

Effective antigen cross-presentation by prostate cancer patients' dendritic cells: implications for prostate cancer immunotherapy

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Despite the potency with which dendritic cells (DCs) are able to utilize the exogenous MHC I antigen cross-presentation pathway to cross-present antigen for the activation of killer T cells in model systems, concern about defects in immune function in cancer patients has led to uncertainty regarding whether immune cells derived from patients can effectively be used to generate tumor vaccines. We have undertaken a careful analysis of the potency of using DCs obtained from prostate cancer patients to cross-present antigen derived from human prostate tumor cells for the activation of antigen-specific T cells. Such DCs can be matured *ex vivo* into functionally active cells and are capable of cross-presenting influenza antigen derived from internalized apoptotic prostate tumor cells. Importantly, we demonstrate effective stimulation of both CD4⁺ and CD8⁺ T cells, as evident by production of IFN- γ , and the ability of CD8⁺ T cells to differentiate into effector CTLs. These results, defining conditions in which prostate cancer patient DCs can efficiently utilize the cross-presentation pathway and in which apoptotic tumor can serve as a source of antigen for DCs to activate T cells, demonstrate that this system warrants clinical study as a potential immunotherapy.

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Introduction

It has been proposed that apoptotic tumor cells may serve as a source of antigen for presentation by the immune system for the activation of effective human tumor immunity.¹ Apoptotic cells are able to deliver antigen to dendritic cells (DCs), where peptide epitopes

derived from these antigens are delivered to both MHC I and MHC II molecules.^{2–5} Loading of MHC I with antigen derived from exogenous material offers a physiologic mechanism for the *in vivo* phenomenon referred to as 'cross-presentation.'⁶ Several studies have shown that the cross-presentation of antigen derived from dying tumor cells and virally infected cells can efficiently load MHC I and MHC II molecules of monocyte derived DCs, which can in turn activate tumor- and virus-specific CD8⁺ and CD4⁺ T cells.^{7,8} To achieve activation of effector CTLs via this pathway, the DC must receive both a maturation stimulus as well as a signal from a CD4 helper T cell (eg CD40L).⁹

Delivery of antigen via cross-presentation as a strategy for immunization confers the advantage of allowing DCs

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to present wide arrays of antigen (both known and unknown) expressed by tumor cells, potentially activating a diverse repertoire of tumor-specific CTLs. Additionally, this approach bypasses the need to determine specific MHC I epitopes, as the DCs do this naturally during the processing of exogenous antigen. Accordingly, DCs cross-presenting apoptotic tumor cells present antigen independent of the patient's HLA haplotype, potentially loading all of the different HLA alleles present on the DC. Moreover, antigen loading via this pathway has been shown to be 3000–10 000 times more efficient than *in vitro* surface loading DCs with peptide, and up to 50 000 times more efficient than whole protein injected intravenously.^{5,10} Finally, by delivering antigen derived from apoptotic tumor cells to autologous DCs, it may be possible to induce effective CD4⁺ helper T cells thus supporting the survival of CD8⁺ memory T cells.⁹

We have applied this approach toward the establishment of a tumor vaccine protocol for prostate cancer patients. Prostate cancer is among the top three cancers afflicting American men, with a prevalence of 8 million,¹¹ and an expected incidence of nearly 189 000 and with approximately 30 200 deaths in 2002 according to the American Cancer Society. While chemotherapy, radiation treatment, and hormonal ablation kill a proportion of tumor cells, the cells that survive do so in a growth-arrested state¹² and it is these cells, resistant to conventional therapy, that a successful tumor-cell vaccine may target. Prostate cancer patients offer several advantages as candidates for new tumor immunotherapies. Patients with rising PSA levels who have failed conventional therapy are at high risk for metastatic disease and death within 5 years.¹³ Furthermore, as chemotherapy has no proven benefit in this patient cohort, these patients offer the chance to test experimental immunotherapy protocols in chemo-naïve individuals, an important issue given the toxic effects of chemotherapy on the immune system.

There is a substantial body of literature suggesting that DCs prepared *ex vivo* from healthy volunteers, and in some instances from cancer patients, have the functional attributes (capable of priming *de novo* T cell responses) of DCs *in vivo* (see Banchereau and Steinman¹⁴ and Bhardwaj¹⁵ for reviews). The specific question of the competence of DCs obtained from prostate cancer patients has been raised in previous studies, yet no systematic screening of DC function in this patient cohort has been reported. Some data have suggested defects in the immune state in prostate cancer patients, and thus raised the question of whether they would be reasonable candidates for immune based anticancer vaccination.¹⁶ In one prostate cancer clinical study, poor functionality of patient DCs has been offered as an explanation for lack of responsiveness to a DC vaccination protocol.¹⁷

Here, we report that prostate cancer patient DCs are capable of generating MHC-peptide complexes from antigen cross-presented from internalized apoptotic prostate cancer cells. Specifically, we demonstrate the ability to reproducibly culture functional DCs derived from prostate cancer patients, monitor the ability of prostate cancer patient DCs to phagocytose and cross-present antigen derived from apoptotic prostate tumor cells, and utilize these DCs to stimulate antigen-specific autologous CD4⁺ and CD8⁺ T cell responses.

Methods

Isolation and preparation of cells

Peripheral blood mononuclear cells (PBMCs), DCs, and T cells were prepared as previously described. Briefly, peripheral blood was obtained from normal donors in heparinized syringes and PBMCs were isolated by sedimentation over Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ, USA). T-cell-enriched and T-cell-depleted fractions were prepared by rosetting with neuraminidase-treated sheep red blood cells. Immature dendritic cells (DCs) were prepared from the T-cell-depleted fraction by culturing cells in the presence of granulocyte and macrophage colony-stimulating factor (GM-CSF, Immunex, Seattle, WA, USA) and interleukin-4 (IL-4, R&D Systems, Minneapolis, MN, USA) for 6 days. Volumes of 1000 U/ml of GM-CSF and 500–1000 U/ml of IL-4 were added to the cultures on days 0, 2 and 4. To generate mature DCs, the cultures were transferred to fresh wells on day 6 and a mixture of 50 ng/ml tumor necrosis factor- α (TNF- α , Alexis Biochemicals, San Diego, CA, USA) and 0.1 μ M prostaglandin E-2 (PGE-2, Sigma Co., St. Louis, MO, USA) was added for an additional 36 h.¹⁹ At day 6, >95% of the cells were CD14⁻, CD83⁻, CD86^{lo}, HLA-DR^{lo} DCs. Upon maturation, on day 8, >90% of the cells were of the mature CD14⁺, CD83⁺, CD86^{hi}, HLA-DR^{hi} phenotype. CD4⁺ and CD8⁺ T cells were further purified to >99% purity by positive selection using the MACS column purification system (Miltenyi Biotech, Auburn, CA, USA).

Induction of apoptotic death

The mouse lymphoma cell line EL4 (ATCC #TIB-39), the human prostate carcinoma cell line LNCaP (ATCC #CRL-1740) and the human prostate carcinoma cell line PC-3 (ATCC #CRL-1435) were used as a source of apoptotic cells as they can all be efficiently infected with influenza virus. The LNCaP, EL4 and PC-3 cells were infected with influenza, incubated for 5 h to allow expression of influenza proteins and washed out of serum containing media. Apoptosis was triggered using a UV-B lamp (Derma Control, Inc., Old Forge, PA, USA) calibrated to provide 120 mJ/cm²/s. Infected and uninfected LNCaP, EL4 and PC-3 cells were harvested after UV-B irradiation at various time points and incubated with carboxyfluorescein-labeled fluoromethyl ketone (FMK)-peptide inhibitors of caspases for 1 h at 37°C (CaspaTag kit, Intergen, Purchase, NY, USA). Cells were then washed three times in PBS. A volume of 5 μ g/ml propidium iodide (PI) was added and samples were acquired and analyzed on a FACS Calibur flow cytometry machine.

In vitro and *in vivo* confirmation of replication incompetence of apoptotic cells

LNCaP and PC-3 cells were exposed to 120 mJ/cm² UV-B and incubated at 37°C for 10–12 h to allow for the initiation of apoptotic death. A total of 10⁶ UV-B-irradiated or live cells were pulsed with 4 μ Ci/ml ³H-thymidine and incubated at 37°C for 12–16 h. Cells were assayed for beta emission and counts per minute (cpm)

were measured. *In vivo* studies were performed using male nude mice (NU/J Foxn^{nu}) (Jackson Laboratories, Bar Harbor, ME, USA) injected subcutaneously in the right flank with either 10¹ live LNCaP or PC-3 cells or 10⁶ apoptotic LNCaP or PC-3 cells. Four mice were injected per group. Injections were prepared with an equal volume of Matrigel (Becton Dickinson, San Jose, CA, USA) to support tumor growth. Tumors were measured using calipers over 5 weeks and average tumor diameter is reported.

Phagocytosis of apoptotic cells

EL4, LNCaP and PC-3 cells were labeled using the red lipid-intercalating fluorescent dye PKH26-GL (Sigma Co., St. Louis, MO, USA), and induced to undergo apoptosis by UVB irradiation. After 10–12 h, allowing time for the cells to undergo apoptosis, they were cocultured with immature DCs that were dyed with the green fluorescent dye PKH67-GL (Sigma Co.), at a ratio of 1:1. FACS Calibur analysis was performed and double positive cells were enumerated as a measure of phagocytosis.²⁰

Allogeneic mixed leukocyte reaction

A total, 2 × 10⁵ purified allogeneic T cells were cultured with mature DCs to give T cell to DC ratios of 10:1, 30:1, 100:1, 300:1, 900:1, 2700:1 and 8100:1 in 96-well flat-bottomed plates for 5 days. At 5 days of incubation, cocultures were pulsed with 4 μCi/ml ³H-thymidine and harvested 16 h later to assay for T-cell proliferation and DC function. T cells were assayed for beta emission and c.p.m. were measured. Triplicate wells were averaged and means are reported. Error bars indicate two standard deviations.

Detection of antigen-specific T cells

ELISPOT assay for IFN-γ release Immature DCs, apoptotic cells and a DC maturation stimulus (TNF-α and PGE-2) were incubated together for 36 h to allow for phagocytosis of the apoptotic EL4 and PC-3 cells, antigen processing and DC maturation. The DCs were collected, counted and plated in wells containing purified T-cell populations precoated with 5 μg/ml of a primary anti-IFN-γ mAb (clone Mab 1-DIK) (Mabtech, Sweden). In all experiments, 2 × 10⁵ T cells were added to 6.6 × 10³ DCs to give a 30:1 T cell:DC ratio. CD40L was added to the CD8⁺ T cell and DC cocultures to bypass the requirement for CD4 help.⁹ The cultures were incubated in the plates for 40–44 h at 37°C. At that time, the ELISPOT plate was developed. Briefly, cells were washed out using mild detergent and the plates were incubated with 1 μg/ml biotin-conjugated IFN-γ mAb (clone Mab 7-B6-1) (Mabtech, Sweden). Wells were next stained using the Vectastain Elite Kit as per the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). Colored spots represented the IFN-γ-releasing cells and are reported as spot forming cells/10⁶ cells. Triplicate wells were averaged and means are reported. Error bars represent two standard deviations.

⁵¹Chromium release assay HLA-A2.1⁺ DCs were cocultured as described above in a 96-well plate with purified syngeneic CD8⁺ T cells and agonistic anti-CD40 antibody (Mabtech, clone SC26) for 7 days. The TAP^{-/-}, HLA-A2.1⁺, class II⁻ T2 cell line (ATCC #CRL-1992) was pulsed for 1 h with 1 μM of the immunodominant influenza matrix peptide, GILGFVFTL and 100 μCi of ⁵¹Cr. Percent cytotoxicity is calculated as compared to spontaneous release (% cytotoxicity = (experimental well ⁵¹Cr release – spontaneous release) / (total release – spontaneous release) × 100%). Specific lysis was determined by subtracting the percent lysis of unpulsed T2 cells. Influenza-infected DCs or matrix peptide pulsed DCs served as controls in all experiments in order to measure the donor's maximal CTL responsiveness to influenza. Background lysis ranged from 0 to 10% for the unpulsed T2 cells.

Results

Infection and generation of apoptotic prostate tumor cells

We tested the ability of the prostate cancer cell lines, LNCaP and PC-3, to package and deliver shared antigens to DCs as measured by effective apoptosis, phagocytosis and subsequent cross-presentation by dendritic cells. In order to develop an effective model system, which would allow us to track antigen cross-presentation and T-cell activation, we infected LNCaP and PC-3 cells with influenza virus and assessed their expression of the influenza M1 (matrix) antigen. FACS analysis revealed that 6 h after infection the M1 antigen could be detected in both LNCaP and PC-3 cells (Figure 1a).

We next confirmed that apoptotic death could be induced in prostate cancer cells following UV-B irradiation. Upon receiving a death stimulus, cells activate caspases and undergo apoptotic death as evident by single positive staining with CaspaTag, which detects activated caspase molecules (Intergen). PI, a fluorescent marker excluded by cells with an intact plasma membrane, intercalates into the DNA of necrotic cells that have lost membrane integrity. UV-B irradiation of 120 mJ/cm² was able to successfully induce apoptosis in LNCaP and PC-3 cells within 24–48 h (Figure 1b). Notably, secondary necrosis, as evident by PI labeling, did not occur under these conditions (Figure 1b) until after 44 h.

A proportion of UV-B-irradiated cells examined at 48 h were neither CaspaTag nor PI positive. For apoptotic tumor cells to be considered as a source of antigen in clinical trials, it would be important to confirm that UV-B-irradiation results in efficient cell killing. We first assessed whether UV-B irradiated cells were able to replicate *in vitro* by measuring ³H-thymidine incorporation at 48 h. Figure 1c demonstrates that no cell division was detectable in UV-B-irradiated LNCaP or PC-3 cells. To examine whether any cells remained viable 48 h after UV-B irradiation, we injected UV-B-irradiated LNCaP and PC-3 cells into nude mice and monitored tumor growth *in vivo*. While 10¹ live LNCaP or PC-3 cells injected into an immunocompromised mouse could both form solid tumors within 5 weeks, 10⁶ injected UV-B

irradiated LNCaP or PC-3 cells did not form any palpable mass even after 3 months of follow-up (Figure 1d). These data demonstrate that this UV-B irradiation protocol renders LNCaP and PC-3 cells replication incompetent, with a viability of less than one in 10^5 tumor cells.

Phagocytosis of apoptotic prostate tumor cells

We next evaluated whether apoptotic LNCaP and PC-3 cells could be effectively phagocytosed by DCs. Monocytes from normal volunteers were differentiated into immature DCs for 6 days and then labeled with a green vital dye. LNCaP and PC-3 cells were stained with a red vital dye prior to exposure to UV-B light. After coculturing immature DCs and apoptotic tumor cells, phagocytosis was measured by FACS analysis, in which green DCs were monitored for their ability to capture and phagocytose red apoptotic LNCaP or PC-3 material. After 4 h of coculture, more than 65% of DCs had phagocytosed the apoptotic LNCaP cells and 40% of DCs

had phagocytosed the apoptotic PC-3 cells (Figure 1e). To demonstrate that the method of uptake was indeed phagocytosis, cocultures were also incubated at 4°C and in EDTA, conditions which are both known to inhibit phagocytosis (Figure 1e). While there was an apparent delay in induction of early apoptotic death, phagocytosis was still effective suggesting that *in vitro* assays for measuring apoptotic death may be less efficient than a DC's ability to recognize early markers for programmed cell death.²¹

Apoptotic prostate tumor cells provide antigen for dendritic cell cross-presentation

We next examined whether apoptotic tumor cells could be processed by DCs to stimulate functional influenza-specific T cells as effectively as previously described cell lines. For these purposes, we developed an ELISPOT assay to examine antigen-specific IFN- γ T-cell responses. In these experiments, we compared the ability of apoptotic LNCaP and PC-3 cells to serve as a source of antigen as compared to apoptotic EL4 cells, a mouse thymoma cell line used as a positive control.⁹ As an additional control, DCs were directly infected with the influenza virus, allowing for antigen processing and presentation via the classical MHC I endogenous pathway. Apoptotic LNCaP and PC-3 were both able to transfer antigen to DCs for the stimulation of influenza specific T cells, and did so as effectively as EL4 cells (Figure 2). We conclude that the observed T-cell responses were influenza antigen-specific and not allogeneic responses to LNCaP or PC-3, as no significant T-cell stimulation was detected in cultures containing DCs presenting apoptotic tumor cells alone.

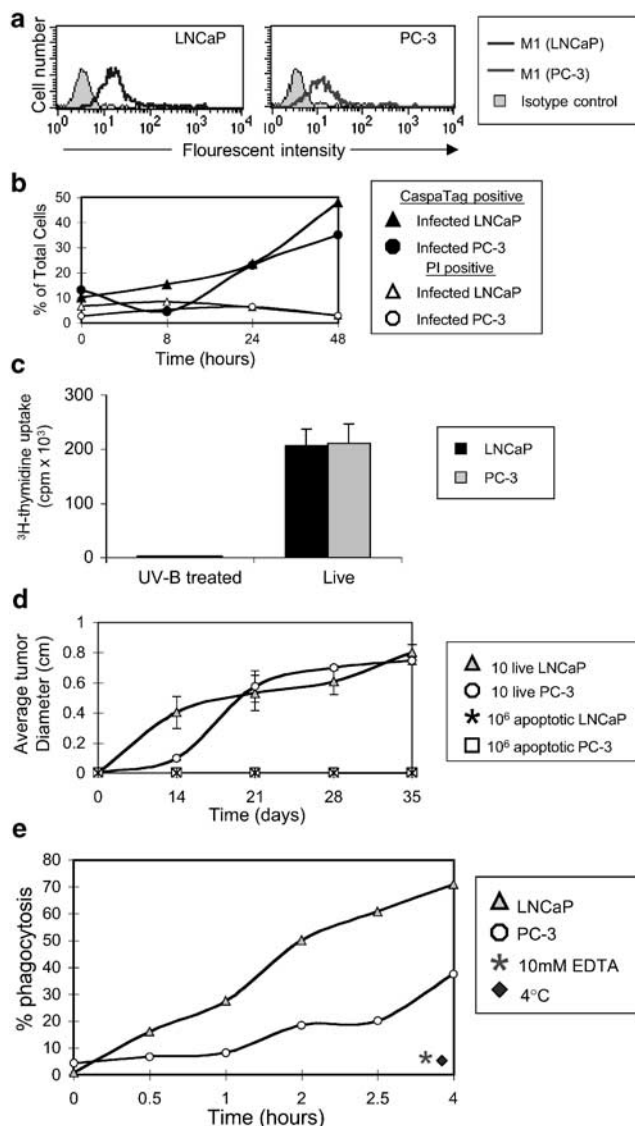


Figure 1 (a) Loading prostate lines with model antigen: influenza matrix peptide. LNCaP and PC-3 cells were washed out of serum containing media and infected with influenza virus (1:2). Successful infection is demonstrated via intracellular FACS analysis for viral antigens (eg M1). (b) Analysis of prostate cell apoptosis and necrosis. LNCaP and PC-3 cells were irradiated with UV-B and stained with CaspaTag fluorescent label and 5 $\mu\text{g}/\text{ml}$ PI as described in Methods. Cells were analyzed by FACS for CaspaTag (apoptotic) vs PI labeling (necrosis). Percent apoptosis over 48 h is reported. Data is indicative of more than 10 experiments. (c) UV-B irradiated LNCaP and PC-3 cells are replication incompetent *in vitro*. LNCaP and PC-3 cells were irradiated with 120 mJ/cm^2 UV-B and assessed at 48 h for ^3H -thymidine incorporation. c.p.m. for live, replicating cells vs apoptotic cells are shown. Triplicate wells were averaged and means are reported. Error bars indicate two standard deviations. (d) Apoptotic LNCaP and PC-3 cells are replication incompetent *in vivo*. Nude mice were injected subcutaneously in the right flank with either 10^7 live LNCaP or PC-3 or 10^6 UV-B irradiated LNCaP or PC-3. Injections were with an equal volume of Matrigel and average tumor diameter over 5 weeks is reported. Error bars indicate two standard deviations (four mice per group). (e) Apoptotic LNCaP cells are successfully phagocytosed by immature DCs. LNCaP and PC-3 cells were labeled with the red vital dye, PKH26 and then exposed to 120 mJ/cm^2 UV-B to induce apoptotic death. After 10–12 h, immature DCs were labeled green with PKH67 and cocultured with the apoptotic LNCaP or PC-3 cells at a ratio of 1:2. Phagocytosis was measured by FACS analysis and green DCs that acquired red apoptotic LNCaP or PC-3 were scored as positive. To confirm that the method of uptake was indeed phagocytosis, cocultures were also incubated at 4°C (diamond) and in EDTA (star), which are both known to inhibit phagocytosis.

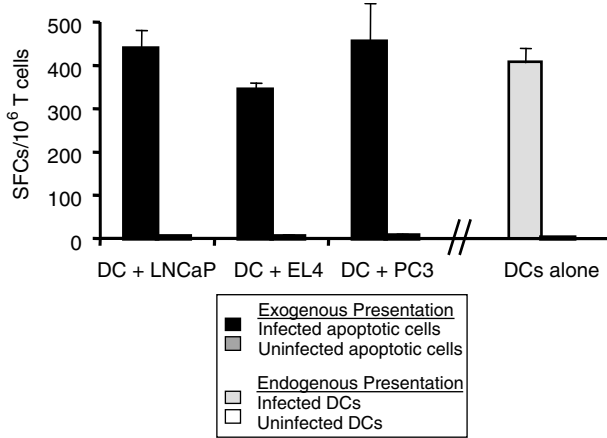


Figure 2 Apoptotic LNCaP transfer antigen to DCs for the activation of specific T cells. LNCaP, PC-3 and ELA cells were infected with influenza and incubated for 5 h to permit expression of viral proteins. Cells were then irradiated with UV-B and cultured for 10–12 hrs to allow apoptotic death to occur. Immature DCs were then cocultured with the apoptotic cells at a ratio of one DC:one apoptotic LNCaP or PC-3 cell or 5 EL4 cells in the presence of TNF- α and PGE-2. DCs were harvested at 36 h and plated with syngeneic bulk T cells (CD4⁺:CD8⁺ = 2:1) in an ELISPOT assay at a ratio of 30 T cells to 1 DC. Directly infected DCs, presenting antigen via the ‘classical’ endogenous MHC I presentation pathway served as a positive control for the generation of influenza-specific T cells. After 40–44 h, the ELISPOT plate was developed and the number of antigen-reactive T cells expressing IFN- γ were counted. In all experiments, uninfected EL4, LNCaP, PC-3 and DCs served as the negative controls for presentation of antigen via the exogenous and endogenous pathways, respectively. Experiments were performed in triplicate wells and mean spot-forming cells (SFCs) per million T cells are reported. Error bars represent two standard deviations. These data are representative of more than 20 experiments.

Prostate cancer patient DCs are functional and immunologically competent

It has been suggested that prostate cancer patients may be immunocompromised.¹⁶ To explore this issue, we examined whether it was possible to differentiate and mature dendritic cells from the peripheral blood monocyte precursors of stage IV prostate cancer patients. These studies were performed using autologous serum, allowing us to assess whether patient serum itself might harbor inhibitory factors. Peripheral blood from six stage IV prostate cancer patients (Table 1) was processed as described in Methods.¹⁸ Briefly, peripheral blood monocytes were obtained by peripheral blood draw or leukapheresis, grown for 6 days in the presence of GM-CSF and IL-4, and matured in the presence of PGE-2 and TNF- α . Data from four representative patients are shown (Figure 3a). In all six patients examined, FACS analyses of DCs revealed that they expressed CD86, CD83, and high levels of HLA-DR, and that they lacked expression of CD14, all of which were consistent with the phenotype of mature DCs. In addition, these cells exhibited a stellate morphology characteristic of mature DCs (data not shown).

We next evaluated whether prostate cancer patient DCs could stimulate allogeneic T cells in a functional assay. The allogeneic mixed-lymphocyte reaction (allo-MLR) assay provides a means for assessing the viability, maturity and function of dendritic cells. By measuring uptake of ³H-thymidine, it was possible to compare the

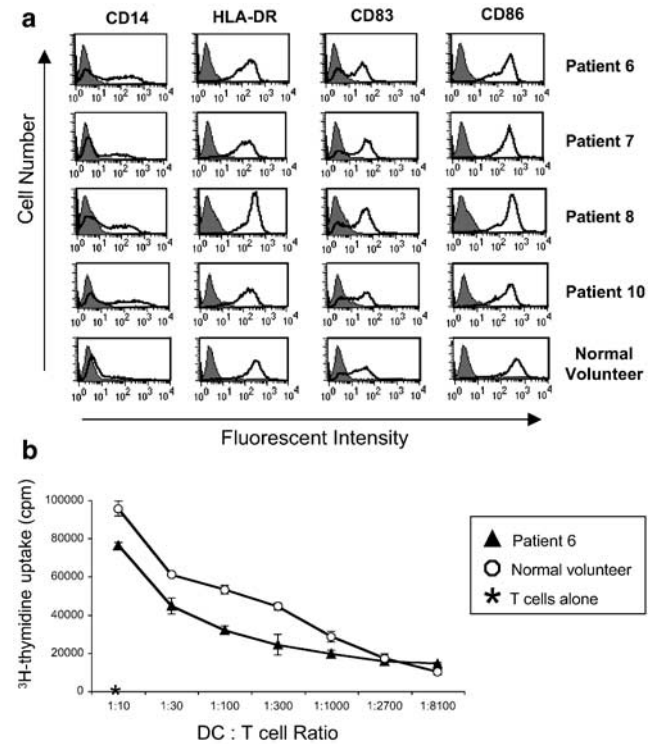


Figure 3 DCs derived from prostate cancer patients are phenotypically and functionally equivalent to normal individuals. (a) Peripheral blood derived monocytes were grown in 1% autologous plasma supplemented with GM-CSF and IL-4. On day 6, TNF- α and PGE-2 were added and after 2 days, cells were tested for maturation phenotype by FACS analysis. Mature DCs are defined as CD14⁻, CD83⁺ with high surface expression of CD86 and HLA-DR. (b) To confirm functional activity, allogeneic T cells were cocultured with mature DCs at DC to T cell ratios of 1:10, 1:30, 1:100, 1:300, 1:1000, 1:2700 and 1:8100. T-cell proliferation was assayed using ³H-thymidine uptake and c.p.m. are shown. Error bars indicate two standard deviations. Data are representative of more than six experiments.

ability of patient and normal volunteer DCs to stimulate T-cell proliferation. The results of these assays demonstrated that prostate cancer patient DCs were nearly as potent as normal volunteer DCs in their ability to present and stimulate T-cell division (Figure 3b); similar results were seen in six of six prostate cancer patients assayed (data not shown). A small difference in the stimulatory capacity of DCs observed (Figure 3b) may relate to differences in the population of precursor cells that were harvested (normal control cells were obtained from buffy coats, while prostate patient cells were obtained by leukapheresis). Taken together, these data confirm that functionally mature DCs can be generated from the peripheral blood of prostate cancer patients.

Prostate cancer patient DCs cross-present apoptotic antigen as efficiently as normal control DCs

To further examine the functionality of prostate cancer patient DCs, we assessed their ability to cross-present antigen derived from prostate cancer cells for the activation of syngeneic T cells. The ELISPOT assay was employed to measure antigen-specific IFN- γ responses of purified CD4⁺ and CD8⁺ T cells in order to evaluate

Table 1 Leukapheresates used in these studies and corresponding clinical histories

Designation	Patient 4	Patient 6	Patient 7	Patient 8	Patient 10	Patient 12
Age (y)	73	55	77	65	59	45
HLS-A2.1	Positive	Positive	Positive	Negative	Positive	Positive
Initial diagnosis	07/79	07/00	08/02	04/96	6/97	9/00
T stage	3	3	2	3c	4	3a
N stage	0	0	0	0	0	0
M stage	0	0	1b	0	0	0
Chemotherapy	Yes	Yes	Yes	No	Yes	No
Hormonal ablation	Yes	Yes	Yes	Yes	Yes	No
Immunotherapy	Yes	Yes	No	Yes	No	No
Radiation	Yes	No	No	Yes	No	Yes
Prostatectomy	No	9/15/96	No	6/23/92	No	10/4/00
Disease status	Clinical metastasis: castrate	Clinical metastasis	Clinical metastasis: castrate	Clinical metastasis	Clinical metastasis: castrate	No clinical metastasis

whether antigen was presented on both MHC class I and MHC class II molecules. DCs directly infected with the influenza virus (expressing MHC I peptide via the classical or endogenous pathway) and DCs that had phagocytosed infected apoptotic EL4 cells (presenting antigen via the cross-presentation or exogenous pathway) served as positive controls, while uninfected DCs and DCs that had phagocytosed uninfected cells served as negative controls. CD8⁺ T cells were incubated with cross-presenting DCs in the presence of agonistic CD40 antibody to substitute for CD4 T cell help.⁹ When apoptotic LNCaP or PC-3 cells infected with influenza were cross-presented by DCs, antigen specific CD8⁺ and CD4⁺ T cell responses were elicited (Figure 4a). Similar results were obtained in six out of six patients (Table 2). We conclude that prostate cancer patient DCs effectively cross-present apoptotic prostate cancer material for the generation of both CD4⁺ and CD8⁺ T cell responses.

Prostate cancer patient DCs can cross-present antigen for the generation of antigen-specific CTLs

Several patients studied were HLA-A2.1 positive, allowing us to evaluate the induction of HLA-restricted effector CTLs by cytotoxicity assays. DCs obtained from HLA-A2.1⁺ prostate cancer patients were cocultured with apoptotic influenza-infected LNCaP and PC-3 cells and these charged DCs were used to stimulate CD8⁺ T cells in the presence of agonistic anti-CD40 antibody. Influenza-infected DCs served as a positive control for classical MHC I endogenous presentation, while uninfected DCs and DCs that had phagocytosed uninfected cells served as respective negative controls. Cytotoxic T-cell activity was assessed by measuring ⁵¹Cr release from T2 cells pulsed with matrix peptide. CD8⁺ T cells stimulated by DCs cross-presenting influenza antigen from apoptotic prostate cancer cells were able to lyse T2 targets that had been pulsed with the HLA-restricted influenza matrix peptide in two out of three patients, but not able to lyse control T2 cells (Figure 5, Table 2). In controls in which PSMA peptides were used, no significant lysis over background was seen (data not shown). These data indicate that prostate cancer patient DCs effectively cross-present influenza antigen derived

from apoptotic prostate tumor cells for the generation of antigen-specific cytotoxic T cells.

Discussion

In previous studies, we have demonstrated that DCs obtained from normal volunteers or patients with paraneoplastic neurologic disorders are able to cross-present antigen for the activation of antigen-specific T-cell responses.²⁻⁴ In the current study, we have extended this work to show that DCs obtained from a general population of prostate cancer patients are able to process antigen derived from apoptotic prostate tumor cell lines for the activation of antigen-specific CD4⁺ and CD8⁺ T cell responses. These data provide a foundation for considering the use of apoptotic tumor cells in vaccination strategies to stimulate tumor immune responses in cancer patients.

Previous studies assessing the effectiveness of immunotherapy in prostate cancer, generally using defined prostate tumor peptides as a source of antigen, have met with mixed success.^{17,22,23} The current study provides the first effort to focus specifically on the ability of apoptotic prostate tumor cells to cross-present tumor antigens to prostate cancer patients autologous DCs. We have identified two prostate cancer cell lines, LNCaP and PC-3, that can be used to cross present antigen. These cells were infected with influenza virus (Figure 1a), establishing an internal positive control to monitor the efficacy of antigen cross-presentation from prostate tumor cells.

We have demonstrated that LNCaP and PC-3 can be rendered apoptotic through UV-B irradiation and can be effectively phagocytosed by functional immature dendritic cells (Figure 1b-d). This is significant in light of a number of reports in which necrotic tumor lysates have been compared with apoptotic tumor cells in preclinical and clinical studies. In these studies, tumor lysates have been prepared using various protocols (such as freeze-thaw), generating a population of poorly defined necrotic and possibly some apoptotic cells, and in some clinical studies, limited responses post vaccine to tumor lysates have been seen in some (eg 3/10 samples of patient peripheral blood lymphocytes;²⁴ see Bhardwaj¹⁵ for a review). Many, but not all, pre-clinical

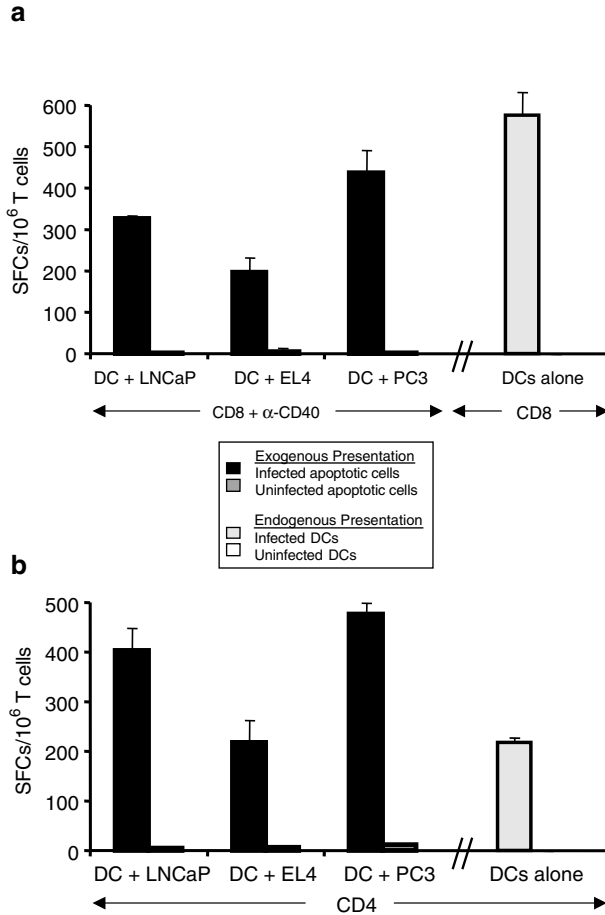


Figure 4 Apoptotic prostate cancer cells transferring antigen to prostate cancer patient DCs activate CD8⁺ and CD4⁺ T cells. EL4, LNCaP, and PC-3 cells were infected with influenza and incubated for 5 h to permit expression of viral proteins. Cells were then irradiated with UV-B and cultured for an additional 10–12 h to allow apoptotic death to occur. Immature DCs were then cocultured with the apoptotic cells at a ratio of one DC: one apoptotic LNCaP or PC-3 cell or 5 EL4 cells in the presence of TNF- α and PGE-2. DCs were harvested at 36 h and plated with highly purified syngeneic CD8⁺ T cells in addition to agonistic CD40 antibody (a) or purified syngeneic CD4⁺ T cells (b) in an ELISPOT assay. Directly infected DCs, presenting antigen via the ‘classical’ endogenous MHC I presentation pathway served as a positive control for the generation of influenza-specific T cells. After 40–44 h, the ELISPOT plate was developed as described in Methods and the number of antigen-reactive T cells expressing IFN- γ were enumerated. In all experiments, uninfected EL4, LNCaP, PC-3 and DCs served as the negative controls for presentation of antigen via the exogenous and endogenous pathways, respectively. Values are the averages of triplicate wells and mean SFCs per million T cells are reported. Error bars represent two standard deviations. ELISPOT data shown from prostate cancer patient 8 is representative of more than eight experiments with six different prostate cancer patients, one renal carcinoma patient and one mesothelioma patient.

studies suggest that apoptotic cells may be somewhat, and perhaps very significantly superior to necrotic tumor lysates in their ability to stimulate T cell responses^{2,25–28} (Darnell et al, unpublished data).

Several explanations may underlie the limited responses seen in tumor lysate-based vaccinations, and the potentially improved response to apoptotic tumor cells. The efficacy of DC cross-presentation to T cells depends critically on the state of the antigen to which the DC is exposed. Apoptotic cells are a natural and potent source

of antigen. Apoptotic cells are phagocytosed by $\alpha v\beta 5$ and CD36⁺ receptors on DCs,²⁰ and then processed and presented to T cells on both MHC class I and II molecules.^{2,3,5,29} The potency of DC cross-presentation of apoptotic cells to T cells depends critically on the ability to present antigen to both CD4⁺ and CD8⁺ T cells. In the absence of CD4⁺ help, DCs cross-presenting antigen tolerize rather than activate CD8⁺ T cells.^{9,30} Therefore, careful attention to the type of cell death induced in tumor cell vaccination, as well as the ability of antigen to activate both CD4⁺ and CD8⁺ T cells, may be of importance in generating reproducible and potent results.

We find that DCs obtained from six out of six prostate cancer patients can be differentiated from peripheral blood monocytes to have a mature surface phenotype as assessed by FACS, and are fully functional, as assessed by allo-MLR (Figure 3). Considering the severity of disease and the toxicity to the immune system of treatments administered in cancer patients, it has been uncertain whether cancer patient DCs are capable of stimulating T-cell mediated immunity. In a study where PSMA peptide-pulsed DCs were used to stimulate antigen-specific T cells in prostate cancer patients, only two of 82 men showed responses.³¹ It has been suggested that the low frequency of immune responses might have resulted in part from poor DC function.¹⁷ In other studies, only 25–35% of advanced stage prostate cancer patients were considered fully immunocompetent, as assessed by DTH responses to diphtheria/tetanus, streptokinase and mumps, and 33% of patients were found to be completely anergic.^{14,23,32} While multiple aspects of cell-mediated immune responses may be defective in prostate cancer patients, these observations underscore the significance of our ability to document the ability to obtain functional DCs from six out of six prostate cancer patients.

We also demonstrate that DCs derived from six out of six prostate cancer patients can phagocytose apoptotic LNCaP and PC-3 cells for the generation of both MHC I and MHC II/peptide complexes and the activation of CD4⁺ and CD8⁺ T cells, as assessed by ELISPOT assay (Figure 4). Moreover, these DCs are able to stimulate CD8⁺ memory T cells into antigen-specific effector CTLs capable of lysing peptide-loaded target cells (Figure 5). We demonstrate here that DCs derived from prostate cancer patients are indeed immunologically competent and are able to activate, and not tolerize, functional T-cell responses in six out of six prostate cancer patients. We conclude that DCs cross-presenting apoptotic tumor cells may form the basis of an effective immune-based clinical vaccination strategy in prostate cancer patients.

Optimizing DC-based clinical trials

DCs play a key role in the presentation of antigen to naive T-cells and the induction of primary immune responses (reviewed in Banchereau and Steinman,¹⁴) and clinical trials using DCs as cancer vaccines have shown promising results.³³ Many of the current DC-based clinical trials in progress take advantage of the few identified tumor-restricted antigens for which MHC I epitopes have been defined; pulsing these peptides onto DCs to elicit tumor-specific cytotoxic T-cell (CTL)

Table 2 Comprehensive ELISPOT and CTL data

(a) ELISPOT ^a				
SFCs/10 ⁶ T cells	DC+infected LNCaP	DC+infected EL4	DC+infected PC-3	Infected DCs
CD8 ⁺ αCD40	289 (38)	293 (35)	357 (40)	507 (45)
CD4 ⁺	274 (50)	277 (48)	439 (26)	193 (37)
(b) CTL ^b				
% Specific lysis	DC+infected LNCaP	DC+infected EL4	DC+infected PC-3	Infected DCs
Patient 6	38 (2)	32 (4)	68 (6)	72 (3)
Patient 7	7 (3)	10 (1)	18 (4)	3 (1)
Patient 10	17 (4)	62 (5)	37 (3)	40 (2)

CTL = cytotoxic T cell; SFCs = spot-forming cells; DC = dendritic cells.

^aELISPOT data from seven prostate cancer patients were averaged and SFCs/10⁶ CD8⁺ T cells or SFCs/10⁶ CD4⁺ T cells are shown. Data are representative of six separate experiments with six prostate cancer patients. Individual values were averages of triplicate wells and average standard deviations are shown in parentheses.

^bCytotoxicity assays were performed as described in Methods. CTL data from three separate prostate cancer patients are shown. Percentage specific lysis of T2 cells pulsed with matrix peptide is reported with standard deviations in parentheses.

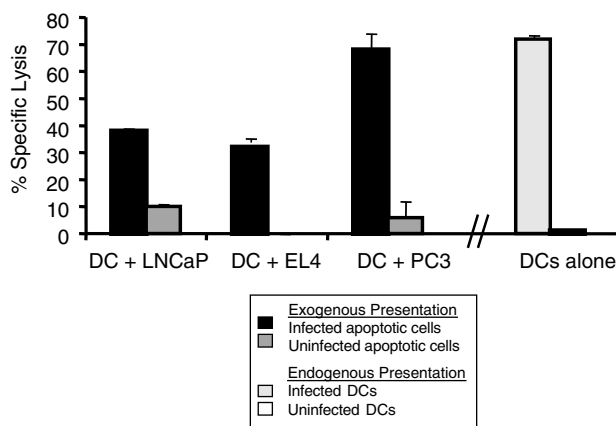


Figure 5 Apoptotic prostate cancer cells transferring antigen to prostate cancer patient DCs activate cytotoxic T cells. EL4, LNCaP, and PC-3 cells were infected with influenza and incubated for 5 h to permit expression of viral proteins. Cells were then irradiated with UV-B and cultured for an additional 10–12 h to allow apoptotic death to occur. Immature DCs were then cocultured with the apoptotic cells at a ratio of one DC:one apoptotic LNCaP or PC-3 cell or five EL4 cells in the presence of TNF-α and PGE-2. DCs were harvested at 36 h and plated with highly purified syngeneic CD8⁺ T cells and agonistic CD40 antibody. Cells were cultured for 7 days and then incubated with matrix peptide pulsed T2 cells or T2 cells alone for 5 h at an effector-to-target ratio of 30:1. Values are averages of triplicate wells and percent specific lysis is reported. Error bars are indicative of two standard deviations. Results of prostate cancer patient 6 are shown. Data are representative of six separate experiments with two prostate cancer patients and four normal volunteers.

responses.^{33,34} Numerous ongoing trials are underway in melanoma, myeloma and other cancers, in which patients are vaccinated with DCs that have been pulsed with the HLA-A2.1 epitopes of tumor-associated antigens, including MAGE-3, Melan A/MART-1 for melanoma, and, in prostate cancer, PSMA-1/2.^{14,33,35} While peptide-pulsed DCs have shown some clinical response in tumor immunotherapy trials, they entail a number of inherent disadvantages addressed by our results. DCs pulsed with peptides from antigens that are not critical for tumor growth may allow tumors to evade lysis by activated CD8⁺ T cells by downregulating expression of the targeted tumor antigen or presentation of the relevant epitope. Such escape has been documented in

melanoma patients treated with MAGE peptide-pulsed DCs, who have shown recurrence of disease with MAGE-negative tumors.^{36,37} Clearly, an attractive feature of our strategy to cross-presenting apoptotic tumor to DCs is its potential to immunize against a broad range of tumor antigens.

Although the use of peptides derived from multiple tumor antigens may help address the problem of tumor escape, it has also proven difficult to identify large numbers of useful tumor antigens. In addition, the use of peptide antigens is hindered by the high degree of MHC polymorphism. Indeed, peptide-DC vaccines are restricted to one HLA haplotype (generally HLA A2.1). This restricts the number of patients eligible for such vaccines, and underutilizes the potential breadth of the immune response to any one antigen. This is particularly in issue in prostate cancer, where African Americans are over-represented, but in whom there is a <20% incidence of the HLA A2.1 allele.³⁸ We have shown that we are able to cross-present apoptotic material derived from whole prostate tumor cells to DCs for the activation of autologous T cells. This offers the possibility of activating a broad spectrum of potentially tumor reactive T cells, allowing immunization without prior knowledge of specific tumor antigens, yet while presenting any one antigen on multiple HLA molecules.

An essential feature of studies utilizing DC-based immunotherapy will be to monitor that antigen cross-presentation indeed results in effective boosting of antigen-specific T cells. We have demonstrated that prostate tumor cells expressing model antigens, may effectively be utilized to provide such a positive control. The ability to follow responses to such a control antigen will allow for accurate conclusions regarding safety and efficacy, thereby providing an important addition to current tumor immunotherapy studies.

Another difficulty with the use of peptide vaccines is that HLA class I-restricted peptides are able to activate antigen-specific CD8⁺ T cells, but not CD4⁺ T cells. This is significant, as CD4⁺ T cells are important for the activation and maintenance of CD8⁺ effector memory cells.^{9,30,39,40} An observation of significant clinical importance to DC immunotherapy is that CD8⁺ T cells become tolerized rather than activated in the absence of such CD4⁺ T cell help.⁹ We show here that apoptotic

prostate tumor cells effectively cross-present antigen to activate both antigen-specific CD4⁺ and CD8⁺ T cells. These observations, taken together with our current results, suggest that the cross-presentation pathway outlined in this paper may prove to be of particular clinical value.

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References

- 1 Darnell RB. Onconeural antigens and the paraneoplastic neurologic disorders: at the intersection of cancer, immunity and the brain. *Proc Natl Acad Sci USA* (1996); **93**: 4529–4536.
- 2 Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* (1998); **392**: 86–89.
- 3 Albert ML et al. Tumor-specific killer cells in paraneoplastic cerebellar degeneration. *Nat Med* (1998); **4**: 1321–1324.
- 4 Albert ML, Austin LM, Darnell RB. Detection and treatment of activated T cells in the cerebrospinal fluid of patients with paraneoplastic cerebellar degeneration. *Ann Neurol* (2000); **47**: 9–17.
- 5 Inaba K et al. Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J Exp Med* (1998); **188**: 2163–2173.
- 6 Bevan MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* (1976); **143**: 1283–1288.
- 7 Albert ML et al. In: Gordon S (ed). *Uptake and presentation of phagocytosed antigens by dendritic cells, Phagocytosis and Pathogens*. JAI Press: Greenwich, CT, Philadelphia 1999; pp 363–378.
- 8 Albert ML, Darnell RB. Paraneoplastic neurologic degenerations: keys to tumour immunity. *Nat Cancer Rev* (2003); **4**: 36–44.
- 9 Albert ML, Jegathesan M, Darnell RB. Dendritic cell maturation is required for the cross-tolerization of CD8⁺ T cells. *Nat Immunol* (2001); **9**: 1–8.
- 10 Li M et al. Cell-associated ovalbumin is cross-presented much more efficiently than soluble ovalbumin *in vivo*. *J Immunol* (2001); **166**: 6099–6103.
- 11 Scardino PT, Weaver R, Hudson MA. Early detection of prostate cancer. *Hum Pathol* (1992); **23**: 211–222.
- 12 Agus DB et al. Prostate cancer cell cycle regulators: response to androgen withdrawal and development of androgen independence. *J Natl Cancer Inst* (1999); **91**: 1869–1876.
- 13 Narain V, Cher ML, Wood Jr DP. Prostate cancer diagnosis, staging and survival. *Cancer Metastasis Rev* (2002); **21**: 17–27.

- 14 Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* (1998); **392**: 245–252.
- 15 Bhardwaj N. Processing and presentation of antigens by dendritic cells: implications for vaccines. *Trends Mol Med* (2001); **7**: 388–394.
- 16 Tjoa B et al. Presentation of prostate tumor antigens by dendritic cells stimulates T-cell proliferation and cytotoxicity. *Prostate* (1996); **28**: 65–69.
- 17 Small EJ et al. Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells. *J Clin Oncol* (2000); **18**: 3894–3903.
- 18 Bender A et al. Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J Immunol Methods* (1996); **196**: 121–135.
- 19 Rieser C et al. Prostaglandin E2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: synergistic activation of interleukin 12 production. *J Exp Med* (1997); **186**: 1603–1608.
- 20 Albert ML et al. Immature dendritic cells phagocytose apoptotic cells via alpha5beta1 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J Exp Med* (1998); **188**: 1359–1368.
- 21 Savill J. Phagocyte recognition of apoptotic cells. *Biochem Soc Trans* (1996); **24**: 1065–1069.
- 22 Heiser A et al. Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors. *J Clin Invest* (2002); **109**: 409–417.
- 23 Lodge PA et al. Dendritic cell-based immunotherapy of prostate cancer: immune monitoring of a phase II clinical trial. *Cancer Res* (2000); **60**: 829–833.
- 24 Geiger JD et al. Vaccination of pediatric solid tumor patients with tumor lysate-pulsed dendritic cells can expand specific T cells and mediate tumor regression. *Cancer Res* (2001); **61**: 8513–8519.
- 25 Yang R, Xu D, Zhang A, Gruber A. Immature dendritic cells kill ovarian carcinoma cells by a FAS/FASL pathway, enabling them to sensitize tumor-specific CTLs. *Int J Cancer* (2001); **94**: 407–413.
- 26 Kotera Y, Shimizu K, Mule JJ. Comparative analysis of necrotic and apoptotic tumor cells as a source of antigen(s) in dendritic cell-based immunization. *Cancer Res* (2001); **61**: 8105–8109.
- 27 Fonteneau JF et al. Characterization of the MHC class I crosspresentation pathway for cell associated antigens by human dendritic cells. *Blood* (2003); **102**: 4448–4455.
- 28 Scheffer SR et al. Apoptotic, but not necrotic, tumor cell vaccines induce a potent immune response *in vivo*. *Int J Cancer* (2003); **103**: 205–211.
- 29 Albert ML, Bhardwaj N. Resurrecting the dead: dendritic cells acquire antigen from apoptotic cells. *Immunologist* (1998); **6**: 194–198.
- 30 Bennett SR et al. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* (1998); **393**: 478–480.
- 31 Salgaller ML et al. Report of immune monitoring of prostate cancer patients undergoing T-cell therapy using dendritic cells pulsed with HLA-A2-specific peptides from prostate-specific membrane antigen (PSMA). *Prostate* (1998); **35**: 144–151.
- 32 Murphy G et al. Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. *Prostate* (1996); **29**: 371–380.
- 33 Nestle FO et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* (1998); **4**: 328–332.
- 34 Rosenberg SA et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med* (1998); **4**: 321–327, (see comments).
- 35 Schadendorf D, Nestle FO. Autologous dendritic cells for treatment of advanced cancer—an update. *Recent Results Cancer Res* (2001); **158**: 236–248.

- 36 Yee C *et al.* Melanocyte destruction after antigen-specific immunotherapy of melanoma: direct evidence of t cell-mediated vitiligo. *J Exp Med* (2000); **192**: 1637–1644.
- 37 Ohnmacht GA *et al.* Short-term kinetics of tumor antigen expression in response to vaccination. *J Immunol* (2001); **167**: 1809–1820.
- 38 Dawson DV *et al.* Ramifications of HLA class I polymorphism and population genetics for vaccine development. *Genet Epidemiol* (2001); **20**: 87–106.
- 39 Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* (1998); **393**: 474–478.
- 40 Schoenberger SP *et al.* T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* (1998); **393**: 480–483.