High Frequencies of Functional Tumor-Reactive T Cells in Bone Marrow and Blood of Pancreatic Cancer Patients

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Abstract

Pancreatic cancer is characterized by aggressive growth and treatment resistance. New approaches include immunotherapeutic strategies but the type and extent of spontaneous immune responses against tumor antigens remains unclear. A dominance of TH2 cytokines in patients' sera reported previously suggests systemic tumor-induced immunosuppression, potentially inhibiting the induction of tumor-reactive T cells. We characterized the localization, frequencies, and functional potential of spontaneously induced memory T cells specific for individual tumor antigens or the tumor-associated antigen mucin-1 in the peripheral blood and bone marrow of 41 pancreatic cancer patients. We found high numbers of tumor-reactive T cells in all bone marrow samples and in 50% of the blood samples. These cells secreted the TH1 cytokine IFN-γ rather than TH2 cytokines upon stimulation with tumor antigens. Although consistently induced during pancreatic cancer, T cells specific for pancreatic antigens were not detected during chronic pancreatitis, suggesting that their evaluation may be of diagnostic use in both diseases. Freshly isolated T cells from cancer patients recognized autologous tumor cells and rejected them *in vitro* and in a xenotransplant model *in vivo*, suggesting their therapeutic potential. Thus, tumor antigen–specific T cell responses occur regularly during pancreatic cancer disease and lead to enrichment of tumor cell–reactive memory T cells in the bone marrow. The bone marrow can therefore be considered an important organ for antitumor immune responses in pancreatic cancer. (Cancer Res 2005; 65(21): 10079-87)

Introduction

Pancreatic cancer is a highly aggressive, treatment refractory disease and the fourth leading cause of cancer death in the U.S. (1). Median survival time is <6 months (2, 3). New approaches which employ the patient's immune system for treatment may improve survival of pancreatic cancer patients in the future. However, pancreatic cancers seem to be capable of immune evasion, immune suppression, or immune modulation. For instance, Fas expression can be depressed or altered in malignant pancreatic tissue, rendering tumor cells more resistant to cytolysis by activated effector T cells (4, 5). Even more importantly, pancreatic tumors were shown to secrete immunomodulatory cytokines such as interleukin (IL)-10 and transforming growth factor-β (TGF-β), which are known to suppress TH1 responses and to support TH2 responses that might down-regulate CTL activity (1, 6). Similarly, previous studies showed a predominance of TH2 cytokines and TH2 T cells in the blood of pancreatic cancer patients, suggesting a general tumor-induced modulation of cellular immune responses (7).

Despite these observations, recent studies with peripheral blood of patients revealed that tumor-specific CTL responses could be generated *in vitro* upon repetitive T cell stimulation, suggesting the presence of tumor-specific CTL precursors in pancreatic cancer patients (8–12). However, a systematic characterization of spontaneous tumor-specific immune responses based on analyses of freshly isolated T cells has not been done to date. Although immune counteracting mechanisms might be especially efficient in the vicinity of solid tumor masses where the tumor microenvironment can limit the effectiveness of circulating antitumor lymphocytes (13) and may also affect immune responses in tumor draining lymph nodes, T cells resident in distant lymphatic organs might be less exposed to local suppressive influences.

Murine bone marrow has recently been shown to be an autonomous organ for T cell priming and generation of memory T cells with specificity for blood-derived and tumor-associated antigens (14) and to be a predominant site of homing, enrichment, and activation of memory T lymphocytes (15, 16). Similarly, in patients with breast cancer, multiple myeloma and malignant melanoma human bone marrow was found to contain large numbers of tumor-reactive memory T cells (17–20). Pancreatic tumor cells have been detected in the blood and bone marrow of 24% to 34% of investigated patients (21, 22) and might provide tumor antigens for induction or maintenance of local specific memory T cell responses.

In the present study, we addressed the question if and to what extent tumor-specific TH1 and cytotoxic effector T cells are generated in pancreatic cancer patients. To this end, we analyzed the presence, frequencies, and the functional properties of memory T cells specific for individual tumor antigens derived from autologous tumor cells or the defined tumor-associated antigen mucin-1 (MUC1), which is overexpressed in >90% of pancreatic cancers.

We compared specific memory responses of freshly isolated T cells from peripheral blood and bone marrow against cultured autologous tumor cells and dendritic cells presenting autologous tumor-associated antigens or synthesized polypeptides containing either the tandem-repeat sequence MUC1 $(137\text{-}157)_5$ (ref. 23) or the signaling sequence MUC1 (1-100) of MUC1. We thereby show for

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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the first time a regular spontaneous induction of tumor-reactive TH1 and cytotoxic T cells in pancreatic cancer patients capable of recognizing antigen-pulsed dendritic cells as well as autologous tumor cells with a predominant enrichment of such cells in the bone marrow.

Materials and Methods

Patients. Bone marrow and peripheral blood samples were taken from 41 patients with primary pancreatic cancer, 11 patients with chronic pancreatitis, and 5 healthy donors. Patients characteristics are depicted in Table 1 (pancreatic cancer) and Table 2 (chronic pancreatitis). Informed consent was obtained from all participants and the protocol was approved by the Ethical Committee at the University of Heidelberg. Bone marrow samples were aspirated from each anterior iliac crest while the patients were still under anesthesia (17), whereas bone marrow of healthy donors was obtained under local anesthesia. The heparinized bone marrow (that may contain some contaminating peripheral blood–derived cells) was subjected to Ficoll gradient (Pharmacia, Uppsala, Sweden) centrifugation and cells in interphase were collected.

Tumors. Tumor samples were obtained after informed consent from patients during surgical resection for preparation of autologous tumor-associated antigens. In addition, tumor tissue could be obtained from some patients to establish primary tumor cell cultures. The fresh tumor samples were immediately transferred into serum-free medium (X-VIVO 20) and stored on ice. After removal of necrotic tissue and fat, tumor biopsies were cut into small pieces, transferred into Petri dishes, and cultured in

Table 1. Characteristics of patients and patient pancreatic tumors

Patient #	Sex	Age	Histologic classification	Grade	Tumor (location)	Node	Metastasis (location)	Operation
1	F	59	ductal adenocarcinoma	×	T_x	×	peritoneal	exploration + bypass
2	F	65	ductal adenocarcinoma	3	T_4 head	0	0	PPPD
3	M	52	ductal adenocarcinoma	2	T_3 head	1	0	Whipple operation
4	M	73	ductal adenocarcinoma	2	T_3 head	0	0	PPPD
5	F	55	ductal adenocarcinoma	×	T_x	×	liver, peritoneal	exploration + bypass
6	M	62	intraductal papillary mucinous cancer	×	T_x	×	liver	exploration + bypass
7	F	77	ductal adenocarcinoma	1	T_4 head	1	0	PPPD
8	M	58	ductal adenocarcinoma	2	T_2 head	1	0	PPPD
9	M	52	ductal adenocarcinoma	×	T_x	×	peritoneal	exploration + bypass
10	M	60	ductal adenocarcinoma	2	T_3 head	1	0	PPPD
11	M	71	ductal adenocarcinoma	2	T_3 head, body	1	0	total pancreatectomy
12	F	59	ductal adenocarcinoma	2	T_3 head	1	0	PPPD
13	F	66	ductal adenocarcinoma	2	T_3 head	0	0	PPPD
14	F	28	ductal adenocarcinoma	×	T_x	×	peritoneal	exploration + bypass
15	F	61	ductal adenocarcinoma	2	T_3 head	1	0	PPPD
16	M	71	ductal adenocarcinoma	2	T_3 head	0	0	Whipple operation
17	F	66	ductal adenocarcinoma	×	T_3 tail	×	liver	left pancreatectomy, liver biopsy
18	M	70	ductal adenocarcinoma	×	T_x	×	liver	exploration + bypass
19	M	60	ductal adenocarcinoma	3	T_3 head	1	0	PPPD
20	M	70	ductal adenocarcinoma	1	T_3 head	1	0	PPPD
21	M	67	ductal adenocarcinoma	×	T_x	×	peritoneal	exploration + bypass
22	F	53	ductal adenocarcinoma	×	T_1 body	×	×	left pancreatectomy
23	M	53	ductal adenocarcinoma	×	T_x	×	liver	exploration, liver biopsy
24	M	70	ductal adenocarcinoma	×	T_3 head	1	0	PPPD
25	M	66	ductal adenocarcinoma	2	T_3 head	1	0	total pancreatectomy
26	M	64	ductal adenocarcinoma	×	T_x	×	liver, peritoneal	exploration liver biopsy
27	F	44	ductal adenocarcinoma	×	T_3	1	0	left pancreatectomy
28	M	79	ductal adenocarcinoma	×	T_x	×	liver, peritoneal	exploration + bypass
29	M	75	adenocarcinoma papilla	2	T_2 head	1	0	PPPD
30	M	14	neuroendocrine cancer	×	T_3 head	×	liver	PPPD + liver biopsy
31	F	41	ductal adenocarcinoma	×	T_4	×	liver	exploration + bypass
32	M	55	ductal adenocarcinoma	3	T_3 head	1	0	Whipple operation
33	M	61	ductal adenocarcinoma	×	T_3 head	1	0	PPPD
34	M	54	ductal adenocarcinoma	×	T_3 head	1	0	PPPD
35	F	72	ductal adenocarcinoma	2	T_3 body	1	0	left pancreatectomy
36	F	65	ductal adenocarcinoma	×	T_3 tail	1	0	left pancreatectomy
37	F	65	ductal adenocarcinoma	3	T_3 tail	1	liver	left pancreatectomy, liver resection
38	M	59	ductal adenocarcinoma	×	T_x	×	liver	exploration + bypass
39	M	65	ductal adenocarcinoma	×	T_x	×	liver, peritoneal	exploration + bypass
40	F	36	ductal adenocarcinoma	×	T_x	×	liver	biopsy
41	M	61	ductal adenocarcinoma	×	T_x	×	peritoneal	exploration + bypass

Abbreviations: PPPD, pylorus-preserving pancreatoduodenectomy; ×, data not available.

Table 2. Clinical data of patients with chronic pancreatitis

Patient #	Sex	Age at operation	Classification	Operation
1	M	50	chronic sclerosing and calcifying pancreatitis	duodenum-preserving pancreatic head resection
2	M	69	chronic fibrosing pancreatitis	left pancreatectomy pancreaticojejunostomy and splenectomy
3	M	36	chronic fibrosing pancreatitis	pancreatic left resection
4	M	63	chronic sclerosing pancreatitis	Whipple operation
5	F	55	chronic calcifying pancreatitis	duodenum-preserving pancreatic head resection
6	M	44	chronic pancreatitis with pancreaticolithiasis	duodenum-preserving pancreatic head resection
7	M	44	chronic sclerosing and calcifying pancreatitis	duodenum-preserving pancreatic head resection
8	M	36	chronic pancreatitis with marked fibrosis and ductal adenocarcinoma	duodenum-preserving pancreatic head resection
9	M	46	chronic pancreatitis with small atrophy and interstitial fibrosis	Whipple operation
10	M	42	chronic sclerosing pancreatitis	pancreatic left resection
11	M	36	chronic sclerosing pancreatitis	duodenum-preserving pancreatic head resection

DMEM + 10% FCS (Sigma, St. Louis, MO). Cultures were split and remaining tissue pieces were removed when tumor cells became confluent. Medium was changed every 2 days. All cultured primary tumor cells grew progressively. Analysis for expression of HLA molecules and cytokeratins revealed that cultures contained mainly epithelial cells. With the exception of one culture which was HLA class I-negative, all tumor cell cultures expressed HLA I molecules. There was hardly any expression of HLA class II molecules. To test their tumorigenicity, we implanted cells from such cultures into nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) mice. All implanted animals developed large, lethal tumors at the injection site, which histologically resembled adenocarcinoma (data not shown).

For transfer into NOD/SCID mice, 5×10^6 tumor cells were injected s.c. in 100 μL PBS per mouse into the flank region. Survival and general performance of mice was monitored daily. Tumor size was measured weekly (volume = length × width^2 × $\pi/6$; ref. 17).

Generation of bone marrow–derived T cells and dendritic cells. To obtain T lymphocytes, bone marrow mononuclear cells were incubated for 13 days in RPMI 1640 (Life Technologies, Eggenstein, Germany) with 10% human AB serum (PromoCell, Heidelberg, Germany), IL-2 (100 units/mL; Chiron, Ratingen, Germany) and IL-4 (60 units/mL; PromoCell) followed by overnight incubation in the same medium without interleukins (17). Depletion of contaminating B lymphocytes and myeloid precursors was done with magnetic beads conjugated with anti-CD19 and anti-CD15 monoclonal antibodies (mAb; both Dynal, Oslo, Norway).

Natural killer cells were removed using anti-CD56 mAbs (Beckman-Coulter, Krefeld, Germany) and magnetic beads conjugated with anti-mouse immunoglobulin mAbs (Dynal). After depletion, the suspension contained 85% to 95% CD3 T cells (~20% were CD8 T cells). To generate dendritic cells, bone marrow mononuclear cells were cultured for 14 days in serum-free X-VIVO 20 medium (Bio Whittaker, Walkersville, MD) with human granulocyte macrophage colony-stimulating factor (50 ng/mL; Behringwerke,

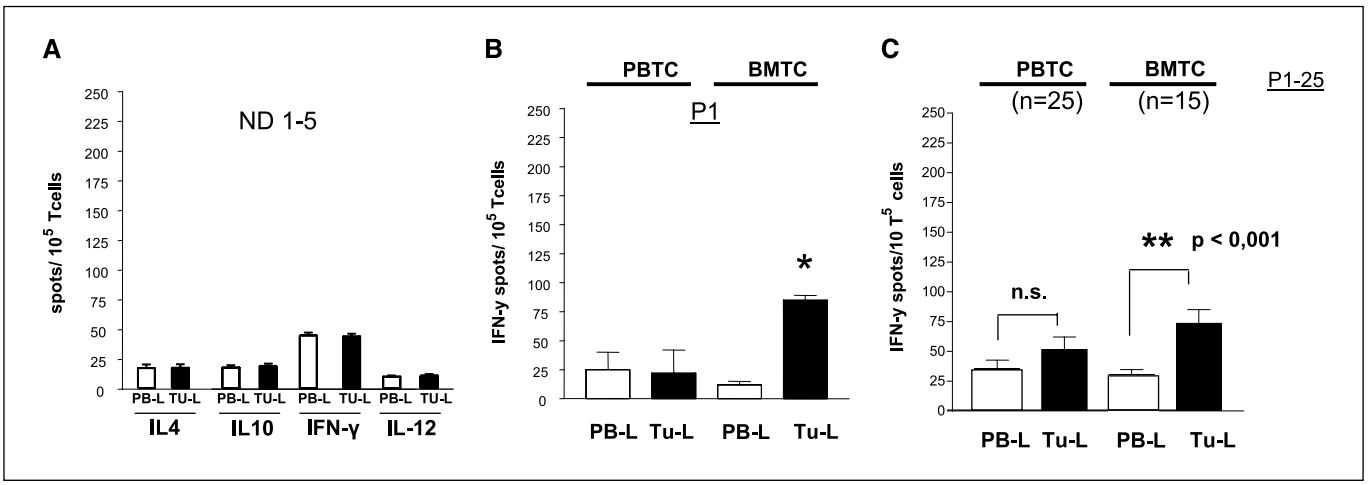

Figure 1. *A,* pancreatic carcinoma lysate does not influence cytokine secretion by T cells from healthy donors. Induction of IL-4, IL-10, IL-12, or IFN-γ secretion by peripheral blood T cells from healthy donors in 40-hour ELISPOT assay upon stimulation with allogeneic lysates from pancreatic cancer (*black columns*) or PBMCs (*white columns*) from the same patient presented by lysate-pulsed autologous dendritic cells. Numbers of cytokine-secreting cells/10^5 purified T cells are shown. *Columns,* means; *bars,* SD from cumulative data of five tested donors. *B,* presence of tumor antigen–reactive memory T cells in bone marrow of a pancreatic cancer patient. IFN-γ ELISPOT analysis of bone marrow T cells (BMTC) and peripheral blood T cells (PBTC) from a pancreatic carcinoma patient (P1). Spot numbers after T cell stimulation with autologous dendritic cells pulsed with autologous tumor lysate (*black columns*) or autologous PBMC lysate (*white columns*); *columns,* mean; *bars,* SD of five wells in each group. One representative out of 25 is shown; *, a significant difference between spot numbers in wells containing tumor lysate compared with control wells. *C,* overall reactivity of T cells from peripheral blood (PBTC) or bone marrow (BMTC) of altogether 25 or 15 corresponding pancreatic cancer patients, stimulated by autologous dendritic cells pulsed with autologous tumor lysate (Tu-L, *black columns*) or autologous PBMC-lysate (PB-L, *white columns*) in short-term IFN-γ ELISPOT assay. *Columns,* mean; *bars,* SD of all tested patients; **; highly significant difference between T cells stimulated with tumor-associated antigen and control antigen; n.s., not significant.

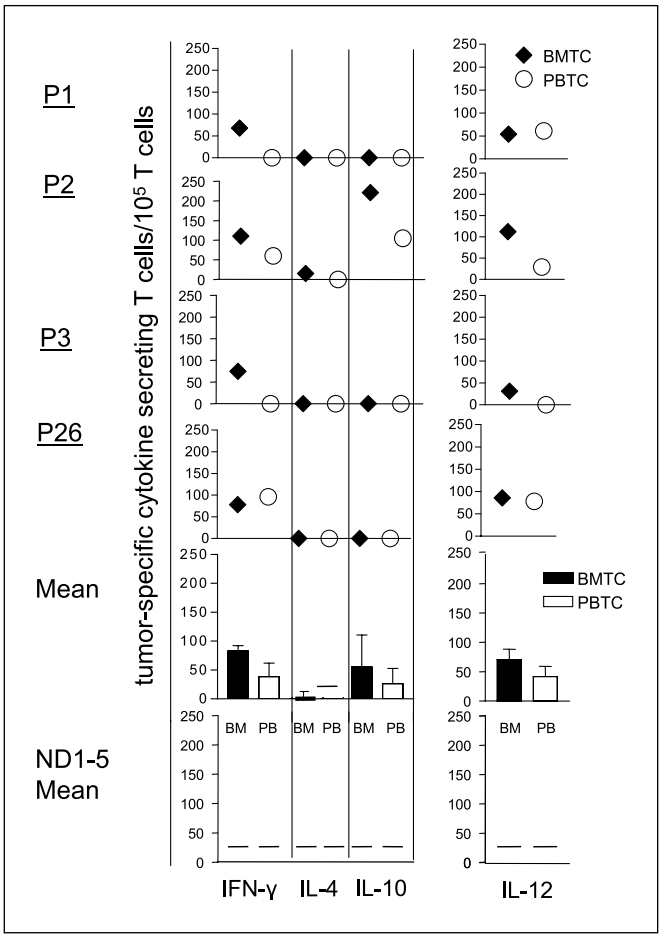

Figure 2. Frequencies of tumor-reactive memory T cells in bone marrow and peripheral blood of pancreatic cancer patients and their capacity to induce antigen-dependent activation of antigen-presenting dendritic cells. IFN-γ, IL-4, IL-10, and IL-12 ELISPOT assays were done using purified T cells from bone marrow (BM or BMTC, ♦/*black columns*) and peripheral blood (PB or PBTC, ○/*white columns*) from four different pancreatic cancer patients (P1-3 and P26) in an autologous setting (using autologous dendritic cells pulsed with autologous tumor- or PBMC-lysates) or from five different healthy donors (ND 1-5) tested in an allogeneic settings (using autologous dendritic cells pulsed with allogeneic tumor- or PBMC-lysates). Values represent the difference between the number of spots observed with the tumor lysates and the PBMC lysates. T cell frequencies in samples containing significantly elevated numbers of cytokine-secreting cells (responders) compared with control wells are shown. Nonresponders are depicted at the bottom line. The lower panels depict mean frequencies of all four patients and all five healthy donors (*thin horizontal lines,* no specific reactivity detected).

Marburg, Germany) and IL-4 (1,000 units/mL; ref. 17). Afterwards, nonadherent dendritic cells were enriched by depletion of contaminating T and B lymphocytes using magnetic beads conjugated with anti-CD3 (Dynal), anti-CD56 (Beckman-Coulter), and anti-CD19 mAbs and pulsed for 20 hours with lysates (200 μg protein/10^6 cells/mL) from autologous tumor (cancer patients), from inflammatory pancreatic tissue (chronic pancreatitis patients), or normal peripheral blood mononuclear cells (PBMC) that were lysed by five freeze/thaw cycles (17) or with the same amount of tetanus toxoid.

IFN-γ ELISPOT assay. IFN-γ producing T lymphocytes were determined as previously described (17). Briefly, dendritic cells pulsed with different lysates or tetanus toxoid were coincubated with autologous T cells (dendritic cell/T cell ratio = 1:5) for 40 hours. The number of IFN-γ spot-forming cells was measured using a microscope Axioplan 2 and KS ELISPOT software (Carl Zeiss Vision, Hallbergmoos, Germany). Spots measured in the presence of dendritic cells pulsed with the autologous PBMC-lysate (as control for lysate-pulsed dendritic cells) or endobulin (as control for tetanus toxoid–pulsed dendritic cells) were considered as nonspecific background (negative control). In some cases, dendritic cells from healthy donors were pulsed with either allogeneic tumor cell lysate or PBMC-lysate from the same patient and analyzed in ELISPOT plates coated with mAbs against IFN-γ, IL-4, IL-10, and IL-12 (Mabtech, Nacka, Sweden) analyzed for respective cytokine secretion as indicators of different endogenous immunomodulating effects of the lysates.

Individuals were designated as responders if the numbers of spots in the presence of dendritic cells loaded with tumor antigens were significantly higher ($P \leq 0.05$ for tumor cell lysates and tetanus toxoid or $P \leq 0.1$ for MUC1-derived peptides) than in negative control wells. The frequency of tumor-reactive bone marrow T lymphocyte was calculated as follows: (spot numbers in wells with tumor peptide–pulsed dendritic cells − spot numbers in negative control wells)/T cell numbers per well.

Mucin-1-derived peptides. Polypeptides containing the tandem-repeat sequence of the MUC1 antigen $(p137\text{-}157)_5$ (ref. 23) and the signaling sequence of MUC1 (p1-100) were synthesized at the peptide facility of the German Cancer Research Center. As negative control antigen for MUC1-derived peptides, human immunoglobulin was used in the same protein concentration.

Cytotoxicity assays. Four-hour ^{51}chromium release assays were done as described (17). Briefly, T cells were stimulated in a coculture with irradiated (100 Gy) autologous tumor cells pretreated with 100 units/mL IFN-γ in a ratio of 5:1 for 3 days. Chromium-labeled autologous tumor cells were used as targets. As control targets, the unrelated, allogeneic tumor cell lines U937 and K562 were used.

For detection of direct cytotoxicity, confluent monolayers of cultured tumor cells were generated in 48-well plates and cocultured with purified peripheral blood T cells in a ratio of 1:5 (test samples) or left untreated (control). For evaluation of antigen specificity, some tumor cultures were blocked with anti-HLA-I mAb prior to coculture with T cells. Dead tumor cells were quantified by trypan blue staining after 48 hours of coculture with autologous peripheral blood T cells.

ELISPOT assays from tumor cell/peripheral blood mononuclear cell cocultures. Tumor cells were cocultured for 48 hours together with unseparated autologous PBMC in a ratio of 1:5 in ELISPOT plates coated with anti-IFN-γ mAb (Mabtech). Detection of spots was done according to the manufacturer's protocol (Mabtech). As controls, aliquots of tumor cells were incubated with anti-HLA I for 1 hour prior to coculture with T cells.

Results

Predominant TH1 profile of tumor-reactive T cells in the bone marrow of pancreatic cancer patients. We assessed the presence and functional character of immune-competent, *ex vivo* tumor antigen–reactive memory T cells in bone marrow or peripheral blood of pancreatic cancer patients using short-term (40 hours) cytokine secretion ELISPOT assays. During such short-term stimulation, antigen-specific cytokine secretion is restricted to memory T cells (data not shown). As a source of individual tumor antigens, we used lysates from autologous tumor cells (test wells) or autologous PBMCs (control wells). Autologous dendritic cells pulsed with such lysates were used without further activation as antigen-presenting cells and allowed for detection of antigen-reactive CD4—as well as CD8 T cells. Allogeneic lysates of pancreatic tumor cells did not induce pronounced cytokine production of T cells (and antigen-presenting cells) from five healthy donors when compared with PBMC lysates from corresponding tumor patients (Fig. 1*A*), demonstrating that potential differences in cytokine contents between tumor- and PBMC lysates are unlikely to account for differences in spot numbers of patients' ELISPOT assays.

In contrast to healthy donors' T cells, T cells from the bone marrow of 15 patients with pancreatic cancer rather than the

peripheral blood of 15 corresponding and 10 additional patients strongly responded to autologous tumor antigen–pulsed dendritic cells with IFN-γ secretion, indicating an enrichment of tumor antigen–reactive memory T cells in that compartment (Fig. 1*B* and *C*; Supplementary Fig. S1).

Analysis of corresponding bone marrow and peripheral blood samples from four patients (Fig. 2; Supplementary Table S1) revealed an enrichment of IFN-γ-secreting T cells in all bone marrow samples. In contrast, tumor-reactive T cells from only one patient secreted IL-10 and IL-4 in response to tumor antigens. This indicates a predominant TH1 profile of tumor-reactive bone marrow T cells. In contrast to bone marrow, we detected tumor antigen–specific memory T cells in corresponding peripheral blood samples of only two of the four patients.

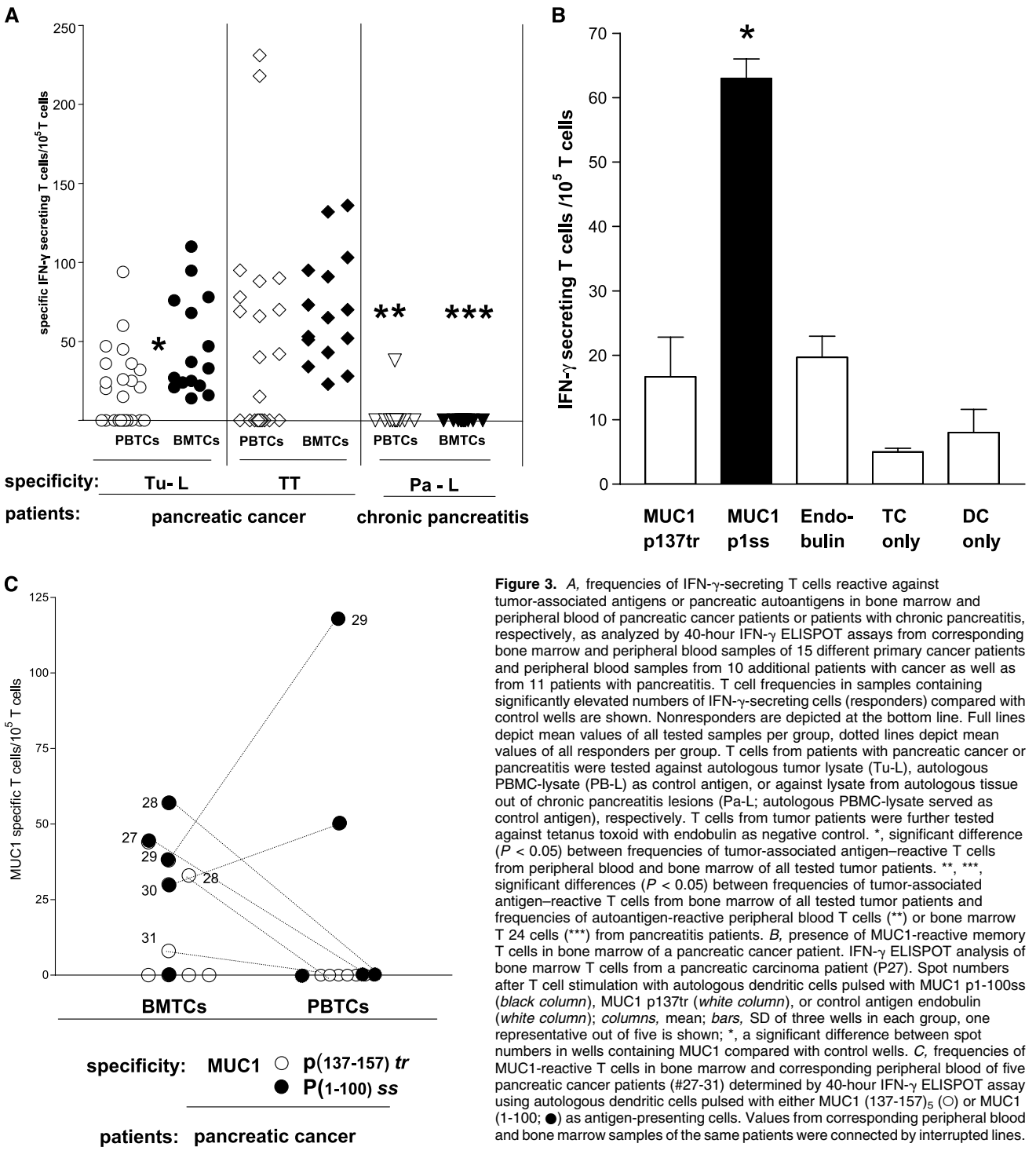

Figure 3. *A,* frequencies of IFN-γ-secreting T cells reactive against tumor-associated antigens or pancreatic autoantigens in bone marrow and peripheral blood of pancreatic cancer patients or patients with chronic pancreatitis, respectively, as analyzed by 40-hour IFN-γ ELISPOT assays from corresponding bone marrow and peripheral blood samples of 15 different primary cancer patients and peripheral blood samples from 10 additional patients with cancer as well as from 11 patients with pancreatitis. T cell frequencies in samples containing significantly elevated numbers of IFN-γ-secreting cells (responders) compared with control wells are shown. Nonresponders are depicted at the bottom line. Full lines depict mean values of all tested samples per group, dotted lines depict mean values of all responders per group. T cells from patients with pancreatic cancer or pancreatitis were tested against autologous tumor lysate (Tu-L), autologous PBMC-lysate (PB-L) as control antigen, or against lysate from autologous tissue out of chronic pancreatitis lesions (Pa-L; autologous PBMC-lysate served as control antigen), respectively. T cells from tumor patients were further tested against tetanus toxoid with endobulin as negative control. *, significant difference ($P < 0.05$) between frequencies of tumor-associated antigen–reactive T cells from peripheral blood and bone marrow of all tested tumor patients. **, ***, significant differences ($P < 0.05$) between frequencies of tumor-associated antigen–reactive T cells from bone marrow of all tested tumor patients and frequencies of autoantigen-reactive peripheral blood T cells (**) or bone marrow T 24 cells (***) from pancreatitis patients. *B,* presence of MUC1-reactive memory T cells in bone marrow of a pancreatic cancer patient. IFN-γ ELISPOT analysis of bone marrow T cells from a pancreatic carcinoma patient (P27). Spot numbers after T cell stimulation with autologous dendritic cells pulsed with MUC1 p1-100ss (*black column*), MUC1 p137tr (*white column*), or control antigen endobulin (*white column*); *columns,* mean; *bars,* SD of three wells in each group, one representative out of five is shown; *, a significant difference between spot numbers in wells containing MUC1 compared with control wells. *C,* frequencies of MUC1-reactive T cells in bone marrow and corresponding peripheral blood of five pancreatic cancer patients (#27-31) determined by 40-hour IFN-γ ELISPOT assay using autologous dendritic cells pulsed with either MUC1 $(137\text{-}157)_5$ (○) or MUC1 (1-100; ●) as antigen-presenting cells. Values from corresponding peripheral blood and bone marrow samples of the same patients were connected by interrupted lines.

TH1 responses are characterized by antigen-specific "cognate" interactions between T cells and antigen-presenting dendritic cells leading to secretion of IL-12 by the dendritic cells (24–26). Similarly, antigen-dependent IL-12 secretion was detected in all four bone marrow samples and in three out of four peripheral blood samples (Fig. 2). In contrast to T cells from pancreatic cancer patients, neither bone marrow nor peripheral blood from five healthy donors contained T cells reacting specifically to tumor antigens or inducing antigen-dependent dendritic cell–activation. Thus, in contrast to previous studies reporting a general predominant TH2 cytokine profile in pancreatic cancer patients, these data indicate a predominant TH1 profile of enriched tumor antigen–reactive memory T cells in the bone marrow.

Tumor antigen–reactive memory T cells are regularly enriched in the bone marrow of pancreatic cancer patients. Short-term IFN-γ ELISPOT assays in the blood of 25 and in corresponding bone marrow from 15 patients revealed the presence of tumor-reactive memory T cells in all (100%) tested bone marrow samples but in only 52% (13 of 25) of blood samples (Fig. 3*A*). The overall numbers of tumor-reactive T cells were significantly higher in bone marrow compared with peripheral blood (Fig. 3*A*; Supplementary Fig. S1) and were comparable to frequencies of T cells reactive against the recall antigen tetanus toxoid (Fig. 3*A*) in the same patients or in healthy donors (data not shown).

This striking enrichment in the bone marrow of IFN-γ-secreting T cells reactive against autologous pancreatic tissue antigens is a unique feature of pancreatic cancer because the bone marrow and blood from a group of 11 patients undergoing pancreatic resection for chronic pancreatitis did not contain memory T cells capable of IFN-γ secretion after activation with autologous pancreatic tissue antigens, although chronic pancreatitis is characterized by phases of strong local and systemic inflammation. Interestingly, the only patient diagnosed for chronic pancreatitis that showed reactivity against autologous pancreas tissue in the peripheral blood contained a small invasive pancreatic carcinoma besides chronic pancreatitis (Fig. 3*A*; Table 2). Thus, the detection of pancreatic antigen-specific TH1 T cells might serve as a useful diagnostic tool to distinguish between pancreatic cancer and chronic pancreatitis in addition to pathologic evaluation.

To evaluate if T cells reactive to a defined tumor-associated antigen are also induced and enriched during the course of pancreatic cancer, we synthesized two 100mer polypeptides from the well-defined tumor-associated antigen MUC1 (23). MUC1 was shown to be expressed in >90% of pancreatic cancers (27). The two polypeptides used contain the signal sequence and the tandem repeat region of MUC1, the latter being described previously to contain HLA-I and -II restricted epitopes that bind to a variety of HLA alleles allowing their application as antigen for functional T cell analyses without selection of distinct HLA types (23). As shown in Fig. 3*B* and *C* (and in Supplementary Fig. S2), we detected functional MUC1-specific T cells in the bone marrow of all five patients but in corresponding peripheral blood samples of only two out of five patients—a finding closely resembling that of the ELISPOT analyses with autologous tumor cell lysates. Although both peptides were recognized by T cells from pancreatic cancer patients, the peptide containing the signal sequence was recognized more frequently.

Direct recognition and rejection of autologous tumor cells by T cells from pancreatic cancer patients. Although we showed the presence and enrichment of functional tumor-specific T cells in bone marrow and peripheral blood of pancreatic cancer patients, their ability to directly recognize life autologous tumor cells remained unclear. We therefore stimulated freshly isolated PBMC from 10 patients with cultured autologous tumor cells for 48 hours in IFN-γ ELISPOT assays. As shown in Fig. 4, the stimulation with live tumor cells induced a significant induction of IFN-γ secretion. Such an effect was blocked partially by anti-HLAI mAb—indicating the presence of HLA-I-restricted T cells capable of recognizing autologous tumor cells. The remaining activity might be either due to replenishment of HLA complexes during the 48-hour coculture, or to natural killer cell activity.

The lytic capacity of purified T cells from altogether eight patients was tested in a 4-hour chromium release assay after a 3-day short-term stimulation by autologous, irradiated tumor cells against autologous tumor cells or the allogeneic cell lines U937 or K562 (as specificity control). Significant specific autologous tumor cell lysis was detected in seven out of eight patients (Fig. 5*A*).

We wondered if pancreatic cancer patients might harbor cytotoxic T cells capable of direct lysis of autologous tumor cells without prior stimulation. We therefore coincubated freshly isolated and purified T cells with autologous tumor cells at a ratio of 5:1 for 48 hours. Under these conditions, tumor cell destruction as quantified by trypan blue staining of tumor cells was observed in all four tested peripheral blood samples (Fig. 5*B*). Blocking of HLA-I significantly reduced the number of dead tumor cells in all experiments, although not completely to control levels, suggesting reexpression of unblocked HLA-I complexes on the tumor cell surface during the culture. These results show that tumor cells can directly reactivate primed T cells, i.e., memory CTL precursors.

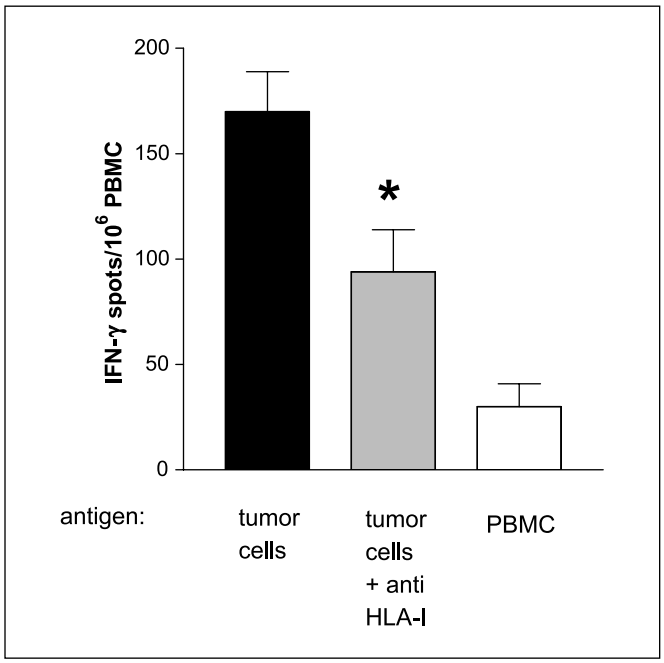

Figure 4. Direct, HLA-I-dependent recognition of autologous tumor cells by memory T cells from pancreatic cancer patients. PBMC from pancreatic cancer patients were analyzed in 40-hour IFN-γ ELISPOT assay for reactivity against cultured autologous tumor cells (T cell/tumor cell ratio, 5:1, *black column*), autologous tumor cells coincubated with anti-HLA-I mAb (*gray column*) or for reactivity against autologous PBMC as control (*white column*). Mean spot numbers and SD from 10 (*black column*) and 5 corresponding peripheral blood samples (*gray* and *white columns*) per 10^6 PBMC are depicted.

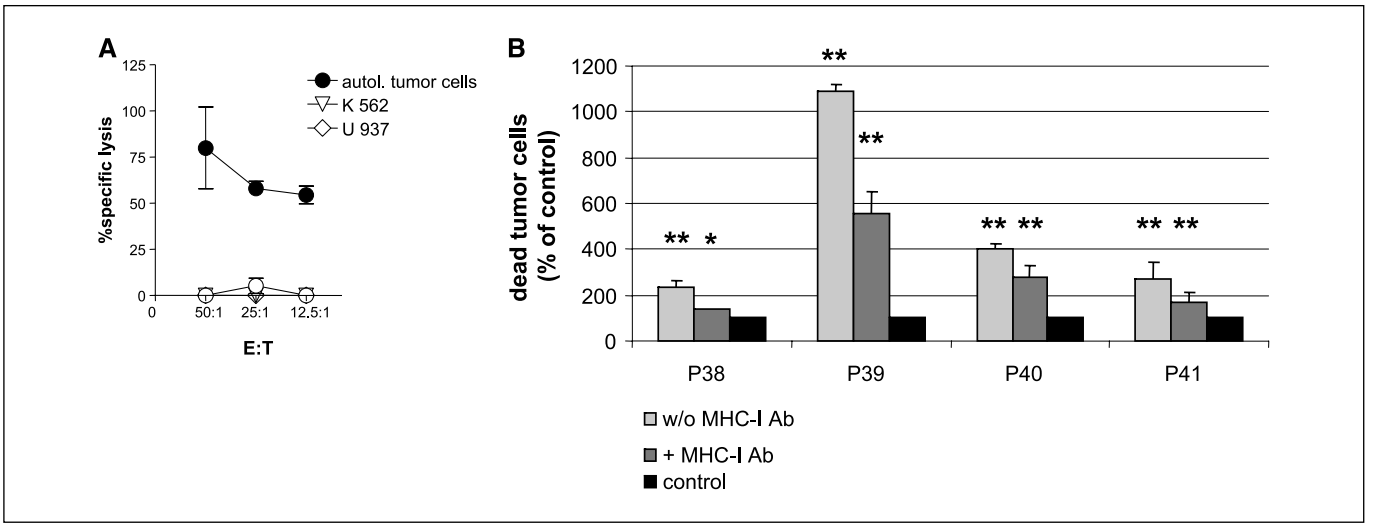

Figure 5. Analysis of CTL activity in T cells from cancer patients against autologous tumor cells. *A,* four-hour ^{51}Cr release assay of purified T cells from a pancreatic cancer patient stimulated in 3-day short-term cocultures with IFN-γ stimulated, autologous tumor cells and tested for lytic activity against unmodified autologous tumor cells (●) or irrelevant allogeneic control tumor lines U937 (promonocytic leukemia, ◇) or NK-sensitive K562 cell line (erythromyeloid leukemia cell line, ▽). One representative out of eight experiments is shown. *B,* HLA-I 25-dependent induction of tumor cell death during 48 hours of coculture with freshly isolated, purified autologous peripheral blood T cells from four different cancer patients. Cultured tumor cells were cocultured with purified peripheral blood T cells in a ratio of 1:5 (test samples, *light gray columns*) or left untreated (control, *black columns*). For evaluation of antigen specificity, some tumor cultures were blocked with anti-HLA-I mAb prior to coculture with T cells (*dark gray columns*). Dead tumor cells were quantified after 48 hours of coculture with autologous T cells by trypan blue staining.

To evaluate the potential therapeutic capacity of reactivated memory T cells derived from pancreatic cancer patients *in vivo*, we transplanted tumor cells from three patients s.c. into NOD/SCID mice. All three patients had detectable tumor-reactive memory T cells in their bone marrow or peripheral blood. Tumor cells from patient P10, which did not express MHC molecules served as a control. All mice had developed progressively growing tumors before treatment. Thirty-five days after tumor injection, 5×10^6 purified T cells from peripheral blood (P8) or bone marrow (P12) of patients were cocultured overnight with autologous dendritic cells, which had been pulsed with lysate from autologous tumor cell lines and applied along with the dendritic cells i.v. to the tumor-bearing mice. As shown in Fig. 6, tumor growth was either not affected in the HLA-negative control (P10), was delayed (P8), or it was strongly inhibited (P12). The differences seen between P8 and P12 could have been due to the large difference in the size of the tumors at the time of the treatment, a tumor of 600 mm^3 (P8) compared with a tumor of 100 mm^3 (P12). The results suggest that reactivated tumor-reactive memory T cells from late-stage pancreatic cancer patients have the potential to exert antitumor effects *in vivo*.

Discussion

This study shows for the first time, for pancreatic cancer patients, the regular existence of tumor-reactive memory T cells in their bone marrow and to a lesser extent in peripheral blood. These cells display a predominant TH1 cytokine profile, secrete IFN-γ upon stimulation by tumor antigen–presenting dendritic cells and are able to kill autologous tumor cells *in vitro* and to reject autologous tumor xenografts, thereby revealing their therapeutic potential.

Previous studies on pancreatic cancer detected T cells from peripheral blood reactive to synthetic or allogeneic tumor-associated antigens upon repeated stimulations (8–11). Here, we analyzed a nonmodified repertoire of freshly isolated T cells and tested it directly for reactivity against autologous antigens upon one short-term restimulation. Because circulating T cells may not be representative for lymphatic organs (which are sites for generation and storage of T cells), we also analyzed T cells from the bone marrow of pancreatic cancer patients. Bone marrow is an intriguing organ for T cell immunity because: (*a*) it is an organ for homing of blood-derived T cells and contains resident dendritic cells and monocytes/macrophages which can function as antigen-presenting cells (14–16); (*b*) it has been recently shown to be a site of naïve T cell priming and memory T cell generation against blood-borne tumor-associated antigens (14); (*c*) in breast cancer patients, bone marrow has been shown to be enriched with therapeutically relevant tumor-reactive memory T cells (17, 18, 24); (*d*) in pancreatic cancer, bone marrow is known to be a homing site for disseminated tumor cells (21, 22); and (*e*) in comparison to tumor-draining lymph nodes, bone marrow is not so directly influenced by immunomodulatory factors from the tumor and may therefore allow polarization of TH1 responses.

Despite an observed predominance of TH2 responses in pancreatic cancer patients which was attributed to the secretion of IL-10 and TGF-β by the tumor microenvironment (7), we found strong cytotoxic and TH1 responses characterized by induction of IFN-γ in the peripheral blood and bone marrow of tested patients. Such TH1 polarization requires release of IL-12 by antigen-presenting cells during the priming of naïve T cells (25, 26). IL-12 is induced in antigen-presenting cells during cognate interactions with TH1 cells (26).

However, its production is suppressed upon stimulation with IL-10 and TGF-β. These cytokines are secreted in the microenvironment of pancreatic tumors. Thus, antigen-presenting cells that take up antigen in the vicinity of solid tumor masses may be influenced to induce tolerance or TH2 responses rather than TH1 responses in the draining lymph nodes. In contrast, the number of

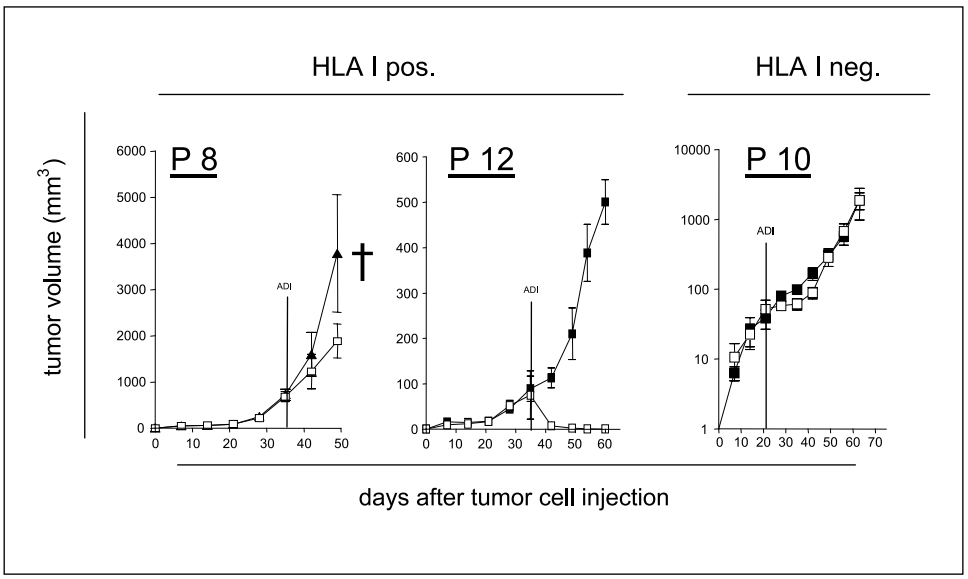

Figure 6. Growth inhibition of xenotransplanted tumors *in vivo* by autologous T cells preactivated with tumor-associated antigen–pulsed dendritic cells. Mice were injected with tumor cells from three different patients. After 35 (P8 and P12) or 21 (P10) days of progressive tumor growth, dendritic cells from the patients were pulsed with lysate from autologous tumor cell lines followed by 20-hour coculture with autologous bone marrow (P12) or peripheral blood (P8) T cells. Then, animals were treated by i.p. injection of 5×10^6 T cells and 1×10^6 dendritic cells (■) or were left untreated (□). As control, animals bearing HLA-I-negative tumors were treated similarly with autologous, tumor-associated antigen–activated T cells, and dendritic cells (P10). Three animals per group and time point were analyzed. *Columns,* mean; *bars,* ± SD of tumor diameters.

disseminated tumor cells found in bone marrow may not be sufficient to secrete enough of these immunomodulating cytokines to affect the generation and maintenance of TH1 cells in that microenvironment.

We hypothesized that the maintenance of a tumor-reactive memory T cell population in the bone marrow is a special property of this compartment. Such memory T cells could be hidden in the bone marrow and thus be out of reach of immunosuppressive influences from the primary tumor. Previous studies on murine models showed that tumor-specific cytotoxic memory T cells were induced in the bone marrow by disseminated tumor cells. These were kept under active control by CD8 memory T cells which prevented the outgrowth of distant metastases (28, 29). It cannot be excluded that pancreatic cancer induces a similar situation of continuous generation of immune competent tumor-reactive T cells controlling disseminated tumor cells in the bone marrow, whereas proportions of these cells upon release into the circulation are lost at the tumor site due to tumor-derived immunosuppressive factors.

Efficient activation of T cells including their TH1 polarization, particularly against low-affinity antigens such as the majority of tumor-associated antigens, requires stimulation by activated, mature antigen-presenting cells. Such activation can be achieved via cognate interactions of antigen-presenting cells via CD40/CD40 L interactions with antigen-specific, activated T cells, by pattern recognition signals present on pathogens, such as bacteria and viruses, or through endogenous Toll-like receptor-activating agents such as heparin sulfate (25, 30). Heparin sulfate is released by a matrix-degrading enzyme, heparanase, which is involved in the metastatic potential of tumor cells and which is strongly overexpressed in many tumors including pancreatic cancer (31). Thus, promalignant tumor cell–derived factors could facilitate or even induce an immune response in the absence of exogeneous inflammatory stimuli.

Besides a potential induction within the bone marrow, circulating tumor-reactive memory T cells induced elsewhere might also accumulate in the bone marrow upon selective homing, where they may acquire an activated state due to an appropriate microenvironment (32, 33). This possibility is supported by the observation of a similar enrichment of tetanus toxoid–reactive T cells in that compartment, whereas long-term persistence of tetanus toxoid in the bone marrow of the patients seems unlikely.

Although tumor-immune memory T cells were found in the bone marrow of all tested pancreatic cancer patients, no such specific cells could be found in the bone marrow of patients with chronic pancreatitis. This difference is not only of theoretical interest but may be very useful as an additional diagnostic test parameter for distinction between chronic pancreatitis and pancreatic cancer, which is currently based solely on histopathologic assessment.

A comparison of tumor-associated antigen–specific T cell responses with those against recall antigens from the same patients revealed similar numbers of memory T cells reactive to tetanus toxoid and tumor-associated antigens in the peripheral blood of cancer patients. The levels of tetanus toxoid–reactive T cells from the blood of healthy donors were similar to those of healthy donors. Therefore, the prevalence of tumor antigen–specific memory T cells in patients with pancreatic cancer can be considered rather high, in the same order as T cells reactive to tetanus toxoid.

In conclusion, our data show for the first time the regular occurrence of tumor antigen–specific T cell responses during the course of pancreatic cancer leading to the generation and enrichment of tumor-immune memory T cells. These T cell responses are of TH1 rather than TH2 type and induce the generation of cytotoxic T cells capable of direct tumor cell recognition and destruction. Because such T cells could be isolated from the bone marrow of all tested patients but only from ~50% of the tested peripheral blood samples, the bone marrow seems to play an important role in the generation and storage of immune-competent, tumor-reactive T lymphocytes.

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