Survivin and PSMA Loaded Dendritic Cell Vaccine for the Treatment of Prostate Cancer

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Dendritic cell (DC)-based vaccines are a promising therapeutic modality for cancer. Results from recent trials and approval of the first DC vaccine by the U.S. Food and Drugs Administration for prostate cancer have paved the way for DC-based vaccines. A total of 21 hormone refractory prostate cancer (HRPC) patients with a life expectancy >3 months were randomised into two groups. DC loaded with recombinant Prostate Specific Membrane Antigen (rPSMA) and recombinant Survivin (rSurvivin) peptides was administered as a subcutaneous (s.c.) injection (5×10⁶ cells). Docetaxel (75 mg/m² intravenous (i.v.)) and prednisone (5 mg, *bis in die (b.i.d.)*) served as control. Clinical and immunological responses were evaluated. Primary endpoints were safety and feasibility; secondary endpoint was overall survival. Responses were evaluated on day 15, day 30, day 60, and day 90. DC vaccination was well tolerated with no signs of grade 2 toxicity. DC vaccination induced delayed-type hypersensitivity reactivity and an immune response in all patients. Objective Response Rate (ORR) by Response Evaluation Criteria in Solid Tumours (RECIST) was 72.7% (8/11) versus 45.4 (5/11) in the docetaxel arm and immune related response criteria (irRC) was 54.5% (6/11) compared with 27.2% (3/11) in the control arm. The DC arm showed stable disease (SD) in 6 patients, progressive disease (PD) in 3 patients, and partial remission (PR) in two patients compared to SD in 5 patients, PD in 6 patients, and PR in none in the docetaxel arm. There was a cellular response, disease stabilization, no adverse events, and partial remission with the rPSMA and rSurvivin primed DC vaccine.

Key words dendritic cell vaccine; prostate cancer; autologous cellular immunotherapy; prostate specific membrane antigen; Survivin

Immunotherapy has recently emerged as a promising treatment modality for management of advanced hormone-refractory prostate cancer (HRPC), in light of new advances in discovery of cancer-specific antigens (tumor-associated antigen (TAA)), and adoptive cellular targeting. Dendritic cell (DC) based adoptive immunotherapy is one such treatment. Dendritic cells are the professional antigen presenting cells that efficiently activate T lymphocytes by presenting antigen to naïve dritic cells are the professional antigen presenting cells that efficiently activate T lymphocytes by presenting antigen to naïve

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and prednisone treatment regimen.

MATERIALS AND METHODS

Patient Population and Study Design A total of 21 patients were enrolled in this randomised, open-label clinical study. Patients were randomised into two study groups. Treatment group consisting of autologous dendritic cell vaccine (rPSMA+rSurvivin) \((n=11)\) and control group consisted of patients receiving docetaxel plus prednisone \((n=11)\). Only patients with HLA-A*0201 allele, minimum expected live span of at least 3 months, a Karnovsky index >60% and serum prostate specific antigen (PSA) between 10–100 ng/mL after radical prostatectomy for T1–T3, N0, M0 prostate adenocarcinoma were included in the study. Gleason score and immuno-histochemistry characterization were determined from prostatectomy specimens. The increase in PSA had to be documented over four consecutive determinations. Bone and pelvic-abdominal scans were performed before inclusion in the study to exclude the presence of any detectable but asymptomatic metastases. Patients received 5 doses of \(5 \times 10^6\) DCs pulsed with combination of rPSMA and rSurvivin (ratio 1 : 1), in each dose and administered as a subcutaneous injection (see study scheme; Fig. 1). The first three doses were administered at a gap of 15 d and last two doses were administered at a gap of 30 d. Control group patients received docetaxel (75 mg/m\(^2\) intravenously over 1 h every 21 d [one cycle]) and prednisone \(5\) mg \(b.i.d\) on days 1–21. Primary end points were feasibility and safety of vaccination with peptide loaded DC and detection of cellular immune responses against the vaccine antigens. Secondary endpoints were overall survival (OS) and objective response rate (ORR). Patients were to be withdrawn from the study in case of unacceptable toxicity, cancer progression suggested by symptoms and confirmed by complementary investigations, or patient’s refusal. The study protocol was approved by the ethics committee of the First Affiliated Hospital of Nanchang University, Jiangxi, China and written informed consent was obtained from all patients before the start of the study. The study was conducted according to the International Conference on Harmonisation (ICH) Good Clinical Practices (GCP) and in compliance with the Declaration of Helsinki 1975 and subsequent amendments.

Generation of Monocyte-Derived DCs The DC vaccines were generated as previously described by Sabado et al.\(^{30}\) Briefly, 1–3\( \times 10^{10}\) peripheral blood mononuclear cells (PBMCs) were collected by a 3 h leukapheresis procedure from each patient, and then placed in a tissue culture flask adherence at a density of \(1 \times 10^6\) cells/cm\(^2\) in the presence of 1% human serum albumin (Baxter, Deerfield, U.S.A.). After 2 h of incubation at 37°C in 5% CO\(_2\), non-adherent cells were removed by rinsing with sterile phosphate buffered saline (PBS). Adherent cells were then resuspended in clinical grade Cell-Gro DC medium (CellGenix, Freiburg, Germany) containing 1000 U/mL GM-CSF (CellGenix, Freiburg, Germany), 50 ng/mL IL-4 (CellGenix, Freiburg, Germany) and were incubated for 5 d. On day 5, DCs were split into 2 aliquots, each aliquot containing rPSMA and rSurvivin peptides respectively. The TAA peptides were separately added at a concentration of 10 µg/mL to an aliquot in 10 mL PBS each for 2 h at 37°C, and then transferred to a single vial. To induce DC maturation, soluble Poly I:C (In-vivoGen, San Diego, U.S.A.) was added in the culture from day 5 to day 7. Afterwards, DCs were washed twice and resuspended in 1 mL PBS. Mature DC (MODC) were characterized for morphology and immune-phenotyping for CD8, CD83, CD86 and CD1a, and tested for sterility. The final formulation contained rPSMA and rSurvivin primed DC in the ratio of 1:1 and total cell concentration of \(5 \times 10^6\) DCs in each dose, all five doses were prepared at a time and were frozen using automated cryopreservation. Microbiologic and endotoxin tests of DCs were performed as per U.S. Pharmacopoeia (USP) before use.

Generation of Recombinant Proteins HLA-A*0201-
binding peptides derived from prostate cancer associated antigens (Table 1) were synthesized and purified (>97% purity) by Jerini Biotools (Berlin, Germany) and obtained as a lyophilized powder. Working solutions were prepared by dissolving 0.6 mg of each peptide first in 30 mL dimethyl sulfoxide (DMSO; WAK Chemie, Bad Soden, Germany) and by subsequently adding 270 mL sterile water, resulting in a final concentration of 2 mg/mL.

**In Vitro Characterisation** Each vial of dendritic cell vaccine was subjected to sterility testing and characterisation for the expression of CD80, CD83, CD86, CD205, CCR7 and HLA-DR. Mycoplasma contamination was checked with the use of a MycoAlert-Mycoplasma detection kit (Lonza, Allendale, U.S.A.). Endotoxin was assessed by means of the kinetic chromogenic limulus amoebocyte lysate test (Lonza).

DC Phenotype Evaluation The phenotype of monocytes, immature and mature DCs were determined by single or two-colour fluorescence analysis. Cells (3×10^6) were resuspended in 50 μL of buffer (PBS, 2% fetal calf serum (FCS), and 1% sodium azid) and incubated for 30 min at 4°C with 10 μL of appropriate fluorescein isothiocyanate or phycoerythrin-labeled mAbs. After incubation, the cells were washed twice and resuspended in 500 μL of assay buffer. The fluorescence was analyzed by a flow cytometer (FACS Calibur, BD Biosciences, U.S.A.). For each sample, 15000 events were acquired, and the percentage of positive cells was reported. Monoclonal antibodies specific for human CD80, CD83, CD86, CD205, CCR7 and HLA-DR (Immunotech, Vienna, Austria), as well as control immunoglobulin (IgG)1 and IgG2a (Benton Dickinson, U.S.A.) were used to characterize DCs.

**Intracellular Interferon Gamma Detection Assay** Intracellular staining for interferon gamma (IFN-γ) released by lymphocytes was performed as described by Kern et al. Briefly, 5×10^6 CD14-depleted peripheral mononuclear cells, obtained before vaccination (T0) and after the fourth vaccination (T4), were cocultured with 1×10^6 mature rPSMA+rSurvivin-pulsed MODCs for 18 h. Monensin (10 μmol; Sigma, Munich, Germany) was added during the last 3 h to block protein secretion. T0 and T4 cells, with and without exposure to rPSMA+rSurvivin, were used as controls. In a parallel set of experiments, 500 ng/mL of ionomycin (Sigma, Munich, Germany) and 50 ng/mL of PMA (Sigma) were added to the cell suspensions.

Table 1. Table Showing Sequence of Recombinant TAA Peptides

<table>
<thead>
<tr>
<th>Peptide code</th>
<th>Sequence</th>
<th>aa position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSMA 154–163</td>
<td>LLHETDSAV</td>
<td>4–12</td>
<td>4, 34</td>
</tr>
<tr>
<td>Survivin 95–104</td>
<td>ELTGEFLKL</td>
<td>95–104</td>
<td>20, 25</td>
</tr>
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</table>

Table 2. Table Showing Demographics of Patients in Each Arm

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>DC vaccine (n=11)</th>
<th>Docetaxel+Prednisone (n=11)</th>
<th>Total (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node only</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Bone only</td>
<td>7</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Bone and lymph node</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Median age, (range), years</td>
<td>66.7 (60–77)</td>
<td>68.8 (63–82)</td>
<td>67.7 (60–82)</td>
</tr>
<tr>
<td>Duration of disease (in months)</td>
<td>55.82</td>
<td>47.72</td>
<td>56.71</td>
</tr>
<tr>
<td>Mean Gleason’s score</td>
<td>7.27</td>
<td>8.3</td>
<td>7.78</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>8</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>ECOG status, n (%)</td>
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<tr>
<td>0</td>
<td>7</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
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</tr>
<tr>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>PSA (ng/mL)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mean</td>
<td>55.14</td>
<td>81.18</td>
<td>68.16</td>
</tr>
<tr>
<td>Range</td>
<td>11–120</td>
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<td>11–269</td>
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<tr>
<td>Lactate dehydrogenase</td>
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</tr>
<tr>
<td>Mean</td>
<td>383.5</td>
<td>297</td>
<td>340.25</td>
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<tr>
<td>Range</td>
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<td>Alkaline phosphatase</td>
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<td>Mean</td>
<td>134</td>
<td>168</td>
<td>151</td>
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<tr>
<td>Range</td>
<td>52–328</td>
<td>65–436</td>
<td>52–436</td>
</tr>
</tbody>
</table>
IgG1 antibodies were used as isotype controls. Samples were analyzed on use of a flow cytometer (FACS Calibur, Becton Dickinson, U.S.A.).

Ratio of CD4+ and CD8+ Cells  Analysis of CD4 and CD8 lymphocyte count was performed according to the procedure earlier described by Bapsy et al.32 Briefly 2–3 mL of peripheral blood was incubated with the following anti-human monoclonal antibodies: anti-CD3-PC5, anti-CD4-FITC, anti-CD8-PE and anti-CD16-FITC (Becton Dickinson). After immunofluorescent staining, the cells were fixed with 1% paraformaldehyde and were then analyzed by means of a FACSCalibur flow cytometer with the use of CellQuest-PRO software (Becton Dickinson). The acquisition and analysis gates were restricted to the lymphocyte gate as determined by their characteristic forward and side-scatter properties. Cell expressing CD markers were acquired and analyzed in the FL1 or FL2 logarithmic scale through the use of the set gates.

Delayed-Type Hypersensitivity Test  The delayed-type hypersensitivity (DTH) skin test was performed with rPSMA+rSurvivin pulsed DCs and unpulsed DCs. On first vaccination and 4th vaccination when appropriate, pulsed DCs were intra-dermally injected into the forearm. A positive skin reaction was defined by a \( > 1.5 \) cm erythema and induration of the skin 48 h after intra-dermal injection.

Response Evaluation After the first administration of the vaccine, patients were monitored for vital signs for 12h and for 4h following the second to fourth vaccination. Follow-up visits were scheduled as follows: For PSA, follow up on day 15, day 60 and day 90; for CD4:CD8 count follow up on day 30 and 90; for DTH follow up on day 0, day 60 and day 90; for immune response (IFN-\( \gamma \)) day 0, day 60 and day 90. For estimating overall survival patients were evaluated from the date of first dose to date of Death. Complete remission (CR) was defined as the lack of any tumour events while near complete remission (NCR) or partial remission referred to 75% and 50% decrease in tumour mass without a new metastatic lesion. Any favourable alteration in patient without any remission criteria was monitored and named as positive alteration (PA). Progressive disease (PD) was defined as any of the following parameters: a PSA rise of more than 50% on two oc-

![Fig. 2. Photomicrograph Showing Morphology of Mature Dendritic Cells on the 8d (40×)](image)

![Fig. 3. FACS Analysis Showing Expression of CD Markers in the Final Product (mDC); (A) CD80; (B) CD83; (C) CD86; (D) CD 205; (E) HLA-DR; (F) CCR7](image)
cations at least 1 week apart, a 25% increase of the diameter of a known metastatic lesion, evidence of new lesions, weight loss of more than 10%, or deterioration in general physical status. Stable disease (SD) was defined as change of the above parameters less than that required for PD. Toxicity was graded according to WHO criteria.

Statistical Evaluation Data were analyzed by paired Student’s t-test, and p values <0.05 were considered statistically significant. Pearson’s correlation was employed to test the correlation between two interval-scaled variables, while Spearman’s rank correlation was employed to test the correlation between two ordinal-scaled variables. The non-parametric Wilcoxon rank sum test was used to compare immunologic outcomes (e.g., CD4/CD8, IFN-γ) between clinical responders and non-responders. Kaplan–Meier analyses for overall survival were performed with GraphPad Prism software (GraphPad Software, San Diego, U.S.A.).

RESULTS

In accordance with the protocol, a total of 21 patients underwent randomisation and were enrolled in the study from February 2011 to November 2011, and were administered autologous DC vaccine. A total of 36 patients were screened for the study, out of which 21 qualified for the inclusion criteria and were randomised into two arms. Eleven patients were included in each arm (n=21) and hence formed intention to treat (ITT). Patient demographics are represented in Table 2. Median age was 67.7 years, and mean baseline PSA was 68.16 ng/mL (range 12–300) (Table 2), with the majority of patients having a Gleason score >7. All 11 patients in the DC vaccine arm could complete all five doses hence, were evaluated for safety and response evaluation. Patients in the control arm completed three cycles (21 d regimen) of docetaxel and prednisone treatment. On-going co-morbid conditions such as hypertension, diabetes, chronic obstructive pulmonary disease and coronary artery disease were controlled with medication.

Phenotype of DCs To determine quality control of the DC formulation, criteria proposed by Bravery et al.33 were followed concerning phenotype and purity. Light microscopy of 8th-d cultured cells showed predominantly mature DCs (Fig. 2). Cells were positive for CD80, CD83, CD86, CD205, CCR7 and HLA-DR (Fig. 3).
As a quality control criterion, a cell viability of 75% was considered as release criteria. Cells were free of microbial contamination and endotoxin.

**DTH Test** To determine Delayed Type Hypersensitivity (DTH) reactivity, rPSMA+rSurvivin-pulsed DCs were injected intradermally into the forearm. A positive DTH reaction was defined as an induration of more than 10 mm in diameter. All patients developed a positive DTH skin test after the first vaccination.

**In Vitro IFN-γ Assay** During the study, IFN-γ production in CD3+ lymphocytes was assessed by flow cytometry in patients from both arms on day 0 and day 60. After coculture with mature MODCs, the expression of IFN-γ in CD3+ cells, which were not stimulated with PMA or ionomycin, was significantly higher in cells obtained after the fourth vaccination (DC vaccine arm) than in cells obtained day 0 \( (p=0.044) \). The Mann–Whitney rank sum test showed that there was a significant increase in day 60 value of IFN-γ in DC vaccine arm as compared with docetaxel arm \( (p=0.048) \) (Fig. 4).

**CD4: CD8 Levels** There was an increasing trend in the mean CD4:CD8 values of day 30 and day 90 (Fig. 5); the Mann–Whitney rank sum test revealed that this increase was not statistically significant \( (p=0.150) \). In majority of the patients the basal values were found <2, which moved towards a value of two.

**Toxicity** DCs were administered as subcutaneous injection at thigh. In all, 55 infusions were administered and all were well tolerated, barring only one incident of temporary exanthema in one patient (Patient ID: 5VR), two days after the third vaccination. The exanthema spontaneously disappeared without additional treatment. At the site of DC injection, a small itching induration was observed in 36.6% (4/11) of patients. Adverse events (AE) were reported in 38.09% (8/21) of patients irrespective of causal relationship to treatment. All AEs were related to the progressive disease and not related to either of the treatment. Three severe adverse events (SAEs) were observed in the docetaxel arm and no SAE were observed in the DC vaccine arm. None of the patient died during the course of treatment (21 d for docetaxel arm and 90 d for DC vaccine arm). Overall DC vaccine was found to be safe and well tolerated with the majority of toxicities being grade 1 fever, chills, fatigue. Serum PSA levels in each patient is represented in Fig. 6. There was no statistically significant change in the PSA levels in either of the arms.

**Response Evaluation** Response rates were summarized as follows: ORR by Response Evaluation Criteria in Solid Tumors (RECIST) was 72.7% (8/11) versus 45.4 (5/11) in Docetaxel arm and immune related response criteria (irRC) was 54.5% (6/11) compared with 27.2% (3/11) in the control arm; 90% confidence interval for ORR was (18.1, 45.3) and (26.5, 51.2) by RECIST and irRC, respectively. Intervals above 0 indicated that ORR was estimated with sufficient precision.
Two patients in the DC vaccine arm showed partial remission (PR) versus none in the docetaxel arm. Stable disease was observed in 6 patients (54%) in the DC vaccine arm versus 5 patients (45%) in the docetaxel arm. Three patients in the DC vaccine arm (27%) showed progressive disease (PD) while 6 (54%) of patients in docetaxel arm showed progressive disease. Overall survival (OS) was measured from the date of randomization to date of death due to any cause. OS was estimated using the Kaplan–Meier survival analysis and represented in Fig. 7. The median OS improved by 11 months (41 months vs. 30 months for controls), an estimated hazard ratio (HR) of 0.56 (95% CI, 0.37–0.85), and stratified log-rank p=0.0051. Median time to HRQoL deterioration was longer in patients assigned to DC vaccine than in those assigned to Docetaxel plus prednisone as assessed by the FACT-P total score (26.3 months for controls [95% CI 11.1–14.0] vs. 7.9 months [7.4–10.6]; HR 0.78, 95% CI 0.66–0.92; p=0.003).

DISCUSSION

The current study has validated the concept of cellular immunotherapy using monocyte derived dendritic cells as a viable treatment option in HRPC. In none of the patient treatment related hematologic, hepatic, renal or neurological toxicity, or autoimmune disease was observed, which shows that autologous DC vaccine was safe. DC was found to meet the quality control specifications according to Sabado et al. The current study has demonstrated that the administration of DC vaccine leads to no major toxicity and is safe. Furthermore, the therapy was well tolerated and feasible to be performed on patients with HRPC. The study achieved its primary endpoint of feasibility and safety of vaccination with peptide loaded DC and detection of cellular immune responses against the vaccine antigens. A survival benefit of 11 months observed in DC vaccine arm and OS of 41 month is higher than reported earlier, sipuleucel-T, the only commercially available dendritic cell vaccine for the treatment of HRPC was found to show an OS improvement of 25.8 months versus 21.7 months in the placebo and no change in time to progression. In study by Thomas–Kasel et al., PSCA and PSA peptide-loaded dendritic cells for the treatment of patients with prostate cancer, was found to show an overall median survival of 13.4 month. Improved OS in the current study may also be attributable to the use of combination of TAA (PSMA and Survivin). Multi epitope strategy has also been earlier used by others in a study by Waeckerle-Men et al., the long-term DC vaccination was associated with an increase in PSA doubling time.

The mature dendritic cells derived from monocytes were found to be expressing CD80, CD83, CD86, CD205, CD209, CCR7 and HLA-DR surface markers consistent with the properties of antigen presenting cells and earlier studies. None of the treated patients receiving DC vaccine had a 50% PSA decrease compared with baseline; previous studies have reported reduced levels of PSA serum in limited cases. On the contrary there was an increase in day 15 and day 60 PSA values; however, a downward trend was observed on day 90. The initial surge in PSA may be attributed to the heightened immune response, observed in various other studies. The PSA values did not correlate with the response as SD and PR patients also showed surge in day 15 and day 60 values of PSA. All patients showed a positive DTH response against the TAA used for priming the DC. Activation of immune response by DC vaccine is also supported by the values of IFN-γ released by CD3 cells; other studies have failed show a statistically significant increase in the release of IFN-γ. The current DC vaccine makes use of PSMA and Survivin as the TAA used for generating a Th1 immune response. The use of purified and defined PSMA and Survivin peptides to prime DCs has already been used for DC based immunotherapy.

CONCLUSION

Therapeutic cancer vaccines have become a reality. The initial failures have increased our understanding of the immune anti tumour response and prompted the development of more potent vaccines and other immunotherapeutic agents that are considerably less toxic than chemotherapies or targeted therapies. Recent trials and approval of first DC vaccine by the U.S. have proven the concept that initiating an active immune response with a therapeutic cancer vaccine can have long-term clinical benefit for patients with cancer. The generally indolent progression of prostate cancer, may explain why vaccines have been more successful in prostate cancer than in other types of cancer. In this study, only patients with advanced tumour stage were treated; however, minimal residual disease may be the optimal clinical setting to apply DC immunotherapy. Several aspects of vaccine optimization, antigen preparation, and method of application are the foci of ongoing and forthcoming studies. If the initially promising results presented here can be confirmed by a larger patient population, then a DC vaccine based on combination of rPSMA and rSurvivin may become sought after option. Several questions related to vaccine manufacturing and quality control, immune monitoring, patient selection and vaccine delivery strategies needs to be addressed. Future studies to address known issues such as tumour escape mechanisms from immune surveillance, for example through down regulation of antigen and major histocompatibility complex (MHC) expression and secretion of immunosuppressive cytokines also needs to be addressed. The current study shows that DCs can be used for adoptive immunotherapy for treatment of patients with prostate cancer.

Conflict of Interest

The authors declare no conflict of interest.

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