Genetically Induced Pancreatic Adenocarcinoma Is Highly Immunogenic and Causes Spontaneous Tumor-Specific Immune Responses

Annette I. Garbe,1 Benjamin Vermeer,1 Jaba Gamrekelashvili,1 Reinhard von Wasielewski,2 Florian R. Greten,3 Astrid M. Westendorf,1 Jan Buer,1 Roland M. Schmid,3 Michael P. Manns,1 Firouzeh Korangy,1 and Tim F. Greten1

Abstract

Treatment options for pancreatic cancer are limited and often ineffective. Immunotherapeutic approaches are one possible option that needs to be evaluated in appropriate animal models. The aim of the present study was to analyze tumor-specific immune responses in a mouse model of pancreatic cancer, which mimics the human disease closely. C57BL/6 EL-TGF-α × Trp53−/− mice, which develop spontaneous ductal pancreatic carcinoma, were generated. EL-TGF-α × Trp53−/− mice developed spontaneous pancreatic tumors with pathomorphologic features close to the human disease. Tumor-specific CD8+ T-cell responses and IgG responses were analyzed in EL-TGF-α × Trp53−/− mice during tumor development and compared with mice with s.c. growing pancreatic tumors. In contrast to spontaneous pancreatic tumors, cell lines generated from these tumors were rejected after s.c. injection into wild-type mice but not in nude or RAG knockout mice. Direct comparison of spontaneous and s.c. injected tumors revealed an impaired infiltration of CD8+ T cells in spontaneous pancreatic tumors, which was also evident after adoptive transfer of tumor-specific T cells. Intratumoral cytokine secretion of tumor necrosis factor-α, IFN-γ, IL-6, and MCP-1 was lower in spontaneous tumors as well as the number of adoptively transferred tumor-specific T cells. Our data provide clear evidence for tumor-specific immune responses in a genetic mouse model for pancreatic carcinoma. Comparative analysis of s.c. injected tumors and spontaneous tumors showed significant differences in tumor-specific immune responses, which will help in improving current immune-based cancer therapies against adenocarcinoma of the pancreas. (Cancer Res 2006; 66(1): 508-16)

Introduction

Adenocarcinoma of the exocrine pancreas is the fourth leading cause of cancer death in the United States (1). Despite efforts in the past 50 years, conventional treatment approaches, such as surgery, radiation, chemotherapy, or combinations of these, have had little effect on the course of this aggressive neoplasm and 5-year survival rates remain below 5% (2). One approach that has shown some promise is immunotherapy (3). Recently, an allogeneic granulocyte macrophage colony-stimulating factor–secreting tumor vaccine was tested in patients who underwent a Whipple procedure. This vaccine was shown to be safe and to induce tumor-specific immunity (4). Immunization with heat shock proteins isolated from pancreatic tumor cells and antibody approaches targeting pancreatic cancer–associated tumor antigens, such as carcinoembryonic antigen, MUC-1, and mutated KRAS, have also undergone clinical testing (5). However, until today, immunotherapeutic approaches have not been tested in a murine model of spontaneous pancreatic adenocarcinoma due to the lack of appropriate murine tumor models (6).

Most immunotherapeutic studies in mice are done using transplantable tumors. However, these experiments do not recapitulate the human disease in any way (7). The tissue that surrounds the tumor, which has been shown to be very important in tumor immunology (8), varies significantly between s.c. injected tumors and tumors growing inside the peritoneal cavity in the gastrointestinal tract. Furthermore, premalignant lesions, which might already induce immune responses (9), cannot be found in s.c. growing tumors. Finally, s.c. tumors usually have different growth kinetics than spontaneously developing tumors (10). Therefore, animal models, which mimic as closely as possible the human cancer for which the therapy or vaccine is designed, should be used (11). There are several transgenic mouse models for spontaneous adenocarcinoma (12), which develop breast and prostate carcinoma. Mice expressing well-characterized human gastrointestinal tumor antigens, such as the carcinoembryonic antigen (CEA) or the adenomatous polyposis coli (APC) gene, have also been described (11).

Different spontaneous pancreatic cancer mouse models have been developed, which either form acinar cell carcinomas (13) or islet cell carcinomas (14–16). By contrast, it has been shown that human ductal pancreatic adenocarcinoma develops through a genetically well-defined multistep progression process (17). Therefore, we decided to use a murine mouse model for spontaneous ductal pancreatic adenocarcinoma, which mimics the human disease very closely.

 Recently, we have described the first murine model for the development of ductal adenocarcinoma of the pancreas (18) with pathomorphologic features and genetic alterations similar to those found in human pancreatic cancer (19). Premalignant tumor lesions can be identified in this model and transgenic mice have been...
shown to develop spontaneous adenocarcinoma of the pancreas within 120 days. Here, we describe tumor-specific cellular and humoral immune responses in EL-TGF-α × Trp53−/− mice after development of spontaneous tumors. In contrast, interestingly, when cell lines were generated in vitro from the spontaneous tumors and s.c. injected, the tumors were rejected. Our data provide clear evidence that spontaneously developing tumors develop tumor-specific immune responses and that s.c. tumor models are poor models for testing cancer vaccines. Finally, our study shows that although spontaneous tumors induce systemic immune responses, these tumors have found mechanisms to evade host immunity.

Materials and Methods

Animals. The p53-deficient mice and the transforming growth factor-α (TGF-α) transgenic mice that express TGF-α-human growth hormone (hGH) fusion gene under the control of the elastase (EL) promotor (EL-TGF-α-hGH transgenic mice, line no. 2261-3) have been previously described (20, 21). EL-TGF-α-hGH transgenic mice were backcrossed for a minimum of 10 generations on C57Bl/6 background. EL-TGF-α × Trp53−/− mice were obtained by crossing EL-TGF-α transgenic mice with Trp53−/− mice. Enhanced green fluorescent protein (EGFP) transgenic mice (22) were obtained from The Jackson Laboratory (Bar Harbor, ME). C57Bl/6 wild-type mice were obtained from Charles River (Sulzfeld, Germany). RAG1−/− mice were kept at Gesellschaft für Biotechnologische Forschung (Braunschweig, Germany). Severe combined immunodeficient beige mice were purchased from The Jackson Laboratory. All mice were kept under specific pathogen-free conditions and all experiments were conducted according to German animal protection guidelines.

Tumor cell lines. The tumor cell lines generated from the spontaneous tumors were named as mPAC for murine adenopancreatic carcinoma. All cell lines were grown in DMEM complete medium (10% FCS, 100 units/mL penicillin, 100 pg/mL streptomycin, 2 mmol/L L-glutamine, 1% NEEA, and 1 mmol/L sodium pyruvate) under standard culture conditions (37°C, 5% CO2). Mycoplasma contamination of cells was excluded by PCR (23).

Western blot analysis. Protein expression analysis in tumors and cell lines was done by Western blot analysis. Equal amounts of total protein were separated on SDS gels and analyzed using a polyclonal guinea pig anti-cytokeratin 8/18 (GP11, Progen, Heidelberg, Germany). Reverse transcription-PCR. Total RNA was isolated from mPAC or control cell lines using RNeasy Kit (Qiagen, Hilden, Germany) according to standard protocols and cdNA was synthesized using reverse transcriptase Superscript II (Life Technologies, Karlsruhe, Germany). Expression of cytokeratin 18 and 19, hGH, and TGF-α was analyzed using specific primers (available upon request).

In vitro growth experiments. To analyze the in vitro growth of the generated cell lines derived from EL-TGF-α × Trp53−/− mice, mPAC were injected s.c. in the hind flank of mice. Tumor development was monitored every other day by measuring the tumor with the metric caliper. Animals did not develop tumors during this time. Histologic analysis of the tumors revealed the typical structures of pancreatic adenocarcinoma with irregular cellular morphology (Fig. 1) (data not shown).

Antibodies and flow cytometry. The monoclonal antibodies against H-2Kb (AF6-88.5), I-A/I-E (M5/114.15.2), CD4 (L3T4; GK1.5), CD8a (Ly-2; 53-6.7), CD25 (PC61) used as FITC, and phycoerythrin were obtained from BD PharMingen (Heidelberg, Germany). Flow cytometry was carried out using a FACSCalibur and CellQuest software (Becton Dickinson, Heidelberg, Germany).

Preparation of single-cell suspensions. Single-cell suspensions were obtained by flushing spleens with DMEM complete medium followed by PBS lysis using erythrocyte lysis buffer (Qiagen) or by disaggregation of mesenteric and inguinal lymph nodes. Cells were washed and passed through a mesh and washed with medium. Single-cell suspensions of tumors were prepared by mechanical dissociation and collagenase/dispace treatment for 45 to 60 minutes at 37°C (Roche Diagnostics, Mannheim, Germany). Cell-cell interactions were disrupted by adding 0.1 mol/L EDTA and cells were washed and passed through a mesh.

CTL assay. mPAC-specific cytotoxicity was assayed in a standard chromium release assay as previously described (24). In brief, mice were immunized by s.c. injection of irradiated (50 Gy) mPAC or irrelevant tumor cell line. After 14 days, pooled mesenteric and inguinal lymph nodes and spleen cells were restimulated for 5 days at 4 × 106 per well in a 24-well culture dish with 1 × 106 mitomycin-treated mPAC (1 mg mitomycin C/1 × 106 cells in 10 mL for 5 hours) in RPMI complete medium containing 50 μmol/L β-mercaptoethanol. Interleukin (IL)-2, 10 units/mL, was added at day 2. After 5 days, the restimulated cells were harvested, washed, and incubated at different ratios with radioactively labeled (100 μCi/106 cells) mPAC or control tumor cell lines. Specific lysis was calculated as follows: (experimental cpm − spontaneous cpm) / (maximum cpm − spontaneous cpm) × 100.

IFN-γ secretion assay. To detect mPAC-specific IFN-γ secretion, the Cytokine Secretion Assay for murine cells (Milenyi Biotech, Bergisch Gladbach, Germany) was used according to the recommendations of the manufacturer. Briefly, 5 × 106 pooled mesenteric and inguinal lymph nodes and spleen cells of EL-TGF-α × Trp53−/− mice or of mice immunized with irradiated mPAC or an irrelevant cell line were stimulated overnight. The next day, IFN-γ secretion by CD8+ T cells was measured and analyzed by fluorescence-activated cell sorting (FACS) according to the instruction of the manufacturer.

Cytometric bead array. To analyze the release of cytokines in tumors, single-cell suspensions were generated from freshly isolated tumors and incubated for 12 hours at 37°C. Supernatants were harvested and cytokine content was determined by cytometric bead array according to the instructions of the manufacturer (BD PharMingen).

Histology. Tumors were immersion fixed in buffered formalin, embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin and eosin. Freshly excised tumors were snap frozen in liquid nitrogen. Sections (6 μm) of frozen tissues were fixed in methanol/acetone and stained with purified anti-CD4 or anti-CD8 antibodies (GK1.5, 53-6.72, BD PharMingen), followed by incubation with rabbit anti-rat immunoglobulin (IgG; DakoCytomation, Copenhagen, Denmark), and visualized using the APAAP system (DakoCytomation). Sections were counterstained with hemalum.

Detection of tumor-specific antibody responses. To determine mPAC-specific serum IgG titers, mPAC and control cell lines were stained using serum (1:50 dilution) obtained from mPAC-bearing mice as primary antibody. A FITC-conjugated goat anti-mouse pan IgG secondary antibody (Southern Biotech, Birmingham, AL) was used to detect bound serum IgG.

Transfer experiments. mPAC-specific T cells were generated from EL-TGF-α × Trp53−/− mice as described above for cytotoxicity analysis. In vitro stimulated cells, 2 × 106, were i.v. injected into mice as indicated. Twelve hours after transfer, tumors were isolated and analyzed as described above.

Results

EL-TGF-α × Trp53−/− mice on C57BL/6 background develop pancreatic tumors. C57BL/6 EL-TGF-hGH mice were crossed with C57BL/6 Trp53−/− mice. As shown in Fig. 1A, 100% of the EL-TGF-α × Trp53−/− mice developed rapidly growing tumors in the pancreas within 120 days after birth. Heterozygous littermates did not develop tumors during this time. Histologic analysis of the pancreatic tumors explanted from C57BL/6 EL-TGF-α × Trp53−/− mice revealed the typical structures of pancreatic adenocarcinoma with irregular cellular morphology (Fig. 1B).

Establishment of murine pancreatic adenocarcinoma cell lines. Cell lines named mPAC (see Materials and Methods) were generated from pancreatic tumors of at least six different EL-TGF-α × Trp53−/− mice and were maintained in culture for more than 20 passages. To confirm the origin of these cell lines, they were analyzed for expression of different cytokeratins and...
expression of TGF-\(\alpha\) and hGH. Western blotting revealed characteristic cytokeratin 8/18 (52.5 and 45.5 kDa) expression as marker for epithelial origin as shown for two of the cell lines mPAC-2 and mPAC-6 in Fig. 1C. This was also confirmed by reverse transcription-PCR (RT-PCR; data not shown). Cytokeratin 19 expression, detected by RT-PCR (174 bp), indicated a ductal origin of mPAC (Fig. 1D). RT-PCR was also done to show TGF-\(\alpha\) expression in the tumor cell lines (Fig. 1D). To confirm that the TGF-\(\alpha\) identified by RT-PCR was not the endogenously expressed TGF-\(\alpha\), but the transgene, expression of hGH was analyzed (Fig. 1E). The 342-bp product specific for hGH could only be found in mPAC but in none of the irrelevant cell lines, such as RMA and NIH3T3, as tested by RT-PCR. As shown in Fig. 1F, FACS analysis using anti-H-2K\(\beta\) and anti-I-A/I-E specific antibodies was done to analyze MHC class I and class II expression on established tumor cell lines. All mPACs analyzed had MHC class I but no class II expression. Individual mPAC showed variable levels of MHC class I expression, ranging from almost negative to highly positive cells.

MHC class I–positive mPAC-6 cells were used for all further experiments unless otherwise mentioned.

Pancreatic tumor cell lines derived from spontaneous pancreatic adenocarcinoma of EL-TGF-\(\alpha\) \(\times\) Trp53\(^{-/-}\) mice are highly immunogenic when subcutaneously transplanted into C57BL/6 wild-type mice. To analyze the immunogenicity of mPACs in vivo, these cells were s.c. injected in immune competent congenic C57BL/6 wild-type mice. Although mPAC grew progressively in vitro, unexpectedly, they were rejected when s.c. injected into mice after 10 to 12 days (Fig. 2A). To prove that this regression is an immune-dependent mechanism, tumor cells were also injected in different immune incompetent mice. Injection of mPAC in immune incompetent RAG1\(^{-/-}\) or nude mice resulted in a slow but progressive outgrowth of the tumor 27 days after injection (Fig. 2A). Similar results were found in severe combined immunodeficient beige mice (data not shown). To analyze if s.c. injected tumor cells were also rejected in tumor-bearing mice, mPAC cells were s.c. injected into 75-day-old EL-TGF-\(\alpha\) \(\times\) Trp53\(^{-/-}\) mice.

Figure 1. EL-TGF-\(\alpha\) \(\times\) Trp53\(^{-/-}\) mice on C57BL/6 background develop malignant pancreatic tumors, from which tumor cell lines were established. A, cumulative tumor incidence in EL-TGF-\(\alpha\) Trp53\(^{-/-}\) mice on C57BL/6 background (\(n = 13\)). B, histologic analysis of a representative pancreatic tumor of an EL-TGF-\(\alpha\) \(\times\) Trp53\(^{-/-}\) mouse on C57BL/6 background stained with H&E (original magnification, \(\times 100\)). C, cytokeratin 8/18 (52.5 and 45.5 kDa) expression of mPAC was shown by Western blot. D, RT-PCR shows cytokeratin 19 expression (174 bp) and the expression of the transgene TGF-\(\alpha\) (158 bp) in mPAC. E, RT-PCR shows expression of hGH (342 bp), which is fused to the EL-TGF-\(\alpha\) construct to produce TGF-\(\alpha\) transgenic mice, in mPAC. F, analysis of MHC class I and class II expression on mPAC cell lines.
transformation had not occurred at this early time point. Therefore, clearly revealed that in contrast to 12-week-old mice, malignant EL-TGF-α tumor growth was measured.

To analyze systemic immune responses, we also analyzed T cells from wild-type mice 2 weeks after s.c. injection of mPAC tumor cells or RMA cells for 14 hours before IFN-γ secretion. The supernatants were analyzed for IFN-γ secretion by ELISA (data not shown).

Lymphocytes of mPAC-immunized mice exhibit cytotoxic activity. Tumor-specific IFN-γ secretion analysis clearly indicated a difference in the number of tumor-specific T cells in mice with spontaneous pancreatic carcinoma and in mice after s.c. injection of tumor cells. To analyze whether this difference would also lead to a different lytic activity of T cells, tumor-specific cytotoxicity of T cells from mice with spontaneous pancreatic adenocarcinoma and with s.c. tumors was analyzed. Two weeks after immunization, T cells from C57BL/6 mice were analyzed in CTL using MHC class I–positive and MHC class I–negative mPAC as targets at the effector-to-target (E/T) ratios indicated. Significant lysis of mPAC tumor cells (42% and 28% at E/T ratios of 100:1 and 11:1) was found only in mice vaccinated with pancreatic carcinoma cells but not after vaccination with an irrelevant cell line (RMA cells), which were used as a negative control in this experiment (Fig. 4A). Additionally, only MHC class I–positive targets were lysed, indicating a CD8–specific T-cell response (Fig. 4B). In contrast, T cells from TGF-α Trp53−/− mice displayed no tumor-specific lysis (Fig. 4C). Finally, splenocytes from mice with spontaneous tumors after s.c. challenge with mPAC tumors were stimulated in vitro and analyzed for their specificity. As shown in Fig. 4D, significant lysis of mPAC tumor cells was observed in these mice.

However, growth kinetics of the injected mPAC cells in tumor-bearing mice was similar to that in wild-type mice (Fig. 2B).

EL-TGF-α × Trp53−/− mice develop tumor-specific cellular immune responses. To analyze systemic immune responses, we next studied antigen specificity of T cells in EL-TGF-α × Trp53−/− mice with and without pancreatic adenocarcinoma. Histologic analysis of pancreas from 6-week-old EL-TGF-α × Trp53−/− mice clearly revealed that in contrast to 12-week-old mice, malignant transformation had not occurred at this early time point. Therefore, we decided to analyze 6- and 12-week-old EL-TGF-α × Trp53−/− mice as well as heterozygous littermate controls. For comparison, we also analyzed T cells from wild-type mice 2 weeks after s.c. injection of mPAC or an irrelevant tumor cell line. T cells were isolated from mice as indicated and were stimulated in vitro with mPAC tumor cells or RMA cells for 14 hours before IFN-γ secretion was determined by FACS. Using mPAC cell line and RMA cells as negative control, we were clearly able to detect 1% tumor-specific CD8+ T cells in mice with spontaneous tumors (Fig. 3A). In contrast, no mPAC-specific T cells were found in non-tumor-bearing 6-week-old EL-TGF-α × Trp53−/− mice, their age-matched EL-TGF-α × Trp53−/− littermate controls, or wild-type mice (Fig. 3A). Moreover, in wild-type mice after s.c. injection of mPAC tumor cells, we were able to detect up to 4% of mPAC-specific CD8+ T cells (Fig. 3B). Tumor specificity of T cells was also confirmed by intracellular cytokine secretion analysis and by ELISA (data not shown).

In vivo growth kinetics of mPAC. A, after s.c. inoculation into immune competent C57BL/6 mice (▲), mPAC-derived tumors grew during the first 10 days and then started to regress. Twenty-one days after injection, no tumor was detectable in C57BL/6 mice. Injection into immune deficient RAG1−/− and nu/nu mice resulted in lagged but progressive outgrowth of the tumor. mPAC-derived tumors grew faster in RAG1−/− than nu/nu mice. B, growth kinetics of s.c. injected mPACs in 75-day-old tumor-bearing EL-TGF-α × Trp53−/− mice. EL-TGF-α × Trp53−/− mice were challenged with mPAC-6 cell line and tumor growth was measured.

Figure 2. In vivo growth kinetics of mPAC. A, after s.c. inoculation into immune competent C57BL/6 mice (▲), mPAC-derived tumors grew during the first 10 days and then started to regress. Twenty-one days after injection, no tumor was detectable in C57BL/6 mice. Injection into immune deficient RAG1−/− and nu/nu mice resulted in lagged but progressive outgrowth of the tumor. mPAC-derived tumors grew faster in RAG1−/− than nu/nu mice. B, growth kinetics of s.c. injected mPACs in 75-day-old tumor-bearing EL-TGF-α × Trp53−/− mice. EL-TGF-α × Trp53−/− mice were challenged with mPAC-6 cell line and tumor growth was measured.

Figure 3. Tumor-specific IFN-γ response. A, pooled splenocytes and lymph node cells of 12-week-old EL-TGF-α × Trp53−/− (top left) and EL-TGF-α × Trp53−/− mice (top right) and of 6-week-old EL-TGF-α × Trp53−/− mice (bottom left) and EL-TGF-α × Trp53−/− mice (bottom middle) were restimulated in vitro with mPAC and analyzed by IFN-γ capture assay. Naïve wild-type were used as controls (top right). FACS analysis revealed that only mice that already bear pancreatic tumors show IFN-γ secretion of CD8+ T cells. B, lymphocytes from mPAC-immunized mice were restimulated in vitro with mPAC and analyzed for IFN-γ secretion. IFN-γ capture assay revealed a strong IFN-γ secretion of CD8+ T cells derived from mPAC immunized mice (left) but not from mice immunized with RMA cells (right).
Protection experiment. Cytokine secretion analysis and cytotoxicity assays showed induction of significant tumor-specific immune responses upon s.c. injection of mPACs. Therefore, EL-TGF-α × Trp53−/− mice were vaccinated by s.c. injection of irradiated mPACs beginning at 6 weeks of age to test if this immune response is strong enough to inhibit development of spontaneous pancreatic tumors in EL-TGF-α × Trp53−/− (Fig. 4E). Three EL-TGF-α × Trp53−/− mice, of ages 6, 7, 9, and 11 weeks, were vaccinated with 1 × 10⁶ mPACs. However, as shown in Fig. 4E, all mice developed spontaneous tumors within 120 days similar to untreated mice (Fig. 1A).

EL-TGF-α × Trp53−/− mice develop tumor-specific IgG antibody responses. Tumor-specific antibody responses have been found in patients with pancreatic carcinoma (25). Therefore, to further validate the immunologic significance of the described tumor model, we analyzed sera from 6- and 12-week-old EL-TGF-α × Trp53−/− mice and their age-matched heterozygous littermates as well as mice after s.c. injection with mPAC for the presence of mPAC-specific antibody responses. A humoral response against mPAC, but not against a control cell line, in the sera of four of nine 12-week-old EL-TGF-α × Trp53−/− mice and a weaker response in the sera of three of seven 6-week-old EL-TGF-α × Trp53−/− mice were analyzed. E, repeated injections of mPACs in 6-week-old EL-TGF-α × Trp53−/− mice (n = 3) did not inhibit development of spontaneous tumors.
could be detected (Fig. 5). As expected, wild-type mice vaccinated with mPAC cells also had a significant mPAC-specific immune response, which was not found in the sera from tumor-free EL-TGF-α Trp53−/− controls. No antibody responses could be detected in the sera of tumor-free heterozygous littermate control mice (0 of 4).

Analysis of infiltrating T cells in spontaneous pancreatic adenocarcinoma and subcutaneous transplanted tumors. Our in vivo experiments clearly showed an immune-mediated rejection of s.c. transplanted tumors whereas in mice with spontaneous pancreatic cancer, the tumors grew progressively. Therefore, we explanted the spontaneous tumors and examined them for the presence of tumor-infiltrating lymphocytes by immunohistochemistry as well as FACS analysis. S.c. transplanted tumors were used as controls. As shown in Fig. 6A, CD4+ T cells were the predominantly infiltrating cells in both tumors whereas significantly less CD8+ T cells were found in spontaneous tumors. To further quantify the number of infiltrating T lymphocytes, single-cell suspensions were prepared from tumors and analyzed by FACS. We were able to quantify cells more accurately and identified 10- to 20-fold more CD8+ T cells in rejected s.c. transplanted tumors (Fig. 6B and C) whereas the difference for CD4+ T cells was less profound. We also analyzed CD25 expression on infiltrating CD4+ T cells in both types of tumors. Interestingly, here we saw that 9% of CD4+ T cells infiltrating s.c. transplanted tumors expressed CD25, in contrast to 1% positive cells in spontaneous tumors (Fig. 6D). Although we have not analyzed the function of these T cells, we have been able to show that the majority of these cells expressed Foxp3 (70% and 90%; Supplementary Data 1), suggesting that these cells could be regulatory T cells infiltrating the tumors.

To analyze the tumor environment as well as the function of tumor infiltrating lymphocytes, we also examined the secretion of various cytokines in tumor explants. Single-cell suspensions were

![Figure 5](image)

**Figure 5.** Humoral responses against mPAC in EL-TGF-α × Trp53−/− mice. Serum from mice as indicated was diluted 1:50 and used to stain mPAC cells (left) and a control tumor cell line (right). An antibody response was observed in mPAC-vaccinated wild-type mice (top) and 12-week-old EL-TGF-α × Trp53−/− mice (second row). Six-week-old EL-TGF-α × Trp53−/− mice exhibit only very slight antibody responses against mPAC (third row), which were not found in 12-week-old tumor-free EL-TGF-α × Trp53−/− littermate controls (bottom).

![Figure 6](image)

**Figure 6.** Tumor infiltration by T cells. A, sections from spontaneous pancreatic tumors (left) and of 7-day-old s.c. injected tumors (right) were analyzed for the presence of CD8+ (top) and CD4+ T cells (bottom). B, FACS analysis of tumor-infiltrating lymphocytes. Tumors were explanted, digested, and tumor-infiltrating cells were analyzed by FACS. A representative FACS of tumor-infiltrating lymphocytes. C, combined data from three independent experiments. D, analysis of CD25 expression on tumor-infiltrating CD4+ T cells.
prepared from explanted tumors and cytokine production was analyzed by cytometric bead array (Fig. 7A). Whereas tumor explants of s.c. injected tumor secreted significant amounts of IFN-γ (18 pg/100 mg tissue), tumor necrosis factor-α (TNF-α; 138 pg/100 mg tissue), IL-6 (7,140 pg/100 mg tissue), and MCP-1 (7,140 pg/100 mg tissue), significantly less was secreted by spontaneous pancreatic tumors (3 pg IFN-γ, 15 pg TNF-α, 183 pg MCP-1, and 591 pg IL-6 per 100 mg of tissue). In summary, only CD4+ T cells, but almost no CD8+ T cells, infiltrated spontaneous tumors and only a low amount of inflammatory cytokines was produced in spontaneous tumors in contrast to s.c. injected tumors.

Adoptive transfer of tumor-specific T cells in mice with spontaneous pancreatic adenocarcinoma. Thus far, our experiments showed a clear humoral and cellular tumor-specific immune response in mice with spontaneous pancreatic tumors. However, in contrast to s.c. injected tumors, these tumors grew progressively and only few CD8+ T cells infiltrated these tumors. Previous studies have shown that tumors develop an intrinsic resistance to leukocyte infiltration, which might impair tumor-specific immune responses (26). This prompted us to adoptively transfer tumor-specific T cells in mice with spontaneous tumors and track them inside the tumors. Because T-cell receptor transgenic T cells are not available for our tumor model, we vaccinated EGFP transgenic C57BL/6 mice (22) with pancreatic tumor cells s.c. and stimulated T cells from these mice in vitro. The use of EGFP transgenic mice, which express green fluorescence protein in all cells, facilitates the detection of transferred T cells into tumor-bearing mice. Additionally, as shown above (Fig. 4), using this approach, we were able to generate tumor-specific, lytic CD8+ T cells. Pancreatic tumor-specific EGFP+ cells were transferred into mice with spontaneous pancreatic tumors and s.c. transplanted tumors. After 12 hours, tumors were explanted and single-cell suspensions were prepared from all tumors for FACS analysis. A clear difference in the number of transferred infiltrating T cells was observed: 5,800 adoptively transferred cells/mg tissue were found in s.c. tumors whereas only 900 cells/mg tissue were found in spontaneous pancreatic tumors (Fig. 7B).

Discussion

In the present study, we have investigated tumor-specific immune responses in a murine model of spontaneous ductal adenocarcinoma of the pancreas. In this model, transgenic mice expressing TGF-α in the pancreas are crossed with p53 knockout mice, resulting in pancreatic tumors, which mimic human tumors closely (19). Therefore, this model is helpful in the identification of new therapeutic options for treatment of pancreatic cancer.

Although cancer vaccines have already been tested in patients with pancreatic cancer (6), only limited preclinical data are available supporting an immunotherapeutic approach for the treatment of pancreatic cancer. In the past, different mouse models for pancreatic cancer have been used. In contrast to the mouse model used in our study, all other mouse models studied thus far are SV40 T antigen–dependant tumor models (11). Whereas the SV40 T antigen provides the opportunity of using SV40 T antigen–specific T cells as well as analyzing antigen-specific immune responses, these mice either develop endocrine pancreatic tumors (26, 27) or acinar adenocarcinomas (13), which differ significantly from human ductal adenocarcinomas (28). In contrast, our study investigates for the first time immune responses in a murine pancreatic tumor model, which mimics the human disease closely.

Until today, most preclinical studies testing immune-based therapies, including whole-cell cancer vaccines, have used transplantable tumors (3). However, host-tumor interactions, which might have a significant effect on the efficiency of immune based therapies, are neglected in these models (29). Therefore, the use of spontaneous tumor models for the evaluation of potential new immunotherapies has been suggested (11). Thus far, most transgenic mouse models used in tumor immunology are based on overexpression of a defined human antigen such as HER-2/neu, CEA, or MUC-1 (30–34), which facilitates the analysis of antigen-specific immune responses. Because the immunodominant tumor antigen in our tumor model was unknown, we first generated tumor cell lines from spontaneous tumors, which were used in analysis of tumor-specific humoral and cellular immune responses. Surprisingly, all of the established murine pancreatic adenocarcinoma cells were rejected after s.c. injection in wild-type mice despite progressive growth in vitro and in vivo in nu/nu and Rag1−/− mice, suggesting an immune-mediated rejection of s.c. injected tumor cells. Injecting mPACs into EL-TGF-α × Trp53−/− mice at different ages revealed important information about the strength of tumor-specific immune responses in these mice. First, whole tumor cell vaccination of 6-week-old EL-TGF-α × Trp53−/−
mice, which are free of pancreatic cancer at this time point, did not delay development of spontaneous pancreatic neoplasms in these mice, suggesting that tumor-specific immune responses induced by s.c. tumors were still not strong enough to impair the development of pancreatic tumors. In addition, rejection of s.c. injected mPACs in tumor-bearing EL-TGF-α × Trp53+/−/C2 mice revealed no evidence for the development of functional tolerance in these mice. Finally, CTL analysis of vaccinated EL-TGF-α × Trp53+/−/C2 mice provided evidence that there is no impairment in the induction of tumor-specific immune responses in these mice. Similar findings have been described in patients with solid tumors (35, 36). Finally, we also provide evidence that it is possible to induce tumor-specific immune responses in tumor-bearing animals; however, s.c. whole-cell vaccinations of immunogenic tumors are not potent enough to inhibit the development of genetically induced tumors in this model.

Our studies clearly show spontaneous tumor-specific immune response in mice with pancreatic tumors and at the same time an immune-mediated rejection of s.c. injected pancreatic tumor cell lines. This observation raises the question why spontaneous tumors progress and s.c. injected tumor cells regress. A series of different experiments was done to look for immunologic differences between spontaneous and s.c. tumors including analysis of tumor infiltrating T cells, cytokine secretion, and adoptive T-cell transfer experiments. A significantly higher number of tumor-infiltrating CD4+ T cells were detected in s.c. tumors when compared with spontaneous pancreatic carcinoma; however, further characterization of these CD4+ T cells did not reveal a significant difference of the phenotype of these cells. Interestingly, a high proportion of these cells expressed CD25 and Foxp3, suggesting a regulatory T-cell phenotype of these cells (37). Tumor-infiltrating CD8+ T cells were found more frequently in s.c. tumors, which were possibly the source of increased cytokine levels in these tumors. Finally, adoptive T-cell transfer experiments were done to investigate whether a difference in T-cell homing could explain the differences observed in spontaneous and s.c. tumors as suggested by others (38) and as observed in MET mice (39). Indeed, a significantly higher number of transferred T cells were found in s.c. tumors, suggesting that T-cell homing could be clearly different in s.c. and spontaneous tumors, leading to the different immune responses observed. One possibility might be that the tumor environment in the spontaneous tumors does not provide inflammatory stimuli for the cells to home and become effector cells.

In summary, our data provide the first insights into tumor-specific cellular and humoral immune responses induced in a novel murine model for ductal pancreatic adenocarcinoma. These extensive studies on mice with spontaneously developing pancreatic adenocarcinoma, as well as on the transplantable mPAC tumor model, provide a powerful tool to characterize the mechanisms leading to the escape of the autochthonous tumors. Our model not only mimics human disease closely but also provides insight into the ability of progressive spontaneous tumors to induce immune impairment. Our findings clearly suggest that the use of transplantable tumors injected s.c., which has long been integral to tumor immunology research, is only a poor model to understand tumor-specific immune responses and, therefore, spontaneous tumor mouse models should rather be used for preclinical testing of possible new therapeutic approaches as suggested by others (11). Furthermore, careful evaluation of the different vaccination studies mentioned above should help develop suitable immunotherapeutic approaches for the prevention and treatment of pancreatic cancer.

References


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Annette I. Garbe, Benjamin Vermeer, Jaba Gamrekelashvili, et al.


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