

# Dendritic cell-based vaccination combined with gemcitabine increases survival in a murine pancreatic carcinoma model

C Bauer, F Bauernfeind, A Sterzik, M Orban, M Schnurr, H A Lehr, S Endres, A Eigler, M Dauer

*Gut* 2007;56:1275–1282. doi: 10.1136/gut.2006.108621

See end of article for authors' affiliations

Correspondence to:  
Dr Marc Dauer,  
Medizinische Klinik  
Innenstadt, University of  
Munich, Ziemssenstr. 1,  
80336 Munich, Germany;  
marc.dauer@med.  
uni-muenchen.de

Revised 20 March 2007  
Accepted 22 March 2007  
Published Online First  
29 March 2007

**Background:** Tumour-specific cytotoxic T lymphocytes (CTLs) can be activated *in vivo* by vaccination with dendritic cells (DCs). However, clinical responses to DC-based vaccination have only been observed in a minority of patients with solid cancer. Combination with other treatment modalities such as chemotherapy may overcome immunoresistance of cancer cells. It has been shown previously that gemcitabine sensitises human pancreatic carcinoma cells against CTL-mediated lysis. Here, a murine pancreatic carcinoma model was used to investigate whether combination with gemcitabine increases therapeutic efficacy of DC-based vaccination.

**Methods:** Bone marrow-derived DCs from C57BL/6 mice were loaded with UV-irradiated, syngeneic Panc02 carcinoma cells and were administered subcutaneously. For prophylactic vaccination, mice were vaccinated three times at weekly intervals prior to tumour challenge with Panc02 cells. Therapeutic vaccination was started when tumours formed a palpable nodule. Gemcitabine was administered intraperitoneally twice weekly.

**Results:** Prophylactic DC-based vaccination completely prevented subcutaneous and orthotopic tumour development and induced immunological memory as well as tumour antigen-specific CTLs. In the subcutaneous tumour model, therapeutic DC-based vaccination was equally effective as gemcitabine (14% vs 17% survival at day 58 after tumour challenge; controls, 0%). Combination of the two strategies significantly increased survival of tumour-bearing mice (50% at day 58 after tumour challenge). DC-based vaccination also prevented death from pulmonary metastatisation after intravenous injection of Panc02 cells.

**Conclusion:** DC-based immunotherapy may not only be successfully combined with gemcitabine for the treatment of advanced pancreatic carcinoma, but may also be effective in preventing local recurrence or metastatisation in tumour-free patients.

Pancreatic carcinoma represents the fifth leading cause of cancer death in the Western world. The 5-year overall survival rate is <5%.<sup>1</sup> Pancreaticoduodenectomy represents the only curative form of treatment. However, only 10–15% of patients diagnosed are eligible for surgical treatment. Chemotherapy with gemcitabine is currently the most effective treatment for advanced pancreatic carcinoma, resulting in a moderate increase in survival compared with 5-fluorouracil (5-FU) treatment.<sup>2,3</sup> Recent trials have failed to show an improvement in survival when gemcitabine is combined with other chemotherapeutic drugs.<sup>4</sup> Therefore, novel strategies are required for the treatment of advanced pancreatic cancer. Pancreatic carcinoma cells can be recognised by tumour-specific T cells.<sup>5,6</sup> Moreover, tumour infiltration with cytotoxic T lymphocytes (CTLs) and T helper cells represents a favourable prognostic factor for patients with pancreatic adenocarcinoma.<sup>7</sup> Tumour-reactive T cells capable of tumour rejection can also be isolated from blood of pancreatic carcinoma patients.<sup>8</sup>

Tumour-specific T cells can be activated by vaccination with dendritic cells (DCs).<sup>9,10</sup> DCs are specialised antigen-presenting cells with the unique capacity to establish primary immune responses.<sup>11</sup> DCs can deliver exogenous antigens into the major histocompatibility complex (MHC) class I processing pathway to activate CTLs, a process termed "cross-presentation".<sup>12</sup> Contact with microbial or inflammatory "danger signals" as well as T cell-derived activation signals induces DC maturation and interleukin (IL)12 secretion.<sup>13,14</sup> IL12 plays a central role in regulating tumour-directed immune responses and stimulates natural killer (NK) cells, CTLs and interferon (IFN) $\gamma$ -producing T helper type 1 (Th1) cells. Vaccination with tumour antigen-loaded DCs has been shown to elicit antitumoural CTL

responses *in vivo* and to induce tumour regression in cancer patients.<sup>9,15–17</sup> Pancreatic carcinoma cells can be rejected *in vivo* after vaccination with tumour antigen-loaded DCs.<sup>18,19</sup> However, tumours are capable of forming an immunosuppressive environment rendering them insensitive to CTLs. Combination with other treatment strategies such as radiation or chemotherapy may break the immunoresistance of cancer cells and enhance the therapeutic efficacy of DC-based vaccination.

Gemcitabine not only exerts direct antitumoural activity, but also mediates immunological effects relevant for tumour immunotherapy.<sup>20–22</sup> Antitumoural CTL responses can be induced by DCs cross-presenting antigens of tumour cells treated with a multidrug regime including gemcitabine.<sup>23</sup> Enhanced cross-presentation of tumour antigens by DCs after gemcitabine treatment also leads to increased tumour recognition by CTLs *in vivo*.<sup>24</sup> Recently, we were able to demonstrate that gemcitabine sensitises human pancreatic carcinoma cells to DC-induced tumour-specific CTL responses.<sup>25</sup> Here, we used a murine pancreatic carcinoma model to investigate whether combination with gemcitabine augments therapeutic efficacy of DC-based vaccination *in vivo*.

**Abbreviations:** CD, cluster of differentiation; CFSE, carboxyfluorescein diacetate-succinimidyl ester; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; 5-FU, 5-fluorouracil; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; MuLV, murine leukaemia virus; NK, natural killer; TGF, transforming growth factor; Th1, T helper type 1; TNF, tumour necrosis factor

## MATERIALS AND METHODS

### Mice, cell lines and media

Animal studies were approved by the Regierung von Oberbayern, Munich, Germany. C57BL/6 mice were from Harlan Winkelmann (Borchen, Germany). Transgenic OT-1 animals from the C57BL/6 background were provided by Professor Brocker (Department of Immunology, University of Munich). OT-1 T cells were grown in RPMI (Biochrome, Berlin, Germany) with 10% fetal calf serum (FCS; Gibco, Invitrogen Cooperation, Paisley, UK), penicillin and streptomycin. The H2-b-positive cell line Panc02 is derived from a methylcholanthrene-induced pancreatic adenocarcinoma in C57BL/6 mice.<sup>26, 27</sup> Panc02 cells were maintained in RPMI with 10% FCS, 2 mM L-glutamine (Life Technologies, Paisley, UK), 100 U/ml penicillin (Sigma, Munich, Germany) and 100 µg/ml streptomycin (Seromed, Jülich, Germany). Prior to co-incubation with DCs, Panc02 cells were irradiated using UV-B light with 0.75 J/cm<sup>2</sup>. The viability of UV-irradiated tumour cells was excluded by light microscopy and thymidine incorporation. The H2-Kb-positive lymphoma cell line EL4 (kindly provided by Professor Enders, Institut für Chirurgische Forschung, University of Munich) was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, penicillin and streptomycin.

### mRNA extraction and reverse transcription-PCR

Samples were cleaned in phosphate-buffered saline (PBS), snap-frozen in liquid nitrogen and stored at -70°C. Total RNA was isolated using the Roche Total RNA Tissue Extraction Kit (Roche, Mannheim, Germany) after homogenising tissue with an Ultra Turrax instrument (Janke und Kunkel, Staufen, Germany). RNA was stored with an equal volume of ethanol at -80°C. The yield and purity of the RNA were determined by spectroscopic analysis. For reverse transcription, M-MLV Reverse Transcriptase (Gibco Life Technologies, Paisley, UK), RNase Inhibitor (Roche, Mannheim, Germany), oligo(dT) primer for cDNA synthesis (Roche, Mannheim, Germany) and dNTP (Promega, Madison, WI, USA) were used. PCR was performed with *Taq* DNA polymerase as recommended by the supplier (Roche, Mannheim, Germany). Primers were manufactured by Applied Biosystems (Weiterstadt, Germany) based on sequences from the literature.<sup>28</sup>

### Monoclonal antibodies and flow cytometry

For phenotypic analysis, DCs were incubated for 30 min at 4°C with 5 µg/2×10<sup>5</sup> cells anti-mouse CD86-FITC monoclonal antibody (mAb; clone GL1), CD11b-PerCP mAb (clone M1/70), CD11c-APC mAb (clone HL3; all from BD Biosciences, San Jose, CA, USA), CD80-PE mAb (clone RMMP1, Caltag, Burlingame, CA, USA), MHC II-PE mAb (clone NIMR-4, Southern Biotech, Birmingham, AL, USA) and appropriate isotype controls. Cells were examined by flow cytometry (FACSCalibur, BD Biosciences, Heidelberg, Germany). Data were analysed using CellQuest software (BD Biosciences, Heidelberg, Germany). MHC class I expression was examined using anti-mouse mAb (H2-Kb, clone AF6-88.5; and H2-Db, clone KH95, both from BD Biosciences, San Jose, CA, USA).

### Peptides

The H2-Kb restricted peptides OVA<sub>257-264</sub>, TRP<sub>2181-188</sub> and p15E<sub>604-611</sub> were from Jerini Peptide Technologies (Berlin, Germany).<sup>29-31</sup>

### Reagents

Gemcitabine (Gemzar®) was from Lilly (Indianapolis, IN, USA). Midazolam (Roche, Basel, Switzerland), medetomidin (Orion Corporation, Espoo, Finland), fentanyl (Janssen-Cilag,

Neuss, Germany) and naloxon (Deltaselect, Dreieich, Germany) were used for anaesthesia.

### Cytokines, growth factors, stimulating agents and ELISAs

Granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN $\gamma$ , IL1 $\beta$ , IL2 and IL4 were from PeproTech (London, UK) and IFN $\alpha$  was from HyCult Biotechnology (Uden, The Netherlands). Lipopolysaccharide (LPS), p:IC, R848 and tumour necrosis factor $\alpha$  (TNF $\alpha$ ) were from Sigma-Aldrich (Steinheim, Germany). Murine ELISA kits for IL12 p70, IL12 p40 and IFN $\gamma$  were from BD Biosciences (San Diego, CA, USA).

### Generation and antigen loading of bone marrow-derived DCs

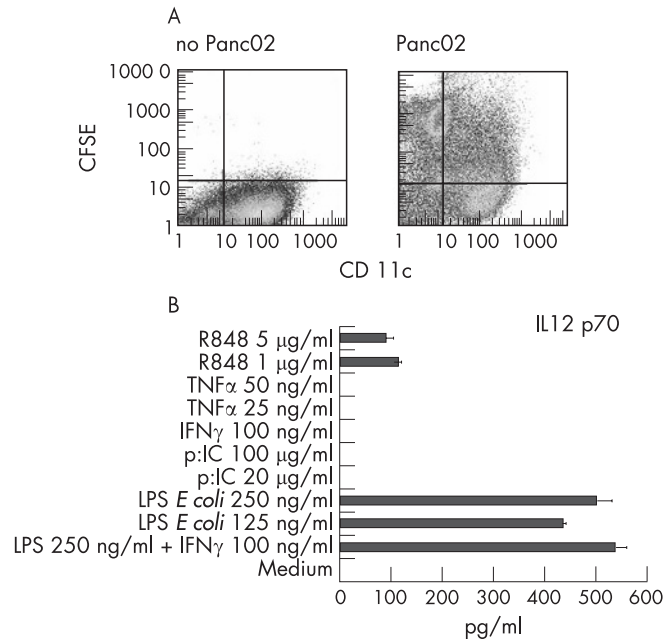
Bone marrow-derived DCs were prepared as described.<sup>32</sup> Bone marrow cells were harvested from murine femur and tibia. Erythrocytes were lysed with ammonium chloride buffer (BD Biosciences, Heidelberg, Germany). Cells were cultured at 5×10<sup>5</sup> cells/ml in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 µg/ml streptomycin, 1 IU/ml penicillin, 20 ng/ml GM-CSF and 20 ng/ml IL4 (DC medium). After 7 days, loosely adherent cells were harvested, and expression of DC markers was quantified by fluorescence-activated cell sorting (FACS). DCs represented 70% of the preparation. Co-incubation with Panc02 cells (referred to as "pulsing" in the following) was carried out at a DC/tumour cell ratio of 10:1. LPS (250 ng/ml) and IFN $\gamma$  (100 ng/ml) were added for the last 24 h. At day 9, loosely adherent cells were harvested, washed and resuspended in RPMI, and analysed by FACS.

### Tumour induction and vaccination strategies

For immunisation, 3×10<sup>5</sup> DCs were injected subcutaneously into the right flank in a final volume of 200 µl RPMI. Mice were challenged with Panc02 cells by contralateral subcutaneous injection (1×10<sup>6</sup> in 200 µl of RPMI). Therapeutic vaccination started after tumours had formed a palpable nodule. Gemcitabine was administered intraperitoneally twice weekly in a concentration of 25 or 50 mg/kg body weight. Tumour growth was expressed as the product of perpendicular diameters. Animals were killed when the tumour size exceeded 400 mm<sup>2</sup> or when there were signs of animal distress observed twice in 48 h. For orthotopic tumour development, the left flank of anaesthetised mice was opened and the spleen mobilised to access the pancreas. A total of 2×10<sup>5</sup> Panc02 cells were injected into the pancreas. Tumour growth was monitored by a LaTheta<sup>TM</sup> in vivo CT scanner for small animals (Zinsser Analytic). Animal CT scans were performed in cooperation with Dr Heinz-Peter Scheuber (TierschutzInformationsZentrum für Biomedical Research, University of Munich) and Adam Glowalla (Zinsser Analytic, Germany).

### In vivo cytotoxicity assay

The assay was performed by transferring carboxyfluorescein diacetate-succinimidyl ester (CFSE)<sup>high</sup> (2 µM)- and CFSE<sup>low</sup> (0.2 µM)-labelled C57BL/6 splenocytes into vaccinated or non-vaccinated mice. The CFSE<sup>high</sup>-labelled cells were pulsed with the p15E peptide (2 µg/ml), whereas the CFSE<sup>low</sup>-labelled cells were left unpulsed. A 1:1 ratio of CFSE<sup>low</sup>- to CFSE<sup>high</sup>-labelled cells was injected intravenously 20 h after injection of the target cells, spleens were removed and the ratio of CFSE<sup>low</sup> to CFSE<sup>high</sup> cells was determined by flow cytometry. Cytotoxicity was calculated by the formula: % cytotoxicity in a given mouse = ((% CFSE<sup>high</sup> vac/%CFSE<sup>low</sup> vac))/((% CFSE<sup>high</sup> control/% CFSE<sup>low</sup> control)).



**Figure 1** Bone marrow-derived dendritic cells (DCs) effectively take up tumour antigen and secrete high levels of interleukin (IL)12 p70 after stimulation with lipopolysaccharide (LPS) and interferon (IFN) $\gamma$ . DCs were derived from bone marrow of C57BL/6 mice by culture with granulocyte-macrophage colony-stimulating factor and IL4. On day 6 of culture, DCs were incubated with UV-irradiated, carboxyfluorescein diacetate-succinimidyl ester (CFSE)-labelled Panc02 cells at a ratio of 10:1. Uptake of tumour cells by CD11c<sup>+</sup> DCs was determined by fluorescence-activated cell sorting (FACS) analysis (A; the results of one representative FACS analysis out of five performed are shown). At day 6 of culture, DCs were stimulated for another 24 h with different stimuli. Culture supernatants were harvested and analysed for IL12 production by ELISA (B; data are the mean (SEM) of two independent experiments).

### In vitro cytotoxicity assay

The <sup>51</sup>chromium-release assay was performed in cooperation with Professor R Wank, Department of Immunology, University of Munich. Target cells were incubated with Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (100 µCi/10<sup>6</sup> target cells) at 37°C for 1 h. Cells (5 × 10<sup>3</sup>/well) were washed three times and co-cultured with effector cells in 96-well round-bottomed plates in a final volume of 200 µl. After 4 h of incubation at 37°C, 50 µl of supernatant were harvested and radioactivity determined by a gamma counter (Wallac Oy, Turku, Finland). Maximum release was assessed by incubation with Triton X-100 (Sigma, Munich, Germany) at a final concentration of 2.5%. Spontaneous release was determined in the absence of effector cells. Specific lysis was calculated according to the formula: specific <sup>51</sup>Cr release = ((experimental counts – spontaneous counts)/(maximal counts – spontaneous counts)) × 100%.

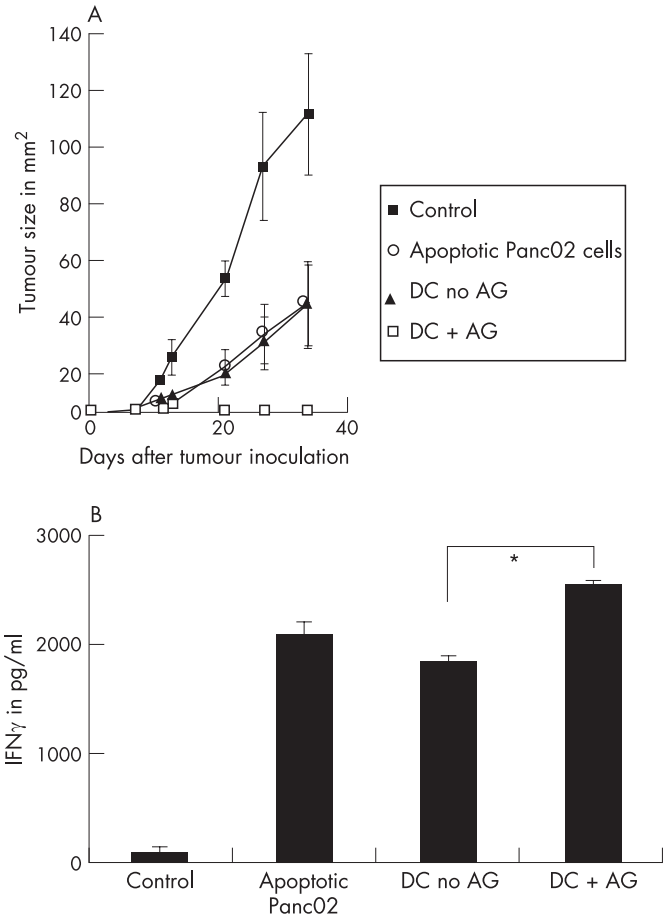
### Statistics

Student's t test was applied to reveal significant differences in tumour protection. We compared the mean tumour size of controls and all treated animals (tumour-free and tumour-positive animals). A value of p < 0.05 was accepted as the level of significance. Kaplan–Meier survival curves were analysed using the Cox proportional hazards model.

## RESULTS

### Vaccination with Panc02-pulsed DCs prevents subcutaneous tumour development

We have previously shown that DCs pulsed with UV-irradiated, apoptotic tumour cells are superior to tumour lysate-pulsed DCs in priming CTLs directed against human pancreatic cancer

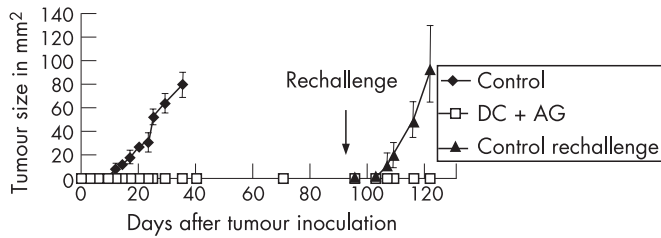


**Figure 2** Prophylactic vaccination with Panc02-pulsed dendritic cells (DCs) completely prevents development of subcutaneous Panc02 tumours. Female 6- to 10-week old C57BL/6 mice were vaccinated subcutaneously with either Panc02-pulsed or unpulsed syngeneic DCs (3 × 10<sup>5</sup>) or UV-irradiated Panc02 cells (1 × 10<sup>5</sup>) three times at weekly intervals, or were left untreated. Seven days after the last vaccination, mice were challenged subcutaneously with 1 × 10<sup>6</sup> Panc02 cells. Subcutaneous tumour growth was monitored at regular intervals and tumour size determined by multiplication of perpendicular diameters (A; data are the mean (SEM) of three animals in each group). In parallel experiments, mice from each group were sacrificed after two vaccinations, and in vitro interferon (IFN) $\gamma$  production of splenocytes determined by ELISA (B; data are the mean (SEM) of two animals in each group). AG, antigen.

cells.<sup>33</sup> This finding has recently been confirmed in the Panc02 tumour model.<sup>34</sup> Thus, we chose UV-irradiated Panc02 cells as a source of tumour antigen. Bone marrow-derived DCs were capable of taking up dead Panc02-cells and secreted IL12 p70 upon stimulation with LPS and IFN $\gamma$  (fig 1). Mice were completely protected from tumour development after three vaccinations with Panc02-pulsed DCs (fig 2A). In the untreated animals, tumours with a mean size of 110 mm<sup>2</sup> developed within 35 days (fig 2A). Tumour size was only moderately reduced in mice receiving UV-irradiated Panc02 cells only or unloaded DCs (mean tumour size after 35 days approximately 40 mm<sup>2</sup>). Tumour protection in mice vaccinated with Panc02-pulsed DCs was associated with higher levels of IFN $\gamma$  secretion in splenocytes isolated after two vaccinations (fig 2B).

### Vaccination with Panc02-pulsed DCs establishes immunological memory and long-term tumour protection

While control animals challenged with Panc02 cells died after 40–50 days, mice that had received prophylactic vaccination with Panc02-pulsed DCs survived more than 3 months without



**Figure 3** Prophylactic vaccination with Panc02-pulsed dendritic cells (DCs) induces immunological memory associated with long-term tumour protection. Female 6- to 10-week old C57BL/6 mice were either vaccinated subcutaneously with Panc02-pulsed DCs ( $3 \times 10^5$ ) three times at weekly intervals or left untreated and challenged subcutaneously with  $1 \times 10^6$  Panc02 cells 7 days after the last vaccination. Tumour size was determined at regular intervals by multiplication of perpendicular diameters. Mice receiving DC vaccination remained tumour free and were rechallenged subcutaneously with  $1 \times 10^6$  Panc02 tumour cells at day 95 after initial tumour inoculation. Previously untreated and unchallenged mice served as a control group (data are the mean (SEM) of three animals in each group). AG, antigen

developing a tumour (data not shown). These mice were rechallenged with Panc02 cells at day 95 after the first tumour challenge. As shown in fig 3, they were still protected from tumour development, as opposed to controls that rapidly developed large tumours (mean tumour size 91 mm<sup>2</sup> at day 26). Vaccinated mice that were rechallenged with Panc02 at day 95 showed long-term survival and were euthanised months later without signs of local or systemic tumour growth (data not shown).

### Vaccination with Panc02-pulsed DCs induces tumour-specific CTLs

Although mice could be protected from tumour development, it remained to be shown that DC vaccination induced a tumour-specific CTL response. The virus-associated murine leukaemia virus (MuLV) env-protein is encoded by the gp70 gene and selectively expressed by murine cancer cells. Figure 4A shows gp70 mRNA expression in Panc02 and C26 colon carcinoma cells derived from Balb/c mice, but not in normal tissue. CFSE<sup>high</sup>-labelled splenocytes from C57BL/6 mice loaded with p15E peptide derived from the MuLV env-protein and CFSE<sup>low</sup>-labelled, unloaded splenocytes were injected into the tail vein of mice either vaccinated three times with Panc02-pulsed DCs or left untreated. By quantifying the relative decrease of p15E-loaded, CFSE<sup>high</sup> splenocytes in vaccinated animals as compared with untreated animals, a specific lysis rate of approximately 12% could be calculated (fig 4B). Thus, Panc02-loaded DCs not only protect animals from tumour development, but also induce immunological memory and a tumour-specific CTL response.

### Panc02 cells are recognised and killed by tumour-specific CTLs

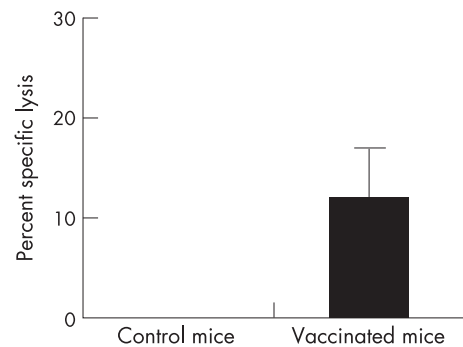
Panc02-derived tumours are poorly immunogenic, similar to human pancreatic adenocarcinoma. Moreover, Panc02 cells express only low levels of MHC class I required for classical CTL-mediated lysis (data not shown). Thus, we analysed whether Panc02 cells could be recognised *in vitro* by antigen-specific CTLs prior to their use in the therapeutic *in vivo* vaccination model. CTLs derived from transgenic OT-1 animals specific for the OVA-epitope SIINFEKL were used.<sup>35, 36</sup> Panc02 cells were loaded with SIINFEKL peptide or an irrelevant control peptide (TRP2) and co-cultured with splenocytes from either wild-type mice or OT-1 mice in a <sup>51</sup>Cr-release assay. EL4 cells loaded with SIINFEKL or an irrelevant peptide were used as control targets. Panc02 cells loaded with SIINFEKL were

A



Control Panc02 Liver Spleen Colon Pancreas LNN C26

B

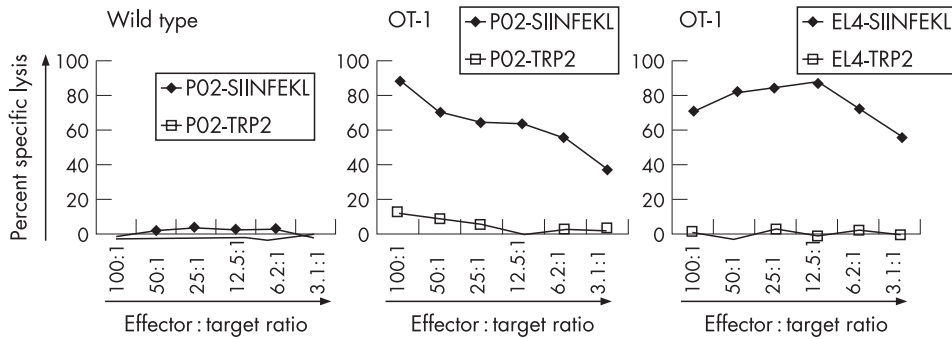


**Figure 4** Vaccination with Panc02-pulsed dendritic cells (DCs) induces tumour antigen-specific cytotoxic T lymphocytes (CTLs). The H2-Kb-restricted peptide p15E is derived from the murine leukaemia virus env-protein encoded by the virus-associated gp70 gene. Expression of gp70 mRNA was determined in Panc02 cells and C26 colon carcinoma cells (derived from the Balb/c mouse strain) as well as in samples from liver, spleen, pancreas and lymph nodes of C57BL/6 mice (A). For detection of p15E-specific CTLs *in vivo*, carboxyfluorescein diacetate-succinimidyl ester (CFSE)<sup>high</sup>-labelled splenocytes from C57BL/6 mice loaded with p15E peptide derived from the MuLV env-protein and CFSE<sup>low</sup>-labelled, unloaded splenocytes were injected into the tail vein of mice either vaccinated three times with Panc02-pulsed DCs or left untreated. After 20 h, spleens were removed and the frequencies of the differently labelled splenocytes were analysed by flow cytometry. By quantifying the relative decrease of p15E-loaded, CFSE<sup>high</sup> splenocytes in vaccinated animals as compared with untreated animals, specific lysis could be determined (B; data are the mean (SEM) of four independent experiments).

specifically lysed by splenocytes from transgenic OT-1 mice (fig 5).

### Combination of DC-based vaccination with gemcitabine increases survival in the Panc02 pancreatic carcinoma model

Four experimental groups with comparable tumour size (approximately 13 mm<sup>2</sup>) were formed 14 days after challenge with Panc02. Mice received either no treatment (control), Panc02-pulsed DCs (DC), gemcitabine (Gem) or Panc02-pulsed DCs plus gemcitabine (DC + Gem). Gemcitabine was given twice weekly, in a dosage of 50 mg/kg, in the combined treatment arm 2 days prior to and 2 days after vaccination with DCs. Therapy was continued for 5 weeks. As shown in fig 6A, animals in the control group had all died 58 days after initiation of therapy. DC-based vaccination alone was almost as equally effective as gemcitabine treatment in preventing death (14% survival at day 58 of therapy vs 17%;  $p = 0.13$  and  $p = 0.08$  for DC vaccine vs control and gemcitabine vs control). In contrast to each treatment strategy applied alone, survival could be significantly increased in the combined treatment arm



**Figure 5** Panc02 cells can be recognised and killed by antigen-specific cytotoxic T lymphocytes in a major histocompatibility complex class I-restricted manner. Panc02 cells were loaded with SIINFEKL or irrelevant TRP2 peptide and co-cultured with splenocytes derived either from wild-type C57BL/6 mice or from transgenic OT-1 mice in a  $^{51}\text{Cr}$ -release assay at effector:target ratios from 3.125:1 to 100:1. EL4 cells loaded with SIINFEKL or irrelevant TRP2 peptide and co-cultured with splenocytes from transgenic OT-1 mice served as positive controls in the  $^{51}\text{Cr}$ -release assay.

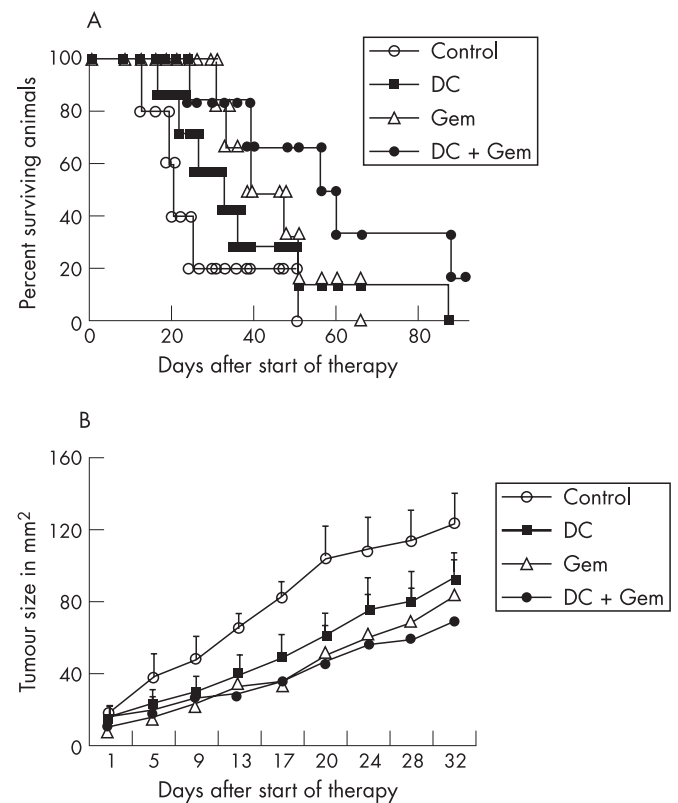
(50% survival at day 58 of therapy;  $p < 0.005$  for combined treatment vs control). Local tumour growth could be reduced in all treatment groups: 32 days after the start of therapy, mean tumour sizes were  $123 \text{ mm}^2$  in the control group,  $91 \text{ mm}^2$  in the DC vaccination group and  $85 \text{ mm}^2$  in the gemcitabine group. In the combined treatment arm, mean tumour size was  $67 \text{ mm}^2$ . However, none of these results reached statistical significance when compared with the control group (fig 6B). The increase in survival of animals treated with DC-based vaccination plus gemcitabine exceeded a solely additive effect. Moreover, gemcitabine treatment alone could not significantly augment survival or reduce local tumour growth. Thus, we hypothesised a synergistic effect of gemcitabine treatment and DC-based vaccination independent from a direct cytotoxic effect of the drug. To test this hypothesis, we reduced the gemcitabine dosage by 50% and repeated the experiment. As shown in fig 7, combination of the DC-based vaccination with gemcitabine increased survival of Panc02 tumour-bearing mice at both dosages of 50 and 25 mg/kg ( $p = 0.01$  and  $p = 0.001$  for DC plus gemcitabine 50 mg/kg vs control, and for DC plus gemcitabine 25 mg/kg vs control). As before, no significant effect on survival could be observed in the groups treated with only one of the two strategies ( $p = 0.98$  and  $p = 0.18$  for DC vaccination alone vs control, and gemcitabine alone vs control).

### Vaccination with Panc02-pulsed DCs prevents metastatisation and orthotopic tumour growth

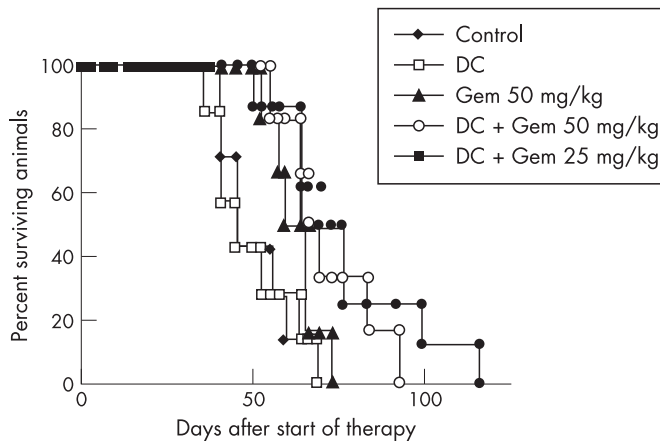
Mice that were left untreated or vaccinated three times with Panc02-pulsed DCs were injected into the tail vein with  $25 \times 10^4$  Panc02 cells 7 days after the last vaccination. As shown in fig 8A, untreated mice died within 60 days. In contrast, immunisation with Panc02-pulsed DCs prevented death in this model. Histopathological analysis confirmed the presence of tumour cells in the lungs of untreated mice (fig 8b). In an orthotopic tumour model, we tested the capacity of the DC vaccination to suppress local tumour growth. Mice were left untreated or vaccinated subcutaneously with  $3 \times 10^5$  Panc02-loaded DCs. Seven days after the last vaccination,  $2 \times 10^5$  Panc02 cells were injected into the pancreas as described in the Materials and methods section. Macroscopic tumours were removed 19 days after tumour induction, and tumour mass was assessed. In the control group, tumours reached a volume of approximately  $400 \text{ mm}^2$  (data not shown) and a mean weight of 400 mg (fig 9, left). Prophylactic vaccination with Panc02-loaded DCs completely prevented growth of orthotopic Panc02 tumours ( $p = 0.014$  for DC vaccination vs control). An experimental small animal CT scanner was used to monitor orthotopic tumour growth in some animals. Three weeks after injection of Panc02 cells into the pancreas, tumours reached approximately 1 cm in diameter in untreated animals (fig 9; right, top).

## DISCUSSION

Here, we show for the first time that DC-based vaccination can be combined with gemcitabine to increase survival in a mouse model of pancreatic cancer. Moreover, we were able to demonstrate that prophylactic DC vaccination can prevent metastatisation and orthotopic growth of murine Panc02 pancreatic carcinoma cells. We chose the Panc02 murine tumour model because its poor immunogenicity resembles one of the characteristic features of human pancreatic carcinoma.<sup>37</sup> However, Panc02 cells can be recognised and



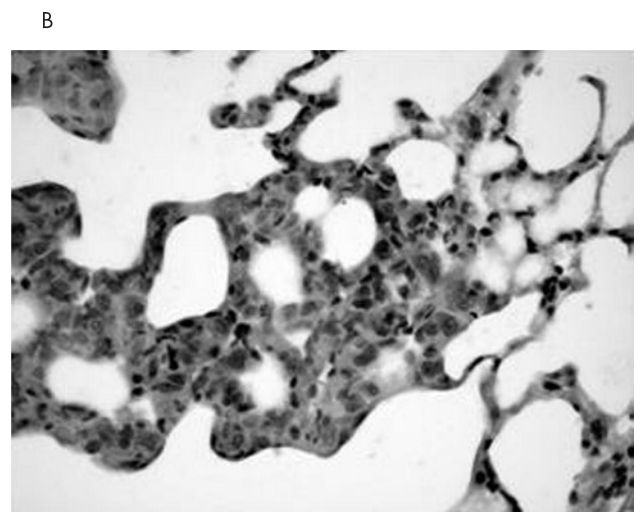
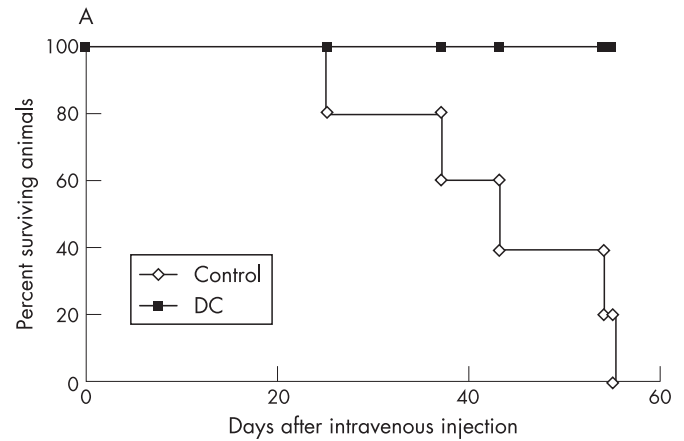
**Figure 6** Combination of dendritic cell (DC)-based vaccination with gemcitabine treatment increases survival in the subcutaneous Panc02 murine pancreatic carcinoma model. Female 6- to 10-week old C57BL/6 mice were challenged subcutaneously with  $0.5 \times 10^6$  Panc02 cells. Fourteen days after tumour inoculation, mice were either left untreated ( $n = 5$ ; control), or were vaccinated with Panc02-pulsed DCs at weekly intervals ( $n = 7$ ; DC), treated with intraperitoneal gemcitabine (50 mg/kg body weight) twice weekly ( $n = 6$ ; Gem) or treated with the combination of DC vaccine and gemcitabine ( $n = 6$ ; DC + Gem). Therapy was continued for 5 weeks. Survival rates in the different treatment groups are depicted as Kaplan-Meier graphs (A). Tumour size was determined at regular intervals by multiplication of perpendicular diameters (B). Data are the mean (SEM) for the number of animals indicated for each group.



**Figure 7** Synergistic effect of dendritic cell (DC)-based vaccination and gemcitabine is preserved after 50% dose reduction of chemotherapy. Female 6- to 10-week old C57BL/6 mice were challenged subcutaneously with  $0.5 \times 10^6$  Panc02 cells. Fourteen days after tumour inoculation, mice were either left untreated ( $n=7$ ), vaccinated with Panc02-pulsed DCs at weekly intervals ( $n=7$ ) or treated with intraperitoneal gemcitabine (Gem 50 mg/kg body weight) twice weekly ( $n=6$ ). Mice treated with the combination of DC vaccine and chemotherapy received either 50 mg/kg body weight ( $n=6$ ) or 25 mg/kg body weight of gemcitabine ( $n=8$ ). Survival rates in the different treatment groups are depicted as Kaplan-Meier graphs.

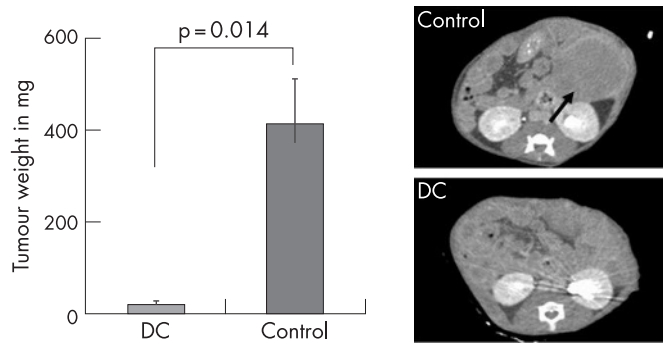
killed by CTLs despite low levels of MHC class I expression, and immunotherapeutic approaches can be effective in this model.<sup>27-38</sup> First, we demonstrated that Panc02-pulsed DCs can establish immunological memory associated with long-term tumour protection and are capable of inducing a tumour-specific CTL response. Of note, peptide-specific CTLs with *in vivo* cytolytic capacity could be induced by DCs loaded with a polyclonal, whole cell-based antigen preparation. Tumour infiltration by CTLs is associated with a better prognosis in pancreatic carcinoma and other solid cancers.<sup>7-39</sup> We were unable to detect increased T-cell infiltration after DC-based vaccination by histopathology, and tumour size could not be significantly reduced in the therapeutic subcutaneous model. However, our data show that DC-based vaccination can prevent death from haematogenic tumour spread and inhibit formation of pulmonary metastases. This is in accordance with findings in the B16 murine melanoma model: although vaccination therapy is unable to prevent local growth of B16 murine melanomas, it is also very effective against pulmonary metastatic disease.<sup>30-40</sup>

Although all mice bearing heterotopic Panc02 tumours died from the disease, some animals tolerated very large subcutaneous tumours for a substantial period of time. In contrast, mice with orthotopic tumours rapidly succumbed to a wasting syndrome after formation of peritoneal metastases. Importantly, prophylactic subcutaneous DC vaccination completely prevented orthotopic tumour growth. Thus, the systemic immune response induced by the DC vaccine is capable of suppressing intraperitoneal tumour growth. Although Takigawa *et al* have previously shown that intraperitoneal injection of tumour lysate-pulsed DCs could inhibit peritoneal spread of orthotopic tumours in a hamster model of pancreatic cancer,<sup>41</sup> this represents an important novel finding. Although not yet formally demonstrated, it appears that circulating tumour-specific CTLs induced by DC vaccination are capable of accessing the peritoneal cavity where tumour cells are recognised and killed. Thus, adjuvant DC-based vaccination may be effective in preventing recurrence from disseminated or local residual tumour cells after pancreaticoduodenectomy in patients with pancreatic carcinoma.



**Figure 8** Prophylactic vaccination with Panc02-loaded dendritic cells (DCs) prevents pulmonary metastatisation after intravenous injection of Panc02 cells. Mice were left untreated or vaccinated three times with Panc02-pulsed DCs. Seven days after the last vaccination,  $25 \times 10^4$  Panc02 cells were injected into the tail vein. As depicted in the Kaplan-Meier analysis, non-vaccinated mice died within 60 days whereas vaccinated mice did not succumb to metastasis formation (A;  $n=5$ ). In untreated mice, lung metastases were found on histopathological analysis (B; one representative analysis of five performed).

Although DC-based vaccination was equally effective as gemcitabine in the therapeutic setting, neither of the two strategies could prevent progression of established tumours nor significantly reduce mortality. While it is known that Panc02 cells are relatively resistant to chemotherapy, the ineffectiveness of the vaccine may be due to an immunosuppressive environment produced by the tumour (eg, by secretion of soluble factors detrimental to T cell-mediated immunity such as IL10 or transforming growth factor (TGF) $\beta$ ), to a limited access of effector cells to tumour tissue or inactivation of tumour-infiltrating lymphocytes, another feature that is similar to human pancreatic cancer.<sup>7-42</sup> If the DC-based vaccine was combined with gemcitabine, the survival rate of tumour-bearing mice could be significantly increased. This is in accordance with previous findings showing that the effectiveness of immune stimulation by *in vivo* CD40 ligation can be augmented by gemcitabine.<sup>24</sup> The exact mechanism by which gemcitabine enhances the effectiveness of the DC-based vaccine in our model remains unknown. In a human *in vitro* model, we were able to show that the drug can sensitise pancreatic carcinoma cells to CTL responses.<sup>25</sup> Others have demonstrated that apoptosis induced by gemcitabine may increase cross-presentation of tumour antigens to CTLs by



**Figure 9** Prophylactic vaccination with Panc02-loaded dendritic cells (DCs) prevents orthotopic tumour growth. Mice were left untreated ( $n=6$ ) or vaccinated subcutaneously with  $3 \times 10^5$  Panc02-loaded DCs ( $n=4$ ). Seven days after the last vaccination,  $2 \times 10^5$  Panc02 cells were injected into the pancreas after laparotomy and mobilisation of the spleen. Tumours were removed 19 days after surgery and tumour weight was determined (left; data are the mean (SEM) for each group). In some animals, orthotopic tumour growth was monitored by CT scanning. Representative abdominal CT scans of an untreated control animal (right, top) and of a vaccinated animal (right, bottom) 19 days after injection of Panc02 cells into the pancreas are shown.

intratumoural DCs.<sup>24</sup> Immunosuppression caused by the chemotherapy applied may limit combination with immunotherapeutic approaches to cancer. However, although inhibition of B cell-mediated immune responses has been described in animal models,<sup>43</sup> gemcitabine can be administered to patients with pancreatic cancer without relevant loss of T cell and DC function.<sup>22</sup> Moreover, preliminary data indicate that gemcitabine may even inhibit Th 2- and specifically augment Th 1-type immune responses in cancer patients.<sup>22</sup> The effect of gemcitabine treatment on the local tumour environment and the frequency and function of tumour antigen-specific CTLs in our model is currently under investigation.

Although clinical trials have demonstrated that tumour-specific immunity can be regularly established by DC-based vaccines, it has also become evident that clinical responses to immunotherapy occur very rarely in patients with gastrointestinal malignancies.<sup>12</sup> Today, researchers try to identify strategies that, on the one hand, help to overcome immunoresistance of tumours and, on the other hand, do not interfere with the activation of a tumour-directed immune response. Increasing evidence suggests that well-established treatment strategies such as radiation, surgical debulking or chemotherapy may be successfully combined with immunotherapeutic approaches.<sup>44-46</sup> Gemcitabine is currently the treatment of choice for patients with irresectable pancreatic carcinoma and is not immunosuppressive. Here, we show that combination with gemcitabine augments therapeutic efficacy of a DC-based vaccine in a murine model of pancreatic cancer. These findings led to the initiation of a phase II trial at our institution investigating combined treatment of patients with advanced pancreatic adenocarcinoma with gemcitabine and an autologous DC-based vaccine.

## ACKNOWLEDGEMENTS

C Bauer, F Bauernfeind and M Dauer were supported by grants from the University of Munich (FoeFoLe No. 481, Promotionsstudium Molekulare Medizin, Gravenhorst-Stiftung). We thank Professor Brocker from the Department of Immunology of the University of Munich for providing transgenic OT-1 animals.

## Authors' affiliations

C Bauer, F Bauernfeind, A Sterzik, M Orban, M Schnurr, A Egler, M Dauer, Section of Gastroenterology, Medizinische Klinik Innenstadt, University of Munich, Ziemssenstr. 1, 80336 Munich, Germany

S Endres, Division of Clinical Pharmacology, Medizinische Klinik Innenstadt, University of Munich, Ziemssenstr. 1, 80336 Munich, Germany  
H A Lehr, Institut Universitaire de Pathologie, Centre Hospitalier Universitaire Vaudois (CHUV), Rue du Bugnon 25, CH-1011 Lausanne, Switzerland

Competing interests: None.

\*These authors contributed equally. This work is part of the doctoral theses of F Bauernfeind and A Sterzik at the University of Munich, Munich, Germany.

## REFERENCES

- Jemal A, Murray T, Ward E, *et al.* Cancer statistics, 2005. *CA Cancer J Clin* 2005;55:10-30.
- Burris HA 3rd, Moore MJ, Andersen J, *et al.* Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997;15:2403-13.
- Burris HA 3rd. Recent updates on the role of chemotherapy in pancreatic cancer. *Semin Oncol* 2005;32(4 Suppl 6):S1-3.
- Louvet C, Labianca R, Hammel P, *et al.* Gemcitabine in combination with oxaliplatin compared with gemcitabine alone in locally advanced or metastatic pancreatic cancer: results of a GERCOR and GISCAD phase III trial. *J Clin Oncol* 2005;23:3509-16.
- Ito M, Shichijo S, Tsuda N, *et al.* Molecular basis of T cell-mediated recognition of pancreatic cancer cells. *Cancer Res* 2001;61:2038-46.
- Schnurr M, Galambos P, Scholz C, *et al.* Tumor cell lysate-pulsed human dendritic cells induce a T-cell response against pancreatic carcinoma cells: an in vitro model for the assessment of tumor vaccines. *Cancer Res* 2001;61:6445-50.
- Fukunaga A, Miyamoto M, Cho Y, *et al.* CD8+ tumor-infiltrating lymphocytes together with CD4+ tumor-infiltrating lymphocytes and dendritic cells improve the prognosis of patients with pancreatic adenocarcinoma. *Pancreas* 2004;28:e26-31.
- Schmitz-Winnenthal FH, Volk C, Z'Graggen K, *et al.* High frequencies of functional tumor-reactive T cells in bone marrow and blood of pancreatic cancer patients. *Cancer Res* 2005;65:10079-87.
- Banchereau J, Palucka AK, Dhodapkar M, *et al.* Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. *Cancer Res* 2001;61:6451-8.
- Schultz ES, Schuler-Thurner B, Stroobant V, *et al.* Functional analysis of tumor-specific Th cell responses detected in melanoma patients after dendritic cell-based immunotherapy. *J Immunol* 2004;172:1304-10.
- Banchereau J, Briere F, Caux C, *et al.* Immunobiology of dendritic cells. *Annu Rev Immunol* 2000;18:767-811.
- Mosolits S, Ullenhag G, Mellstedt H. Therapeutic vaccination in patients with gastrointestinal malignancies. A review of immunological and clinical results. *Ann Oncol* 2005;16:847-62.
- Sallusto F, Cella M, Danieli C, *et al.* Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* 1995;182:389-400.
- Heuffer C, Koch F, Stanzl U, *et al.* Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. *Eur J Immunol* 1996;26:659-68.
- Nestle FO, Aljaghi S, Gilliet M, *et al.* Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 1998;4:328-32.
- Schuler-Thurner B, Dieckmann D, Keikavoussi P, *et al.* Mage-3 and influenza-matrix peptide-specific cytotoxic T cells are inducible in terminal stage HLA-A2.1+ melanoma patients by mature monocyte-derived dendritic cells. *J Immunol* 2000;165:3492-6.
- Banchereau J, Ueno H, Dhodapkar M, *et al.* Immune and clinical outcomes in patients with stage IV melanoma vaccinated with peptide-pulsed dendritic cells derived from CD34+ progenitors and activated with type I interferon. *J Immunother* 2005;28:505-16.
- Akiyama Y, Maruyama K, Nara N, *et al.* Antitumor effects induced by dendritic cell-based immunotherapy against established pancreatic cancer in hamsters. *Cancer Lett* 2002;184:37-47.
- Stift A, Friedl J, Dubsky P, *et al.* In vivo induction of dendritic cell-mediated cytotoxicity against allogeneic pancreatic carcinoma cells. *Int J Oncol* 2003;22:651-6.
- Hou JM, Liu JY, Yang L, *et al.* Combination of low-dose gemcitabine and recombinant quail vascular endothelial growth factor receptor-2 as a vaccine induces synergistic antitumor activities. *Oncology* 2005;69:81-7.
- Nowak AK, Robinson BW, Lake RA. Synergy between chemotherapy and immunotherapy in the treatment of established murine solid tumors. *Cancer Res* 2003;63:4490-6.
- Plate JM, Plate AE, Shott S, *et al.* Effect of gemcitabine on immune cells in subjects with adenocarcinoma of the pancreas. *Cancer Immunol Immunother* 2005;54:915-25.
- Correale P, Cusi MG, Del Vecchio MT, *et al.* Dendritic cell-mediated cross-presentation of antigens derived from colon carcinoma cells exposed to a highly cytotoxic multidrug regimen with gemcitabine, oxaliplatin, 5-fluorouracil, and leucovorin, elicits a powerful human antigen-specific CTL response with antitumor activity in vitro. *J Immunol* 2005;175:820-8.

- 24 **Nowak AK**, Lake RA, Marzo AL, *et al*. Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. *J Immunol* 2003;**170**:4905–13.
- 25 **Dauer M**, Herten J, Bauer C, *et al*. Chemosensitization of pancreatic carcinoma cells to enhance T cell-mediated cytotoxicity induced by tumor lysate-pulsed dendritic cells. *J Immunother* 2005;**28**:332–42.
- 26 **Corbett TH**, Roberts BJ, Leopold WR, *et al*. Induction and chemotherapeutic response of two transplantable ductal adenocarcinomas of the pancreas in C57BL/6 mice. *Cancer Res* 1984;**44**:717–26.
- 27 **Schmidt T**, Ziske C, Marten A, *et al*. Intratumoral immunization with tumor RNA-pulsed dendritic cells confers antitumor immunity in a C57BL/6 pancreatic murine tumor model. *Cancer Res* 2003;**63**:8962–7.
- 28 **Zeytin HE**, Patel AC, Rogers CJ, *et al*. Combination of a poxvirus-based vaccine with a cyclooxygenase-2 inhibitor (celecoxib) elicits antitumor immunity and long-term survival in CEA.Tg/MIN mice. *Cancer Res* 2004;**64**:3668–78.
- 29 **Rotzschke O**, Falk K, Stevanovic S, *et al*. Exact prediction of a natural T cell epitope. *Eur J Immunol* 1991;**21**:2891–4.
- 30 **Bloom MB**, Perry-Lalley D, Robbins PF, *et al*. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. *J Exp Med* 1997;**185**:453–9.
- 31 **Yang JC**, Perry-Lalley D. The envelope protein of an endogenous murine retrovirus is a tumor-associated T-cell antigen for multiple murine tumors. *J Immunother* 2000;**23**:177–83.
- 32 **Heckelsmiller K**, Beck S, Rall K, *et al*. Combined dendritic cell- and CpG oligonucleotide-based immune therapy cures large murine tumors that resist chemotherapy. *Eur J Immunol* 2002;**32**:3235–45.
- 33 **Schnurr M**, Scholz C, Rothenfusser S, *et al*. Apoptotic pancreatic tumor cells are superior to cell lysates in promoting cross-priming of cytotoxic T cells and activate NK and gammadelta T cells. *Cancer Res* 2002;**62**:2347–52.
- 34 **Kim HS**, Choo YS, Koo T, *et al*. Enhancement of antitumor immunity of dendritic cells pulsed with heat-treated tumor lysate in murine pancreatic cancer. *Immunol Lett* 2006;**103**:142–8.
- 35 **Hogquist KA**, Jameson SC, Bevan MJ. The ligand for positive selection of T lymphocytes in the thymus. *Curr Opin Immunol* 1994;**6**:273–8.
- 36 **Hogquist KA**, Jameson SC, Heath WR, *et al*. T cell receptor antagonist peptides induce positive selection. *Cell* 1994;**76**:17–27.
- 37 **Turnquist HR**, Kohlgraf KG, McIlhenny MM, *et al*. Tapasin decreases immune responsiveness to a model tumor antigen. *J Clin Immunol* 2004;**24**:462–70.
- 38 **Schmidt J**, Ryschich E, Sievers E, *et al*. Telomerase-specific T-cells kill pancreatic tumor cells in vitro and in vivo. *Cancer* 2006;**106**:759–64.
- 39 **van Beek J**, zur Hausen A, Snel SN, *et al*. Morphological evidence of an activated cytotoxic T-cell infiltrate in EBV-positive gastric carcinoma preventing lymph node metastases. *Am J Surg Pathol* 2006;**30**:59–65.
- 40 **Winter H**, Hu HM, McClain K, *et al*. Immunotherapy of melanoma: a dichotomy in the requirement for IFN-gamma in vaccine-induced antitumor immunity versus adoptive immunotherapy. *J Immunol* 2001;**166**:7370–80.
- 41 **Takigawa Y**, Akiyama Y, Maruyama K, *et al*. Antitumor effect induced by dendritic cell (DC)-based immunotherapy against peritoneal dissemination of the hamster pancreatic cancer. *Cancer Lett* 2004;**215**:179–86.
- 42 **von Bernstorff W**, Voss M, Freichel S, *et al*. Systemic and local immunosuppression in pancreatic cancer patients. *Clin Cancer Res* 2001;**7**(3 Suppl):925s–32s.
- 43 **Nowak AK**, Robinson BW, Lake RA. Gemcitabine exerts a selective effect on the humoral immune response: implications for combination chemo-immunotherapy. *Cancer Res* 2002;**62**:2353–8.
- 44 **Picozzi VJ**, Kozarek RA, Traverso LW. Interferon-based adjuvant chemoradiation therapy after pancreaticoduodenectomy for pancreatic adenocarcinoma. *Am J Surg* 2003;**185**:476–80.
- 45 **Broomfield S**, Currie A, van der Most RG, *et al*. Partial, but not complete, tumor-debulking surgery promotes protective antitumor memory when combined with chemotherapy and adjuvant immunotherapy. *Cancer Res* 2005;**65**(17):7580–4.
- 46 **Correale P**, Cusi MG, Tsang KY, *et al*. Chemo-immunotherapy of metastatic colorectal carcinoma with gemcitabine plus FOLFOX 4 followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients. *J Clin Oncol* 2005;**23**:8950–8.