Original Article

Dendritic Cell-Based Immunotherapy Induces Transient Clinical Response in Advanced Rat Fibrosarcoma – Comparison with Preventive Anti-tumour Vaccination

(dendritic cells / immunotherapy / cancer immunotherapy / chemotherapy)

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Abstract. In this study we present the models of preventive and therapeutic vaccination of sarcoma-bearing rats with dendritic cells that present tumour antigens from killed tumour cells. We present the characteristics of dendritic cell-based vaccine and its capacity to induce anti-tumour immune response both *in vitro* and *in vivo*. We show that preventive vaccination efficiently prevents tumour growth. On the other hand, vaccination of rats with established tumours did not lead to eradication of the tumours. Despite the induction of a vigorous immune response after administration of dendritic cell-based vaccine and transient decrease in tumour progression, tumours eventually resumed their growth and animals vaccinated with dendritic cells succumbed to cancer. In both settings, preventive and therapeutic, dendritic cell-based vaccination induced a vigorous tumour-specific T-cell response. These results argue for the timing of cancer immunotherapy to the stages of low tumour load. Immunotherapy initiated at the stage of minimal residual disease, after reduction of tumour load by other modalities, will have much better chance to offer a clinical benefit to cancer patients than the immunotherapy at the stage of metastatic disease.

Introduction

Combination of surgery, chemotherapy and radiotherapy greatly improved the prognosis of cancer patients. Despite that this approach results in significant reduction of tumour mass, a small population of precursor tumour cells or cancer stem cells often survives and subsequently gives rise to a new population of tumour cells that leads to relapse. Clearly, other treatment modalities should be sought in order to improve the clinical outcome. With recent rapid advances in the understanding of biology of the immune response and the importance of an anti-tumour immune response for a long-term prognosis of cancer, immunotherapy has emerged as another treatment modality with the potential to contribute to further improvements in the survival.

Among the approaches explored for the induction of the anti-tumour immune response, use of dendritic cells (DCs) is probably the most promising. DCs are considered to be the most efficient, "professional", antigen-presenting cells (APCs) that have the ability to prime naïve T cells and initiate primary immune responses (Banchereau et al., 1998). DCs exist in two functionally and phenotypically distinct stages, immature and mature. Immature DCs have high endocytic activity, specialize in antigen capture and processing, and reside in peripheral tissues. Immature DCs play a crucial role in the induction and maintenance of peripheral tolerance. Upon exposure to pathogen-derived products or innate pro-inflammatory signals, DCs lose their phagocytic activity and migrate to draining lymph nodes while becoming mature DCs. Mature DCs have high antigen-presenting capability and T-cell stimulatory capacity due to the expression of high levels of antigen-presenting, adhesion and co-stimulatory molecules as well as other DC-specific markers, such as CD83 and DC-LAMP (Spisek et al., 2004; Steinman et al., 2007).

Discovery of the key role of DCs in initiating anti-tumour response evoked intensive effort to design immunotherapeutic trials in which activated mature DCs presenting tumour antigens would induce specific anti-tumour response. Use of whole killed tumour cells as a source of tumour antigens is one of the most often tested strategies for several reasons. This approach does not require prior knowledge of potent tumour rejection

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Abbreviations: APC – antigen-presenting cell, DC – dendritic cell, PMBC – peripheral blood mononuclear cell.

antigens. Presentation of peptides derived from tumour cells on both MHC class I and class II leads to the induction of a broad immune response. Use of autologous tumour cells provides a full spectrum of tumour-specific antigens, including patient-specific antigens resulting from random mutations. Recent studies support the evidence that DCs loaded with apoptotic cells can induce strong antigen-specific immune response *in vitro* and *in vivo*. The ability of tumour antigen-pulsed mature DCs to elicit protective immunity against cancer has been reported in various human tumours such as malignant melanoma, leukaemia, renal cell carcinoma, prostate cancer, breast cancer and hepatic cancer (Berard et al., 2000; Spisek et al., 2002; Blattman et al., 2004). There is a large number of animal models where administration of tumour vaccine prevents tumour growth upon subsequent challenge with live tumour. However, clinical trials attempting to induce an effective immune response in patients with cancers have only had a limited success. Although administration of anti-tumour vaccines usually induced a detectable anti-tumour specific immune response, the impact on the tumour progression was limited (Rosenberg et al., 2004).

In this study we present models of preventive and therapeutic vaccination of sarcoma-bearing rats with dendritic cells that present tumour antigens. We present characteristics of the DC-based vaccine and its capacity to induce anti-tumour immune response both *in vitro* and *in vivo*. We show that the preventive vaccination efficiently prevents growth of the tumours. On the other hand, vaccination of rats with established tumours did not lead to eradication of the tumours. Despite the induction of a vigorous immune response after administration of DC-based vaccine and transient decrease in tumour progression, tumours eventually resumed their growth and animals vaccinated with DCs succumbed to cancer.

Material and Methods

Animals

Lewis rats (4–6 weeks old) were housed and bred according to the institutional guidelines.

Cell lines and culture media

K2 rat fibrosarcoma cells were cultured in MEM medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Cambrex, Charles City, IA). For cell cultures, RPMI+10% FCS was used. Cells were routinely checked for mycoplasma infection by Hoechst-33258 (Invitrogen, Carlsbad, CA) labelling.

Generation of rat dendritic cells

Monocyte-derived DCs were prepared as follows. Peripheral blood mononuclear cells (PBMCs) were purified on Ficoll-Hypaque gradient (Amersham Pharmacia Biotech, Uppsala, Sweden), lymphocytes were depleted by adhesion in culture for 4 h, and adherent cells were cultured for 5 days with 250 pg/ml of rat IL-4 and 10 ng/ml of rat GM-CSF (Masse et al., 2004). Immature DCs were co-cultured overnight with killed K2 cells in complete RPMI at a ratio of 1 : 1. To avoid adhesion of cells to plastic culture flasks, immature DCs were seeded on plates pre-coated with 10 mg/ml poly-2-hydroxyethyl methacrylate (Sigma, Lyon, France). For DC maturation, cells were cultured for 6 h with 25 μg/ml of Poly I:C (Sigma).

Quantification of p70 IL-12 production

DC supernatants were harvested 24 h after addition of Poly I:C and stored at –20 °C. Concentrations of IL-12 p70 were determined by ELISA (Invitrogen).

Flow cytometry experiments

For analysis of the DC phenotype, 2×10^5 cells were incubated with CD80/B7.1 and CD 86 mAbs and diluted in PBS/BSA 0.3% for 30 min at 4 °C. Antibodies were purchased from Pharmingen (San Diego, CA). Specific MAbs were detected using an antimouse IgG antibody conjugated to FITC (Immunotech, Marseille, France). After incubation, cells were rinsed, re-suspended in PBS and acquired in FACSCalibur. Data were analysed by FlowJo software (Treestar, Ashland, OR). For intracellular cytokine staining, splenocytes were re-stimulated *in vitro* with killed tumour cells in the presence of 5 μg/ml of brefeldin A (Sigma-Aldrich, Prague, Czech Republic) at 37 °C for 6 h. The cells were first stained with CD8 mAb, fixed, permeabilized with cytofix-cytoperm buffer (BD Biosciences, Franklin Lakes, NJ), and stained with PE-conjugated mAb to IFN-γ.

In vitro induction of tumour-specific T cells

K2 cells were detached with 0.05% trypsin (Lonza, Vierviers, Belgium), washed and killed by UV irradiation (312 nm for 10 min). Immature DCs (day 5) were fed tumour cells at a DC-tumour cell ratio of 1 : 1 for 6 h and engulfment of tumour cells was confirmed by confocal microscopy as described previously. Tumour cell-pulsed DCs were then stimulated by 25 μg/ml of Poly I:C for 6 h and used for stimulation of splenic T cells. K2-loaded mature DCs were added to T cells at a ratio of 1 : 10. IL-2 (25 IU/ml) was added on day 3 of culture. On day 7, T cells were re-stimulated by K2-loaded DCs and analysed for production of IFN-γ by intracellular staining as described previously (Spisek et al., 2007).

In vivo administration of DC-based vaccine, therapeutic and preventive tumour vaccination assays

For tumour protection experiments, 5×10^5 mature DCs pulsed with killed K2 cells were injected intravenously (i.v.). Seven days later, 2×10^6 live K2 cells were inoculated subcutaneously (s.c.). Tumour cell growth

was measured with calipers every other day. Rats were scored positive for tumour as soon as tumours became palpable and grew progressively.

For therapeutic vaccination assays, 2×10^6 live K2 tumour cells were injected subcutaneously in the right flank. Three weeks later when tumours became palpable and reached approx. the size of 180 mm^3, rats were immunized with 5×10^5 mature DCs loaded with killed K2 cells. DCs were injected s.c. in the vicinity of tumours. Tumour cell growth was measured with calipers. Rats were killed when tumour size exceeded 1500 mm^3. Five rats per experimental group were used in each experiment.

Immunomonitoring, detection of K2 fibrosarcoma-specific T cells induced by DC-based vaccination

Splenocytes were collected prior to vaccination and at various time points after administration of the DC-based vaccine. Frequency of K2 sarcoma-specific T cells was analysed by IFN-γ. ELISPOT (AID, Strassberg, Germany). Pre- and post-vaccination samples were thawed and 2×10^5 splenocytes seeded in 96-well ELISPOT plate with 2×10^4 K2 cell-pulsed or unpulsed mature DCs. After overnight incubation, the number of IFN-γ-producing T cells was analysed according to the manufacturer's instructions in ELISPOT reader (AID).

Statistical analysis

Each *in vitro* experiment was done at least three times. Results are expressed as means ± SD. For tumour protection experiments, Kaplan-Meier survival curves were constructed. Differences in mean tumour volume between groups of the *in vivo* experiment (two independent experiments) were compared using Mann-Whitney test.

Results

Generation of mature DCs loaded with killed K2 fibrosarcoma tumour cells

We first generated monocyte-derived immature DCs from PBCMs. Five days of culture in the presence of rat IL-4 and GM-CSF led to differentiation of a uniform population of cells with typical morphology of immature DCs (Fig. 1A). The capacity of immature DCs to be activated by pathogen-derived stimuli was tested by stimulation with bacterial LPS or by addition of a syn-

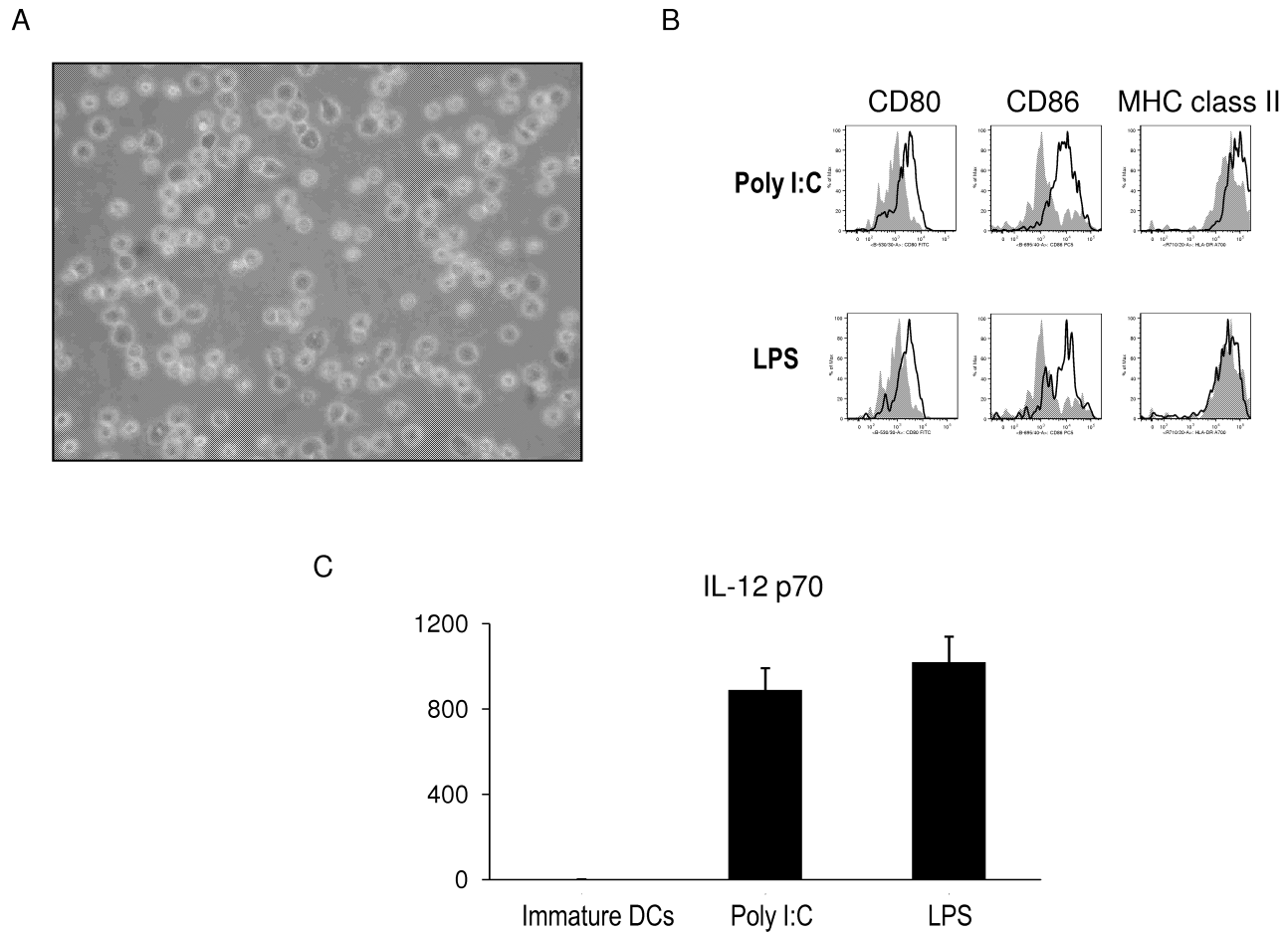

Fig. 1. Characteristics of dendritic cells used for anti-tumour vaccination. Immature DCs were generated in RPMI+10% FCS medium and activated by Poly I:C for 24 h. A – morphology of immature DCs; B – phenotype of immature DCs (grey) and DCs activated for 24 h by Poly I:C or LPS (black line); C – cytokine production by Poly I:C-activated DCs.

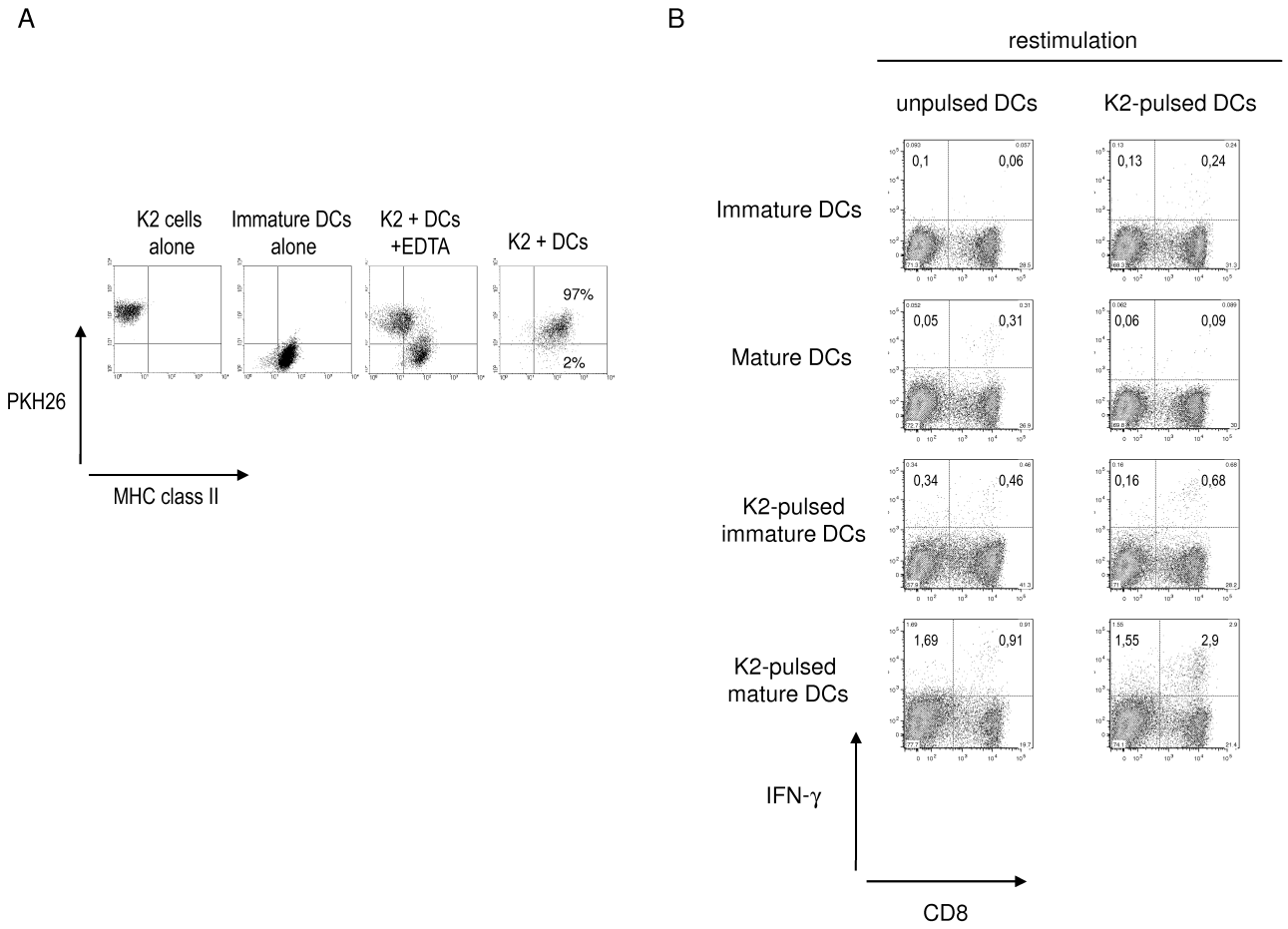

Fig. 2. DCs delivered to sarcoma-bearing rats captured killed tumour cells and induced tumour-specific T-cell responses *in vitro*. A – phagocytosis of killed K2 rat fibrosarcoma cells by immature DCs. Internalization of tumour cells was verified by flow cytometry. Killed apoptotic tumour cells were labelled by PKH-26 and co-cultured with immature DCs labelled with monoclonal antibody against HLA-II; B – *in vitro* expansion of K2 fibrosarcoma-specific T cells. Immature DCs were loaded with killed K2 cells, activated by Poly I:C and used as stimulators of autologous T cells. After 7 days of culture, T cells were re-stimulated and the frequency of K2-specific IFN-γ-producing T cells was analysed by intracellular flow cytometry.

thetic analogue of viral RNA, Poly I:C. Twenty-four-hour culture with these two potent activation signals induced significant up-regulation of maturation-associated co-stimulatory molecules (CD80, CD86) and molecules associated with antigen presentation, such as class II HLA molecules (Fig. 1B). DCs stimulated with Poly I:C and LPS also produced high quantities of Th1-polarizing cytokine IL-12 p70 (Fig. 1C).

For use in cancer immunotherapy, in addition to maturation, DCs have to present tumour antigens. In this study, we used killed tumour cells as a source of tumour antigens. We thus tested whether immature DCs can phagocytose killed K2 rat fibrosarcoma tumour cells. Immature DCs avidly captured K2 cells killed by UVA irradiation as documented by flow cytometry (Fig. 2A). Mature DCs loaded with killed K2 cells were then used as stimulators for the induction of tumour-specific T cells in the *in vitro* experiments. After two weeks of stimulation of autologous splenocytes with DCs loaded with killed K2 cells, we detected significant induction and expansion of tumour-specific lymphocytes, predominantly CD8 T cells. Immature DCs were inefficient in stimulation of tumour-specific T cells (Fig. 2B).

In vivo cancer immunotherapy in preventive and therapeutic settings

After showing the efficacy of mature DCs pulsed with killed tumour cells to induce tumour-specific T cells *in vitro*, we set to evaluate the effect of DC vaccination in two *in vivo* models. In the preventive vaccination model, rats were first vaccinated with DC-based vaccine and then challenged with live tumour cells two weeks later. In the therapeutic vaccination model, rats were vaccinated with DCs loaded with K2 cells three weeks after the challenge with tumour cells, at the time the tumours had already been established. As shown in Fig. 3A, rats vaccinated with mature DCs loaded with killed K2 cells in the preventive settings were completely protected against subsequent challenge with live tumor cells. Vaccination with unpulsed DCs or killed K2 tumour cells alone led to no protection (Fig. 3A). On the

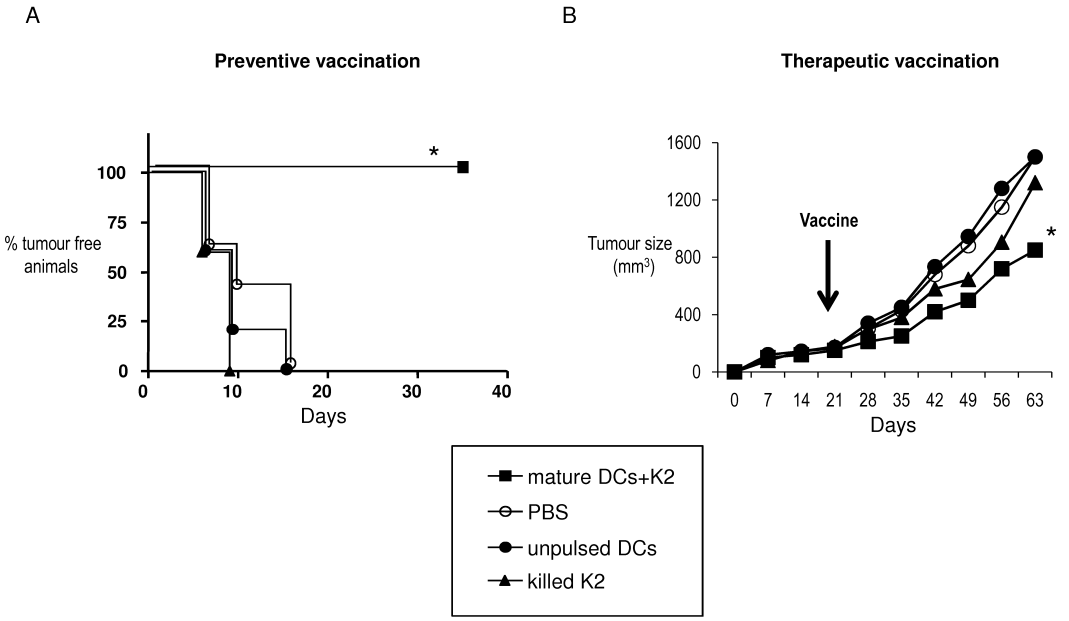

Fig. 3. Effect of vaccination with mature DCs loaded with killed K2 rat fibrosarcoma cells. A – for tumour protection experiments, 5×10^5 mature DCs pulsed with killed K2 cells were injected i.v. Seven days later, 2×10^6 live K2 cells were inoculated s.c. Tumour cell growth was measured with calipers every other day. Rats were scored positive for tumour as soon as the tumours became palpable and grew progressively. Kaplan-Meier survival curves were constructed and $P < 0.05$ was considered to be statistically significant,*; B – for therapeutic vaccination assays, 2×10^6 live K2 tumour cells were injected subcutaneously into the right flank. Three weeks later, when tumours became palpable and reached approx. the size of 180 mm^3, rats were immunized with 5×10^5 mature DCs loaded with killed K2 cells. DCs were injected s.c. in the vicinity of tumours. Tumour cell growth was measured with calipers. Rats were killed when tumour size exceeded 1500 mm^3. Five rats per experimental group were used in each experiment. *, $P < 0.05$.

other hand, administration of DC-based vaccine in the therapeutic settings did not induce tumour regression. Although tumour growth in rats vaccinated with mature DCs pulsed with killed K2 cells was significantly slower than in control rats (Fig. 3B), tumours grew progressively even in the vaccinated rats and these eventually succumbed to the tumour.

We analysed splenocytes of rats vaccinated with DC-based vaccine at various time points pre- and post-vaccination for the presence of K2-specific T cells by IFN-γ ELISPOT. Both preventive and therapeutic vaccination led to a significant increase in the frequency of K2-specific T cells (Fig. 4). We have not observed a significant difference in the frequency of tumour-specific T cells

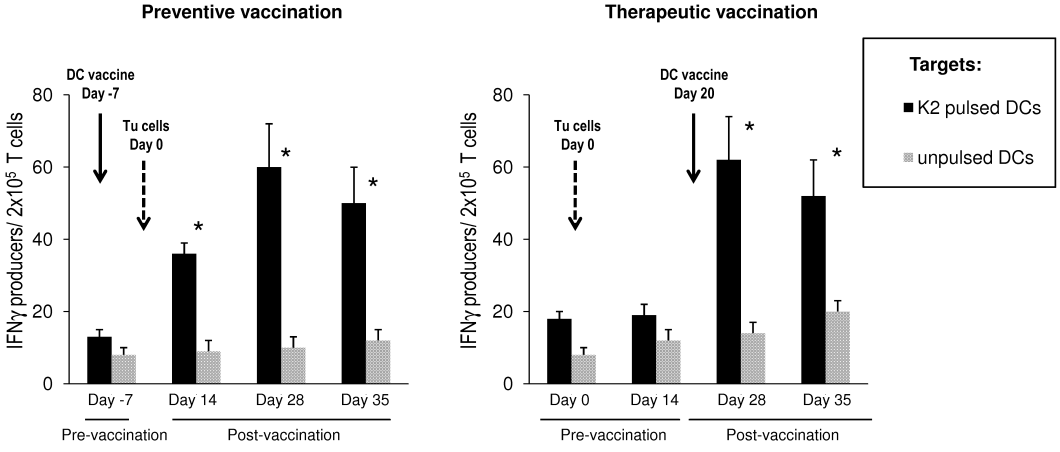

Fig. 4. In vivo expansion of tumour-reactive T cells after vaccination with DCs loaded with killed K2 cells in preventive (A) and therapeutic (B) DC vaccination. PBMCs were re-stimulated overnight *in vitro* with Poly I:C-matured DCs loaded with killed K2 cells. Frequency of IFN-γ-producing cells was quantified by an ELISPOT assay. Data shown are mean/SD of triplicates after subtraction of spots induced by unpulsed DCs. *P value for comparison with pre-vaccination samples, $P < 0.05$.

between the groups of preventive and therapeutic vaccination.

Discussion

Recent studies in mice and humans have convincingly documented that the immune system plays a crucial role in the control of tumorigenesis (Dunn et al., 2002). However, clinical trials attempting to induce an effective immune response in patients with cancers have only had a limited success. Although administration of anti-tumour vaccines usually induced a detectable anti-tumour specific immune response, the impact on the tumour progression was limited (Gilboa, 2004). Opponents of anti-tumour immunization often claim that the results of animal studies failed to translate in human settings as clinical trials with anti-tumour vaccines have been unsuccessful. This is, however, a big misconception. There is a fundamental difference between successful vaccines against cancer in animal models and clinical trials conducted in humans. Vaccines in animal models have been largely tried and proved effective in the preventive setting (preventive vaccination). In contrast, vaccination against cancer has generally been attempted in the context of therapy (therapeutic vaccination), in patients who already have advanced, often metastatic tumours and received extensive chemotherapy prior to cancer immunotherapy. In fact, the lack of therapeutic benefit in clinical trials is not surprising given the severe immunosuppression in these patients (Spisek, 2006; Spisek and Dhodapkar, 2006).

In our study, we tested the efficiency of DC-based vaccination in two different scenarios, in the model of preventive and therapeutic vaccination. We used a previously established protocol based on the use of mature DCs pulsed with killed tumour cells as a source of tumour antigens (Spisek et al., 2002; Tobiasova et al., 2007). We administered mature DCs in order to induce a specific anti-tumour T-cell response. In the preventive settings, anti-tumour vaccination prior to tumour challenge protected all animals from the tumour growth. On the other hand, vaccination initiated at the stage of advanced disease only slowed down the kinetics of tumour growth but failed to eradicate the tumour. In both models, DC vaccination induced robust tumour-specific T-cell response, as shown by ELISPOT.

A current view on the interaction between the immune system and transforming tumour cells has been formulated by G. P. Dunn, L. J. Old and R. D. Schreiber. They propose to distinguish three distinct stages in the process of cancer evolution: elimination, equilibrium and escape (Dunn et al., 2004a,b). This hypothesis has now been supported by strong experimental evidence (Koebel et al., 2007). Most often, a transformed tumour cell will be recognized and destroyed and the process will terminate in the elimination phase. In the equilibrium phase, the host immune system and any surviving tumour cell variants enter into the state of dynamic equilibrium. The enormous plasticity of cancer cell arising from increasing genetic instability may eventually give rise to new phenotypes that have reduced immunogenicity and evolved various mechanisms for the evasion from the control of the immune response. Tumours, for example, often induce expression or production of factors such as transforming growth factor beta (TGF-β) and IL-10, which may suppress or attenuate the anti-tumour immune response. Through production of these immunosuppresive factors, tumours may condition local DCs to induce suppressive T cells, such as $FoxP3^+$ Tregs and IL-13-producing $CD4^+$ T cells (Marincola et al., 2000; Ghiringhelli et al., 2005; Aspord et al., 2007).

The cancer immunoediting model predicts that patients in the equilibrium phase would benefit the most from cancer immunotherapy, while in patients with advanced cancer (in the escape stage) immunotherapy should not play a major role because the tumour has escaped from the control of the immune system. However, the majority of cancer patients are diagnosed at late stages of the disease and it is important to seek strategies for effective cancer immunotherapy in this cohort. The goal of immunotherapy in advanced cancer patients does not have to be the complete eradication of tumour cells, but rather a reversal from the escape phase back to the equilibrium stage. This would allow the immune response to keep the residual tumour cells in check and restore the balance between the host and the population of tumour cells. To reverse the balance back towards the equilibrium stage, tumour load should be reduced prior to the initiation of immunotherapy by other treatment modalities. Appropriate combination of tumour mass reduction (by surgery and/or chemo/radiotherapy) and neutralization of tumour-induced immunosuppression might set the right conditions for the induction of anti-tumour immune response by the active immunotherapy of choice. Simple removal of the tumour mass has been described to reverse tumour-induced immune tolerance in animals with metastatic breast cancer. Furthermore, certain chemotherapeutics, such as anthracyclins and bortezomib, have been shown to induce immunogenic cell death characterized by the translocation of heat-shock proteins from the endoplasmic reticulum to the plasma membrane (Obeid et al., 2007; Spisek and Dhodapkar, 2007; Spisek et al., 2007, Zitvogel et al., 2008). Pioneer studies also suggested that chemotherapy and immunotherapy can synergize, as prior immunotherapy sensitizes tumour cells to subsequent chemotherapy. Two clinical studies, in end-stage small cell lung cancer and glioblastoma multiforme in which DC vaccines were administered before salvage chemotherapy, have reported increased response rates to chemotherapy administered after DC vaccination (Yu et al., 2001; Wheeler et al., 2004; Antonia et al., 2006).

Taken together, implementation of modern cancer immunotherapeutic strategies into the management of cancer patients is highly desirable as there is now ample evidence that the immune system has the capacity to recognize and eliminate neoplastic cells. Based on the above-reviewed premises, immunotherapy should be

initiated in patients with tumour cell load reduced by other treatment modalities. It is conceivable that a rational combination of immunotherapy, surgery and chemotherapy could substantially improve the prognosis of cancer patients. Immunotherapy initiated at the stage of minimal residual disease will have much better chance to offer a clinical benefit to cancer patients than immunotherapy at the stage of metastatic disease. Researchers, immunologists and oncologists together need to make effort to design such combined therapeutic protocols including different treatment modalities and to determine the optimal time schedule.

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