we were able to demonstrate the expansion of OFA-specific TCR V $\beta$ 22 CTLs, which were already present in TILs, by both OFA-specific and RCC-specific T cells (Fig. 4, *arrow 3*). No significant correlation between the banding patterns of G250-specific CTLs, TILs, and RCC-specific CTLs could be observed. Consistent with the ELISPOT analyses described above, the SSCP pattern of T cells stimulated by DCs transfected with RCC RNA contained hTERT- and OFA-specific T cells as a subcomponent but was more diverse when compared with T cells specific for known RCC antigens or TILs, but was predominantly directed against antigens unique to total tumor RNA.

It appears that bands of similar size commonly emerge among the RCC- and GFP-specific T-cell lanes. This, however, is expected because, in every individual, environmental exposure to pathogens and other antigens can stimulate clonal expansion of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, thus leading to background common bands.

Interestingly, we further show that the clonality of TILs is quite different from that of the RCC-specific T cells. A possible explanation for this finding is that only small numbers of TILs represent, in fact, antigen-specific effector cells, potentially explaining the largely dis-

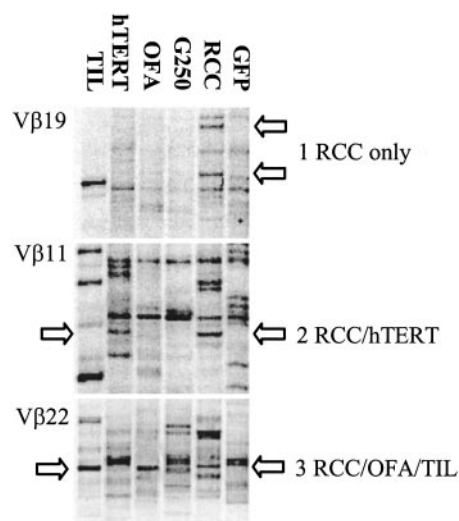


Fig. 4. SSCP analysis of TCR V $\beta$  utilization. PBMCs from a renal cancer patient were stimulated twice with autologous DCs transfected with renal tumor RNA (RCC), hTERT RNA (hTERT), G250 RNA (G250), or OFA RNA (OFA) to generate CTLs. PCR using V $\beta$ -specific primers and a fluorescent C $\beta$ -specific primer was performed followed by SSCP analysis using cDNA from CTL lines. Autologous renal tumor tissue (TIL) or CTLs generated against GFP were used as controls. As exemplified for V $\beta$ 11, V $\beta$ 22, and V $\beta$ 19, the following SSCP banding patterns were identified (see arrows): 1, patterns unique for RCC-specific CTLs; 2, patterns present in both hTERT- and RCC-specific CTLs; 3, patterns present in RCC-specific CTLs, OFA-specific CTLs, and TILs.

appointing results of TIL therapy in a metastatic renal cancer setting. In conclusion, our data suggest that tumor RNA-transfected DCs are capable of stimulating a broad set of T-cell specificities, including those directed against hTERT and OFA.

**Clinical Responses.** In this exploratory pilot study, we were unable to reliably assess potential vaccine-induced clinical responses, because 8 of 10 study subjects elected to undergo other therapies within 1–8 months after completion of the third and final vaccination cycle. Second-line treatment included either IL-2 therapy ( $n = 6$ ), surgery ( $n = 1$ ), and/or palliative irradiation ( $n = 1$ ). Among the entire group, 3 of 10 patients are confirmed to have died from malignancy, whereas the remaining 7 patients are alive as of April 2002 with documented stable or slowly progressing, but asymptomatic, disease. The calculated mean survival among the entire group was  $19.8 \pm 3.1$  months. Of the two patients (patients 7 and 8) who did not undergo adjunct therapy, one (patient 8) died 2 months after DC therapy, and one subject (patient 7) experienced disease stabilization lasting for more than 22 months.

## DISCUSSION

We have previously shown that tumor RNA-transfected DCs represent an effective strategy to stimulate primary and polyclonal T-cell responses from PBMCs of renal and prostate cancer patients *in vitro* (16). Furthermore, the results of a Phase I clinical trial indicated that PSA RNA-transfected DCs are capable of stimulating potentially therapeutic antitumor immunity in prostate cancer patients (7). However, in this study, the vaccine-induced T-cell responses were restricted to epitopes on the PSA molecule and did not target the many, potentially more relevant, antigens expressed by the primary tumor. Therefore, we sought to investigate a DC-based, polyvalent vaccine strategy geared for the induction of tumor-specific T-cell responses directed against a broad spectrum of tumor antigens by using antigen in the form of total tumor RNA.

Our results clearly demonstrate the feasibility of this approach associated with no apparent adverse events or dose-limiting toxicities. As commonly observed in immunotherapy studies that include nephrectomy as a trial component (20), five patients were withdrawn from the study because of rapid and symptomatic disease progression after surgery or during the vaccination phase. Our results also indicate that such a strategy is not only feasible and safe but, moreover, capable of stimulating potentially therapeutic tumor-specific T-cell responses in the vaccinated subjects. Significant increases of renal tumor-specific T cells were observed in six of seven evaluable subjects after three vaccination cycles as determined by ELISPOT analysis. Furthermore, we could show in one instance that the vaccine-induced, tumor-specific CTLs were capable of lysing autologous tumor cells. These results are remarkable in view of the fact that many questions as to the optimal dose, frequency, and route are still unanswered and remain to be addressed in future trials.

In this study, we used a different manufacturing protocol than in the previous PSA RNA trial by transfecting thawed and reconstituted immature DCs with renal tumor RNA on the day of administration rather than using RNA-transfected, cryopreserved, thawed, and reconstituted cells for injection. This change resulted in significantly lower cell numbers of RNA-transfected DCs that were available for vaccination and, therefore, precluded dose escalation to the high-dose level, a dose level determined feasible in our PSA RNA study. The reason for this phenomenon can be explained by the fact that the recovery of immature and cryopreserved DCs is suboptimal because of phenotypic instability, increased cell death, and increased plastic adherence during the reconstitution phase, all resulting in significantly lower numbers of phenotypically immature DCs after reconstitution.

Moreover, we and others have shown that transfection with naked RNA provides a partial maturation stimulus to DCs (7, 18) leading to increased expression of the activation markers CD83 and CD86 and resulting in a more robust and stable phenotype than DCs that were not exposed to RNA or other maturation stimuli. DC maturation has also been shown to be accompanied by increased potency in stimulating T cells, the acquisition of migratory properties, and the secretion of Th-1 type cytokines such as IFN- $\gamma$ . We, therefore, believe that the RNA-transfected DCs used in this clinical study were already committed to maturation and thus capable of priming T-cell reactivities *in vivo*, notwithstanding the fact that even more efficient T-cell priming could have been achieved by vaccination with fully and stably matured DCs.

The results of prior clinical trials using peptide-loaded DCs indicate that the induction of clinically effective immune responses will require the induction of polyclonal immune responses attributable to the development of antigen escape mutants (21). As a consequence, more recent trials focused on strategies loading DCs with class I-restricted peptide mixtures (22), or on combining class I- and class II-specific peptides (23), with the goals of further optimizing immune responses and of avoiding the emergence of tumor escape. Here, we show that the immune responses stimulated by RCC RNA-transfected DCs are polyclonal in nature and contain reactivities against antigens, which collectively may be necessary to stimulate potent antitumor immunity. Our data suggest that the individual T-cell responses directed against the self-antigens OFA, hTERT, and G250 make up the bulk of the renal tumor-specific polyclonal response; however, this is inconceivable, and the data suggest rather that the ELISPOT measurements of individual antigen-specific T-cell reactivities are not additive.

In this study, we were unable to reliably assess potential vaccine-induced clinical responses because the majority of subjects (8 of 10) elected to undergo secondary therapies most commonly applied in the form of IL-2 protocols. Tumor-related mortality in the study subjects was unexpectedly low with only 3 of 10 patients dying from disease after a mean follow-up of 19.8 months, whereas the expected survival in this patient group rarely exceed 9–12 months (24). As of April 2002, only in two surviving subjects a complete disappearance of (single) metastatic lesions was observed after adjunct surgical intervention (patient 1) or adjuvant high-dose IL-2 therapy (patient 5), but not after vaccination alone. All of the other subjects who are still alive experienced stabilization of metastatic disease or attenuated tumor growth. Although it is speculative whether DC therapy or other factors may have contributed to patient survival, several DC-based trials, including our own, suggest that the hallmark of a clinical response to DC vaccination may not necessarily represent measurable tumor reduction, but rather, the establishment of stable disease potentially resulting in improved survival in the absence of any toxicity (7, 25).

In summary, active immunotherapy with RCC RNA-transfected DCs represents a novel approach for RCC immunotherapy. Our data provide evidence on vaccine safety and *in vivo* bioactivity of RNA-loaded DCs in patients with metastatic RCC, although the proof for clinical benefit remains to be established in future clinical trials. This study establishes the foundation for additional optimization and refinements of the RNA-loaded DC strategy with the ultimate goal of developing therapeutic vaccines for cancer patients.

## ACKNOWLEDGMENTS

We thank Penny Triplett and Kay Walker for their editorial assistance.

## REFERENCES

- Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1998. *CA Cancer J. Clin.*, 48: 6–29, 1998.



2. Holtl, L., Rieser, C., Papesh, C., Ramoner, R., Herold, M., Klocker, H., Radmayr, C., Stenzl, A., Bartsch, G., and Thurnher, M. Cellular and humoral immune responses in patients with metastatic renal cell carcinoma after vaccination with antigen pulsed dendritic cells. *J. Urol.*, *161*: 777–782, 1999.
3. Fong, L., and Engleman, E. G. Dendritic cells in cancer immunotherapy. *Annu. Rev. Immunol.*, *18*: 245–273, 2000.
4. Raychaudhuri, S., and Rock, K. L. Fully mobilizing host defense: building better vaccines. *Nat. Biotechnol.*, *16*: 1025–1031, 1998.
5. Heiser, A., Dahm, P., Maurice, M., Yancey, D., and Vieweg, J. Human dendritic cells pulsed with renal tumor RNA stimulate tumor specific CTL *in vitro* without inducing autoimmune toxicity. *Cancer Res.*, *61*: 411–419, 2001.
6. Heiser, A., Maurice, M. A., Yancey, D. R., Dahm, P., Pruitt, S. K., Boczkowski, D., Nair, S. K., Ballo, M. S., Gilboa, E., and Vieweg, J. Induction of polyclonal prostate cancer specific cytotoxic T lymphocytes using dendritic cells transfected with amplified tumor RNA. *J. Immunol.*, *166*: 2953–2960, 2001.
7. Heiser, A., Coleman, D. M., Dannull, J., and Vieweg, J. Dendritic cells transfected with prostate specific antigen RNA induces PSA specific T cell responses in metastatic prostate cancers. *J. Clin. Investig.*, *159*: 2002.
8. McKiernan, J. M., Buttyan, R., Bander, N. H., de la Taille, A., Stifelman, M. D., Emanuel, E. R., Bagiella, E., Rubin, M. A., Katz, A. E., Olsson, C. A., and Sawczuk, I. S. The detection of renal carcinoma cells in the peripheral blood with an enhanced reverse transcriptase-polymerase chain reaction assay for MN/CA9. *Cancer (Phila.)*, *86*: 492–497, 1999.
9. Suenaga, M., Soda, H., Oka, M., Yamaguchi, A., Nakatomi, K., Shiozawa, K., Kawabata, S., Kasai, T., Yamada, Y., Kamihira, S., Tei, C., and Kohno, S. Histone deacetylase inhibitors suppress telomerase reverse transcriptase mRNA expression in prostate cancer cells. *Int. J. Cancer*, *97*: 621–625, 2002.
10. Andrews, D. M., Leary, C. P., Hishii, M., Shen, J., and Kurnick, J. Use of single stranded conformational polymorphism (SSCP) for analysis of the T cell receptor. *In: J. R. Oksenberg (ed.), The Antigen T Cell Receptor: Selected Protocols and Applications*, pp. 326–365. Austin: Landes Bioscience, 1996.
11. Yassai, M., Naumova, E., and Gorski, J. Generation of TCR spectratypes by multiplex PCR for T cell repertoire analysis. *In: J. R. Oksenberg (ed.), The Antigen T Cell Receptor: Selected Protocols and Applications*, pp. 373–404. Austin: Landes Bioscience, 1996.
12. Pastorek, J., Pastorekova, S., Callebaut, I., Mornon, J. P., Zelnik, V., Opavsky, R., Zat'ovicova, M., Liao, S., Portetelle, D., Stanbridge, E. J., *et al.* Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment. *Oncogene*, *9*: 2877–2888, 1994.
13. Van Den Eynde, B. J., Gaugler, B., Probst-Keppler, M., Michaux, L., Devuyt, O., Lorge, F., Weynants, P., and Boon, T. A new antigen recognized by cytolytic T lymphocytes on a human kidney tumor results from reverse strand transcription. *J. Exp. Med.*, *190*: 1793–1800, 1999.
14. Scharnhorst, V., van der Eb, A. J., and Jochemsen, A. G. WT1 proteins: functions in growth and differentiation. *Gene*, *273*: 141–161, 2001.
15. Zelle-Rieser, C., Barsoum, A. L., Sallusto, F., Ramoner, R., Rohrer, J. W., Holtl, L., Bartsch, G., Coggin, J. J., and Thurnher, M. Expression and immunogenicity of oncofetal antigen-immature laminin receptor in human renal cell carcinoma. *J. Urol.*, *165*: 1705–1709, 2001.
16. Heiser, A., Dahm, P., Yancey, D., Maurice, M., Boczkowski, D., Nair, S. K., Gilboa, E., and Vieweg, J. Human dendritic cells transfected with RNA encoding prostate specific antigen stimulate prostate specific CTL responses *in vitro*. *J. Immunol.*, *164*: 5508–5514, 2000.
17. Weissman, D., Ni, H., Scales, D., Dude, A., Capodici, J., McGibney, K., Abdool, A., Isaacs, S. N., Cannon, G., and Kariko, K. HIV gag mRNA transfection of dendritic cells (DC) delivers encoded antigen to MHC class I and II molecules, causes DC maturation, and induces a potent human *in vitro* primary immune response. *J. Immunol.*, *165*: 4710–4717, 2000.
18. Ni, H., Capodici, J., Cannon, G., Communi, D., Boeynaems, J. M., Kariko, K., and Weissman, D. Extracellular mRNA induces dendritic cell activation by stimulating tumor necrosis factor- $\alpha$  secretion and signaling through a nucleotide receptor. *J. Biol. Chem.*, *277*: 12689–12696, 2002.
19. Su, Z., Vieweg, J., Weizer, A. Z., Dahm, P., Yancey, D., Turaga, V., Higgins, J., Boczkowski, D., Gilboa, E., and Dannull, J. Enhanced induction of telomerase-specific CD4(+) T cells using dendritic cells transfected with RNA encoding a chimeric gene product. *Cancer Res.*, *62*: 5041–5048, 2002.
20. Fallick, M. L., McDermott, D. F., LaRock, D., Long, J. P., and Atkins, M. B. Nephrectomy before interleukin-2 therapy for patients with metastatic renal cell carcinoma. *J. Urol.*, *158*: 1691–1695, 1997.
21. Jager, E., Ringhoffer, M., Altmannsberger, M., Arand, M., Karbach, J., Jager, D., Oesch, F., and Knuth, A. Immunoselection *in vivo*: independent loss of MHC class I and melanocyte differentiation antigen expression in metastatic melanoma. *Int. J. Cancer*, *71*: 142–147, 1997.
22. Banchereau, J., Palucka, A. K., Dhodapkar, M., Burkeholder, S., Taquet, N., Rolland, A., Taquet, S., Coquery, S., Wittkowski, K. M., Bhardwaj, N., Pineiro, L., Steinman, R., and Fay, J. Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. *Cancer Res.*, *61*: 6451–6458, 2001.
23. Schuler-Thurner, B., Schultz, E. S., Berger, T. G., Weinlich, G., Ebner, S., Woerl, P., Bender, A., Feuerstein, B., Fritsch, P. O., Romani, N., and Schuler, G. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. *J. Exp. Med.*, *195*: 1279–1288, 2002.
24. Gleave, M. E., Elhilali, M., Fradet, Y., Davis, I., Venner, P., Saad, F., Klotz, L. H., Moore, M. J., Paton, V., and Bajamonde, A. Interferon  $\gamma$ -1b compared with placebo in metastatic renal-cell carcinoma. Canadian Urologic Oncology Group. *N. Engl. J. Med.*, *338*: 1265–1271, 1998.
25. Fong, L., Brockstedt, D., Benike, C., Breen, J. K., Strang, G., Ruegg, C. L., and Engleman, E. G. Dendritic cell-based xenoantigen vaccination for prostate cancer immunotherapy. *J. Immunol.*, *167*: 7150–7156, 2001.