Intratumoral Immunization with Tumor RNA-Pulsed Dendritic Cells Confers Antitumor Immunity in a C57BL/6 Pancreatic Murine Tumor Model

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ABSTRACT

Pancreatic tumors lack adequate recruitment of immunocompetent cells, especially dendritic cells (DCs). We have shown previously that coculturing of natural killer-like T cells with DCs transfected with pancreatic tumor cell line-derived RNA reverses pancreatic carcinoma cell resistance by directly triggering natural killer-like T lymphocytes in vitro. In the present study, we tested triggering of specific T lymphocytes in vivo by using an immunocompetent mouse strain (C57BL/6).

Syngenic, bone marrow-derived DCs were pulsed with tumor RNA derived from the pancreatic cell line Panc02. This cell line is a ducal pancreatic adenocarcinoma and shows high resistance to every known class of clinically active antitumor agent. Panc02 cells were implanted orthotopically via ultrasound guidance and led to pancreatic tumor formation in all of the mice. Thereafter, tumor RNA-pulsed DCs were injected intratumorally.

Intratumoral administration of tumor RNA-pulsed DCs induced significantly more potent protective immunity than s.c. or i.v. administration. It was significantly more effective than administration with unpulsed DCs or DCs pulsed with a control tumor RNA derived from a lymphomatosus cell line (EL4). The antitumor effect was caused by induction of antigen-specific T lymphocytes as shown by additional in vitro studies.

Results favor intratumoral injection of tumor RNA-pulsed DCs for immunotherapy of pancreatic cancer.

INTRODUCTION

Ductal pancreatic adenocarcinoma is the fourth leading cause of cancer death in the Western world. Unfortunately, recent advances in diagnostics, staging, and therapy in pancreatic carcinoma of exocrine origin have not resulted in significant improvements in long-term survival within the past 3 decades (1). Median survival for all affected patients does not exceed 2 years with a 5-year survival of 10–15% (2). Thus, new approaches are necessary to improve the outcome of patients with exocrine pancreatic cancer.

Dendritic cells (DCs) are the antigen-presenting cells that are highly effective at initiating T cell-mediated immune response (3). Immature DCs efficiently capture and process antigen in peripheral tissues followed by migration to regional lymph nodes. In the environment of the regional lymphoid organ, they interact with the T cells via costimulatory molecules, adhesion molecules, and MHC influenced concomitantly by certain cytokines (4). Changes in phenotype and function enable DCs to potently activate T cells and to induce antigen-specific immune responses.

Immunotherapy has developed to a feasible tumor specific therapy. Different strategies have included animal and clinical trials with agents to non-specifically boost immunity, factors to augment specific immunity, transfer of lymphokine-activated killer cells and transfer of expanded populations of tumor-infiltrating lymphocytes. Vaccination strategies addressed the composition of the antigen, whether a defined tumor antigen (purified tumor antigens, recombinant peptide tumor antigens) or an unfractioned mixture of tumor derived antigens (tumor extracts) and the form in which the antigen should be presented (peptides or a nucleic acid) (5). Recently, the use of RNA has been used in tumor vaccination protocols (6). The use of RNA has several potential advantages. RNA can be effectively amplified from a very small number of cells, thus, unlike tumor-extract vaccines, only a small amount of tumor is needed to prepare the material for vaccination. Furthermore, it is not necessary to know the molecular nature of the putative tumor antigen(s). Also, unlike DNA-based vaccines, there is little danger of incorporation of RNA sequences into the host genome.

We have shown previously that coculturing of natural killer (NK)-like T cells with DCs that had been transfected with pancreatic tumor cell line-derived RNA reverses pancreas carcinoma cell resistance by directly triggering NK-like T lymphocytes in vitro (7). To test whether specific T lymphocytes can also be induced in an in vivo system, we used an orthotopic exocrine ductal pancreatic carcinoma model in immunocompetent mice for additional immunotherapeutic experiments. In contrast to other antigen-presenting cells, antigen-pulsed DCs can be administered in situ to prime naive T-helper and CTLs without additional adjuvants. Although direct evidence is limited, several findings suggest that intratumoral (i.t.) DCs play an important role in antitumor immune responses (8). For example, increased numbers of i.t. DCs are associated with better outcomes in patients with a variety of carcinomas (9–12). Evidence also suggests that increasing the number of i.t. DCs in cancer patients with immunomodifiers is beneficial (10). In contrast, especially in patients with pancreatic carcinoma, it has been shown that DCs, if at all present, were located outside the margin of the tumor (11). Although DCs have been shown to acquire antigen from tumor cells in vitro and in vivo, it is not known whether the DCs observed within malignant tissue acquire tumor-associated antigen and migrate to initiate effective antitumor T-lymphocyte responses in vivo. The tumor microenvironment may lack the appropriate proinflammatory signals to differentiate DC precursors. Tumors may actively suppress DCs, which may be a central mechanism to escape immunosurveillance. On the basis of emerging evidence that in vitro physical interaction between DCs and tumor cells is fundamental to the induction of therapeutic immunity, and that DCs are capable of ingesting apoptotic tumor cells and acquiring tumor-associated antigens to induce class I restricted CTLs in vitro (12, 13), we hypothesized that i.t. administration of bone marrow-derived DCs can induce a cytotoxic T-cell response against the tumor.

Therefore, the current study was conducted to test whether i.t. injected tumor RNA-pulsed DC-based immunotherapy can be achieved in an immunogenic murine model of established orthotopic exocrine ductal pancreatic cancer.
INTRATUMORAL DC ADMINISTRATION IN PANCREATIC CANCER

MATERIALS AND METHODS

Mice. Male and female 6–10-week-old mice of the strain C57BL/6 (h-2b) were purchased from Charles River Inc. (Sulzfeld, Germany). Five to nine animals were kept in each cage. Animals were exposed to 12-h-light/12-h-darkness cycles, and standard food and water were provided on request. All of the animal studies were approved by our Institutional Animal Care and Use Committee and were conducted in accordance with the principles and procedures approved by the local regulatory agency (Bezirksregierung Köln: 23.203.2-BN 22, 26/00).

Cell Lines and Culture Medium. PANC02 (kindly provided by Dr. Guillermino Mazzolini, University de Navarra, Pamplona, Spain) is a highly tumorigenic murine pancreatic tumor cell line with ductal morphology that was derived in 1984 from a methylcholanthrene-induced tumor growing in a C57BL/6 mouse (14). It is a ductal adenocarcinoma of the pancreas that has been shown to produce rapidly growing local tumors after s.c. inoculation, which are highly resistant to every known class of clinically active antitumor agent (15). The cell line is tumorigenic after injection of only 10⁵ pancreatic cells into nude or immunocompetent C57BL/6 mice and leads to death from progressive disease in all of the treated animals within a few weeks. PANC02 cells were maintained in RPMI cell culture medium (Life Technologies, Inc., Berlin, Germany) supplemented with 10% FCS (PAA, Co, Germany), 100 units/ml penicillin, and 100 μg/ml streptomycin (Seromed, Jülich, Germany) and incubated at 37°C under 5% CO₂. Cells were passaged by brief trypsinization with 0.025% trypsin. EL4 (kindly provided by Dr. Volker König, Virchow Klinikum, University of Berlin, Berlin, Germany) was established from a lymphoma induced in a C57BL/6 mouse by 9,10-dime-thyl-1,2-benzanthracene. The EL4 cell line grows in suspension and was maintained in DMEM (Life Technologies, Inc.) with 10% FCS. EL4 was given i.p. It also grows in the adjacent area of the pancreas when given into the tail of the pancreas. For better comparison with DC inoculation, we also gave EL4 cells s.c., growing in all of the injected animals.

mAbs and Flow Cytometry. Two-color immunostaining was performed with the use of FITC- and phyceroerythrin-conjugated monoclonal antibodies (mAbs). The following antitumor mAbs and appropriate isotype controls were used: DC characterization: MHC class II, CD14, CD40, CD54, CD80 (PharMingen, Hamburg, Germany), and CD86/B7-2 (Sero tec, Düsseldorf, Germany). DCs were prepared using CD4, CD8, Ly6G, CD45R/B220 (all from Southern Biotechnology Associates, Inc., Birmingham, AL), CD83, and MHC class I H-2Kb (from Beckmann Coulter, Krefeld, Germany). Cells were incubated for 30 min at 4°C with 5 μg/2 × 10⁵ cells rat antimouse mAbs. After washing with PBS containing 0.5% bovine serum, FITC-conjugated mouse antirat IgG (Dianova, Hamburg, Germany) was added for 20 min at 4°C. After washing in PBS, samples were analyzed immediately using an EPICS Profile II flow cytometer (Coulter Electronics, Inc., Hialeah, FL).

Generation of Bone Marrow-Derived DCs. DCs were prepared as described with minor modifications (16). Bone marrow leukocytes flushed from mouse tibia and femur were depleted of erythrocytes by incubation in 0.9% ammonium chloride for 3 min at 37°C, and depleted of T and B lymphocytes and granulocytes by incubation (30 min, 4°C) with rat antimouse CD4, CD8, Ly6G, and CD45R mAbs (Serotec), followed by incubation (15 min; 4°C) with goat antirat IgG conjugated to magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and separation in a magnetic field. Unbound cells were cultured in DC medium consisting of RPMI 1640 containing 10% FCS, 1% l-glutamine, and antibiotics supplemented with recombinant human granulocyte macrophage-colony stimulating factor (200 units/ml; Roche, Mannheim, Germany) and interleukin 4 (Cell Sciences, Norwood, MA). Leukocyte suspension cells were harvested, and expression of MHC class II, CD40, CD54, CD80, and CD86 was quantified by flow cytometry (d1, d3, d5, and d7). In some experiments on day 7 or 9, DCs were additionally stimulated with 5–60 ng/ml murine tumor necrosis factor α (R&D Systems, Minneapolis, MN).

Pulsing of Antigen-Presenting Cells. Pulsing of DCs (5 × 10⁵) with RNA was routinely performed in serum-free RPMI (Life Technologies, Inc.). DCs were washed twice with RPMI. Pulsing was done in six-well plates. The cationic lipid 1,2-dioleyloxy-3-(trimethylammonio)-propan (Boehringer, Mannheim, Germany) was used to deliver RNA into cells (17). According to the manufacturer’s protocol, the content of endotoxin in the cationic lipid complex 1,2-dioleyloxy-3-(trimethylammonio)-propan and the RNA preparation is 0%. A control with 1,2-dioleyloxy-3-(trimethylammonio)-propan alone showed no difference in maturation of DCs (measured by flow cytometry; data not shown). RNA and 1,2-dioleyloxy-3-(trimethylammonio)-propan in a ratio of 1:2.5 were mixed at room temperature for 20 min. The amount of total RNA was 10 μg. The complex was added to the DCs on day 3 after preparation in a total volume of 3–3.5 ml/well, lightly shaken at 37°C for 1 h, and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO₂. Pulsing was stopped after 24 h by changing the medium. DCs were used for tumor treatment on day 6 following preparation after carefully washing twice with PBS.

In Vivo Tumor Inoculation and In Vivo Immunization. Tumor inoculation via ultrasound guidance has been developed by our group (18). Inoculation of tumor cells via high-resolution ultrasound (Sono CT; Philips Medical Systems, Andover, MA) was performed transcutaneously. After induction of anesthesia, animals were placed in a supine position. The abdomen was carefully shaved. For acoustic coupling, ultrasound gel (Maas, Versmold, Germany) centrifuged for 2 min at 2000 × g was used. Transverse horizontal imaging planes were obtained and modified to allow visualization of the long axis of the pancreas. To achieve the greatest benefit from real-time compound imaging, the potential focal lesions were placed in the central region of the image to take advantage of the complete component frame overlap. Image analysis was performed using a calibrated software tool (HDI-Lab, version 1.9.1; Philips Ultrasound, Bothell, WA). The tumor injection via ultrasound was compared with tumor cell inoculation via transabdominal operation with upper left line abdominal incision, tumor cell inoculation, closure of the abdominal cavity, and subsequent tumor size measurements via repetitive laparotomies every 2–4 days. There was a highly significant correlation between the rate of successful tumor inoculation and high accuracy for measuring tumor volume between these two methods. Tumor inoculation (d3 after DC preparation), DC inoculation (d6), and measuring of tumor size (d3, d5, d7, d9, d11, and d14) were performed by high-resolution ultrasound. Tumor cells of 1 × 10⁵–1 × 10⁶ in 0.5 ml of tumor cell suspension were injected into the tail of the pancreas. Pancreatic tumors were treated by saline solution, 10⁵ unpulsed DCs, 5 × 10⁵ DCs pulsed with EL4-derived tumor DNA, or 5 × 10⁶ DCs pulsed with PANC02-derived tumor RNA. A series of six animals was processed with the aforementioned procedure, and each animal was individually examined on days 3, 5, 7, 9, 11, and 14 for measurement of tumor size. Animals were killed when the tumor reached a volume set before the therapy according to the local animal committee requirements or when they became moribund before the expected tumor volume. C57BL/6 mice were immunized by one of three different routes of vaccination: i.t., i.v., and s.c. EL4 cells grew i.p., and in the adjacent structure of the pancreatic tail and after s.c. inoculation. EL4 RNA-pulsed DCs were given i.p. into tumors in the region of the pancreas and into s.c. tumors.

Detection of DCs in Pancreatic Tumors. The 3-day-old established pancreatic tumors of three C57BL/6 mice were injected in vivo under ultrasound control or removed and injected ex vivo under vision with 5 × 10⁵ DCs prepared from syngenic C57BL/6 mice, both within a 5-min period, to confirm that the DCs were injected i.t. Immediately after the injection, a single-cell suspension of each tumor was prepared, and the DCs were enumerated through the positive coexpression of MHCII, CD80, CD86, and CD14 (PharMingen) by flow cytometry analysis.

Antigen-Specific T-Lymphocyte Proliferation and Function. T cells were isolated on day 10 by passing spleen from tumor-bearing C57BL/6 mice, who had been treated with unpulsed DCs, DCs pulsed with PBS, or DCs pulsed with EL4 or PANC02 RNA, through a 70-μm cell strainer (BD Falcon; Becton Dickinson, Heidelberg, Germany), followed by lysis of erythrocytes (Ortho Diagnostic Systems, Neckargemünd, Germany) and magnetic bead conjugate-mediated depletion of B lymphocytes and granulocytes with rat and mouse CD45R and Ly6G mAbs (Serotec). T cells were 60–80% pure as determined by flow cytometry. Bone marrow-derived DCs (5 × 10⁵, unpulsed or pulsed on day 3 with PBS or tumor-derived RNA) were cocultured (day 7) with 1 × 10⁶ T cells for 7 days in culture medium with granulocyte macrophage colony-stimulating factor (100 units/ml) and interleukin 2 (40 ng/ml). After coculture T-cell proliferation assay, analysis of IFN-γ-producing immunologic effector cells and cytotoxicity assay were performed.

T-Cell Proliferation Assay. A 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure proliferation. This is a proliferation assay offering comparable results using [3H]thymidine incorporation. MTT is cleaved by living cells to yield a dark blue formazan product. This process requires active mitochondria, and not even freshly dead cells cleave significant amounts of MTT. Briefly, bone marrow-derived DCs of day 7 were cocultured with 2 × 10^5 T cells, and 72 h at various ratios and plated in 96-well round-bottomed microtiter plates (2 × 10^3/200 µl). After coculturing, 20 µl of MTT stock were added to each well. Plates were incubated in a 5% CO₂ incubator at 37°C for 5 h, media were removed, and 200 µl of DMSO were added to each well. Within 1 h, absorbency was measured at 450 nm.

Analysis of IFN-γ-Producing Immunologic Effector Cell Cultures by Magnetic Cell Sorting Cytokine Secretion Assay. Antigen-specific T-lymphocyte function was evaluated by IFN-γ-producing effector cells determined by MACS cytokine secretion assay (Miltenyi) and carried out according to the manufacturer’s instructions. In brief, after spleen preparation, B-lymphocyte and granulocyte depletion IFN-γ-catch reagent were attached to the cell surface via CD45 antibody. Secreted IFN-γ binds to the catch reagent during a 45-min incubation period. IFN-γ-specific detection antibody conjugated to phycoerythrin was added, and antiphycocerythrin microbeads were used for enrichment on Vario MACS columns.

Cytotoxicity Assay. A nonradioactive cytotoxicity assay, which offers sensitivity comparable with 51Cr release cytotoxicity assay, was used to measure cytotoxic activity (Perkin-Elmer, Rodgau-Jugenheim, Germany). This method is based on loading target cells with an acetoxymethyl ester of fluorescence enhancing ligand (BATDA). The ligand quickly penetrates the cell membrane. Within the cell, the ester bonds are hydrolyzed to form a hydrophilic ligand (TDA), which no longer passes the membrane. After cytolyis, the ligand is released and introduced to a Eu solution. The Eu and the ligand form a high fluorescent and stable chelate (EuTDA). The measured signal correlates directly with the amount of lysed cells. Experiments were performed according to the manufacturer’s protocol. Briefly, target cells (PANC02; 1 × 10^6) were plated in triplicates in a U-bottomed 96-well tissue culture plate and incubated for 2 h (37°C; 5% CO₂ humidified atmosphere) with various ratios of E:T cells. After incubation, 20-µl aliquots from all of the wells were transferred to a fresh 96-well plate. To each well of the plate, 180 µl of the Eu solution mix were added and incubated at room temperature for 15 min on a shaker. Fluorescence data were collected using a 96-well plate in a time-resolved fluorometer (Wallac Oy, Turku, Finland). Maximum release of Eu was performed by incubation of the target cells with the supplied lysis buffer. Target cells without effector cells were used as negative control (spontaneous release). Specific release was calculated as percentage cytotoxicity = experimental release (counts) / spontaneous release (counts) / maximum release (counts) - spontaneous release (counts) of target cells.

CD4, CD8, Granulocyte, and NK Cell Proliferation. Mice were divided into four different groups (each n = 4): healthy (not treated), tumor-bearing mice (not treated), unpulsed DC-treated mice, and tumor (PANC02) RNA-pulsed DC-treated mice. Tumor-bearing mice were treated with a tumor suspension of PANC02 cells. Treated mice received their DCs on day 3. All of the mice were killed on day 7, and the spleens were removed. T-cell isolation was performed as described previously, but no depletion was performed. Cells were incubated with the corresponding antibodies for 15 min on ice. Cells were then washed with PBS containing 1% BSA. Dual-color flow cytometric analysis was performed on a Coulter Epics XL cytometer. Data were collected from 30,000 cells and analyzed. Cells were phenotyped with the following monoclonal markers: CD4, CD8 granulocytes mAb, and NK1.1 mAb (all from Beckman Coulter).

Statistics. Student’s t test was applied to reveal significant differences in tumor protection by stimulated DCs. We compared the mean tumor size of mice (not treated), nonpulsed DC-treated mice, and tumor (PANC02) RNA-pulsed DC-treated mice. Tumors, as expected, grew rapidly. Wang et al. (22) also described PANC02 as extremely aggressive after implantation as manifested by progressive growth in the pancreas, peritoneal dissemination, and distant metastasis to multiple organs, including the liver and lungs.

We observed that potent CTL can be induced by coculture of tumor RNA-pulsed relatively immature DCs in vitro (7). Therefore, we adopted these findings to an in vivo study using an orthotopic cancer model and addressed two theories. We hypothesized that pancreatic tumor growth might be decreased by (a) local injection of DCs pulsed with tumor RNA inducing (b) consecutive generation of tumor-specific CTL.

Effect of tumor RNA-pulsed DCs of antigen-specific T lymphocytes on cytotoxic activity was first shown in vitro. Therefore, 4 days of coculture of splenic-derived lymphocytes from tumor-bearing and treated mice, each 7 days of age, with unpulsed DCs or DCs pulsed with EL4 RNA or PANC02 RNA led to a significant increase in cytotoxic activity as measured in an Eu assay using PANC02 cells as targets (Fig. 1). For example, lyses at an E:T ratio of 50:1 showed 46.6 ± 12.5% for PANC02-pulsed DCs compared with 16.3 ± 4.2% for EL4 RNA-pulsed DC-treated mice (P < 0.05).

The effect of tumor RNA-pulsed DCs on T-cell proliferation of antigen-specific T lymphocytes in vitro was shown because DCs pulsed with PBS or DCs pulsed with RNA derived from EL4 cell line had no effect on proliferation of T cells gained from the spleen of tumor-bearing and i.t.-treated mice (Fig. 2). Intratumoral injection of tumor RNA-pulsed DCs caused a significant proliferation of T cells gained from the spleen (Fig. 2), whereas we detected no significant proliferation from i.v. or s.c. tumor RNA-pulsed DCs (data not shown). At a high DC number (DC:T-cell ratio, 1:10), EL4 RNA-pulsed DCs only marginally activated T cells. Even T-cell proliferation induced by unpulsed DCs was higher. In contrast, PANC02 RNA-pulsed DCs induced high T-cell proliferation, and there was a 2.7-fold increase between these two preparations. Similarly, at lower DC numbers (DC:T-cell ratios, 1:160), we observed no marked differences in the capacity of the respective DCs to induce T-cell proliferation. At a DC:T-cell ratio of 1:40, PANC02-pulsed DCs induced a 5.5-fold increase compared with EL4 RNA-pulsed DCs, but PANC02-pulsed DCs were only marginally more effective at low DC numbers (DC:T-cell ratio, 1:160) than EL4 RNA-pulsed DCs.
supernatants were assayed for IFN-γ or cocultured with tumor RNA-pulsed DCs for 3 days, and the treated mice on day 5. The splenocytes were then either left unpulsed or RNA-pulsed DCs. Lymphocytes were gained from the spleen from RNA-pulsed DC, i.v., or s.c. with either PANC02 RNA- or EL4 RNA-pulsed DCs. Tumor-bearing mice were treated with i.t. tumor RNA-pulsed DCs. Results represent means; bars, ±SE. Similar results were obtained in three separate experiments.

Fig. 2. Tumor RNA pulsing enhances T-cell stimulatory effects of DCs in a specific dependent fashion. DCs were pulsed with water, left unpulsed, pulsed with EL4 (lymphoma) RNA, and PANC02 RNA. After 24 h, pulsing was stopped by changing the medium and cocultured with splenic T cells in a 96-well microtiter plate at the indicated ratio for 72 h. On day 3 of coculturing, cells were used for an MTT assay. Fluorescence at a 20:1 ratio was set 100%. Proliferation of lymphocytes was increased significantly after pulsing with PANC02 RNA compared with EL4, unpulsed DCs, EL4 RNA-pulsed DCs, and water at 20:1 ratio. Results represent means of three independent experiments; bars, ±SE.

We then evaluated the lymphocytes gained from the spleen of immunized mice for the presence of an antigen-specific T helper type 1 (Th-1) cytokine response shown by IFN-γ secretion to tumor RNA-pulsed DCs. Tumor-bearing mice were treated with i.t. tumor RNA-pulsed DC, i.v., or s.c. with either PANC02 RNA- or EL4 RNA-pulsed DCs. Lymphocytes were gained from the spleen from treated mice on day 5. The splenocytes were then either left unpulsed or cocultured with tumor RNA-pulsed DCs for 3 days, and the supernatants were assayed for IFN-γ (Fig. 3). We observed significant IFN-γ secretion from splenocytes when tumor RNA-pulsed DC vaccine was administered i.t. (Fig. 3), and we did not detect IFN-γ secretion from i.v. or s.c. tumor RNA-pulsed DCs. In native effector cell populations, 4.1 ± 1.1 pg IFN-γ-producing cells could be detected (Fig. 3). Coculture of immunologic effector cells with EL4 RNA-pulsed DCs led to an increase of IFN-γ-secreting effector cells to 5.9 ± 1.3 pg. After coculture with tumor RNA-pulsed DCs, the percentage of IFN-γ-producing immunologic effector cells did not increase when using i.v. or s.c. immunization. In contrast, PANC02 RNA-pulsed DCs from mice immunized i.t. produced significant amounts of IFN-γ (211 ± 11.0 pg; P < 0.05) compared with EL4 RNA-pulsed DCs also from orthotopically induced tumors. Others have shown that the route of DC vaccine administration may also have a qualitative influence on the Th-1/Th-2 cytokine response profile. In a study by Morikawa et al. (23), bone marrow-derived DCs were shown to stimulate a Th-1 cytokine response in the draining lymph nodes when administered s.c. and a Th-2 cytokine response when administered i.v. This favorably matches the results by Tanaka et al. (24), who also found that the cytolytic activity of the effector cells was tumor specific, and CD8 and MHCI restricted.

Comparing amounts of CD4, CD8, granulocytes, and NK cells after tumor challenge showed that tumor RNA-pulsed DC (given i.t.)-treated mice had significantly more CD8 cells than tumor-bearing mice (P < 0.0001), unpulsed (P < 0.0001), and healthy mice (P = 0.0001). CD4 cells did not increase significantly after i.t. immunization with tumor RNA-pulsed or unpulsed DCs compared with tumor-bearing mice. Healthy mice had significantly more CD4 cells than those treated with pulsed DCs (P = 0.002). Granulocyte

Fig. 3. After immunization with i.t., s.c., or i.v. tumor RNA-pulsed DCs, and spleens from tumor-bearing mice were removed, cells were prepared for preparation of splenic tumor-specific CD8 T lymphocytes and tested for IFN-γ secretion by ELISA. PANC02 RNA-pulsed DCs induced a more potent, specific Th-1-type cytokine response than EL4 RNA-pulsed DCs. Furthermore, i.v. or s.c. immunization was ineffective in inducing a Th-1-type cytokine response. Results represent means of three independent experiments; bars, ±SE. Statistical significance of differences: i.t. versus i.v., P < 0.05; i.t. versus s.c., P = 0.01.

Fig. 4. Amounts of CD4, CD8, granulocytes (granulo), and NK (NK1.1) cells in the spleen after i.t. immunization were measured via flow cytometry after 7 days in healthy mice (A), 7 days after tumor inoculation (B), and 4 days after treating with unpulsed DCs (C) and tumor RNA-pulsed DCs (D). Results represent means of two independent experiments; bars, ±SE.
Similar results were obtained in three separate experiments. Results represent means; bars, ±SE. Similar results were obtained in three separate experiments.

amounts increased significantly after tumor inoculation, treatment with unpulsed DCs, and tumor RNA-pulsed DCs compared with healthy mice (each $P < 0.05$). Unpulsed DCs induced significantly more NK1.1-positive cells than pulsed DCs ($P = 0.042$; Fig. 4).

We next assessed the influence of the tumor RNA-pulsed DCs on the ability to induce protective antitumor immunity in vivo. C57BL/6 mice were immunized s.c., i.v., or i.t. with a $5 \times 10^5$ dose of tumor RNA-pulsed DCs on day 3. As shown in Fig. 5A, only treatment with i.t. DC administration induced significant antitumor immunity compared with nonimmunized mice ($P \leq 0.05$) and could abrogate the established tumor shown by significantly reduced tumor volume. Three of six mice (50%) showed complete remission of the tumor with significant prolongation of survival. In contrast, i.v. and s.c. injections were ineffective in preventing the growth of orthotopic pancreatic carcinoma (Fig. 5A; $P \geq 0.05$) and in preventing metastasis (Fig. 5B; $P \geq 0.05$). Orthotopic injection of $5 \times 10^5$ DCs generated protective immunity indicating that the efficacy of orthotopic immunization is approximately five times greater than s.c. or i.v. immunization. Treatment with unpulsed DCs also generated a small but less pronounced inhibition of tumor growth (data not shown).

There appears to be some controversy regarding the optimal route of immunization with a DC vaccine and the optimal source of DC. Whereas three studies found that i.v. application was superior to s.c. or i.p. (25, 26), one study described better effects after s.c. immunization (27). According to the literature, application into the lymph nodes may be a superior route of DC vaccine administration (28). None of these studies compared their application techniques with i.t. injection as was done by our group. In addition, our data stress the crucial role of the application mode. The potency of protective immunity induced by i.t. immunization is approximately five times higher than with s.c. or i.v. immunization (Fig. 5A). These results indicate that i.t. injection of tumor RNA-pulsed DCs is the optimal approach. In fact, no mice in the i.t.-treated group had to be killed because of tumor progress. Furthermore, we showed a protective immunity against liver metastases compared with mice immunized s.c. or i.v. (Fig. 5B). Recently, Tong et al. (8) reported that i.t. injection of DCs generated from bone marrow of normal mice considerably enhanced the effect of a systemic chemotherapy in an s.c. colon adenocarcinoma model. The strong antitumor effect in mice was related to the generation of tumor-specific CTL (8). Tanaka et al. (24) investigated antitumor effects of i.t. administration of DCs in mice after low-dose chemotherapy and found complete remission of the treated tumor. There is only one human study dealing with our treatment strategy. Triozzi et al. (29) treated patients with advanced solid tumors with i.t. injection of in vitro-derived DCs, and showed biological activity in terms of lymphocyte infiltration associated with DCs and necrosis.

With respect to source (bone marrow-derived DCs, spleen-derived DCs, and lymphatic system-derived DCs), results concerning inducing immunity are equivocal, and to date we cannot consider any method of DC generation to be superior. Intimate contact between tumor cells and immunologic effector cells appears to be the most important.

To evaluate the tumor specificity of tumor RNA-pulsed DC vaccination and to determine whether these findings can be attributed to a specific antitumor effect, similar experiments were conducted with EL4, a lymphomatous C57BL/6-derived tumor cell line. Fig. 6 demonstrates the effectiveness of orthotopic immunization of different unpulsed and tumor RNA-pulsed DCs. DCs themselves are able to induce cytotoxic effects as shown by tumor volume reduction (30). Because of the rapid cytotoxic effects observed here, we performed coculture experiments of DCs and PANC02 in vitro with different ratios and were not able to detect any realistic ratio to inhibit PANC02 growth (data not shown). The antitumor effect, at least in vivo, can only further be enhanced with PANC02-derived tumor RNA-pulsed
DCs, as shown by significant antitumor immunity compared with EL4-derived tumor RNA-pulsed DCs (P < 0.05). Untreated mice, mice treated with EL4 tumor RNA, those treated with nonpulsed DCs, and groups treated with PBS had to be killed because of tumor progression, following requirements of the local animal committee. The mice treated with PANC02 RNA-pulsed DCs were all killed on day 31, according to the local animal committee requirements. There was no difference in delivering EL4-pulsed DCs i.p. or s.c. (data not shown). This indicates that it is a specific immune reaction, whereas there was no effect against the syngeneic lymphomatous tumor cell line EL4.

In summary, we demonstrated that i.t. injection of a tumor RNA-pulsed DC vaccine generates a potent antitumor immune response. Additional studies are warranted to establish whether this approach can be transferred to humans with pancreatic cancer.

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