

Massive expansion of regulatory T-cells following interleukin 2 treatment during a phase I-II dendritic cell-based immunotherapy of metastatic renal cancer

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Received February 26, 2009; Accepted May 6, 2009

DOI: 10.3892/ijo_00000368

Abstract. Cytotoxic chemotherapy is ineffective in metastatic renal cancer. However, systemic administration of interleukin 2 (IL-2) or infusion of dendritic cells (DCs) loaded with tumor extracts can lead to some response rates with concomitant survival improvements. We report the results of a phase I-II pilot study combining DCs and IL-2 where six patients were included. DCs were derived from bone marrow CD34⁺ cells and loaded with autologous tumor extracts. CD34-DC vaccines were infused subcutaneously at day 45, 52, 59, 90 and 120 following surgery in combination with IL-2, that was subsequently administrated after the 3rd and 4th DC vaccinations. Preparation of tumor extracts and CD34-DCs were satisfactory in all patients but one. Due to rapid tumor progression, one patient was excluded before vaccination. In the 4 remaining patients, two received 3 vaccinations, while the 2 others received 5 vaccinations and the full IL-2 treatment. No adverse effect due to the vaccinations was observed. A specific immune response against autologous tumor cells

was observed in the 2 patients who completed the treatment. Interestingly, these 2 patients had a more prolonged survival than the patients receiving 3 vaccinations. Importantly, a transient and massive increase of circulating natural regulatory T-cells (nTregs) was evidenced in 3 patients following IL-2 administration. Overall, the use of CD34-DC vaccines is feasible, safe and non-toxic. A specific anti-tumor immune response can be detected. However, our data highlights that IL-2 is a potent inducer of nTregs *in vivo* and as such may have a negative impact on cancer immunotherapy.

Introduction

Cytotoxic chemotherapy is usually ineffective for metastatic renal cancer and particularly renal cell carcinoma (RCC), which have a poor prognosis with a median survival of less than a year. Systemic administration of cytokines such as interleukin 2 (IL-2) (1-4), interferon- α (5) or both (6,7) can lead to response rates varying from 7 to 26% with concomitant improvements of survival (8). This suggests that renal cancer could be a good candidate for immune-based therapies. Likewise, cellular immunotherapy approaches have been proposed including allogeneic stem cell transplantation (9), *ex vivo* expansion of lymphokine activated killer cells (LAK) and tumor infiltrating lymphocytes (TIL) (8,9) and, more recently, vaccination with dendritic cells (DCs). DCs are powerful antigen-presenting cells (APCs) that play a major role in initiating primary immune responses against microbial, tumoral or self antigens. They elicit T-cell immune responses by presenting antigens on major histocompatibility complex (MHC) class I and class II molecules to both naive and memory CD8 and CD4

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Key words: immunotherapy, dendritic cells, renal cell carcinoma, CD34⁺ cells, interleukin 2, regulatory T-cells

T-cells (10-12). The possibility to generate *in vitro* functional DCs from peripheral blood monocytes (13-16) or from CD34⁺ progenitors obtained from cord blood (17-19), bone marrow (BM) or cytokine mobilized peripheral blood (20-23) has opened a new field of vaccine-based immunotherapy for cancer. Thus, various DC-based clinical trials have been performed for treatment of metastatic RCCs using either tumor lysate-pulsed DCs (24-26), tumor cell-DC hybrids (27-29), tumor RNA-transfected DCs (30) or more recently DCs loaded with HLA-A2-restricted MUC1-derived peptides (31). These clinical trials performed with monocyte-derived DCs (Mo-DCs) provide evidence of relatively good biological immune anti-tumor responses, but low clinical responses. Improvements can be expected by using DCs derived from CD34 progenitors (CD34-DCs) instead of Mo-DCs (32-35). CD34-DCs that are less homogenous than Mo-DCs, contain two major myeloid DC subsets, i.e. Langerhans cells and interstitial dermal-type DC, and seem able to achieve a large immune response (36,37). Interestingly, DC-based immunotherapy can be also combined to IL-2 administration (24,38) or to anti-angiogenic drugs that have recently proven their efficiency in the treatment of advanced RCCs (39).

Therefore, we designed a pilot study in which 6 patients with advanced metastatic renal cancer received tumor extract-pulsed CD34-DC vaccines in combination with IL-2 administered subcutaneously (s.c.) after the 3rd and the 4th vaccination (for protocol design see Fig. 1). We introduced the IL-2 treatment in combination with vaccination because it is commonly accepted that IL-2 is a good adjuvant treatment that can trigger/reinforce anti-cancer effector immune responses.

DCs were produced from CD34⁺ purified cells, taking into account the opportunity of the general anesthesia required for surgery to harvest bone marrow cells. Thus, we showed the feasibility of producing large amount of DCs generated from BM CD34⁺ cells in a closed system using clinical grade reagents and GMP conditions. Vaccination was well tolerated and could induce specific anti-tumor responses. However, we surprisingly observed that IL-2, albeit routinely used in the treatment of advanced RCC, resulted in a massive increase of natural regulatory T-cells (nTregs) with the potential to hamper vaccination efficacy.

Materials and methods

Patients characteristics, eligibility criteria and study design. Patients were included in this multi-center study upon the following criteria: >18 age <70 years; inclusion at time of diagnosis (i.e. prior to any treatment); primary tumor volume of at least 10 cm³ when estimated by tomodensitometry; RCCs with clear-cell histopathology and bi-dimensionally measurable metastatic lesions; Karnofski's performance status >60; expected survival of >6 months. Exclusion criteria were: patients with history of other cancers, autoimmune diseases or infections such as HIV, HBV, HCV; presence of brain metastasis; solid organ allograft with immunosuppressive treatment (corticosteroids and/or other immunosuppressive drugs); pregnancy; other immunotherapy or chemotherapy within 4 weeks prior to inclusion. All patients gave a written informed consent. The study was approved by an Institutional

Review Board and the 'Agence Française de Sécurité Sanitaire des Produits de Santé' (AFSSaPS) in September 2002. The clinical characteristics of the patients are presented in Table I. The design of the trial is summarized in Fig. 1. In brief, bone marrow (BM) cells were harvested under general anesthesia immediately after total nephrectomy. BM-CD34⁺ cells were purified and used for production of CD34-DCs while tumor extracts were prepared from the tumor. Four to six weeks after surgery, DCs generated in a closed system were pulsed with tumor extracts and injected into patients. For each patient, 6 productions of pulsed-CD34-DCs were carried out, 5 of them were utilized for vaccinations and the remaining one being used for immunomonitoring assays. The treatment schedule was as follows: eligible patients received at least 5 vaccinations of 2-4x10⁵ tumor extract-pulsed DCs per kg of body weight, suspended in 5 ml of NaCl 0.9%. The vaccine was administered s.c. in a single injection site. Treatment was repeated every week for the first three vaccinations and monthly for vaccination 4 and 5. Immunomonitoring was assessed before the 1st injection, after the 3rd vaccination and one month after the final vaccination by collecting peripheral blood (PB) for functional analysis of T-cells. The patients also received s.c. recombinant human IL-2 (Proleukin®, Chiron Corp, Amsterdam, The Netherlands) during 2 cycles of 4 weeks (i.e. 5 injections of 18x10⁶ IU at week 1, 2 injections of 18x10⁶ IU + 3 injections of 9x10⁶ IU at week 2, 3 and 4) after the 3rd and the 4th DC-injections according to standard clinical practices.

Clinical grade reagents. The culture medium used throughout this study was RPMI-1640 without phenol red (Invitrogen Corporation, Paris, France) containing 10% secured human AB serum (hABs purchased from Etablissement Français du Sang, Rhône-Alpes, France) and 2 mM L-glutamine. The following human recombinant cytokines approved for clinical use were: Stem Cell Factor (SCF, Amgen, Thousand Oaks, CA, USA), Flt3-Ligand (Flt3-L, Immunex, Seattle, WA, USA), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF, Leucomax®, Schering Plough, Levallois-Perret, France), Interleukin 4 (IL-4, Strathmann, Hamburg, Germany), Tumor Necrosis Factor α (TNF α , Boehringer Mannheim, Mannheim, Germany).

Preparation of autologous tumor extracts and establishment of tumor cell lines. Fresh tumor samples obtained from primary tumors were minced into 1-2 mm³ fragments and placed in RPMI-1640 under sterile conditions. Single cell suspensions were obtained by digesting the samples in 50 ml of RPMI containing 0.75 mg/ml collagenase (Liberase®, Roche diagnostics, Meylan, France) and 30 IU/ml DNase (Pulmozyme®, Roche Laboratories). After, 4 to 5 cycles of digestion, cells were centrifuged at 400 x g for 12 min, counted and cell viability evaluated after trypan blue exclusion. An aliquot of the cell suspension was stained by May-Grünwald-Giemsa and the cell content evaluated by light microscopy examination. Some of tumor cell suspensions were immediately cryopreserved and others were cultured at 2x10⁵ cells/ml in DMEM containing 10% fetal calf serum (FCS) for 10 days and frozen to use them in immunomonitoring assays (see below).

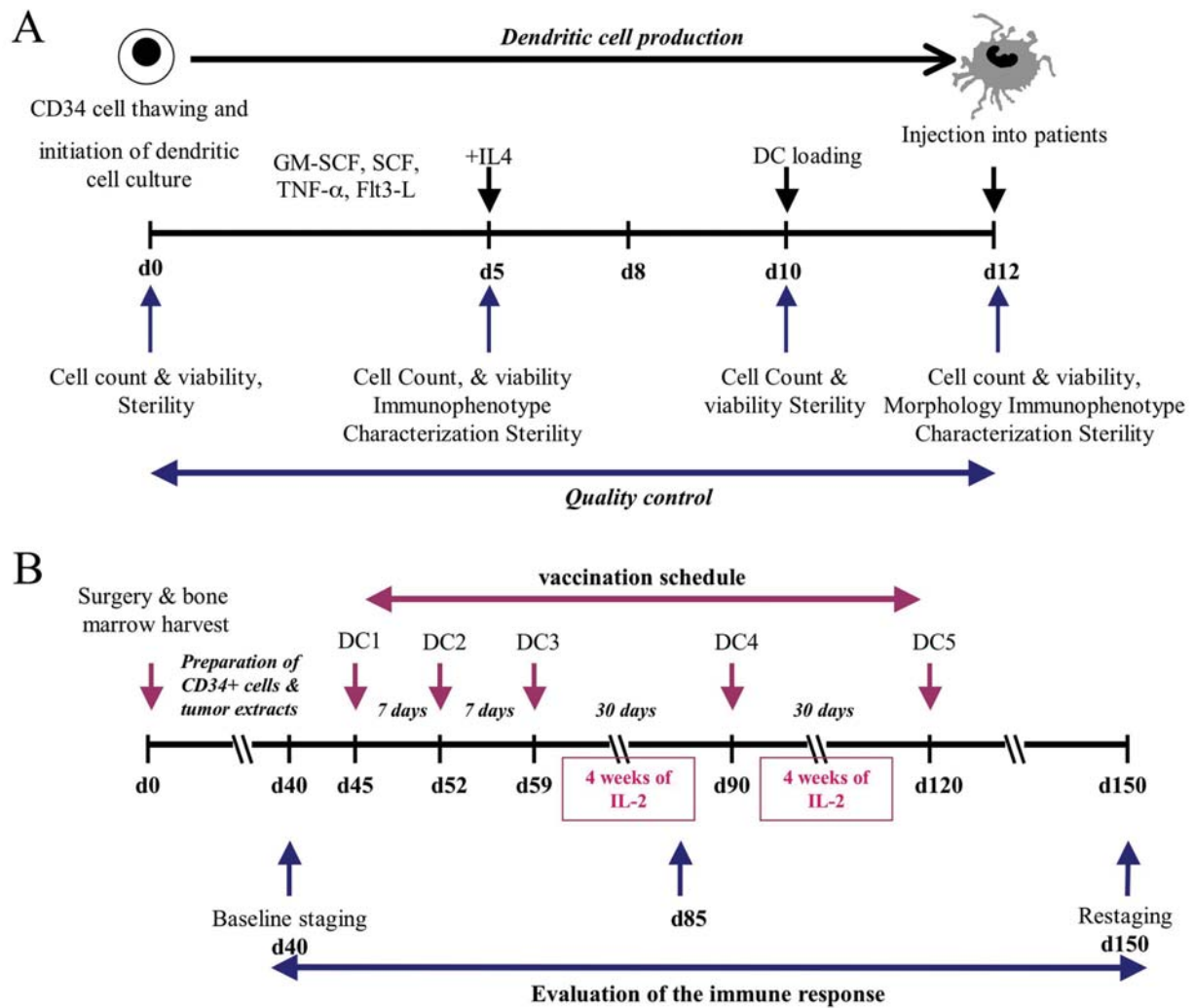


Figure 1. Scheme of the production of DCs (A), study design and vaccination schedule (B). (A) Schematic representation of the different steps for the production of tumor extract-loaded DCs derived from CD34⁺ cells including the different quality controls carried out in process and on the final cell product. (B) Schematic representation of the clinical trial proposed to the patients and of the different time points for the evaluation of the immune response.

Table I. Patients characteristics and disease status before surgery.

Patients UPN	Ages/gender	Type	Primary tumor			Evaluable metastases
			Tumor staging ^a	Nodal status ^a	Furhmann grading	
101	37/F	RCC	PT3	N2	IV	Chest
201	55/M	RCC	PT2	Nx	IV	Chest, bone
401	68/F	RCC	PT3a	Nx	III-IV	Chest, liver, adrenal gland
402	56/M	RCC	PT3	N0	III	Chest
501	50/M	RCC	PT3a	Nx	III-IV	Bone
502	43/M	Sarcoma	ND	ND	ND	Bone

RCC, renal cell carcinoma; F, female; M, Male and ND, not determined. ^aAccording to Bostwick and Murphy (40).

For tumor extracts, cells were conditioned in 50 ml cryocyte bags (Nexell international, Wommel, Belgium), lysed by 4 to 5 freeze (liquid nitrogen) and thaw (room temperature) cycles. Lysis was monitored by light microscopy on the percentage of viable cells after trypan blue exclusion. Tumor

extracts were then centrifuged at 1,000 x g for 12 min, supernatants were collected and protein contents determined. Microbiologic examination of extracts was also performed. Tumor extracts were aliquoted and stored at -30°C until use.

Positive selection of CD34⁺ cells from BM samples. Bone marrow harvests were collected in 500 ml heparinized culture bags. All manipulations were carried out in closed system under standardized operating procedures (SOP). Cells were washed in PBS, platelets were removed and mononuclear cells (MNCs) were separated by Ficoll density centrifugation using a COBE™ 2991 cell washer (COBE BCT, Lakewood, CO, USA). Then, cells were washed twice and processed for positive CD34⁺ selection according to the manufacturer's instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany), with minor modifications during the cell labeling. After Ficoll gradient, MNCs were transferred in 600 ml transfer pack (TP) bags (Baxter, Maurepas, France), then centrifuged at 400 x g at 20°C for 10 min. The supernatant was discarded into TP bags using a plasma extractor, then the cell pellet was carefully resuspended and removed into 150 ml TP bags for labeling. The cells were washed again in PBS/EDTA buffer (Miltenyi Biotec) supplemented with 1% of human serum albumin (HSA, LFB, Courtaboeuf, France). Up to 1x10¹⁰ MNC were resuspended in 90 ml of PBS/EDTA buffer + 1% HSA (wash buffer) and incubated at room temperature in the presence of human gamma globulin (Tegeline 50 mg/ml, LFB) at a final concentration of 1.5 mg/ml for 15 min. CliniMacs CD34⁺ reagent (7.5 ml) was added to each 1x10¹⁰ MNC and cells were incubated at room temperature for 30 min. The cells were then washed twice in complete buffer and resuspended in wash buffer to give a final volume of 100 ml containing <2x10⁷ MNC/ml. The cells were transferred into a single 600 ml TP bag (Baxter) that was connected to the CliniMacs device (Miltenyi Biotec). The device was used following the manufacturer's SOP using the described washing buffer. The cells were retained after separation in 42-45 ml of washing buffer into 150 ml TP bags. Positively selected CD34⁺ cells were fractioned in 10 ml of buffer + 4% HSA and cryo-preserved in liquid nitrogen in 50 ml cryocyte bags (Baxter) until use. Cell aliquots were taken before and after selection for various quality controls such as cell counts and viability staining with trypan blue, FACS analysis, determination of clonogenic growth and microbiologic examination.

Production in closed system of pulsed DCs derived from CD34⁺ cells. At day 0, for initiation of culture, a CD34⁺ aliquot was thawed and washed twice in complete buffer (4% HSA). CD34⁺ cells were then cultured at 10⁵ cells/ml in 85 cm² X-Fold bag (Nexell international) in RPMI-1640 without phenol red supplemented with L-glutamin, 10% (vol/vol) hABs and containing 300 ng/ml SCF, 300 ng/ml Flt3-L, 100 ng/ml GM-CSF and 5 ng/ml TNF α . The bags were identified and incubated in a humidified 5% CO₂ atmosphere at 37°C. At day 5, 8, and 10, cells were harvested, washed in cold 1X PBS. Then, the cell pellet was carefully resuspended in culture medium and counted. The cells were replated at 2.5-5x10⁵ cells/ml in culture medium containing the same cytokines than at day 0 except that Flt3-L was used at 50 ng/ml and that IL-4 was added at 50 ng/ml. At day 10, cells were pulsed with autologous tumor extracts (100 μ g/10⁶ cells) in complete medium at room temperature for 4 h. Cells were then loaded with 50 μ g/ml Keyhole Limpet Hemocyanin (KLH, Vacmune®, Biosyn, France) for at least 2 h. After pulsing, DCs were cultured in bag for 48 additional hours. At

day 12, pulsed DCs were washed twice and conditioned in a 5 ml final volume of 0.9% NaCl. While different quality controls were performed during the production process and on the final cell product (see below), vaccine release criteria were: i) negative bacterial culture 48 h prior to injection, ii) cell number 2-4x10⁵ cells/kg of body weight, iii) cell viability \geq 85%, iv) CD1a expression \geq 30% or HLA-DR \geq 50% in cell preparation vaccine as determined by immunophenotypic analysis. When pulsed DCs were not used for vaccination, they were aliquoted and frozen for immunomonitoring. In at least one DC preparation per patient, part of the DC culture was not pulsed at day 10. This was done in order to obtain aliquots of non-pulsed DCs that will be also used for immunomonitoring, as well as for evaluating the eventual toxicity of tumor extracts on DCs.

Quality controls for evaluation of CD34 selection. Numeration of CD34⁺ cells and clonogenic progenitors were performed before and after selection by flow cytometry and semi-solid culture assays, respectively.

CD34 counts. The numeration and the viability of CD34⁺ progenitors was performed by flow cytometry using the Stem-Kit (Beckman Coulter, Villepinte, France) according to the manufacturer's instructions. This kit contains a set of reagents including CD34 and CD45 mAbs and Stem-Count Fluorospheres, and allows the direct determination of the absolute count of CD34⁺ cells. Exclusion of dead cells was achieved using the 7-aminoactinomycin D intercalating DNA dye.

Semi-solid culture assays for clonogenic progenitors. Cells (10⁵ and 10⁴ cells/ml) were plated in methylcellulose cultures before and after CD34 selection, respectively. For granulocyte-macrophage colony forming unit (CFU-GM) assay, methylcellulose medium supplemented with appropriate cytokines (Methocult H4435, StemCell Technologies, Meylan, France) was used. For dendritic cell colony forming unit assay (CFU-DC), cells were cultured in methylcellulose medium (Methocult H4230, StemCell Technologies) supplemented with 50 ng/ml GM-CSF, 50 ng/ml SCF, 50 ng/ml Flt3-L, 50 ng/ml IL-4 and 12 ng/ml TNF α as described elsewhere (41). Cells were incubated at 37°C in a humidified incubator containing 5% CO₂ during 12 and 14 days for CFU-DC assay and CFU-GM assay, respectively. The colonies were then visualized and scored with an inverted microscope.

Quality controls of the production of pulsed-DCs. For each production of pulsed DCs, different quality controls were performed including cell morphology, immunophenotyping and mixed leukocyte reaction (MLR).

Cell morphology. At day 5 and 12, cell aliquots were washed twice with 1X PBS, adjusted at a concentration of 5x10⁵ DC/ml and 2-5x10⁵ cells were spun onto microscope slides. These cytopsin preparations were stained with May-Grünwald-Giemsa and analyzed by light microscopy.

Immunophenotyping of DCs. After 12 days of culture, cells were double stained with the following murine anti-human

mAbs directly conjugated either to fluorescein isothiocyanate (FITC) or to phycoerythrin (PE): CD1a-FITC (Ortho Diagnostic, Raritan, NJ, USA); CD1a-PE, CD14-FITC and CD83-PE (Beckman Coulter); CD40-FITC, CD54-PE, CD80-PE, CD86-PE and HLA-DR-PE (BD Biosciences, Mountain View, CA, USA). Negative controls were irrelevant isotype-matched mAbs. Briefly, 10^5 cells were incubated with the appropriate mAbs at 4°C for 20 min, washed twice in PBS/2% FCS and then fixed in PBS containing 1% paraformaldehyde before analysis by flow cytometry.

Mixed leukocyte reaction (MLR). The functional ability of produced pulsed DCs to stimulate allogeneic T-cells was assessed by MLR, as described elsewhere (41).

Clinical follow-up of the patients. Follow-up was designed to evaluate both safety and efficiency of the pulsed DC/Proleukin® treatment. First, during the five month after the 1st DC-vaccination, an intensive follow-up was engaged, appreciating both tolerance and initial anti-tumor efficiency. Then, an additional long-term follow-up, until the end of the study, was started to evaluate possible progression disease and overall survival.

Tolerance was appreciated both immediately after each vaccination and during the intervals between the DC injections by recording any potential physical or biological toxic adverse effect according to the World Health Organization criterias and by evaluating the performance status according the Karnofski's index. Potential auto-immune disorders were evaluated by physical examination and laboratory analysis before the 1st and 4th vaccinations and after the 5th vaccination. At these times, blood sample were collected for anti-nuclei, -DNA, -tissus, -microsome, -thyroglobulin, -TSH receptor and -peroxydase auto-antibodies, and also for Latex and Waaler-Rose reactions and free-T4 and TSH hormones as well as for immunomonitoring assays.

Immunomonitoring

Delayed-type hypersensitivity (DTH). Delayed-type hypersensitivity skin tests were performed with autologous DCs pulsed or not with either tumor extracts (5 µg), keyhole limpet hemocyanin (KLH, 5 µg) or tuberculin as recall antigen. Viable DCs (5×10^5) were injected intradermally before the 1st vaccination, after the 3rd vaccination and one month after the final vaccination. A positive skin reaction was defined as >5 mm diameter erythema and induration appearing 48-72 h after intradermal injection.

Characterization of T-cell subsets. For cell surface staining, various combinations of the following murine anti-human mAbs directly conjugated either to FITC, phycoerythrin (PE), PerCP or Allophycocyanine (APC) were used: CD3, CD4, CD8, CD25, CD27, CD28, CD45RA, CD45RO, CD62L, CD127, CCR7, HLA-DR. Negative controls were appropriate irrelevant isotype-matched mAbs. Briefly, cells were incubated with the appropriate mAbs at 4°C for 20 min, washed twice in PBS containing 2% of FCS, then fixed in PBS containing 1-4% paraformaldehyde before analysis by flow cytometry using a FACScalibur (Becton-Dickinson).

Detection of nTregs in PBMC collected from patients was performed at different time points: before vaccination at day 40, after the 3rd vaccination and the 1st cycle of IL-2 at day 85, and one month after the 2nd cycle of IL-2 and the 5th vaccination at day 150. nTregs are known to be CD3⁺CD4⁺ cells expressing high levels of CD25 (CD25^{hi}), the transcription factor Foxp3, and low levels of CD127 (CD127^{low/-}) (42,43). Thus, PBMC were thawed, simultaneously stained with CD3, CD4, CD25 mAbs and either CD127 mAbs or with rat anti-human FOXP3-APC (PCH101 clone, eBioscience, San Diego, CA, USA). Rat IgG2a APC was used as isotype control (eBiosciences).

Detection of anti-tumoral responsive cells. Detection of tumor-responsive cells was based on the frequency of interferon-γ (IFN-γ) and perforin producing cells. Tumor extract-pulsed DCs and non-pulsed DCs were thawed and co-cultured with autologous PBMCs (DC/PBMCs ratio = 1/10) collected from patients at different time points. Cells were co-cultured in RPMI containing 10% hABs, IL-2 (20 U/ml) and IL-7 (10 ng/ml, Cytheris, Vanves, France) in 24-well plate (10^6 cells/well, 2 ml/well) for 2 weeks, half medium and cytokines being renewed every 3-4 days. Then, cells were co-cultured with autologous RCC tumor cells obtained as described above at two different effector/tumor cells ratio (E/T: 25/1 and 10/1) for 5 h in RPMI containing 10% hABs and 1 µg/ml GolgiStop (Becton-Dickinson) to block cytokine secretion. Cells alone or stimulated with PHA + ionomycin were used as negative and positive controls, respectively. The cells were then harvested, washed and stained with CD3-PerCP and CD8-APC (both from Becton-Dickinson). Cells were then permeabilized with Cytotfix/Cytoperm plus (Becton-Dickinson) for 30 min followed by two washes in Perm/Wash solution (Becton-Dickinson). Intracellular staining was then performed with anti-IFN-γ-FITC and perforin-PE or a matched isotype control antibody for 30 min, washed twice in Perm/Wash solution, fixed in 2% paraformaldehyde and analyzed by flow cytometry using a FACScalibur (Becton-Dickinson).

For cell immunophenotyping and intracellular cytokine detection at least 10,000 and 100,000 gated events were collected in a listmode file, respectively. Then, analyses were carried out using CellQuest (Becton-Dickinson) or FlowJow (Tree Star, San Carlos, CA, USA) software. Results were expressed as a percentage of positive cells and/or by median of fluorescence intensity (MFI) by comparison to negative controls.

Results

Tumor samples and bone marrow harvests from the 6 patients included in this study were obtained and processed.

Purification of CD34⁺ cells from bone marrow harvests. The mean volume of BM harvests and the mean number of cells were 667.2 ± 58.7 ml and $11.8 \pm 1.1 \times 10^9$, respectively. Results of the CD34 purification are presented in Table II. After platelet removal, MNCs were enriched by Ficoll gradient allowing to obtain between 1.75 to 5.1×10^9 MNCs except for patient P#101. Indeed, this patient had a severe inflammatory

Table II. Clinical grade production of CD34⁺ bone marrow cells.

Patients UPN	Before purification				After purification							
	Total no. of cells (x10 ⁹)	% of CD34 ⁺	Total no. of CD34 ⁺ cells (x10 ⁶)		CFU-GM	CFU-DC	% of CD34 ⁺	Total no. of CD34 ⁺ cells (x10 ⁶)		Recovery	CFU-GM	CFU-DC
101	0.23	4	9.1		0.56	0.18	81	0.54		5.9	ND	ND
201	5.1	2	102		0.52	0.15	97	78.1		76.6	13	3.4
401	1.75	5.8	101.5		1.01	0.23	93.4	41		40.4	11.5	4.6
402	2.6	3.7	94.7		1.09	0.24	92	47.5		50.2	25.2	4.9
501	3.7	2.8	103.6		0.39	0.15	87.3	40.8		39.4	18.4	4.4
502	3.1	8.2	255		0.71	0.26	91.6	71.6		28.2	13.6	5.2

syndrome favoring BM clotting that in turn resulted in technical adverse problems and subsequently to an insufficient number of CD34⁺ cells (0.54x10⁶ cells). For the 5 remaining patients, the mean number and the mean percentage of CD34⁺ cells obtained after purification were 55.8±8x10⁶ cells and 92.3±1.6%, respectively. Functional study of myeloid (CFU-GM) and dendritic (CFU-DC) progenitors indicates that the purification process led to 25±6-fold and 22.5±1.8-fold enrichments for CFU-GM and CFU-DC, respectively. Overall, the purification of BM-CD34⁺ cells using the CliniMacs device was satisfactory in 5 out of 6 patients. Then, CD34⁺ cells were aliquoted and stored in liquid nitrogen.

Preparation of autologous tumor extracts. Cell suspensions from tumor samples were obtained for all patients following nephrectomy. Then, tumor extracts were prepared. The criteria for releasing tumor extracts in order to yield sufficient numbers of pulsed DCs for 5 vaccinations were as follows: i) number of viable cells after 4-5 freeze and thaw cycles ≤1%; ii) sterility of the product; iii) protein content ≥30 mg. While the size and weight of each tumor sample that we could use for extract preparation depended upon the size of the primitive tumor, the number of cells present in cell suspensions and the protein contents of the extracts did not appear to correlate to the size of tumor samples due to tumor heterogeneity in term of necrosis and vascularization (Table IIIA). Cell suspensions were characterized by morphology examination (Table IIIB). The results indicate that cell suspensions contained at least 50% (50-90%) of tumor cells. Thus, the preparation of tumor extracts was satisfactory in all patients as the protein content, varying from 31.4 to 161.7 mg, was sufficient enough for pulsing at least 3x10⁸ DCs at day 10 of culture.

Clinical grade production of CD34-DCs pulsed with tumor extracts. As mentioned above, the number of CD34⁺ cells obtained in patient P#101 was not sufficient enough and for this reason production of DCs was not further carried out. Patient P#502 was excluded from the study due to the sarcomatous histopathological type of his primary tumor despite a satisfactory production of both CD34⁺ cells and tumor extracts. Thus, clinical grade production of DCs was performed in 4 out of 5 eligible patients. The recovery of CD34⁺ cells after thawing was ≥70% giving 11.4±1.5x10⁶, 4±0.7x10⁶, 5.1±0.8x10⁶ and 6±0.4x10⁶ CD34⁺ cells for patients P#201, P#401, P#501 and P#402, respectively. The mean growth curves for each patient showing the proliferation and differentiation of CD34⁺ into DCs are presented in Fig. 2A. Cells first proliferate giving rise to a mean number of 41-58x10⁶ and 17.8-20.7x10⁶ cells at day 8 and 10, respectively. After day 8-10, cell proliferation decreases while the differentiation process increases. The results concerning the amount of DCs and the number of vaccinations that we could produce for each patient at day 12 are summarized in Table IIIC. Thus, 16 clinical grade productions used for vaccinations were prepared giving rise to 20.6±2.4x10⁶ pulsed-DCs from 7.4±0.9x10⁶ CD34⁺ cells. In addition, productions of unpulsed and pulsed-DCs were also performed for each patient in view of the immunomonitoring particularly for DTH tests. Overall, these data indicate the feasibility of the cryopreservation and

Table III. Clinical grade production of tumor extracts and CD34-DCs.

A, Generation of tumor extracts from tumor samples					
Patients UPN	Weight of tumor samples (g)	Tumor cell suspension (x10 ⁹)	No. of tumor cells used for extracts (x10 ⁶)	Protein content (mg)	
101	5.2	0.26	216	33.9	
201	25.1	1.09	997	161.7	
401	57.4	1.27	900	93.7	
402	17.3	0.37	340	31.4	
501	3	0.16	148.4	69.2	
502	17.2	1.63	1414	104.3	

B, Cytological analysis of tumor cell suspensions					
Percent (%) of total cells					
Patients UPN	Lymphocytes	Macrophages	Siderophages	Polymorphonuclear cells	Tumor cells
201	30%	<10%	<10%	<5%	60-70%
401	<10%	<10%	<5%	<1%	60%
402	<10%	<5%	<5%	<10%	80-90%
501	20%	10-15%	5-10%	3%	50%
502	<10%	<1%	<1%	<1%	90%

C, CD34-DCs					
Production of CD34-DCs for vaccinations (x10 ⁶)					
Patients UPN	Production 1	Production 2	Production 3	Production 4	Production 5
201	24.6 (11.2)	19.8 (9.5)	10.9 (10)	2.8 (9.1)	37.3 (17.7)
401	11.28 (3.5)	30.5 (5.8)	29.8 (3.9)	ND	ND
402	18.3 (6.5)	17.4 (6.8)	21.3 (5.9)	12.5 (5.4)	39.1 (7.7)
501	15.4 (6.7)	20.9 (4.7)	17.7 (3.9)	ND	ND

In brackets, are the number of CD34⁺ cells seeded in culture for generating DCs. ND, Not done.

differentiation of BM-CD34⁺ into DCs in a reproducible manner.

Immunophenotyping and characterization of dendritic cells in the final clinical grade cell products. At day 12 of culture, viable cells were counted and cell aliquots were stained with various mAbs and analyzed by flow cytometry in order to assess the DC content of each production. Table IVA displays for each patient the mean percentage \pm SEM of different markers that are known to be expressed by DCs at day 12 of culture. Importantly, >75% of the cells expressed HLA-DR. 25-45% of the cells also expressed the CD80 and CD86 costimulatory molecules and 30-70% were CD40-positive. Since CD1a was expressed at day 5 of culture by >30% of the cells (not shown), this marker which strongly varied from one patient to another, decreased after day 8 of culture and particularly after loading of DCs with tumor extracts. The relatively low levels of expression of co-stimulatory molecules

and CD1a are due to the fact that cultures were carried out in the presence of hABs instead of FCS and IL-4, respectively. As observed by others (35), CD34-derived DCs weakly expressed CD83. Finally, <10% of the cells expressed CD14 as the cultures were carried out in the presence of IL-4 (19). Analysis of Giemsa-stained cytopins showed the presence within final cell products of cells with typical DC morphology (not shown). The functionality of DC to stimulate T-cells was assessed in an MLR assay (Fig. 2B)

Vaccination of the patients, safety and tolerance. As we were able to fulfill the releasing criteria for each DC production, cell products were injected as described in Fig. 1. As shown in Table IIIC, P#201 and P#402 received 5 vaccinations, the mean of viable cells injected per kg of body weight were $2 \pm 0.5 \times 10^5$ and $2.3 \pm 0.4 \times 10^5$, respectively (Table IVB). Patients P#401 and P#501 only received 3 vaccinations because of progressive tumor growth and/or requirement of other

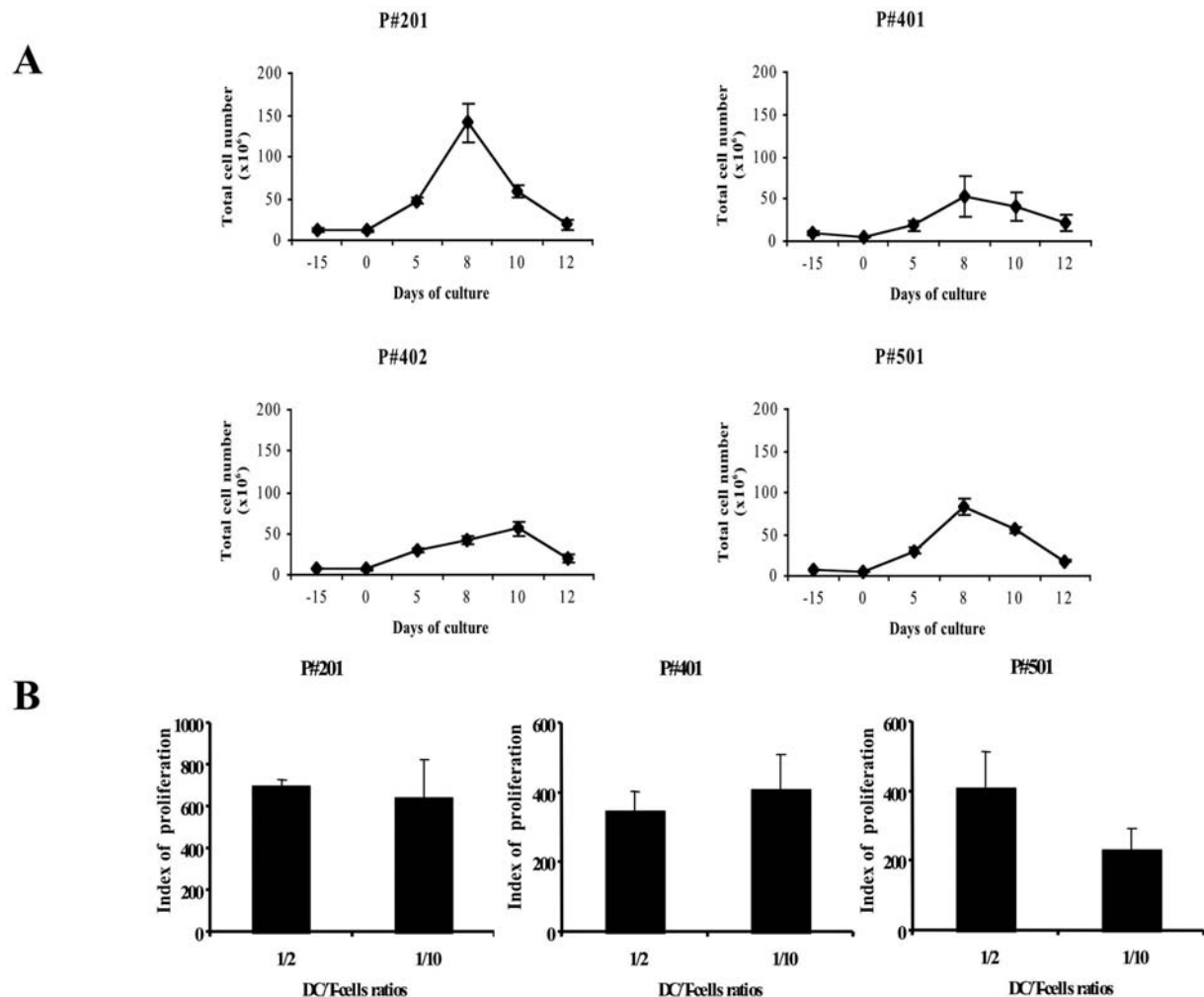


Figure 2. Growth curves (A) and functional study (B) of DCs differentiated from BM-CD34⁺ cells. (A) Each curve is the mean \pm SEM of n (see Table IIIC) DC productions expressed in total cell number for patient #201, #401, #501 and #402, respectively. (B) Stimulation of allogeneic T-cells by CD34-DCs. DCs and allogeneic T-cells were co-cultured in triplicate at different DC/T-cell ratio (1/2, 1/10) for 5 days, and T-cell proliferation was measured by tritiated thymidine incorporation as described elsewhere (41). Results for each patient are presented as the mean of 2-3 different MLR, each corresponding to separate DC production. The results have been normalized by presenting T-cell proliferation as an index of proliferation calculated as follows: [DC/T-cells mixed cultures (cpm) - T-cells cultured alone (cpm)]/[T-cells cultured alone (cpm)]. The mean number of cpm at a 1/2 ratio for patient 201, 401 and 501 were 78450 ± 2850 , 78850 ± 12300 and 86650 ± 17200 , respectively.

treatments that will excluded these patients from the present study. Indeed, P#401 developed a cardiac failure associated with an acute renal failure and P#501 had a medullary compression due to cervical metastasis which required high doses of corticoids in view of surgery. These two patients were injected with $2.7 \pm 1.2 \times 10^5$ and $2.5 \pm 0.2 \times 10^5$ per kg of body weight, respectively (Table IVB). Thus, the total number of viable cells injected into the patients ranged from 47.7 to 73.1×10^6 cells. In the four patients receiving pulsed-DCs, initial and delayed tolerance was excellent without clinical nor biological adverse effect due to the vaccinations. No patient developed erythema at the injection site nor systemic toxicity following vaccination. No biological nor clinical manifestations of auto-immune disease developed in these patients.

Follow-up of the patients and study of the immunological responses

Delayed-type hypersensitivity. In order to evaluate DTH which reflects the *in vivo* T-cell response, patients were

injected intradermally with either tuberculin as a recall antigen, KLH, tumor extracts alone, unloaded DCs or DCs loaded with tumor extracts before vaccination (d40), after the 3rd vaccination and the first round of IL-2 (d85) and one month after the 5th vaccination and IL-2 treatment (d150). In none of the patients receiving 3 or 5 vaccinations, a positive DTH could be observed. The absence of positive DTH for tuberculin in all patients except one (patient #201) strongly suggests that patients were immuno-depressed.

Evidence of tumor reactive CD8⁺ T-cells. Blood samples from the 2 patients who had completed the full vaccination protocol were tested for the presence of tumor extract reactive CD8⁺ T-cells (Fig. 3). The results showed an increase of the percentage of IFN- γ and perforin-positive CD8⁺ T-cells after restimulation with tumor-extract pulsed DCs by comparison to negative controls (i.e. PBMC stimulated with non-pulsed DCs) as soon as after 3 vaccinations and 4 weeks of IL-2. These increases were still detectable, albeit less important, one month after the full treatment was achieved. These data

Table IV. Characterization of dendritic cell vaccines.

A, Immunophenotype								
Patients UPN	HLA-DR	CD1a	CD80	CD86	CD40	CD83	CD14	n
201	76.2±1.7	16.2±0.4	29.9±16.7	27.9±0.3	33.6±0.5	9.7±0.6	2.3±1.1	5
401	99.2±0.1	30.4±2.0	32.5±0.8	44.8±1.1	68.9±1.1	17.8±0.5	9.5±2.5	3
402	87.8±1.7	4.4±0.4	26±2.3	36.8±2.2	51.6±2.4	11.2±1.8	5±1.1	5
501	86.9±1.6	10.9±3.5	25.4±6.7	40±6.8	57.1±4.1	15.6±0.3	4.6±0.2	3

Expression of each cell surface marker is presented as mean percentage ± SEM. n, number of productions.

B, Number of injected cells

Patients UPN	Total cell no. of injected cells (x10 ⁶)	Mean of injected cells (x10 ⁶)	Total cell no./Kg (x10 ⁵)	Mean of cells/Kg (x10 ⁵)	No. of vaccinations
201	68.3	13.6±3.3	10	2±0.5	5
401	55.3	18.4±8.5	8.2	2.7±1.2	3
402	73.1	14.6±2.6	11.7	2.3±0.4	5
501	47.7	15.9±1.4	7.7	2.5±0.2	3

Numbers are mean percentage ± SEM of n vaccinations.

indicate that vaccination of metastatic RCC patients with tumor-pulsed DCs derived from CD34⁺ cells can elicit a specific anti-tumor biological immune response.

Effect of vaccination and IL-2 on T-cell subsets. No significant imbalance among naive and memory T-cell subsets was observed before, during and after vaccination (data not shown). Before vaccination, the study of nTregs in 3 patients indicated that the frequency of CD3⁺CD4⁺FOXP3⁺ T-cells (2.2, 2.6 and 4.6%) remained in the normal range as compared to a control group of healthy donors (3±0.7%, n=9). At day 85 (one month after 3 vaccinations and just after the first 4 weeks of IL-2), a striking increase of nTregs was observed (Fig. 4). Interestingly, the percentage of CD3⁺CD4⁺FOXP3⁺ cells returned to baseline levels one month (day 150) after the complete treatment (i.e. 5 vaccinations and two cycles of 4 week IL-2). The characterization of nTregs based on CD25^{hi} or CD127^{low/-} expression gave rise to similar data (not shown). These data strongly suggest that IL-2 treatment may transiently increase the percentage of circulating nTregs in RCC patients.

Clinical outcome. One patient (P# 501) progressed rapidly during the round of vaccinations (cervical and dorsal bone metastasis) and was excluded from the study after the 3rd vaccination to start a second line of treatment by interferon-α and conventional chemotherapy. He died of progressive disease 10 months after diagnosis. In the three patients who received Proleukin®, moderate to elevated transient fever was observed. This was clearly linked to the course of this cytokine treatment. In one patient (P#201), this led to reduction of the Proleukin dosage for the last four weeks of the treatment. In another patient (P#401), the Proleukin induced saline inflation

leading to severe cardiac dysfunction and subsequent acute renal failure regressing upon arrest of Proleukin. This patient died from pulmonary embolism.

No tumor regression was evidenced in treated patients. However, in the two patients who received five DC vaccinations, one (P#201) displayed a stable disease during the rounds of DC vaccinations, and then evidenced pulmonary progression shortly after. He died 16 month after initial diagnosis. The other (P#402) also had stable disease for 6 months from the 1st DC vaccination, then he had pulmonary progression of the disease 6 weeks after the 5th DC vaccination. At this time, he received an anti-angiogenic treatment followed by conventional chemotherapy. However, the disease continued to progress and the patient died 24 months after initial diagnosis.

Discussion

In this study we have shown the feasibility of producing large amount of clinical-grade CD34⁺-derived DCs pulsed with tumor extract under GMP conditions and the absence of toxicity after vaccination of metastatic RCC patients with these cell products. Most of the DC-based clinical trials have been carried out using Mo-DCs, and only few of them have used CD34-DCs (23,35), CD34⁺ cells being obtained from G-CSF-mobilized peripheral blood hematopoietic stem cells. In this study, we have used BM CD34-DCs, BM cells being harvested during the general anesthesia. This approach can be proposed to cancer patients requiring surgery and will also have the advantage of avoiding the use of G-CSF. Indeed, besides its transient and uncomfortable side effects (i.e. pain and fever), G-CSF can favor tumor growth, as some tumor cells express

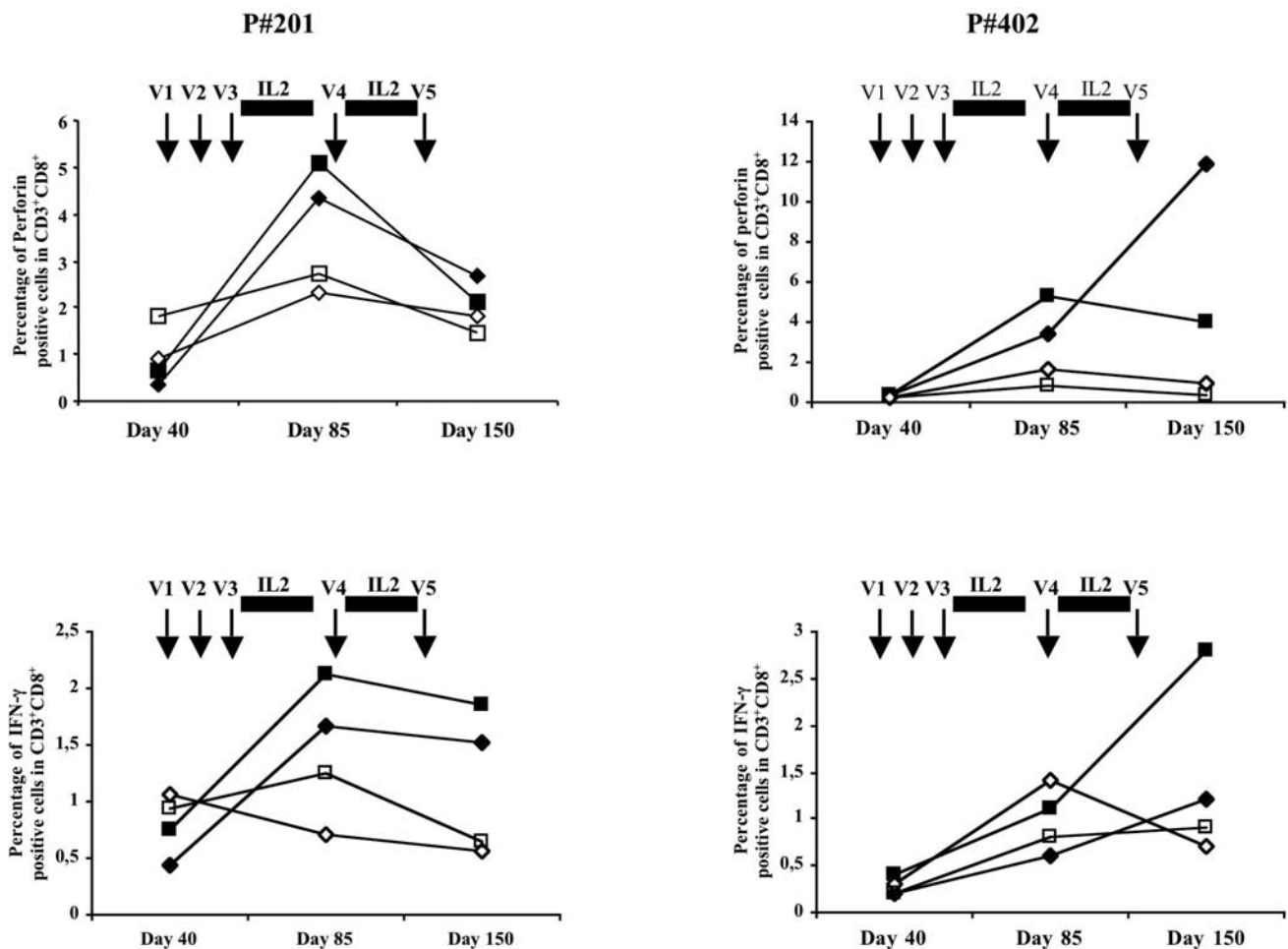


Figure 3. Kinetics of the biological immune response. Evaluation of the anti-tumoral responses was based on the frequency of CD3⁺CD8⁺ cells producing IFN- γ or perforin. PBMC, collected at different time points (day 40, day 85 and day 150), were co-cultured either with autologous tumor extract-pulsed DCs (filled symbols) or unpulsed DCs (open symbols) in the presence of low doses of IL-2 and IL-7 for 2 weeks. Then, primed cells were restimulated by autologous tumor cells derived from RCC at two E/T ratio: 25/1 (diamond symbols) and 10/1 (square symbols) for 5 h. Then, production of IFN- γ and perforin was determined by flow cytometry after gating analysis on CD3⁺CD8⁺ cells. Data presented are from the two patients that received the full vaccination treatment and arrows represent the schedule of the vaccinations. ♦, T-cells primed with tumor extract-pulsed DCs and co-cultured with target cells at 25/1 ratio. ■, T-cells primed with unpulsed DCs and co-cultured with target cells at 25/1 ratio. ◇, T-cells primed with tumor extract-pulsed DCs and co-cultured with target cells at 10/1 ratio. □, T-cells primed with unpulsed DCs and co-cultured with target cells at 10/1 ratio.

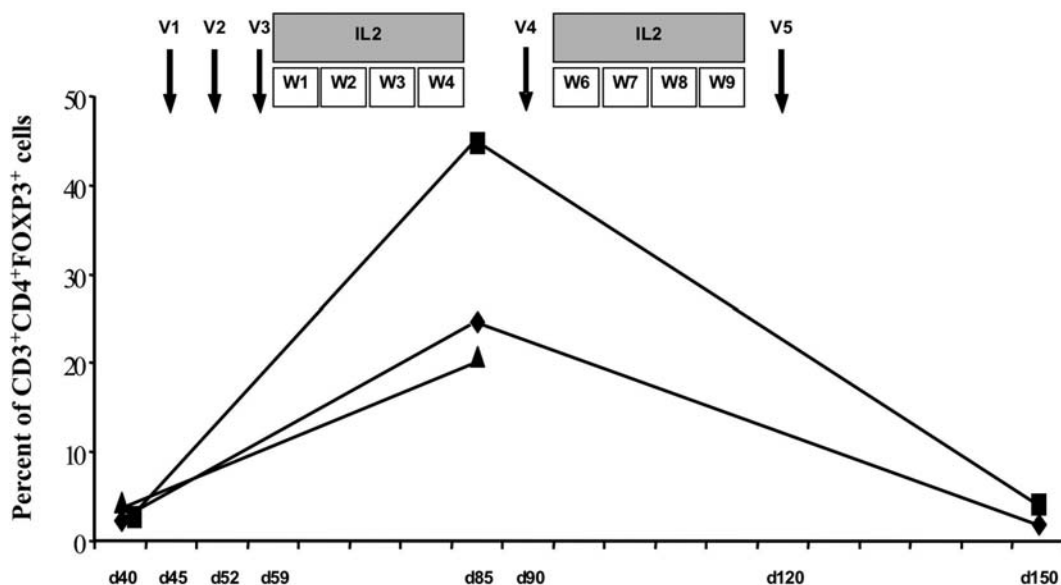


Figure 4. Follow-up of nTreg during the protocol study. nTreg were identified by flow cytometry based on FOXP3 expression gated within CD3⁺CD4⁺ T-cells. Arrows represent the schedule of the vaccinations. Symbols for each patient studied are: ♦ for patient # 201; ▲ for patient # 401; ■ for patient # 402.

such growth factor receptors (44) and also disturb the balance between different T-cell subsets (45,46). In our study, we observed a decrease of the cell growth after 8 days of culture (see Fig. 2A), yet allowing injecting an average amount of 2.27×10^5 cells/kg (see Table IIIB). According to the data reported by Banchereau *et al* (35), it seems possible to load CD34-DCs at day 8 and to inject them into the patients at day 9 to achieve an efficient immune response. Thus, it should be worthwhile to test this setting in a future clinical trial in order to increase the number of injected cells and to decrease the culture duration. In addition, the sequenced exposure of CD34⁺ cells to various cytokines (i.e. Flt3-L plus IL-6 and then GM-CSF) seems important to favor their differentiation into tumor-competent DCs (47). Although our CD34-DCs were produced in the presence of Flt3-L and GM-CSF, IL-6 was not used. Nevertheless, an anti-tumor immunological response was evidenced in the two patients having received the full vaccination treatment. This was demonstrated, in the absence of specific and well-identified tumor associated antigen, by generating for each patient a continuous tumor cell line. Thus, we were able to obtain, by using CD34-DCs loaded with total tumor cell extracts, an immune response specifically directed against autologous tumor cells. This indicates that our final DC product can induce T-cell stimulation rather than tolerance.

However, while we did not observe an increased immune responses after the 4th and the 5th vaccination, we believe that this was due to the fact that patients received their IL-2 treatment after the 3rd vaccination. Indeed, we observed that the percentage of circulating nTregs massively increased after the first cycle of IL-2 administration that followed the 3rd DC vaccination. They represented >30% of the whole CD4 T-cell population.

Our data are in agreement with previous studies (48,49) indicating that high-dose bolus IL-2 administration increased peripheral blood nTregs as well as nTregs in the tumor microenvironment (50) in patients with metastatic melanoma or RCC. Although we observed that the percentage of nTregs rapidly returned to baseline levels one month after the cessation of IL-2, it is thus striking that a cytokine initially given for boosting effector immune responses may actually drive primarily the nTreg expansion. Our observation may have major implication since there are numerous pieces of evidence that nTregs play a key role in tumor development in mice and humans (51). In the 2 patients receiving both 5 DC vaccines and IL-2, no tumor progression was observed during the treatment. Nevertheless, our data suggest that IL-2 administration may counterbalance the beneficial effect of the DC vaccines for inducing an anti-tumor T-cell response. Our results should be taken into consideration in future DC-based clinical trials and IL-2 administration avoided particularly in advanced RCC patients where the percentage of circulating nTregs seems to be basically increased (52).

Overall, we report the possibility of using BM-CD34⁺ cells for producing large number of clinical grade DCs that can be injected into cancer patients without any toxic effect. Because only few patients could undergo the complete protocol due to the severity of their pathology, DC-based immunotherapy should be envisioned in a more favorable context. Indeed, prognosis of metastatic RCC patients has been recently and

considerably improved by anti-angiogenic drug therapy (53). However, eliciting an efficient anti-tumor immune response to eliminate residual tumor cells and/or to control tumor growth still remains a challenge. Thus, one can propose to combine anti-angiogenic drug therapies with DC-based immunotherapy and *in vivo* Treg depletion (54) in order to reinforce the immune stimulation (51,55). Indeed, *in vivo* Treg depletion both in different animal tumor models (56) and in cancer patients (57) has been proven to enhance the anti-tumor immune response. Other strategies consisting of either inhibiting indoleamine 2,3-dioxygenase (IDO) (58) or blocking B7-H1 (59), two pathways involved in immune suppression in cancer, would also be of interest.

Furthermore, directing the immune response to recently identified tumor-associated antigens such as MUC1 (38), carbonic anhydrase IX-G250MN (CA9) (60) or survivin (61) in RCC cancer patients should also be progressed in order to improve the efficacy of such cell therapy strategies. Our data also prompt to consider re-evaluating the use of IL-2 in gene and cell therapies of cancer.

Acknowledgements

We thank Dr Olivier Rixe (AP-HP, Groupe Hospitalier Pitié Salpêtrière, Department of Oncology, Paris, France) for helpful discussions. We thank Virginie Leclercq, Céline Pellé, Anne Gaignerie, Pierre Cheraud, Marie-Christine Burland, Véronique Bon-Durand for their expert technical assistance. This study was granted by the 'Projet Hospitalier de Recherche Clinique (PHRC)' and M.C. was financially supported by PHRC.

References

1. Gore ME, Galligioni E, Keen CW, *et al*: The treatment of metastatic renal cell carcinoma by continuous intravenous infusion of recombinant interleukin-2. *Eur J Cancer* 30A: 329-333, 1994.
2. Rosenberg SA, Yang JC, Topalian SL, *et al*: Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *JAMA* 271: 907-913, 1994.
3. Fyfe G, Fisher RI, Rosenberg SA, Sznol M, Parkinson DR and Louie AC: Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy. *J Clin Oncol* 13: 688-696, 1995.
4. Yang JC, Sherry RM, Steinberg SM, *et al*: Randomized study of high-dose and low-dose interleukin-2 in patients with metastatic renal cancer. *J Clin Oncol* 21: 3127-3132, 2003.
5. Pyrhonen S, Salminen E, Ruutu M, *et al*: Prospective randomized trial of interferon alfa-2a plus vinblastine versus vinblastine alone in patients with advanced renal cell cancer. *J Clin Oncol* 17: 2859-2867, 1999.
6. Negrier S, Escudier B, Lasset C, *et al*: Recombinant human interleukin-2, recombinant human interferon alfa-2a, or both in metastatic renal-cell carcinoma. *Groupe Français d'Immunothérapie. N Engl J Med* 338: 1272-1278, 1998.
7. Negrier S, Perol D, Ravaud A, *et al*: Randomized study of intravenous versus subcutaneous interleukin-2, and IFNalpha in patients with good prognosis metastatic renal cancer. *Clin Cancer Res* 14: 5907-5912, 2008.
8. van Spronsen DJ, de Weijer KJ, Mulders PF and De Mulder PH: Novel treatment strategies in clear-cell metastatic renal cell carcinoma. *Anticancer Drugs* 16: 709-717, 2005.
9. Tykodi SS, Warren EH, Thompson JA, *et al*: Allogeneic hematopoietic cell transplantation for metastatic renal cell carcinoma after nonmyeloablative conditioning: toxicity, clinical response, and immunological response to minor histocompatibility antigens. *Clin Cancer Res* 10: 7799-7811, 2004.

10. Banchereau J and Steinman RM: Dendritic cells and the control of immunity. *Nature* 392: 245-252, 1998.
11. Steinman RM, Inaba K, Turley S, Pierre P and Mellman I: Antigen capture, processing, and presentation by dendritic cells: recent cell biological studies. *Hum Immunol* 60: 562-567, 1999.
12. Banchereau J, Paczesny S, Blanco P, *et al*: Dendritic cells: controllers of the immune system and a new promise for immunotherapy. *Ann NY Acad Sci* 987: 180-187, 2003.
13. Zhou LJ and Tedder TF: CD14⁺ blood monocytes can differentiate into functionally mature CD83⁺ dendritic cells. *Proc Natl Acad Sci USA* 93: 2588-2592, 1996.
14. Palucka KA, Taquet N, Sanchez-Chapuis F and Gluckman JC: Dendritic cells as the terminal stage of monocyte differentiation. *J Immunol* 160: 4587-4595, 1998.
15. Cao H, Verge V, Baron C, *et al*: In vitro generation of dendritic cells from human blood monocytes in experimental conditions compatible for in vivo cell therapy [see comments]. *J Hematother Stem Cell Res* 9: 183-194, 2000.
16. Dauer M, Obermaier B, Herten J, *et al*: Mature dendritic cells derived from human monocytes within 48 hours: a novel strategy for dendritic cell differentiation from blood precursors. *J Immunol* 170: 4069-4076, 2003.
17. Caux C, Massacrier C, Dezutter-Dambuyant C, *et al*: Human dendritic Langerhans cells generated in vitro from CD34⁺ progenitors can prime naive CD4⁺ T cells and process soluble antigen. *J Immunol* 155: 5427-5435, 1995.
18. Rosenzweig M, Camus S, Canque B, Guigon M and Gluckman JC: The influence of different cytokine schedules on human dendritic cell (DC) differentiation from cord blood CD34⁺ cells. *Blood* 88 (Suppl 1): 154a, 1996.
19. Caux C, Massacrier C, Vanbervliet B, *et al*: CD34⁺ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to granulocyte-macrophage colony-stimulating factor plus tumor necrosis factor alpha: II. Functional analysis. *Blood* 90: 1458-1470, 1997.
20. Siena S, Di Nicola M, Bregni M, *et al*: Massive ex vivo generation of functional dendritic cells from mobilized CD34⁺ blood progenitors for anticancer therapy. *Exp Hematol* 23: 1463-1471, 1995.
21. Bernhard H, Disis ML, Heimfeld S, Hand S, Gralow JR and Cheever MA: Generation of immunostimulatory dendritic cells from human CD34⁺ hematopoietic progenitor cells of the bone marrow and peripheral blood. *Cancer Res* 55: 1099-1104, 1995.
22. Strunk D, Rappersberger K, Egger C, *et al*: Generation of human dendritic cells/Langerhans cells from circulating CD34⁺ hematopoietic progenitor cells. *Blood* 87: 1292-1302, 1996.
23. Mackensen A, Herbst B, Chen JL, *et al*: Phase I study in melanoma patients of a vaccine with peptide-pulsed dendritic cells generated in vitro from CD34(+) hematopoietic progenitor cells. *Int J Cancer* 86: 385-392, 2000.
24. Oosterwijk-Wakka JC, Tiemessen DM, Bleumer I, *et al*: Vaccination of patients with metastatic renal cell carcinoma with autologous dendritic cells pulsed with autologous tumor antigens in combination with interleukin-2: a phase I study. *J Immunother* 25: 500-508, 2002.
25. Holtl L, Zelle-Rieser C, Gander H, *et al*: Immunotherapy of metastatic renal cell carcinoma with tumor lysate-pulsed autologous dendritic cells. *Clin Cancer Res* 8: 3369-3376, 2002.
26. Pandha HS, John RJ, Hutchinson J, *et al*: Dendritic cell immunotherapy for urological cancers using cryopreserved allogeneic tumour lysate-pulsed cells: a phase I/II study. *BJU Int* 94: 412-418, 2004.
27. Marten A, Renoth S, Heinicke T, *et al*: Allogeneic dendritic cells fused with tumor cells: preclinical results and outcome of a clinical phase I/II trial in patients with metastatic renal cell carcinoma. *Hum Gene Ther* 14: 483-494, 2003.
28. Avigan D, Vasir B, Gong J, *et al*: Fusion cell vaccination of patients with metastatic breast and renal cancer induces immunological and clinical responses. *Clin Cancer Res* 10: 4699-4708, 2004.
29. Barbuto JA, Ensina LF, Neves AR, *et al*: Dendritic cell-tumor cell hybrid vaccination for metastatic cancer. *Cancer Immunol Immunother* 53: 1111-1118, 2004.
30. Su Z, Dannull J, Heiser A, *et al*: Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells. *Cancer Res* 63: 2127-2133, 2003.
31. Wieriecky J, Mueller M and Brossart P: Dendritic cell-based cancer immunotherapy targeting MUC-1. *Cancer Immunol Immunother* 55: 63-67, 2006.
32. Mortarini R, Anichini A, Di Nicola M, *et al*: Autologous dendritic cells derived from CD34⁺ progenitors and from monocytes are not functionally equivalent antigen-presenting cells in the induction of melan-A/Mart-1(27-35)-specific CTLs from peripheral blood lymphocytes of melanoma patients with low frequency of CTL precursors. *Cancer Res* 57: 5534-5541, 1997.
33. Meierhoff G, Krause SW and Andreesen R: Comparative analysis of dendritic cells derived from blood monocytes or CD34⁺ hematopoietic progenitor cells. *Immunobiology* 198: 501-513, 1998.
34. Ferlazzo G, Wesa A, Wei WZ and Galy A: Dendritic cells generated either from CD34⁺ progenitor cells or from monocytes differ in their ability to activate antigen-specific CD8⁺ T cells. *J Immunol* 163: 3597-3604, 1999.
35. Banchereau J, Palucka AK, Dhodapkar M, *et al*: Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. *Cancer Res* 61: 6451-6458, 2001.
36. Banchereau J, Ueno H, Dhodapkar M, *et al*: Immune and clinical outcomes in patients with stage IV melanoma vaccinated with peptide-pulsed dendritic cells derived from CD34⁺ progenitors and activated with type I interferon. *J Immunother* 28: 505-516, 2005.
37. Cao T, Ueno H, Glaser C, Fay JW, Palucka AK and Banchereau J: Both Langerhans cells and interstitial DC cross-present melanoma antigens and efficiently activate antigen-specific CTL. *Eur J Immunol* 37: 2657-2667, 2007.
38. Wieriecky J, Muller MR, Wirths S, *et al*: Immunologic and clinical responses after vaccinations with peptide-pulsed dendritic cells in metastatic renal cancer patients. *Cancer Res* 66: 5910-5918, 2006.
39. Sciarra A, Gentile V, Salciccia S, Alfarone A and Di Silverio F: New anti-angiogenic targeted therapy in advanced renal cell carcinoma (RCC): current status and future prospects. *Rev Recent Clin Trials* 3: 97-103, 2008.
40. Bostwick DG and Murphy GP: Diagnosis and prognosis of renal cell carcinoma: highlights from an international consensus workshop. *Semin Urol Oncol* 16: 46-52, 1998.
41. Movassagh M, Baillou C, Cosset FL, Klatzmann D, Guigon M and Lemoine FM: High level of retrovirus-mediated gene transfer into dendritic cells derived from cord blood and mobilized peripheral blood CD34⁺ cells. *Hum Gene Ther* 10: 175-187, 1999.
42. Liu W, Putnam AL, Xu-Yu Z, *et al*: CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4⁺ T reg cells. *J Exp Med* 203: 1701-1711, 2006.
43. Seddiki N, Santner-Nanan B, Martinson J, *et al*: Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 203: 1693-1700, 2006.
44. Tachibana M, Miyakawa A, Nakashima J, *et al*: Autocrine growth promotion by multiple hematopoietic growth factors in the established renal cell carcinoma line KU-19-20. *Cell Tissue Res* 301: 353-367, 2000.
45. Francke A, Piao W, Lauber J, *et al*: G-CSF as immune regulator in T cells expressing the G-CSF receptor: implications for transplantation and autoimmune diseases. *Blood* 102: 734-739, 2003.
46. Rutella S, Zavala F, Danese S, Kared H and Leone G: Granulocyte colony-stimulating factor: a novel mediator of T cell tolerance. *J Immunol* 175: 7085-7091, 2005.
47. Cohen PA, Koski GK, Czerniecki BJ, *et al*: STAT3- and STAT5-dependent pathways competitively regulate the pan-differentiation of CD34pos cells into tumor-competent dendritic cells. *Blood* 112: 1832-1843, 2008.
48. Ahmadzadeh M and Rosenberg SA: IL-2 administration increases CD4⁺ CD25(hi) Foxp3⁺ regulatory T cells in cancer patients. *Blood* 107: 2409-2414, 2006.
49. van der Vliet HJ, Koon HB, Yue SC, *et al*: Effects of the administration of high-dose interleukin-2 on immunoregulatory cell subsets in patients with advanced melanoma and renal cell cancer. *Clin Cancer Res* 13: 2100-2108, 2007.
50. Jensen HK, Donskov F, Nordmark M, Marcussen N and von der Maase H: Increased intratumoral FOXP3-positive regulatory immune cells during interleukin-2 treatment in metastatic renal cell carcinoma. *Clin Cancer Res* 15: 1052-1058, 2009.

51. Curiel TJ: Regulatory T cells and treatment of cancer. *Curr Opin Immunol* 20: 241-246, 2008.
52. Cesana GC, DeRaffele G, Cohen S, *et al*: Characterization of CD4⁺CD25⁺ regulatory T cells in patients treated with high-dose interleukin-2 for metastatic melanoma or renal cell carcinoma. *J Clin Oncol* 24: 1169-1177, 2006.
53. Choueiri TK, Bukowski RM and Rini BI: The current role of angiogenesis inhibitors in the treatment of renal cell carcinoma. *Semin Oncol* 33: 596-606, 2006.
54. Powell DJ Jr, Attia P, Ghetie V, Schindler J, Vitetta ES and Rosenberg SA: Partial reduction of human FOXP3⁺ CD4 T cells in vivo after CD25-directed recombinant immunotoxin administration. *J Immunother* 31: 189-198, 2008.
55. Holtl L, Ramoner R, Zelle-Rieser C, *et al*: Allogeneic dendritic cell vaccination against metastatic renal cell carcinoma with or without cyclophosphamide. *Cancer Immunol Immunother* 54: 663-670, 2005.
56. Ghiringhelli F, Larmonier N, Schmitt E, *et al*: CD4⁺CD25⁺ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol* 34: 336-344, 2004.
57. Dannull J, Su Z, Rizzieri D, *et al*: Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest* 115: 3623-3633, 2005.
58. Katz JB, Muller AJ and Prendergast GC: Indoleamine 2,3-dioxygenase in T-cell tolerance and tumoral immune escape. *Immunol Rev* 222: 206-221, 2008.
59. Curiel TJ, Wei S, Dong H, *et al*: Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med* 9: 562-567, 2003.
60. Uemura H, Fujimoto K, Tanaka M, *et al*: A phase I trial of vaccination of CA9-derived peptides for HLA-A24-positive patients with cytokine-refractory metastatic renal cell carcinoma. *Clin Cancer Res* 12: 1768-1775, 2006.
61. Mahotka C, Krieg T, Krieg A, *et al*: Distinct in vivo expression patterns of survivin splice variants in renal cell carcinomas. *Int J Cancer* 100: 30-36, 2002.