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Research Article

Anti–Tumor Necrosis Factor Therapy Inhibits Pancreatic Tumor Growth and Metastasis

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Abstract

Chronic inflammation has been implicated in the pathogenesis of many severe autoimmune disorders, as well as in diabetes, pulmonary diseases, and cancer. Inflammation accompanies most solid cancers including pancreatic ductal adenocarcinoma (PDAC), one of the most fatal cancers with surgery being the only curative therapeutic approach currently available. In the present work, we investigated the role of the major proinflammatory cytokine tumor necrosis factor α (TNFα) in the malignancy of PDAC cells *in vitro* and *in vivo*. *In vitro*, TNFα strongly increased invasiveness of Colo357, BxPc3, and PancTuI cells and showed only moderate antiproliferative effect. TNFα treatment of mice bearing orthotopically growing PDAC tumors led to dramatically enhanced tumor growth and metastasis. Notably, we found that PDAC cells themselves secrete TNFα. Although inhibition of TNFα with infliximab or etanercept only marginally affected proliferation and invasiveness of PDAC cells *in vitro*, both reagents exerted strong antitumoral effects *in vivo*. In severe combined immunodeficient mice with orthotopically growing Colo357, BxPc3, or PancTuI tumors, human-specific anti-TNF antibody infliximab reduced tumor growth and metastasis by about 30% and 50%, respectively. Importantly, in a PDAC resection model performed with PancTuI cells, we found an even stronger therapeutic effect for both anti-TNF compounds. Infliximab and etanercept reduced the number of liver metastases by 69% and 42%, respectively, as well as volumes of recurrent tumors by 73% and 51%. Thus, tumor cell–derived TNFα plays a profound role in malignancy of PDAC, and inhibition of TNFα represents a promising therapeutic option particularly in adjuvant therapy after subtotal pancreatectomy. [Cancer Res 2008;68(5):1443–50]

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a fatal disease, evidenced by the fact that the annual mortality rates are close to the incidence rates (1). The characteristic features of PDAC cells are uncontrolled proliferation, high metastatic potential, and resistance to chemotherapy, radiotherapy, and immunotherapy (2). Among the therapeutic options for PDAC patients, only surgical resection offers the chance for cure, but this can only be performed in 10% to 15% of patients (3). Although in experienced centers, the standard duodenopancreatectomy (Kausch-Whipple's procedure) for resection of PDAC is associated with a mortality rate below 5%, the prognosis for these patients still remains poor. Even after tumor resection with histologically free margins (R0 resection), local recurrence develop within 2 years in 80% of the patients (4). Little is known about the precise mechanisms of PDAC recurrence, but it occurs especially at the site of primary resection or in the liver (3, 5). This implies that at the time of diagnosis, subclinical metastases or disseminated tumor cells are already present in many patients with no evidence of advanced disease (6) and might change their biological behavior upon surgical manipulation. In accordance with the latter, we recently observed a 3-fold increase in the number of liver and spleen metastases in an orthotopic xenotransplant animal model in mice after pancreatic tumor resection (7, 8). Several *in vitro* and *in vivo* studies with other tumor entities also pointed to a crucial role of surgery-associated inflammatory reactions for local tumor recurrence (9–11). It has been shown that surgery-associated inflammatory reactions are not limited to the resection area but spread out systemically (12, 13). Even enhanced tumor recurrence at distant sites has been observed after surgery (14). Elevated levels of the proinflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor α (TNFα) in the peripheral blood after major abdominal surgery have been described (12, 13). Moreover, enhanced pancreatic tumor cell adhesion to microvascular endothelial cells has been observed after application of IL-1β and TNFα (15, 16). It might be therefore reasonable to hypothesize that proinflammatory mediators such as the aforementioned cytokines play an important role in the hematogenic metastasis of cancer cells after surgical trauma. Pancreatic carcinomas are usually infiltrated with a variety of immune cells including macrophages (17), and increased levels of TNFα were detected in blood of patients suffering from PDAC (18, 19). Moreover, it has been shown that pancreatic tumor cells can secrete TNFα, and this endogenous TNFα confers protection against TNFα- and Adriamycin-mediated apoptosis (20). Thus, there is evidence that PDAC cells *in situ* are regularly exposed to either endogenous (produced in an autocrine fashion) or exogenous (produced by immune or stromal cells) TNFα, pointing to a possible involvement of this cytokine not only in the development and progression of PDAC but also in surgery-associated tumor recurrence and metastasis.

In our present study, we have analyzed the effects of exogenous and tumor cell–derived TNFα on PDAC cells growth and

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

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invasiveness *in vitro* and *in vivo*. To inhibit TNFα, we have used the established TNF-neutralizing drugs infliximab (Remicade™) and etanercept (Enbrel™). We show in a murine orthotopic xenotransplantation model that tumor recurrence and metastasis after surgical resection of PDAC is substantially driven by tumor cell–derived TNFα. Furthermore, we show a significant contribution of endogenous TNFα to the growth and invasiveness of primary PDAC tumors. Therefore, our results strongly suggest that infliximab and etanercept that are broadly used for treatment of chronic inflammatory diseases will also be beneficial in PDAC treatment especially after pancreaticoduodenectomy.

Materials and Methods

Cell culture. Human pancreatic adenocarcinoma cells PancTuI, Colo357, and BxPc3 were cultured in RPMI 1640, supplemented with 10% FCS, 2 mmol/L glutamine, and 1 mmol/L sodium pyruvate. For stimulation experiments, cells were treated with following reagents: TNFα (Knoll AG), etanercept (Enbrel; Wyeth Pharma), or infliximab (Remicade; Essex Pharma). For animal experiments, cells were trypsinized, resuspended in Matrigel (BD Bioscience) at a concentration of 10^6 cells/mL, and stored on ice until injection.

Proliferation assays. Proliferation was determined by cell counting as well as by measurement of [^{3}H]thymidine incorporation. Cell counting was performed using a CASY TT-cell counter (Schärfe System) according to the manufacturer's instructions. For measurement of [^{3}H]thymidine incorporation, cells were seeded in 96-well plates treated with TNFα, etanercept, infliximab, or left untreated for 24 or 48 h and labeled with [^{3}H]thymidine (370 kBq/μL; Amersham-Buchler) for 3 h. Subsequently, cells were harvested and counted in a liquid scintillation counter.

JAM assay. For determination of DNA fragmentation, JAM assay was performed accordingly to the previously published protocol (21). The percentage of the target cell viability measured as percentage of high molecular weight DNA retained on glass fiber filters was calculated as: % viability = $(E/S) \times 100$, where E (experimental) is cpm of retained DNA in the presence of apoptosis-inducing agent under study, and S (spontaneous) is cpm of retained DNA of untreated control cells.

Electrophoretic mobility shift assay. For detection of the AP1 and nuclear factor-κB (NF-κB) activity, nuclear extracts were prepared as described (22). Electrophoretic mobility shift assays (EMSA) were performed by analyzing 5 μg of nuclear extract with the Gelshift AP1 or NF-κB family (Carcinoma) kits (Active Motif) following the instructions of the manufacturer. The samples were separated on native 6% polyacrylamide gels in low-ionic strength buffer (0.25 × Tris-borate-EDTA) and visualized by autoradiography.

Urokinase-type plasminogen activator and IL-8 ELISA. For determination of urokinase-type plasminogen activator (uPA) and IL-8 concentrations in culture supernatants, uPA (American Diagnostica) or IL-8 immunoassays (R&D Systems) were used according to the provided protocols.

Gelatin zymography. The analysis of the levels of matrix metalloproteinases (MMP) in cell culture supernatants was performed by zymography as described previously (23).

Invasion assay. The invasive potential of tumor cells was determined using a trypan blue dye–based model for cell invasion (23). Briefly, KiF-5 fibroblasts were seeded in a 24-well plate (2.5×10^5 cells per well). After 4 days, cells were rinsed with PBS, permeabilized with 500 μL DMSO for 1 h at room temperature, washed twice with PBS, and overlayed with tumor cells (2×10^4 per well in culture medium). After 24 h, the medium was removed and cells received fresh culture medium with or without TNFα (50 ng/mL), etanercept (10 μg/mL), or infliximab (10 μg/mL). After additional 24 to 48 h, cells were washed with PBS, stained for 15 min with 0.2% trypan blue (Invitrogen), washed twice with PBS, and photographed. Because trypan blue stained the dead permeabilized cells, the fibroblasts layer could be distinguished from the living carcinoma cells. The observed areas of unstained cells represent regions where fibroblasts were displaced or digested by invasive tumor cells.

Orthotopic xenotransplantation of human PDAC cells and tumor resection. Four-week-old female severe combined immunodeficient beige (SCID/bg) mice weighing 14 to 19 g were obtained from Harlan-Winkelmann. The mice were allowed to become acclimatized for 10 days and housed in a sterile environment, in which bedding, food, and water were autoclaved (Scantainer). Animal experiments and care were in accordance with the guidelines of institutional authorities and approved by local authorities [number, V 362-72241.121 (16-1/06) and V 312-72241.121-7 (6-1/07)].

To study the effect of exogenously added TNFα on PDAC tumors *in vivo*, tumor- bearing mice were randomly assigned into two groups (n = 10 each). Mice of one group were treated i.p. with 10 μg TNFα (200 μL) on days 3, 6, 9, and 12 after tumor cell inoculation, whereas control mice were injected using the same scheme with a 0.9% saline (200 μL). All animals were sacrificed 31 days postoperatively, and organs and tumors were examined as described previously (24).

To determine the relevance of tumor cell–derived TNFα, mice were orthotopically inoculated with BxPc3, Colo357, and PancTuI cells and i.p. treated either with infliximab (10 μg/g body weight) on days 3, 10, 17, and 24 after tumor cell inoculation or with etanercept (5 μg/g body weight) on days 3, 6, 10, 13, 17, 20, 24, and 27.

Tumor resection experiments were done with mice bearing PancTuI tumors. Ten days after inoculation of tumor cells, relaparotomy was performed and the tumor-bearing pancreas was carefully mobilized and resected by subtotal pancreatectomy as described by us in detail before (7, 8). All mice survived the resection procedure and were randomly assigned to four treatment groups: TNFα group (n = 10), with application of 10 μg TNFα per animal and injection on days 3, 6, 10, and 13 after tumor resection; infliximab treatment group (n = 10), with application of infliximab on days 3, 10, 17, and 24 after subtotal pancreatectomy; and etanercept treatment group (n = 10), where the medicament was applied on days 3, 6, 10, 13, 17, 20, 23, and 27 after resection. Control mice (n = 10) were injected with an equivalent volume of 0.9% saline (200 μL) at the same days. In an additional experimental setting with three groups (n = 10 each), infliximab treatment was administered 3 days before resection ("preinfliximab") in direct comparison to the standard schedule of infliximab (see above) and a control group. Animals were sacrificed, and organs were preserved on day 41 after tumor cell inoculation.

Magnetic resonance imaging. Imaging was performed 28 days after tumor resection. High resolution magnetic resonance imaging (MRI) data were acquired on a clinical 3 Tesla whole-body MR system (Intera; Philips Medical Systems). A dedicated small-animal solenoid receive coil (Philips Research) with an integrated heating system to regulate body temperature of mice during magnetic resonance examination was used for signal acquisition. The magnetic resonance protocol consisted of a short survey scan and a coronal T2-weighted two-dimensional turbo spin-echo sequence (TR 6551 ms; TE 90 ms; FOV 90 × 45 mm; matrix 384 × 192; NSA 3) with an in-plane resolution of 230 μm × 230 μm and a slice thickness of 700 μm. Total scanning time was 12 min and 39 s.

Statistical analysis. *In vivo* data were analyzed using SPSS 11.0 (SPSS, Inc.). P value was owed to the skewed data distribution (tested by Shapiro-Wilk test); different groups were analyzed nonparametrically by Mann-Whitney U test. As two series of animal experiments were performed, the global level of significance was reduced from 0.05 to 0.025, according to Bonferroni's inequality correction for multiple tests.

Results

TNFα enhances invasiveness of PDAC cells *in vitro* and *in vivo*. To determine the effects of TNFα on PDAC cells, we analyzed proliferation, apoptosis, and invasive growth of three PDAC cell lines BxPc3, Colo357, and PancTuI upon treatment with TNFα. We found that TNFα reduced growth of BxPc3 and Colo357 cells by ~40% but showed no effect on the growth of PancTuI cells (Fig. 1*A*). The growth inhibitory effect of TNFα in BxPc3 and Colo357 cells resulted from a combination of apoptosis induction and inhibition of proliferation as was evident from analysis of DNA fragmentation

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(JAM) and [^{3}H]thymidine incorporation (Fig. 1*B*). In contrast to the rather moderate influence on cell growth, TNFα strongly enhanced the invasive properties of all three cell lines as shown by an *in vitro* invasion assay detecting the ability of tumor cells to invade and digest a monolayer of fibroblasts (Fig. 1*C*). Accordingly, the expression of proinflammatory and invasion-promoting proteins, such as IL-8, uPA, and MMP9, was clearly up-regulated by TNFα in these cell lines (Fig. 1*D*). To further prove the relevance of our findings *in vivo*, we treated mice bearing orthotopically xenotransplanted Colo357 and BxPc3 cells with TNFα on days 3, 6, 9, and 12 after tumor cell inoculation and analyzed tumor growth and metastasis. We observed strong enhancement of tumor growth in animals treated with TNFα (Fig. 2*A*). Furthermore, in agreement with the *in vitro* data, TNFα clearly increased the metastatic potential of both analyzed cell lines (Fig. 2*B*).

PDAC cells secrete TNFα. Under *in vivo* conditions, tumor cells may be exposed to TNFα delivered either by tumor-associated cells (infiltrating monocytes, lymphocytes, and other stromal cells) or the tumor cells themselves. To determine whether PDAC cells produce TNFα, we analyzed cell culture supernatants of BxPc3, Colo357, and PancTuI cells. Indeed, as shown in Fig. 3, all three PDAC cell lines secrete picogram amounts of TNFα. To analyze the role of tumor cell–derived TNFα, we first determined the effect of the clinically widely used TNFα inhibitors etanercept and infliximab on PDAC cell growth and invasiveness *in vitro*. We found that the inhibition of TNF only weakly affected the PDAC cell proliferation (Fig. 4*A*) and had a marginal effect on invasiveness in the fibroblast-based *in vitro* invasion assay (data not shown). However, etanercept and infliximab reduced the level of NF-κB and AP1 in Colo357 (Fig. 4*B*) and, furthermore, lowered constitutive production of IL-8 and uPA in Colo357 and PancTuI cells (Fig. 4*C*).

Inhibition of TNFα reduces pancreatic tumor growth and metastasis. To clarify whether etanercept and infliximab might display more potent effects *in vivo*, we analyzed the putative

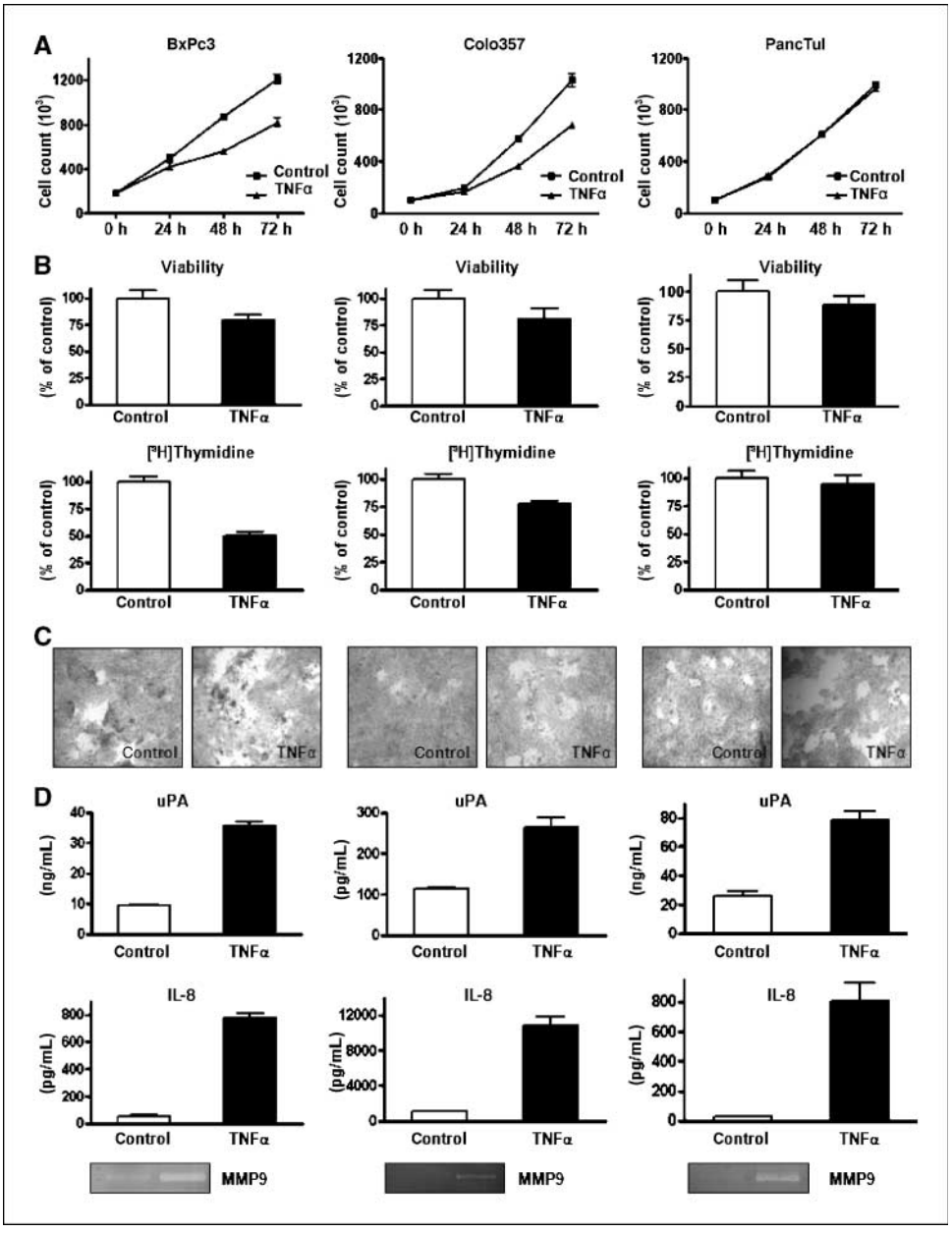

Figure 1. TNFα affects pancreatic tumor cell growth and invasion *in vitro*. *A,* pancreatic tumor cells were cultured in the absence or presence of TNFα (50 ng/mL). After 24, 48, and 72 h, cell number was determined from every sample by cell counting. *Points,* mean from three experiments; *bars,* SD. *B,* effect of TNFα on viability and proliferation of PDAC cells. Cells were treated for 48 h with TNFα (50 ng/mL). Apoptosis (*left*) and proliferation (*right*) were analyzed by JAM assay and [^{3}H]thymidine incorporation assay, respectively. *Columns,* mean (n = 8); *bars,* SD. *C,* TNFα induces invasiveness *in vitro*. BxPc3 and Colo357cells were treated for 48 h and PancTuI for 24 h with TNFα. Invasion assay was performed as described in Materials and Methods. Results shown are representative for at least four independent experiments performed in duplicate. *D,* TNFα induces secretion of uPA, IL-8, and MMP9 in PDAC cells. Cells were treated for 16 h with 50 ng/mL TNFα. uPA and IL-8 amounts were measured in cell culture supernatants by uPA and IL-8 ELISA tests. *Columns,* mean (n = 3); *bars,* SD. Levels of MMPs in cell culture supernatants were determined by zymography (see Materials and Methods).

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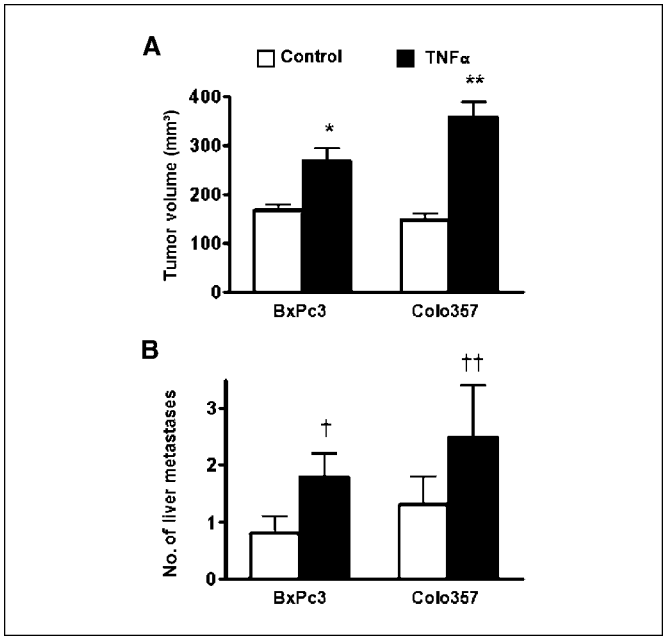

Figure 2. TNFα stimulates tumor growth and metastasis. Pancreatic tumor cells were inoculated into the pancreas of the SCID/bg mice. Tumor volumes (*A*) and number of liver metastases (*B*) were determined 28 d after inoculation. Control mice (n = 10 for each cell line) received 0.9% saline, whereas TNFα-treated mice (n = 10 for each cell line) received TNFα (10 μg) on days 3, 6, 9, and 12 after tumor cell inoculation. *, P = 0.002; **, P = 0.002; †, P = 0.002; ††, P = 0.042.

tumor-promoting activity of tumor cell–derived TNFα in two experimental settings. In one scenario that mimics the palliative situation, we treated tumor-bearing mice with etanercept or infliximab and analyzed the effects of the TNFα inhibition on tumor formation and metastasis (Fig. 5). In a second scenario, we wanted to address the clinical situation after surgery. Therefore, we performed a subtotal pancreatectomy 10 days after tumor cells inoculation and studied the role of TNFα in tumor recurrence and metastasis (Fig. 6).

In the experimental settings reflecting the palliative situation (Fig. 5*A*), we found that blockade of TNFα by infliximab reduced tumor volume by 20% to 30% in every cell line tested, whereas a significant inhibition of tumor growth by etanercept was only observed in BxPc3 cells (Fig. 5*B*). The number of liver metastases was highest in the control group (1.4 liver metastases per mouse) in Colo357 tumor–bearing mice, whereas the mean numbers of liver metastases in mice with BxPc3 and PancTuI tumors were only 0.8 and 0.76, respectively (Fig. 5*C*). Infliximab significantly (P = 0.032) reduced the number of liver metastases in all cases, whereas the administration of etanercept did not show a significant effect, although in Colo357-bearing mice, an inhibitory trend was noticed (Fig. 5*C*).

To investigate the effect of TNFα and TNFα-blockade on tumor recurrence and metastasis after subtotal pancreatectomy, mice bearing PancTuI tumors were treated according to the scheme shown in Fig. 6*A*. For monitoring tumor growth, two mice of each group were examined by MRI. The scans supported the decision for the appropriate time point for sacrifice. The MRI scans on day 28 after tumor resection and corresponding conventional pictures of mice after necropsy on day 31 are shown in Fig. 6*B*. PancTuI tumors appeared in MRI as irregularly shaped lesions in all groups. All lesions showed heterogenous signal intensities with hyperdense areas in T2. Furthermore, the scans revealed that tumor volumes were increased in TNFα and decreased in infliximab- and etanercept-treated animals. After euthanasia of all animals, we analyzed the recurrent tumor growth and metastasis. All animals developed local recurrent tumors. Upon TNFα treatment, we observed a massive increase in recurrent tumor volume and strong enhancement of metastasis (Fig. 6*C* and *D*). Tumor volume was six times higher compared with control animals, and the number of liver metastases per animal was doubled.

More importantly, inhibition of tumor cell–derived TNFα by infliximab or etanercept conversely resulted in a severe reduction of both recurrent tumor growth and metastasis. The volume of the recurrent tumor was reduced by 73% by infliximab and by 51% by etanercept (Fig. 6*C*). The mean number of liver metastases decreased from 2.6 in control mice to 0.8 per mouse in infliximab-treated animals and to 1.5 per mouse in etanercept-treated animals (Fig. 6*D*). To investigate the effect of preoperative inhibition of TNFα, 10 mice received infliximab additionally 3 days before primary tumor resection. However, this regimen did not further inhibit recurrent tumor growth or metastasis compared with the group with only postoperative infliximab treatment (Fig. 6*C* and *D*). Nevertheless, it confirmed the data by showing drastic growth inhibition of the recurrent tumor and reduced numbers of metastases also in this group.

Discussion

Immune cells and inflammatory processes can have quite diverse effects on cancer development. Both innate and adaptive immunity contribute to tumor surveillance and, thus, elicit antitumoral potential (25). Yet, immune cells can also constitute a tumor microenvironment favoring angiogenesis, antiapoptosis, cell migration, and metastasis and, thus, potentially contribute to cancer development and progression (26–28). In fact, there are accumulating clinical and experimental evidences that chronic inflammation promotes tumorigenesis and enhances growth and spread of tumors (29). Accordingly, there is a correlation between chronic pancreatitis and the development of pancreatic carcinoma resulting in an increased risk (30, 31). Notably, severe cancer-associated complications such as cachexia and pain result from the action of inflammatory mediators.

In many inflammatory scenarios, the cytokine TNFα plays a central or even essential role. In accordance with its overwhelming importance for inflammatory processes, several lines of evidence point to a tumor-promoting function of TNFα. Previously, it has been shown that overexpression of TNFα confers invasive

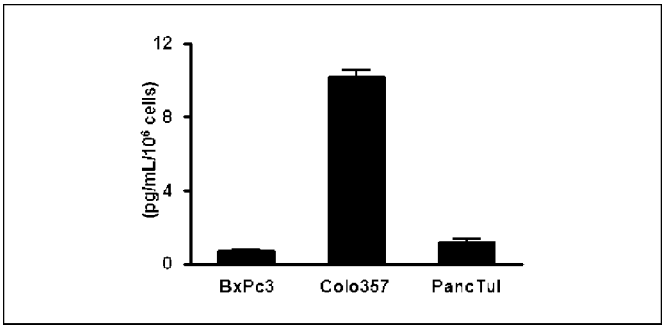

Figure 3. Pancreatic tumor cells secrete TNFα. Cell culture supernatants were analyzed for the presence of TNFα, using high sensitivity TNFα ELISA (R&D Systems). The concentrations were normalized to the cell numbers determined in parallel. *Columns,* mean of three independent experiments; *bars,* SD.

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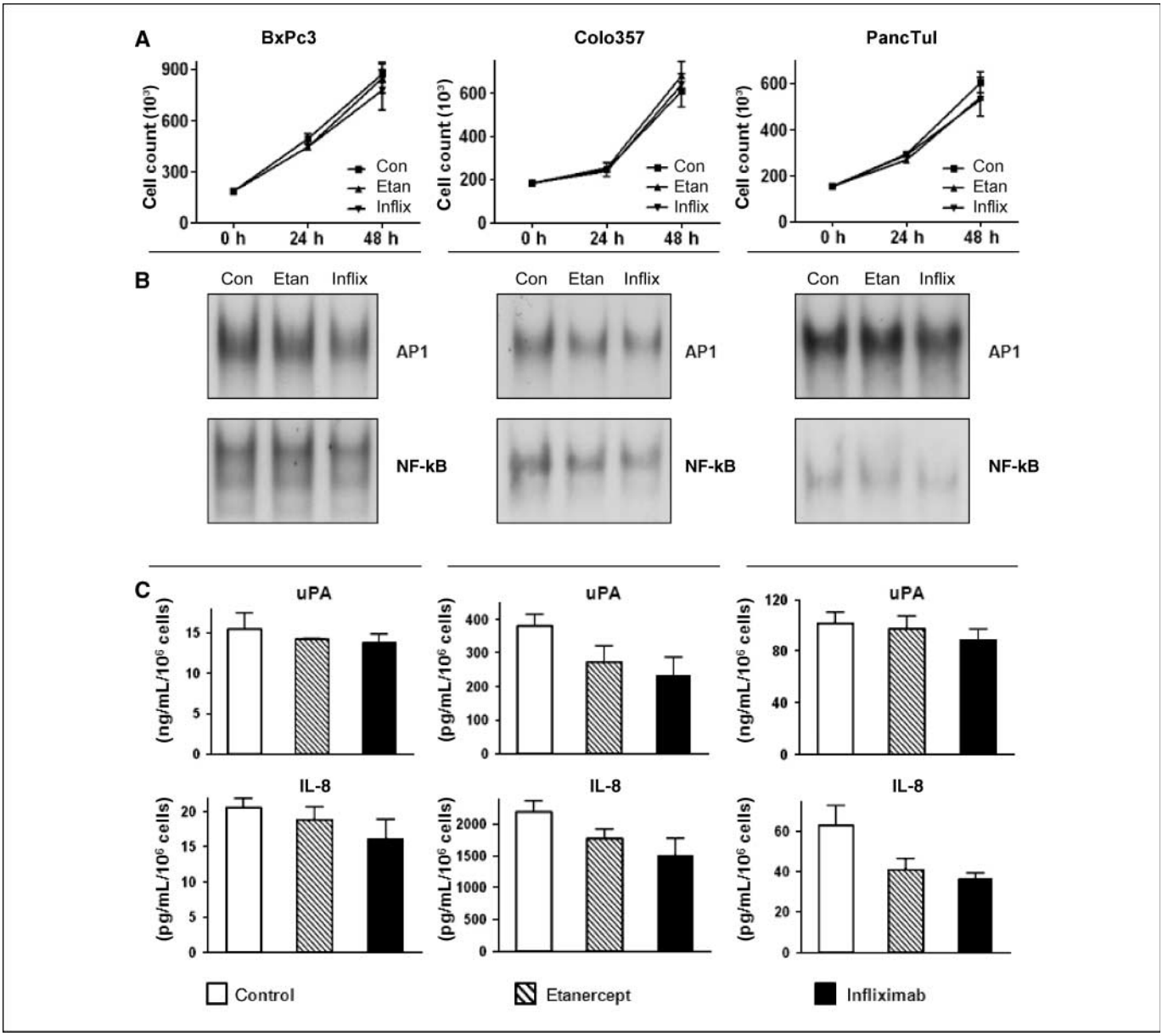

Figure 4. Effects of TNFα inhibition on PDAC cells biology. Cells were cultured in the presence or absence of etanercept (*etan*; 10 μg/mL) or infliximab (*inflix*; 10 μg/mL). *A,* proliferation curves were obtained by cell counting. *Points,* mean of three independent experiments; *bars,* SD. *B,* activity of AP1 and NF-κB were analyzed by EMSA. *C,* amounts of uPA and IL-8 were determined in cell culture supernatants of untreated cells or cells treated for 24 h with etanercept or infliximab using uPA and IL-8 ELISA and normalized to cell numbers determined in parallel. *Columns,* mean of three independent experiments; *bars,* SD. *con,* control.

properties in a cell type–dependent manner in nude mice (32–34). A crucial role of TNFα in initiation of skin tumors and hepatic carcinogenesis is further evident from studies of TNFα and TNFR1 knockout mice (35–37). A variety of tumor-promoting effects of TNFα was further shown *in vitro* and include protection against physiologic and pharmacologic apoptosis inducers, induction of angiogenic factors, enhancement of tumor cell motility, activation of oncogenic pathways, and triggering of epithelial to mesenchymal transition (27, 38).

The NF-κB pathway is regularly and robustly activated by TNFα and mediates many of the protumoral effects of TNFα. NF-κB activation has been reported in PDAC cell lines and primary tumor samples, and furthermore, increased TNFα levels were found in patients suffering from pancreatic cancer (18, 19, 39). Moreover, a recent study showed that the TNFα-processing enzyme ADAM17 is aberrantly expressed in PDAC and contributes to the invasiveness of pancreatic tumor cell lines *in vitro* (40). We therefore analyzed the putative protumoral role of TNFα in PDAC cells malignancy. We found that treatment of BxPc3, Colo357, and PancTuI with TNFα results in dramatic enhancement of invasiveness both *in vitro* and *in vivo* in an orthotopic xenotransplantation model in SCID mice. Interestingly, all three cell lines secreted picogram amounts of TNFα. Inhibition of tumor cell–derived TNFα with infliximab or etanercept showed only weak effects on cell proliferation, invasiveness, as well as on NF-κB and AP1 activity *in vitro*. In contrast, strong effects were observed under *in vivo* conditions where all three tumor cell lines showed reduced tumor growth and a reduced number of liver metastases when the mice

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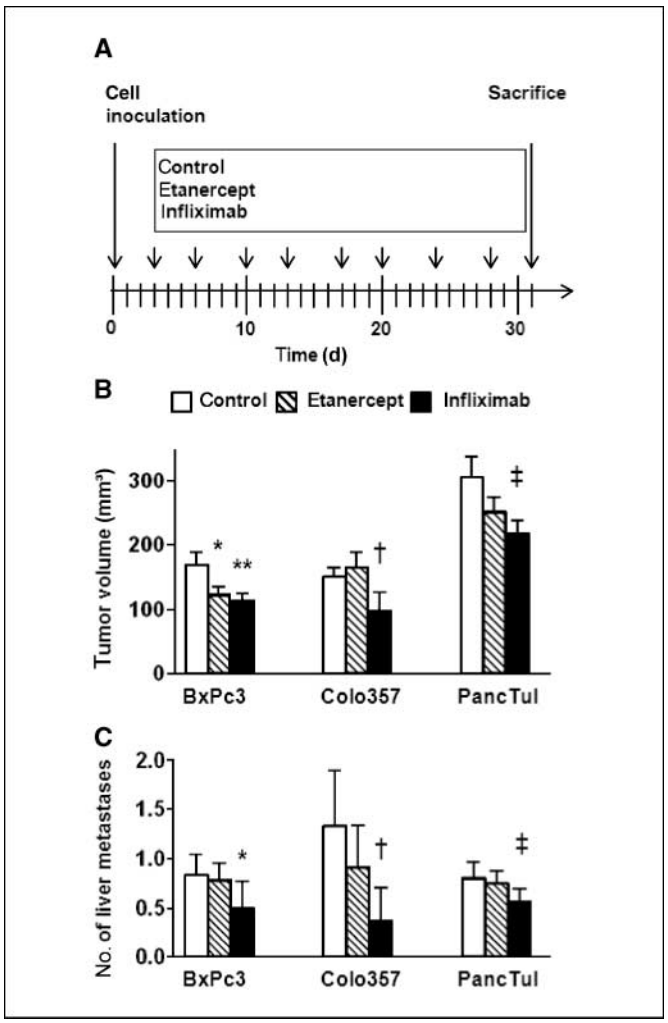

Figure 5. Inhibition of TNFα reduces PDAC tumor growth and metastasis. Mice (n = 10 per group for each cell line) were treated i.p. with 0.9% saline, with infliximab (10 μg/g body weight), or with etanercept (5 μg/g body weight) as shown in the scheme (*A*). Tumor volumes (*B*) and the number of metastases (*C*) were determined 28 d after tumor cell inoculation. The significances of the data were as follows: *, P = 0.046; **, P = 0.042; †, P = 0.047; and ‡, P = 0.022 for tumor volume (*middle*); and *, = 0.026; †, = 0.037; and ‡, P = 0.02 for the metastases.

were repeatedly treated with anti-TNFα agents. To determine if the cellular origin of tumor-promoting TNFα was mouse or human, we used infliximab, an anti-TNFα antibody that has been shown to bind and inhibit exclusively human and chimpanzees but not rodent TNFα (41). In contrast, etanercept binds both human and murine TNFα. Thus, because infliximab showed stronger anti-tumor properties than etanercept in our *in vivo* experiments, data suggest that tumor cell–derived TNFα contributes to the malignancy of pancreatic tumor cell lines in SCID/bg mice. The relatively moderate effect of TNFα neutralization on NF-κB and AP1 activity in the *in vitro* experiments is presumably related to the fact that activation of these pathways is not only mediated by endogenous TNFα but also by other independently regulated proinflammatory factors. Indeed, it has been shown that an autoregulatory feedback loop of IL-1α induction and NF-κB activation acts in pancreatic cancer cells, leading to the constitutive production of uPA (42). Furthermore, an *in vitro* culture system does not accurately represent the *in vivo* situation in which an intensive cross-talk between cancer cells and their environment is present, and even picogram amounts of TNFα secreted by tumor cells may induce strong expression of different cytokines in the tumor adjacent region. Taken together, the data we obtained in mice clearly indicate that in the *in vivo* situation,

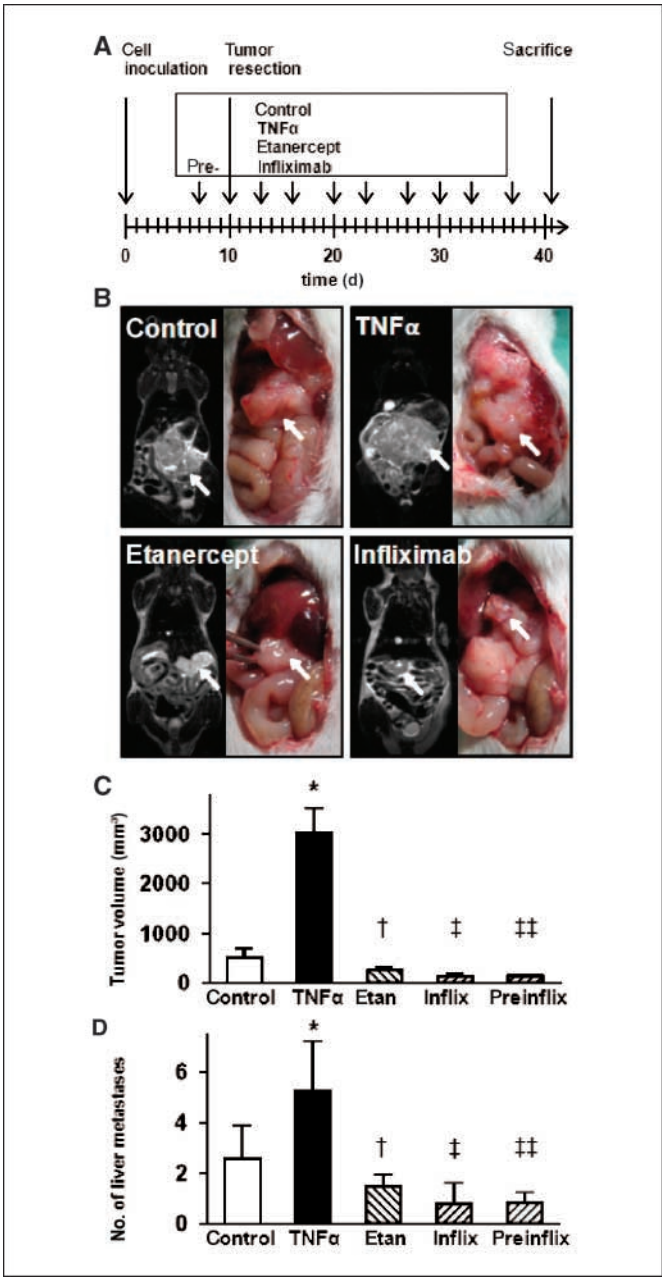

Figure 6. Inhibition of TNFα after subtotal pancreatectomy inhibits tumor regrowth and metastasis. Primary tumors were resected by subtotal pancreatectomy 10 d after inoculation of 10^6 PancTul cells, and mice were treated i.p. with 0.9% saline (control; n = 20), with TNFα (n = 10; 10 μg/mouse), with infliximab (n = 20; 10 μg/g body weight), or with etanercept (n = 10; 5 μg/g body weight) as shown in the scheme (*A*). One group of animals [preinfliximab (*preinflix*); n = 10)] was treated with infliximab additionally 3 d before the tumor resection (*A*). MRI scans and corresponding necropsy pictures are shown in *B*. *Arrows,* recurrent tumor. The recurrent tumor volume (*C*) and the number of metastases (*D*) were determined on the day 31 after primary tumor resection. The significances of the data were as follows: *, P < 0.001; †, P < 0.001; ‡, P < 0.001; and ‡‡, P = 0.008 for tumor volume (*C*); and *, P = 0.001; †, P = 0.039; ‡, P < 0.001; and ‡‡, P < 0.001 for the metastases (*D*).

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inhibition of TNF exerts much more potent effects, possibly by affecting interaction of tumor cells with their environment rather than just acting on the tumor cells themselves.

The strong *in vivo* activity of tumor cell–derived TNFα has very recently been shown by Kulbe et al. (43) for ovarian cancer cells inoculated into the peritoneum of nude mice. The authors showed that tumors derived from cells in which TNFα production was blocked by RNA interference technology were significantly smaller and less invasive than tumors derived from corresponding wild-type cells. However, in line with our own results, inhibition of endogenous TNFα had no influence on the tumor cell growth *in vitro*.

Surgical resection is the most efficient therapeutic option for PDAC patients but is unfortunately often followed by local tumor recurrence and metastasis. As surgical manipulations are unavoidably associated with wounding and consecutive inflammatory reactions, we were especially interested in a potential role of endogenous TNFα in tumor recurrence and surgical trauma–induced metastasis. Upon resection of primary tumors, we observed a prominent role of tumor cell–derived TNFα on recurrent tumor growth and metastasis significantly exceeding the contribution of endogenous TNFα to the development and malignancy of primary PDAC tumors. Using immunohistochemistry, we determined much stronger expression of TNFα in recurrent tumors than in primary tumors (see Supplementary Fig. S1 and Supplementary Table S1). Infliximab and, to a lesser extent, etanercept strongly reduced the size of the recurrent tumor and also considerably diminished the number of metastases. Again, as infliximab, in contrast to etanercept, neutralizes only human and not murine TNFα, PDAC-derived TNFα must be the crucial trigger of the TNF-dependent enhancement of tumor recurrence and metastasis observed in our experimental model. However, an additional role of TNFα produced by tumor-associated murine stroma and tumor-infiltrating cells is very likely because TNFα is a target gene of the NF-κB pathway, and it induces its own expression. In fact, we found an enhanced stroma-localized TNFα expression in all analyzed recurrent tumors (see Supplementary Fig. S1 and Table S1). It is therefore possible that PDAC-derived TNFα secondarily stimulates the production of TNFα in the tumor-associated microenvironment, and thus, a mutual amplification loop leads to the observed dramatically enhanced malignancy of tumor cells after primary tumor resection.

Because inhibition of TNFα did not completely block the tumor recurrence and metastasis, other proinflammatory cytokines such as IL-1β (44) and IL-6 (45) may also be involved in these processes. Further experiments are necessary to identify the effect of these factors.

The stronger inhibitory effects of infliximab than that of etanercept observed by us in all experimental settings may also reflect different TNF-binding characteristics recently shown for these agents. Infliximab binds and neutralizes transmembrane TNFα more efficiently than etanercept. Concerning soluble TNFα (sTNFα), infliximab binds stably to both monomeric and trimeric sTNFα. In contrast, etanercept binds exclusively to trimeric sTNFα, forming complexes of reduced stability (46).

Clinical trials with TNFα-blocking agents are currently under way in patients with solid cancers. Thus far, these trials show that the TNFα blockers are well-tolerated and this is in good accordance with the experience from anti-TNFα treatment of patients with chronic inflammatory diseases (for review see ref. 47). Therefore, the results presented in our study strongly suggest that surgical resection of PDAC combined with an anti-TNF therapy represents a promising novel therapeutic option. Corresponding adjuvant clinical trials should therefore be pursued.

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