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

C. Bauer[#1], F. Bauernfeind[#1], A. Sterzik[#], M. Orban[#], M. Schnurr[#], H.A. Lehr[+], S. Endres[*], A. Eigler[#], M. Dauer[#2]

Section of Gastroenterology[#] and Division of Clinical Pharmacology[*], Medizinische Klinik Innenstadt, University of Munich, Ziemssenstr. 1, 80336 Munich, Germany
Institut Universitaire de Pathologie[+], Centre Hospitalier Universitaire Vaudois (CHUV), Rue du Bugnon 25, CH – 1011 Lausanne, Switzerland

[1] C. Bauer and F. Bauernfeind contributed equally to this manuscript. This work is part of the doctoral theses of F. Bauernfeind and A. Sterzik at the University of Munich, Munich, Germany.

[2]Adress correspondence to Dr. Marc Dauer, Medizinische Klinik Innenstadt, University of Munich, Ziemssenstr. 1, 80336 Munich, Germany. Phone: 0049-89-51602291; Fax: 0049-89-51604576; email: marc.dauer@med.uni-muenchen.de

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Abbreviations: CD, cluster of differentiation; CFSE, carboxyfluorescein diacetate-succinimidyl ester; CTL, cytotoxic T lymphocytes; DC, dendritic cells; 5-FU, 5-fluorouracil; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, Interleukin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NK, natural killer; TGF, transforming growth factor; Th1, T helper type 1; TNF, tumor necrosis factor

Abstract

Background: Tumor-specific cytotoxic T lymphocytes (CTL) can be activated *in vivo* by vaccination with dendritic cells (DC). 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. We have previously shown that gemcitabine sensitizes human pancreatic carcinoma cells against CTL-mediated lysis. Here, we used a murine pancreatic carcinoma model to investigate whether combination with gemcitabine increases therapeutic efficacy of DC-based vaccination. Methods: Bone marrow-derived DC 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 in weekly intervals prior to tumor challenge with Panc02 cells. Therapeutic vaccination was started when tumors formed a palpable nodule. Gemcitabine was administered intraperitoneally twice weekly. Results: Prophylactic DC-based vaccination completely prevented subcutaneous and orthotopic tumor development and induced immunological memory as well as tumor antigen-specific CTL. In the subcutaneous tumor model, therapeutic DC-based vaccination was equally effective as gemcitabine (14 % vs. 17 % survival at day 58 after tumor challenge; controls: 0 %). Combination of the two strategies significantly increased survival of tumor-bearing mice (50 % at day 58 after tumor challenge). DC-based vaccination also prevented death from pulmonary metastatization after i.v.-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 metastatization in tumor-free patients.

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Introduction

Pancreatic carcinoma represents the 5th leading cause of cancer death in the Western world. The 5-year overall survival rate is less than 5 %.[1] Pancreaticoduodenectomy represents the only curative form of treatment. However, only 10 to 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 to 5-fluorouracil (5-FU) treatment.[2, 3] Recent trials have failed to show an improvement in survival when gemcitabine is combined with other chemotherapeutic drugs.[4] Therefore, novel strategies are required for the treatment of advanced pancreatic cancer. Pancreatic carcinoma cells can be recognized by tumor-specific T cells.[5, 6] Moreover, tumor infiltration with cytotoxic T cells (CTL) and T helper cells represents a favourable prognostic factor for patients with pancreatic adenocarcinoma.[7] Tumor-reactive T cells capable of tumor rejection can be also isolated from blood of pancreatic carcinoma patients.[8]

Tumor-specific T cells can be activated by vaccination with dendritic cells (DC).[9, 10] DC are specialized antigen-presenting cells with the unique capacity to establish primary immune responses.[11] DC can deliver exogenous antigens into the major histocompatibility complex (MHC) class I processing pathway to activate CTL, a process termed "cross presentation".[12] Contact to microbial or inflammatory "danger signals" as well as T cell-derived activation signals induces DC maturation and interleukin (IL)-12 secretion.[13, 14] IL-12 plays a central role in regulating tumor-directed immune responses and stimulates natural killer (NK) cells, CTL and interferon (IFN)-γ-producing T helper type 1 (Th1) cells. Vaccination with tumor antigen-loaded DC has been shown to elicit antitumoral CTL responses *in vivo* and to induce tumor regression in cancer patients.[9, 15-17] Pancreatic carcinoma cells can be rejected *in vivo* after vaccination with tumor antigen-loaded DC.[18, 19] However, tumors are capable of forming an immunosuppressive environment rendering them insensitive to CTL. Combination with other treatment strategies such as radiation or chemotherapy may break immunoresistance of cancer cells and enhance the therapeutic efficacy of DC-based vaccination.

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

Material 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 C57BL/6 background were provided by Prof. Brocker (Department of Immunology, University of Munich). OT-1 T cells were grown in RPMI with 10% FCS, penicillin and streptomycin. The H2-b positive cell line Panc02 is derived from a methylcholanthrene-induced pancreatic adenocarcinoma in C57BL/6 mice.[26, 27] Panc02 cells were maintained in RPMI (Biochrome, Berlin, Germany) with 10% FCS (Gibco, Invitrogen Cooperation, Paisley, UK), 2 mM L-glutamine (Life Technologies, Paisley, Scotland), 100 units/ml penicillin (Sigma, Munich, Germany) and 100 µg/ml streptomycin (Seromed, Jülich, Germany). Prior to

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coincubation with DC, Panc02 cells were irradiated using UV-B light with 0.75 J/cm^2. Viability of UV-irradiated tumor cells was excluded by light microscopy and thymidine incorporation. The H2-Kb positive lymphoma cell line EL4 (kindly provided by Prof. Enders, Institut für Chirurgische Forschung, University of Munich) was maintained in DMEM medium with 10% FCS, penicillin and streptomycin.

mRNA extraction and RT-PCR

Samples were cleaned in 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 was 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, Wisconsin, USA) were used. PCR was performed with TaqDNA polymerase as recommended by the supplier (Roche, Mannheim, Germany). Primers were manufactured by Applied Biosystems (Weiterstadt, Germany) based on sequences from the literature.[28]

mAbs and flow cytometry

For phenotypic analysis, DC were incubated for 30 min at 4°C with 5 µg/2 x 10^5 cells anti-mouse CD86-FITC mAb (clone GL1), CD11b-PerCP mAb (clone M1/70) , CD11c-APC mAb (clone HL3; all from BD Biosciences, San Jose, CA) CD80-PE mAb (clone RMMP1, Caltag, Burlingame, CA), MHC ll-PE mAb (clone NIMR-4, Southern Biotech, Birmingham, Alabama), and appropriate isotype controls. Cells were examined by flow cytometry (FACSCalibur, BD Biosciences, Heidelberg, Germany). Data were analyzed 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).

Peptides

The H2-Kb restricted peptides OVA$_{257-264}$, TRP2$_{181-188}$ and p15E$_{604-611}$ were from Jerini Peptide Technologies (Berlin, Germany).[29-31]

Reagents

Gemcitabine (Gemzar®) was from Lilly (Indianapolis, Indiana). 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) was from PeproTech (London, UK), IFN-α from HyCult Biotechnology (Uden, Netherlands), IFN-γ, IL-1β, IL-2 and IL-4 from PeproTech (London, UK). Lipopolysaccharide (LPS), p:IC, R848 and tumor necrosis factor-α (TNF-α) were from Sigma-Aldrich (Steinheim, Germany). Murine ELISA kits for IL-12p70, IL-12p40 and IFN-γ were from BD Biosciences (San Diego, CA).

Generation and antigen-loading of bone marrow-derived DC

Bone marrow-derived DC were prepared as described.[32] 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 × 10^5 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 (PreproTech, London, U.K.) and 20 ng/ml IL-4 (PreproTech, London, U.K.) (DC medium). After 7 days, loosely adherent cells were harvested, and expression of DC markers was quantified by FACS. DC represented 70 % of the preparation. Coincubation with Panc02 cells (referred to as "pulsing" in the following) was

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carried out DC/tumor cell ratio of 10 : 1. LPS (250 ng/ml) and IFN-γ (100 ng/ml) were added for the last 24 hours. At day 9, loosely adherent cells were harvested, washed and resuspended in RPMI and analyzed by FACS.

Tumor indution and vaccination strategies

For immunization, 3×10^5 DC were injected s.c. into the right flank in a final volume of 200 µl RPMI. Mice were challenged with Panc02 cells by contralateral s.c. injection (1×10^6 in 200 µl RPMI). Therapeutic vaccination started after tumors had formed a palpable nodule. Gemcitabine was administered i.p. twice weekly in a concentration of 25 mg or 50 mg/kg body weight. Tumor growth was expressed as the product of perpendicular diameters. Animals were killed when tumor size exceeded 400 mm^2 or when there were signs of animal distress observed twice in 48 h. For orthotopic tumor development, the left flank of anaesthesized mice was opened and the spleen mobilized to access the pancreas. 2×10^5 Panc02 cells were injected into the pancreas. Tumor growth was monitored by a LaTheta^{TM} *in vivo* CT scanner for small animals (Zinsser Analytic). Animal CT scans were performed in cooperation with Dr. med. vet. Heinz-Peter Scheuber (TierschutzInformationsZentrum for 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)^{high} (2 µM)- and CFSE^{low} (0.2 µM)-labelled C57BL/6 splenocytes into vaccinated or non-vaccinated mice. The CFSE^{high}-labelled cells were pulsed with the p15E-peptide (2 µg/ml), whereas the CFSE^{low}-labelled cells were left unpulsed. A 1:1 ratio of CFSE^{low}- to CFSE^{high}-labelled cells was injected i.v. 20 h after injection of the target cells, spleens were removed and the ratio of CFSE^{low} to CFSE^{high} cells was determined by flow cytometry. Cytotoxicity was calculated by the formula: % cytotoxicity in a given mouse = ([% CFSE^{high} vac / %CFSE^{low} vac]) / (]% CFSE^{high} control / % CFSE^{low} control]).

In vitro cytotoxicity assay

^{51}Chromium-release assay was performed in cooperation with Prof. Dr. R. Wank, Department of Immunology. University of Munich. Target cells were incubated with Na$_2$^{51}CrO$_4$ (100 µCi/10^6 target cells) at 37°C for 1 h. Cells (5×10^3/well) were washed three times and cocultured 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 (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 51Cr release = ([experimental counts – spontaneous counts]/[maximal counts – spontaneous counts]) x 100 %.

Statistics

Student's t test was applied to reveal significant differences in tumor protection. We compared the mean tumor size of controls and all treated animals (tumor-free and tumor-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 DC prevents subcutaneous tumor development
We have previously shown that DC pulsed with UV-irradiated, apoptotic tumor cells are superior to tumor lysate-pulsed DC in priming CTL directed against human pancreatic cancer cells.[33] This finding has recently been confirmed in the Panc02 tumor model.[34] Thus, we chose UV-irradiated Panc02 cells as a source of tumor antigen. Bone marrow-derived DC

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were capable of taking up dead Panc02-cells and secreted IL-12p70 upon stimulation with LPS and IFN-γ (fig. 1). Mice were completely protected from tumor development after 3 vaccinations with Panc02-pulsed DC (fig. 2A). In the untreated animals, tumors with a mean size of 110 mm^2 developed within 35 days (fig. 2A). Tumor size was only moderately reduced in mice receiving UV-irradiated Panc02 cells only or unloaded DC (mean tumor size after 35 days approximately 40 mm^2). Tumor protection in mice vaccinated with Panc02-pulsed DC was associated with higher levels of IFN-γ-secretion in splenocytes isolated after two vaccinations (fig. 2B).

Vaccination with Panc02-pulsed DC establishes immunological memory and long-term tumor protection
While control animals challenged with Panc02 cells died after 40 to 50 days, mice that had received prophylactic vaccination with Panc02-pulsed DC survived more than three months without developing a tumor (data not shown). These mice were rechallenged with Panc02 cells at day 95 after the first tumor challenge. As shown in figure 3, they were still protected from tumor development as opposed to controls that rapidly developed large tumors (mean tumor size 91 mm^2 at day 26). Vaccinated mice that were rechallenged with Panc02 at day 95 showed long-term survival and were euthanized months later without signs of local or systemic tumor growth (data not shown).

Vaccination with Panc02-pulsed DC induces tumor-specific cytotoxic T cells
Although mice could be protected from tumor development, it remained to be shown that DC vaccination induced a tumor-specific CTL response. The virus-associated 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^{high}-labelled splenocytes from C57BL/6 mice loaded with p15E peptide derived from the MuLV env-protein and CFSE^{low}-labelled, unloaded splenocytes were injected into the tail vein of mice either vaccinated three times with Panc02-pulsed DC or left untreated. By quantifying the relative decrease of p15E-loaded, CFSE^{high} splenocytes in vaccinated animals as compared to untreated animals, a specific lysis rate of approximately 12 % could be calculated (fig. 4B). Thus, Panc02-loaded DC not only protect animals from tumor development, but also induce immunological memory and a tumor-specific CTL response.

Panc02 cells are recognized and killed by tumor-specific CTL
Panc02-derived tumors are poorly immunogenic, similar to human pancreatic adenocarcinoma. Moreover, Panc02 cells express only low levels MHC class I required for classical CTL-mediated lysis (data not shown). Thus, we analysed whether Panc02 cells could be recognized *in vitro* by antigen-specific CTL prior to their use in the therapeutic *in vivo*-vaccination model. CTL derived from transgenic OT-1 animals specific for the OVA-epitope SIINFEKL were used.[35, 36] Panc02 cells were loaded with SIINFEKL peptide or an irrelevant control peptide (TRP2) and cocultured either with splenocytes from wildtype mice or from OT-1 mice in a ^{51}Cr-release assay. EL4 cells loaded with SIINFEKL or an irrelevant peptide were used as control targets. Panc02 loaded with SIINFEKL were 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 tumor size (approximately 13 mm^2) were formed 14 days after challenge with Panc02. Mice either received no treatment (control), Panc02-pulsed DC (DC), gemcitabine (Gem) or Panc02-pulsed DC 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 two days after vaccination with DC. Therapy was continued for 5 weeks. As shown in figure 6A, animals in the control group had all died 58 days after

initiation of therapy. DC-based vaccination alone was almost 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 (50 % survival at day 58 of therapy; p < 0.005 for combined treatment vs. control). Local tumor growth could be reduced in all treatment groups: 32 days after start of therapy, mean tumor sizes were 123 mm^2 in the control group, 91 mm^2 in the DC-vaccination group and 85 mm^2 in the gemcitabine group. In the combined treatment arm, mean tumor size was 67 mm^2. However, none of these results reached statisitical significance when compared to the control group (figure 6 B). 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 tumor growth. Thus, we hypothesized 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 figure 7, combination of the DC-based vaccination with gemcitabine increased survival of Panc02-tumor bearing mice at both dosages of 50 mg/kg 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 DC prevents metastatization and orthotopic tumor growth
Mice that were left untreated or vaccinated three times with Panc02-pulsed DC were injected into the tail vein with 25 x 10^4 Panc02 cells seven days after the last vaccination. As shown in figure 8A, untreated mice died within 60 days. In contrast, immunization with Panc02-pulsed DC prevented death in this model. Histopathologic analysis confirmed the presence of tumor cells in the lungs of untreated mice (fig. 8b). In an orthotopic tumor model, we tested the capacity of the DC vaccination to suppress local tumor growth. Mice were left untreated or vaccinated subcutaneously with 3 x 10^5 Panc02-loaded DC. Seven days after the last vaccination, 2 x 10^5 Panc02 cells were injected into the pancreas as described in the material and methods section. Macroscopic tumors were removed 19 days after tumor induction and tumor mass was assessed. In the control group, tumors reached a volume of approximately 400 mm^2 (data not shown) and a mean weight of 400 mg (fig. 9, left). Prophylactic vaccination with Panc02-loaded DC completely prevented growth of orthotopic Panc02 tumors (p = 0.014 for DC-vaccination vs. control). An experimental small animal CT scanner was used to monitor orthotopic tumor growth in some animals. Three weeks after injection of Panc02 cells into the pancreas, tumors 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 metastatization and orthotopic growth of murine Panc02 pancreatic carcinoma cells. We chose the Panc02 murine tumor model because its poor immunogenicity resembles one of the characteristic features of human pancreatic carcinoma.[37] However, Panc02 cells can be recognized and killed by CTL despite low levels of MHC class I expression and immunotherapeutic approaches can be effective in this model.[27, 38] First, we demonstrated that Panc02-pulsed DC can establish immunological memory associated with long-term tumor protection and are capable of inducing a tumor-specific CTL response. Of note, peptide-specific CTL with *in vivo*-cytolytic capacity could be induced by DC loaded with a polyclonal, whole cell-based antigen preparation. Tumor infiltration by CTL is associated with a better prognosis in

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pancreatic carcinoma and other solid cancers.[7, 39] We were unable to detect increased T-cell infiltration after DC-based vaccination by histopathology and tumor size could not be significantly reduced in the therapeutic subcutaneous model. However, our data show that DC-based vaccination can prevent death from hematogenic tumor 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.[30, 40]

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

Although DC-based vaccination was equally effective as gemcitabine in the therapeutic setting, neither of the two strategies could prevent progression of established tumors 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 tumor (e.g. by secretion of soluble factors detrimental to T cell-mediated immunity such as IL-10 or TGF-β), to a limited access of effector cells to tumor tissue or inactivation of tumor-infiltrating lymphocytes, another feature that is similar to human pancreatic cancer.[7, 42] If the DC-based vaccine was combined with gemcitabine, survival rate of tumor-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.[24] 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 sensitize pancreatic carcinoma cells to CTL responses.[25] Others have demonstrated that apoptosis induced by gemcitabine may increase cross presentation of tumor antigens to CTL by intratumoral DC.[24] 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,[43] gemcitabine can be administered to patients with pancreatic cancer without relevant loss of T cell and DC function.[22] Moreover, preliminary date indicate that gemcitabine may even inhibit Th 2 and specifically augment Th 1 type immune responses in cancer patients.[22] The effect of gemcitabine treatment on local tumor environment and the frequency and function of tumor antigen-specific CTL in our model is currently under investigation.

Although clinical trials have demonstrated that tumor-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.[12] Today, researchers try to identify strategies that, on the one hand, help to overcome immunoresistance of tumors and, on the other hand, do not interfere with the activation of a tumor-directed immune response. Increasing evidence suggests that well-established treatment strategies such as radiation, surgical debulking or chemotherapy may successfully combined with immunotherapeutic approaches.[44-46] 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

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therapeutic efficacy of a DC-based vaccine in a murine model of pancreatic cancer. These findings lead 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.

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Statement of competing interests

None to declare.

Figure legends

Figure 1: Bone marrow-derived DC effectively take up tumor antigen and secrete high levels of IL-12p70 after stimulation with LPS and IFN-γ.
DC were derived from bone marrow of C57BL/6 mice by culture with GM-CSF and IL-4. On day 6 of culture, DC were incubated with UV-irradiated, CFSE-labelled Panc02 cells at a ratio of 10:1. Uptake of tumor cells by CD11c$^+$ DC was determined by FACS analysis (A; the results of one representative FACS analysis out of five performed are shown). At day 6 of culture, DC were stimulated for another 24 h with different stimuli. Culture supernatants were harvested and analyzed for IL-12 production by ELISA (B; data are the mean ± SEM of 2 independent experiments).

Figure 2: Prophylactic vaccination with Panc02-pulsed DC completely prevents development of subcutaneous Panc02 tumors.
Female 6 to 10-week old C57BL/6 mice were either vaccinated s.c. with Panc02-pulsed or unpulsed syngeneic DC (3 x 10^5) or UV-irradiated Panc02 cells (1 x 10^5) for three times in weekly intervals or left untreated. Seven days after the last vaccination, mice were challenged s.c. with 1 x 10^6 Panc02 cells. Subcutaneous tumor growth was monitored in regular intervals and tumor size determined by multiplication of perpendicular diameters (A; data are the mean ± SEM of 3 animals in each group). In parallel experiments, mice from each group were sacrificed after two vaccinations and *in vitro* IFN-γ production of splenocytes determined by ELISA (B, data are the mean ± SEM of 2 animals in each group).

Figure 3: Prophylactic vaccination with Panc02-pulsed DC induces immunological memory associated with long-term tumor protection.
Female 6 to 10-week old C57BL/6 mice were either vaccinated s.c. with Panc02-pulsed DC (3 x 10^5) for three times in weekly intervals or left untreated and challenged s.c. with 1 x 10^6 Panc02 cells 7 days after the last vaccination. Tumor size was determined in regular intervals by multiplication of perpendicular diameters. Mice receiving DC vaccination remained tumor-free and were re-challenged s.c. with 1 x 10^6 Panc02 tumor cells at day 95 after initial tumor inoculation. Previously untreated and unchallenged mice served as a control group (data are the mean ± SEM of 3 animals in each group).

Figure 4: Vaccination with Panc02-pulsed DC induces tumor antigen-specific cytotoxic T cells.

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The H2-Kb-restricted peptide p15E is derived from the MuLV 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 CTL *in vivo*, CFSE[high]-labelled splenocytes from C57BL/6 mice loaded with p15E and CFSE[low]-labelled, unloaded splenocytes were injected into the tail vein of mice either vaccinated three times with Panc02-pulsed DC 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[high] splenocytes in vaccinated animals as compared to untreated animals, specific lysis could be determined (B; data are the mean ± SEM of 4 independent experiments).

Figure 5: Panc02 cells can be recognized and killed by antigen-specific CTL in an MHC class I-restricted manner.
Panc02 cells were loaded with SIINFEKL or irrelevant TRP2 peptide and cocultured with splenocytes either derived form wildtype C57BL/6 mice or from transgenic OT-1 mice in a ^{51}Cr-release assay at E/T ratios from 3.125 : 1 to 100 : 1. EL4 cells loaded with SIINFEKL or irrelevant TRP2 peptide and cocultured with splenocytes from transgenic OT-1 mice served as positive controls in the ^{51}Cr-release assay.

Figure 6: Combination of 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 s.c. with 0.5 x 10^6 Panc02 cells. Fourteen days after tumor inoculation, mice were either left untreated (n=5; control), or vaccinated with Panc02-pulsed DC in weekly intervals (n=7; DC), or treated with i.p. 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 five weeks. Survival rates in the different treatment groups are depicted as Kaplan-Meier graphs (A). Tumor size was determined in 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 DC-based vaccination and gemcitabine is preserved after 50 % dose reduction of chemotherapy.
Female 6 to 10-week old C57BL/6 mice were challenged s.c. with 0.5 x 10^6 Panc02 cells. Fourteen days after tumor inoculation, mice were either left untreated (n=7), vaccinated with Panc02-pulsed DC in weekly intervals (n=7) or treated with i.p. gemcitabine (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.

Figure 8:
Prophylactic vaccination with Panc02-loaded DC prevents pulmonary metastatization after i.v. injection of Panc02 cells.
Mice were left untreated or vaccinated three times with Panc02-pulsed DC. Seven days after the last vaccination, 25 x 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 histopathologic analysis (B; one representative analysis of 5 performed).

Figure 9:
Prophylactic vaccination with Panc02-loaded DC prevents orthotopic tumor growth.
Mice were left untreated (n=6) or vaccinated subcutaneously with 3 x 10^5 Panc02-loaded DC (n=4). Seven days after the last vaccination, 2 x 10^5 Panc02 cells were injected into the pancreas after laparatomy and mobilization of the spleen. Tumors were removed 19 days after surgery and tumor weight was determined (left; data are the mean ± SEM for each

group). In some animals, orthotopic tumor 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.

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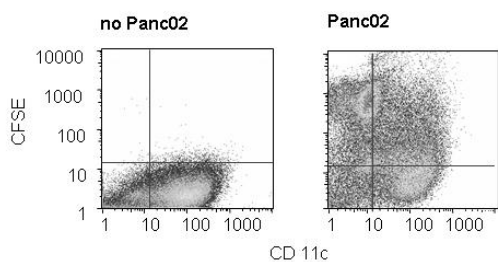
Fig. 1A

Fig. 1B

Fig. 2A

Fig. 2B

Tumor size in mm²

Fig. 3

Gp70 env mRNA expression

Fig. 4A

Percent specific lysis

Fig. 4B

15
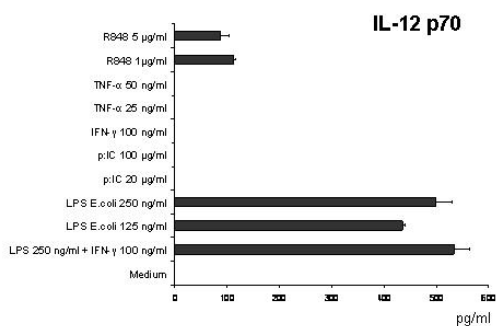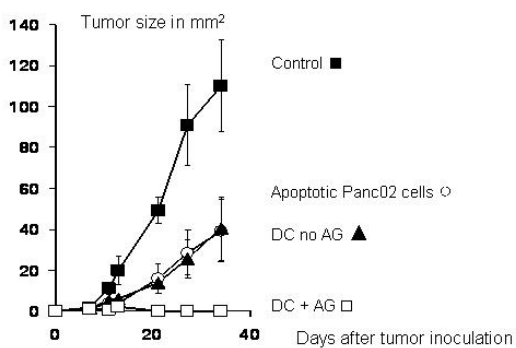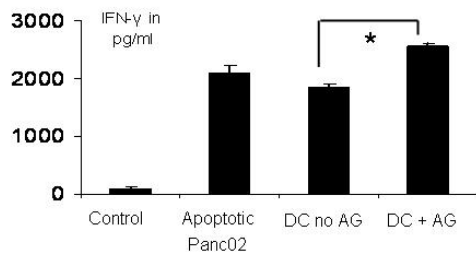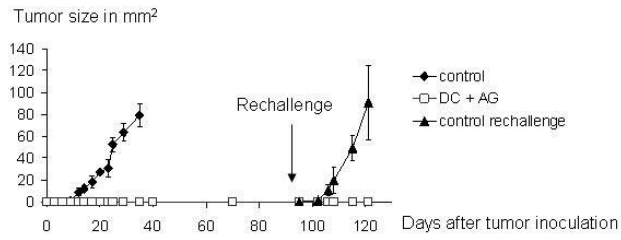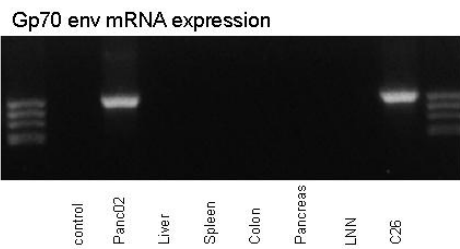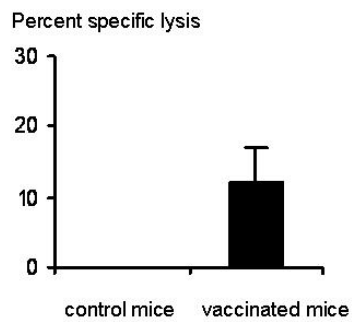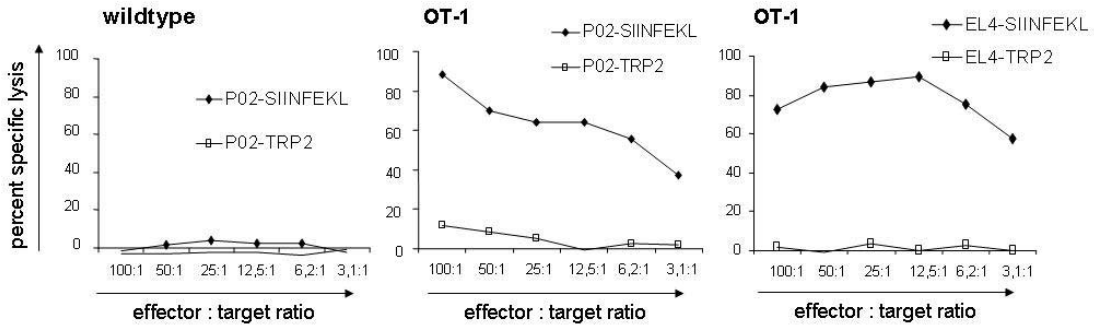
Fig. 5
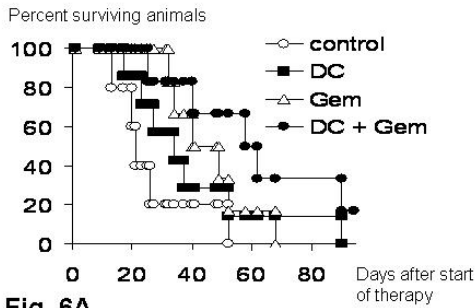
Fig. 6A

Fig. 6B

Fig. 7

Fig. 8A

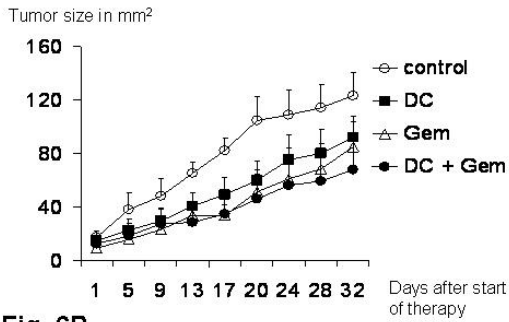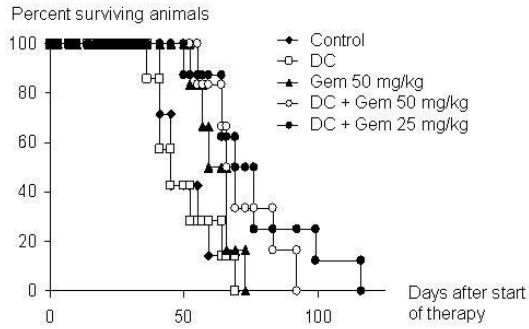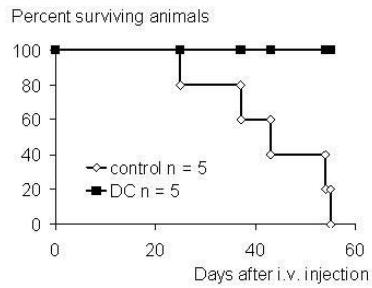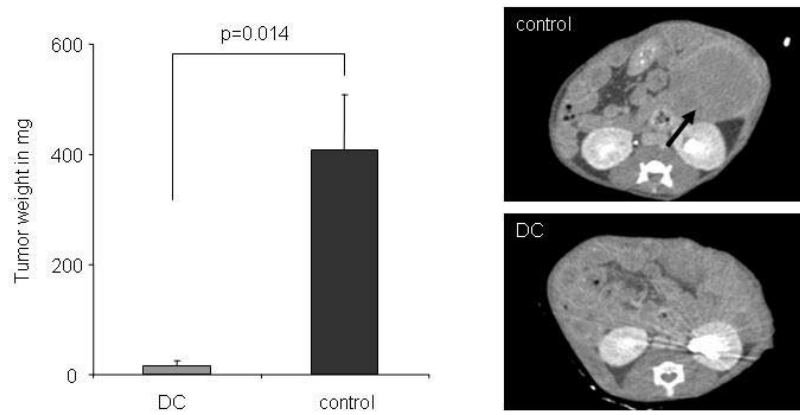**Fig. 8B**

Fig. 9

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

Christian Alexander Bauer, Franz Bauernfeind, Alexander Sterzik, et al.

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