New gene-immunotherapy combining TRAIL-lymphocytes and EpCAMxCD3 bispecific antibody for tumor targeting

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Abbreviations: bsAb: bispecific antibody; Control-lymphocytes: non-transduced primary lymphocytes; EpCAMxCD3: bsAb EpCAMxCD3; EpCAM: epithelial cell adhesion molecule; GEM-BxPc-3: gemcitabine-selected BxPc-3 daughter cells; Lymphocytes: pre-activated, non-transduced lymphocytes; MOCK-lymphocytes: mock transduced primary lymphocytes; mTRAIL: membrane-bound TRAIL; PBMCs: peripheral blood mononuclear cells; sTRAIL: soluble TRAIL; TRAIL: tumor necrosis factor-related apoptosis inducing ligand; TRAIL-lymphocytes: lentiviral TRAIL-transduced primary lymphocytes; 3D: three-dimensional.

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Statement of Translational Relevance

Our proof of concept study is the first step for further development of a systemic gene-immunotherapeutic approach. Autologous patient lymphocytes will be transduced \textit{ex vivo} to TRAIL-overexpressing lymphocytes, which are in turn guided by bispecific antibody EpCAMxCD3 to the tumor microenvironment. Both components induce cell death by different signaling pathways, which enhances tumor cell death even in cells with cancer stem cell characteristics. In addition this approach may sensitize for conventional cytotoxic therapy since both EpCAMxCD3 and TRAIL-lymphocytes induce an inflammatory response. The clinical feasibility of such a gene-immunotherapy may be understood as an initial aid to support endogenous immune responses and the effect of cytotoxic tumor therapy.
Abstract

Purpose: To enhance T cell responsiveness toward cancer cells we overexpressed TRAIL in lymphocytes, since this death ligand induces tumor-specific apoptosis. To increase contact time of lymphocytes with tumor cells and thereby of TRAIL with its death receptors, lymphocytes were linked to the CD3 arm of bispecific antibody EpCAMxCD3, to guide the lymphocytes to tumor cells positive for the cancer stem cell marker EpCAM/ESA.

Experimental Design: Lymphocytes were transduced with TRAIL lentivirus and the anti-tumor effect in presence and absence of EpCAMxCD3 was evaluated in vitro and in xenograft studies using EpCAM-positive pancreatic and prostate cancer cells. Results: Compared to control lymphocytes, TRAIL-lymphocytes increased cytotoxicity and further induced expression of several apoptosis-related molecules. Co-transplantation of TRAIL-lymphocytes and tumor cells in mice or peritumoral injection of TRAIL-lymphocytes in larger xenografts retarded growth and induced apoptosis. Combination of TRAIL-lymphocytes with EpCAMxCD3 potentiated tumor eradication by enhancing anti-apoptotic and anti-proliferative signaling and by decreasing tumor vasculature. Intratumoral cyst formation was involved and associated with enhanced chemokine secretion and infiltration of mouse macrophages, suggesting contribution of an inflammatory host response. Most importantly, tumorigenicity of pancreatic cancer cells with cancer stem cell features resistant to conventional chemotherapy was strongly reduced. Conclusions: This gene-immunotherapeutic approach may be a new tool to support endogenous immune responses toward cancer even in its advanced stages.
**Introduction**

Pancreatic adenocarcinoma and advanced prostate cancer are aggressive malignancies with poor prognosis (1). Nontoxic new approaches are needed to improve overall and progression-free survival. A promising strategy is the use of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), which is a member of the TNF superfamily of death receptor ligands. TRAIL is a transmembrane protein that is cleaved into soluble TRAIL (sTRAIL). Preclinical data show that sTRAIL is a cancer cell–specific molecule exerting antitumor activity *in vitro* and in mice (2, 3). Phase 1/2 clinical trials have demonstrated a favorable toxicity profile for sTRAIL but limited evidence of antitumor activity. This is probably due to short exposure of tumor cells to low TRAIL concentrations and to sTRAIL’s short half-life (4, 5). Strategies to enhance the therapeutic activity of TRAIL include cell-based transportation of the full-length, membrane-bound (m)TRAIL (6-8). Two examples of cell-based approaches are the retransfusion of mTRAIL-transfected tumor-infiltrating T cells of renal cell carcinoma patients (9), and of tumor-infiltrating T cells pre-incubated with recombinant TRAIL-fusion proteins anti-CD3:TRAIL and K12:TRAIL (10). However, the delivery of mTRAIL to tumors using human primary lymphocytes or other immune effector cells from peripheral blood has not been explored so far.

Another novel immunotherapeutic strategy uses bispecific antibodies (bsAb), which are artificial molecules with a dual specificity for antigens. Many bsAb constructs directed against the CD3 molecules on lymphocytes and against distinct tumor-associated antigens are currently in preclinical and clinical testing. Her2/neu, EGFR, CEA, EpHA2, CD33, CD19 and epithelial cell adhesion molecule (EpCAM/ESA/CD326) are frequently chosen targets for such bsAb (11). A prominent example for an EpCAM-directed reagent represents the trifunctional antibody catumaxomab (12), which enhances the anti-tumor activity by redirecting T cells via CD3 and Fcg receptor I/III-positive accessory cells to the tumor. Results of a prospective randomized phase II/III trial indicate prevention of malignant ascites...
in patients with epithelial cancer resistant to conventional chemotherapy (13). Recently, we demonstrated the effectiveness of our bsAb EpCAMxCD3 (HEA125xOKT3), which redirected T cells to EpCAM-positive tumor cells, increased the duration of contacts between T cells and tumor cells and reduced malignant ascites in patients with advanced ovarian cancer (14, 15).

Importantly, EpCAM is overexpressed by the majority of epithelial tumors and derived metastases, but not by normal cells (16). EpCAM-positive tumor entities include pancreatic and prostate adenocarcinomas (17, 18). Increased EpCAM expression indicates a poor prognosis as shown e.g. in breast and gallbladder carcinomas (19, 20). Remarkably, EpCAM, also known as ESA, is suggested as a cancer stem cell marker (21-23), present in several tumor entities including pancreatic (23, 24) and prostate (25) cancer. As shown by Simeone and coworkers the ESA^+ population from human resected pancreatic tumor tissue formed tumors in mice to a much higher frequency than ESA^- cells (23). The same group demonstrated that the marker combination CD44^+CD24^-ESA^+ was most potent in tumor formation in mice compared to CD44^-CD24^-ESA^- . Therefore EpCAM (ESA) alone or in combination with CD24 and CD44 is a relevant marker for pancreatic cancer stem cells.

In the present study we stably overexpressed mTRAIL in primary human lymphocytes by lentiviral transduction and found an increased anti-tumor effect involving reduction of tumorigenicity of pancreatic cancer cells with stem cell character, which was potentiated by combination with EpCAMxCD3.

Materials and Methods

Primary and established cell lines. Human peripheral blood mononuclear cells (PBMCs) and skin fibroblasts were obtained from healthy donors following approval by the ethical
committee of the University of Heidelberg. Established human cell lines BxPc-3 (pancreatic adenocarcinoma), PC-3 (prostate adenocarcinoma), HEK 293T (kidney epithelial) were from ATCC and authenticated throughout the culture by the typical morphology. GEM-BxPc-3 were obtained by continuous incubation of BxPc-3 cells during several months in increasing concentrations of gemcitabine up to 100 nM leading to selection of gemcitabine resistant cells with enhanced cancer stem cell marker expression as we described recently (26).

**Antibodies and reagents.** EpCAMxCD3 (HEA125xOKT3) and CD19xCD3 (HD37xOKT3) bsAb were produced by hybrid-hybridoma technique as described (27). Parental anti-human EpCAM hybridoma (HEA125; IgG1) was raised in our laboratory. Hybridoma OKT3 (IgG2A) directed against the e-chain of the CD3 molecule was purchased from ATCC. Gemcitabine was from Fresenius Kabi (Bad Homburg, Germany).

**Transduction of lymphocytes.** PBMCs (3 x 10⁶ cells per ml) were activated as described (15) and transduced at MOI 10 in 24-well plates pre-coated with retronectin (TaKaRa, Saint-Germain-en-Laye, France), followed by incubation at 37°C for 24 h. This procedure was repeated after 24 h. After transduction, lymphocytes were incubated in complete cell culture medium supplemented with IL-2 (100 U/ml). Lymphocytes were used for experiments at day five after transduction.

**Analysis of apoptosis in vitro.** To detect apoptosis induction by TRAIL-lymphocytes BxPc-3 cells were grown on chamber slides. TRAIL- or MOCK-lymphocytes were added to 70% confluent BxPc-3 cells. 24 hours later, lymphocytes were removed and attached BxPc-3 cells were fixed in aceton. Staining for active caspase-3 was performed with rabbit anti-human pAb (R&D Systems, Abingdon, UK). The signal was enhanced using a Vectastain ABC Elite kit. DAB was used as a chromogen.
Xenograft tumors. In the first experiment a combination of BxPc-3 or PC-3 tumor cells (5 x 10^6) and TRAIL-lymphocytes (5 x 10^6) in PBS were transplanted s.c. to five-week-old NOD SCID mice in a volume of 100 μl. Three days later animals were randomized to five groups (n = 5 per group). EpCAMxCD3 (0.01 or 0.1 mg/kg), irrelevant bsAb CD19xCD3, parental bivalent mAb anti-human CD3 (OKT3), anti-human EpCAM (HEA125) mAb (1 mg/kg) or PBS were injected i.p. All mice were given 1 mg/ml of doxycycline (Sigma) and 5% glucose in the drinking water. Five injections of EpCAMxCD3 or control mAbs were administered i.p. every third day. In a second experiment, BxPc-3 cells (5 x 10^6) were engrafted and 12 days later, when the tumors reached a volume of 30-40 mm^3, TRAIL-lymphocytes (5 x 10^6) were preloaded with EpCAMxCD3 (1 mg/ml) for 1 h and then transplanted s.c. around the tumor. Animals were randomized to three groups (n = 5 per group) and received i.p. injections of EpCAMxCD3 at a dose of 0.01 mg/kg, 0.1 mg/kg or PBS. Controls received EpCAMxCD3 at a dose of 0.1 mg/kg or PBS only. In a third experiment, GEM-BxPc-3 cells were left untreated or were incubated with TRAIL-lymphocytes or with TRAIL-lymphocytes preloaded with EpCAMxCD3 (1 mg/ml) for 2 h at an effector:target ratio of 10:1. Lymphocytes were removed by washing and 100 μl viable Gem-resistant BxPc-3 cells (6 x 10^3) were transplanted to each of four NMRI Fox nu/nu mice/treatment group in 50% matrigel/PBS followed by monitoring of tumor engraftment (tumor take). Tumor size was measured and analyzed as described (26). The animal experiments were approved by the Ethical Committee.

Analysis of xenograft tissue. Frozen 6 μm xenograft tissue sections were examined by histochemistry and immunohistochemistry using standard avidin-biotin technique with a Vectastain ABC Elite kit (Vector, Burlingame, CA, USA).
Real-time RT-PCR. mRNA was isolated from frozen tumor samples, converted to cDNA by standard techniques and PCR was performed with the LightCycler FastStart DNA Sybr GreenI kit (RAS) according to the protocol provided in the parameter specific kits. The transcript number was calculated from a standard curve, obtained by plotting known input concentrations of four different plasmids at log dilutions to the PCR-cycle number (CP) at which the detected fluorescence intensity reaches a fixed value. The calculated copy numbers were normalized according to the expression of Cyclophilin B.

Viability, colony formation and ALDH1 activity. These features were measured as described in our recent publication (26).

Statistical analysis. Data are presented as the mean ± SD. Student’s t-test and χ² test were used to evaluate the difference between groups. Significance of growth kinetics over time was evaluated using variance analysis with repeated measurement and Post-Hoc test for comparison of groups after Dunnett. P < 0.05 was considered statistically significant.

Results

TRAIL-lymphocytes induce apoptosis in tumor cells. While resting lymphocytes express only minimal amounts of TRAIL, expression is enhanced to a median level upon lymphocyte stimulation (28). To further increase expression to therapeutic active levels we cloned membrane TRAIL into a lentiviral vector. Upon lentiviral transduction of lymphocytes and activation, TRAIL expression was measured by FACS-analysis. While TRAIL expression in activated lymphocytes transduced with control vector was 7%, the level was further increased to 57% by the lentiviral TRAIL construct (Supplementary Figure 1A online). Control experiments demonstrate that mainly CD4⁺ and CD8⁺ cells were transduced and that lentiviral transduction does not affect lymphocyte function (Supplementary Figure 1B, C online). Next,
BxPc-3 and PC-3 were used as targets, since these cells express TRAIL receptor 1 and 2 and are sensitive to recombinant sTRAIL (Supplementary Figure 1E, F online). In addition, both cell lines harbor tumor cells with cancer stem cell features and express EpCAM (15, 29, 30). To examine the effect of TRAIL-lymphocytes to apoptosis, we cultured BxPc-3 cells alone or together with pre-activated TRAIL- or MOCK-lymphocytes. Twenty-four hours later the typical apoptotic blebbing of dying cells was observed and caspase-3 activity was measured by immunofluorescence staining followed by counting positive cells. While the basal apoptosis induction was 5%, MOCK-lymphocytes increased it to 60%. This strong apoptosis induction already by control lymphocytes may be due to extensive activation by anti-CD3 mAb and IL-2 prior to co-incubation. This most probably led to spontaneous release of cytotoxic substances from granules as perforins and granzyme B, which are responsible for cell death. Apoptosis was further increased by TRAIL-lymphocytes to 80% suggesting additional specific induction of apoptosis by TRAIL (Figure 1A). These data were confirmed by measurement of apoptosis by propidium iodide staining and FACS analysis 4 h after co-incubation (Figure 1B). To investigate suitability of TRAIL-lymphocytes in a model reflecting the tumor microenvironment, we established 3D tumor reconstructs by embedding BxPc-3 cells together with lymphocytes and fibroblasts in collagen matrix. Seventy-two hours later supernatants and cleared lysates of 3D gels were collected and subjected to protein array analysis. Compared to control 3D constructs harboring BxPc-3 cells and MOCK-lymphocytes, expression of apoptosis-related proteins was increased in the presence of TRAIL-lymphocytes (Supplementary Figure 2A, B online). Marked upregulation of procaspase-3 and of cleaved caspase-3 occurred, confirming our in vitro results (compare Fig. 1A and data not shown). In addition, enhanced expression of the pro-apoptotic proteins cytochrome c, FADD, CD95/TNFSF6, HTRA2/Omi and SMAC/Diablo was strongly activated. Activation of expression of the anti-apoptotic proteins Catalase, HIF-1α, HO-1, HO-2, Hsp27, Hsp60 and Survivin was also observed, although the level of upregulation was
rather low in most cases. To exclude the possibility that the effects of TRAIL-lymphocytes could be tumor type or cell line specific, we performed experiments with PC-3 prostate cancer cells, which confirmed the above described results (Data not shown). These data suggest that TRAIL-lymphocytes are superior to MOCK-lymphocytes in inducing apoptosis in tumor cells.

**Delivery of TRAIL-lymphocytes by EpCAMxCD3 increases retardation of xenografts.**

In order to enforce the anti-tumor activity of TRAIL-lymphocytes we performed combination experiments with EpCAM since our recent study demonstrated that this bsAb retards growth of BxPc-3 xenografts and induces cell death by mediating enhanced contact time between lymphocytes and tumor cells associated with the release of Granzyme B, Perforin, IFN-γ, TNF-α, IL-10 and TGF-1ß (Supplementary Table 2 online) (15). To investigate the effect of EpCAMxCD3 to cytotoxicity of TRAIL-lymphocytes *in vivo*, immunodeficient mice were transplanted subcutaneously with EpCAM-positive BxPc-3 or PC-3 tumor cells in presence or absence of non-transduced or TRAIL-lymphocytes. Three days later, EpCAMxCD3, or parental antibody and irrelevant bsAb control antibodies were i.p. injected five times in three day intervals. Compared to non-transduced lymphocytes, TRAIL-lymphocytes induced tumor growth retardation in BxPc-3 tumors, while the effect in PC-3 xenografts was not significant (Figure 2A). The TRAIL effect was potentiated in both tumor entities by combination with EpCAMxCD3, which was administered three days after transplantation in low doses of 0.1 and 0.01 mg/kg. While in PC-3 cells both doses reduced tumor sizes below the levels obtained by TRAIL-lymphocytes alone, only the higher dose of 0.1 mg/kg further increased the TRAIL-effect in BxPc-3 cells. Parental or irrelevant control antibodies did not change tumor growth as expected (Data not shown). At day 23 tumors were resected and the weight was determined, which confirmed tumor growth kinetics (Figure 2B, Data not shown). Metastases in lung, liver or spleen were not detectable in either treatment group as examined.
by macroscopic inspection, FACS-analysis and immunohistochemistry of tissues for expression of human MHC class I antigen and for human cytokeratins 4, 5, 6, 8, 10, 13, and 18. Body weight or general condition of mice was not affected by tumor growth and treatment (Data not shown).

**Delivery of TRAIL-lymphocytes by EpCAMxCD3 inhibits proliferation, angiogenesis and induces apoptosis and inflammation.** For evaluation of mechanisms involved in xenograft retardation we performed immunohistological staining for proliferation (Ki-67), vessel density (CD31) and apoptosis (TUNEL, active caspase-3) using frozen tissue sections obtained at day 23 after treatment. Therapeutic effects to these parameters were obvious for combination treatment, but were less pronounced for TRAIL-lymphocytes alone (Figure 3 and data not shown). This was expected and may be due to the rather late time point after treatment at which apoptosis induction by the relatively insensitive TUNEL and active caspase-3 measurements may be not detectable anymore. To overcome these assay-specific limitations we examined mRNA expression of apoptosis- and inflammation-related genes in tumor tissue by RT-PCR. The most prominent changes induced by TRAIL-lymphocytes were downregulation of the death receptors TRAIL-R2 and CD95, downregulation of the anti-apoptotic gene survivin and upregulation of the anti-apoptotic genes Hsp27 and Hsp70, as well as upregulation of the inflammation-related genes HIF-1α, NF-κB and VEGF (Supplementary Table 1). Combination with EpCAMxCD3 enhanced expression of the pro-apoptotic genes Bax, TRAIL, p21, TNF-R1 and re-stored TRAIL-R2 expression. However, combination treatment induced also an anti-apoptotic response, since Bcl-2, Bcl-xL, cIAP1, cIAP2, XIAP, Survivin, Hsp27 and Hsp70 were upregulated along with HIF-1α, NF-κB and VEGF. These results suggest that combination of TRAIL-lymphocytes with EpCAMxCD3 induces more pronounced tumor retardation and changes in expression of genes involved in anti-apoptotic, pro-apoptotic and inflammation signaling.
**EpCAMxCD3 potentiates TRAIL-lymphocyte-induced retardation of larger tumors.** To investigate the effect of TRAIL-lymphocytes to larger tumors we transplanted BxPc-3 cells and treated the mice after xenograft tumors had developed 12 days later. TRAIL-lymphocytes alone or TRAIL-lymphocytes preloaded with EpCAMxCD3 were injected s.c. around the tumor. Injections of EpCAMxCD3 were repeated three times in three-day intervals. At day 23 after treatment tumor xenografts were resected and evaluated. TRAIL-lymphocytes, EpCAMxCD3 and both together had induced large liquid areas within the tumor tissue due to voluminous cyst formation (Figure 4A). These cysts were not due to hemorrhage and did not contain cell debris as determined by macroscopic and microscopic inspection (Data not shown). Also, such cysts had already been observed in the former experiment presented in Fig. 3, although less pronounced, which may be due to the earlier treatment. We assume that these cysts are due to induction of inflammation by both, TRAIL-lymphocytes and EpCAMxCD3, as suggested by the results of the RT-PCR experiment, in which induction of expression of inflammation-related genes was found (Compare Supplementary Table 1). Although no significant differences in relative tumor size were detectable (Figure 4B), analysis of vessel density by CD31-staining revealed a strong and significant anti-angiogenic effect induced by both treatment schedules (Figure 4C). Differences in apoptosis between control and treatment groups at this late time point were not detectable by staining for active caspase-3 and TUNEL assay according to the results of Figure 3. Together, the most obvious effect by which TRAIL-lymphocytes alone or combined with EpCAMxCD3 induce tumor retardation is by liquefying the tumor tissue in intratumoral cysts and inhibition of tumor angiogenesis.

**Tumor tissue may be lysed by TRAIL-lymphocyte- and EpCAMxCD3-induced inflammation and associated cyst formation.** To evaluate involvement of infiltrating mouse
macrophages to inflammation and cyst formation, we used BxPc-3 tumor tissue obtained from the experiment shown in Figure 3 and stained it with mouse specific F4/80- and CD68 antibodies. TRAIL-lymphocytes and EpCAMxCD3 alone or in combination strongly promoted infiltration of tumor islets with F4/80- and CD68-positive macrophages (Figure 5). In contrast, macrophages in tumors, which are derived from mice treated with Control-lymphocytes were localized predominantly in collagen-rich stroma surrounding BxPc-3 tumor islets. Macrophages could be detected at the periphery of the islets only occasionally. These results were confirmed by examination of tumor tissue obtained from the experiment shown in Fig. 4 (Data not shown). Interestingly, such a cyst formation was not observed by treatment of BxPc-3 xenografts with control-lymphocytes and EpCAM alone (15). Thus, these results suggest that TRAIL-lymphocytes induce an inflammatory host response, which is increased by EpCAMxCD3, and inflammation is most likely the reason for cyst formation.

Inhibition of tumor take of cancer stem-like cells by TRAIL-lymphocytes and EpCAMxCD3 is associated with inhibition of proliferation and induction of apoptosis.

Finally, we analyzed whether TRAIL-lymphocytes and EpCAMxCD3 may have the potential to eliminate tumorigenicity of highly therapy resistant cancer cells with cancer stem cell characteristics. We used gemcitabine-resistant BxPc-3 cells (GEM-BxPc-3), which we recently selected by continuous treatment with increasing concentrations of gemcitabine for several weeks. This resulted in enhanced expression of the cancer stem cell markers CD44/CD24 (26). While GEM-BxPc-3 compared to parental BxPc-3 cells are resistant toward gemcitabine, they are still sensitive for TRAIL-induced cytotoxicity as measured by MTT assay (Figure 6A). In support of the cancer stem cell character of GEM-BxPc-3 cells we found that these cells possess higher colony-forming capacity and ALDH1 activity compared to parental cells (Supplementary Figure 3A, B online). Correspondingly, co-incubation of GEM-BxPc-3 for 24 h with TRAIL-lymphocytes at an effector:target ratio of 10:1 led to
specific activation of caspase-3 as determined by immunofluorescence microscopy (Supplementary Figure 3C online). Likewise, the number of viable cells after treatment with TRAIL-lymphocytes was reduced compared to cells treated with MOCK-lymphocytes as found by detection of morphology by light microscopy. For evaluation of tumorigenicity we treated GEM-BxPc-3 cells with TRAIL-lymphocytes alone or pre-loaded with EpCAMxCD3. Two hours later viable cells were counted and 6 x 10^3 surviving cells were transplanted to mice (Figure 6B, C). While non-pretreated GEM-BxPc-3 cells rapidly formed tumors within 14 days in all of four transplanted mice, only three mice of the TRAIL-lymphocyte- and only one mouse of the TRAIL-lymphocyte plus EpCAMxCD3-pretreated groups developed tumors at this particular time point. Retarded onset of tumor growth in a fourth mouse transplanted with GEM-BxPc-3 cells pretreated with TRAIL-lymphocytes started at day 21. A second mouse of the TRAIL-lymphocyte plus EpCAMxCD3 treatment group developed a tumor at day 17 and no further tumor development in this group was observed until the end of the experiment at day 35. Most importantly, the percentage of Ki-67-positive proliferating tumor cells was lower and the percentage of apoptotic active caspase 3-positive cells was higher in tumors derived from TRAIL-lymphocyte-pretreated cells, while combination with EpCAMxCD3 further increased these effects (Figure 6D). These results suggests a long-term effect of TRAIL-lymphocytes and EpCAM-CD3 to tumorigenic cells, which might not been eliminated immediately but over time. We did not detect changes in the cancer stem cell marker CD24/CD44 expression profile in tumors derived from TRAIL-lymphocyte-treated cells and were not able to measure it in EpCAMxCD3 combination treatment due to incompatibility of secondary antibodies with EpCAMxCD3 antibody (Data not shown). Nevertheless, reduced tumor take along with reduced proliferation and enhanced apoptosis strongly suggest that tumorigenic properties are targeted by TRAIL-lymphocytes with enhanced effect after combination with EpCAMxCD3.
Discussion

The present study shows that overexpression of mTRAIL in primary human lymphocytes increases T cell responsiveness toward cancer cells of pancreas and prostate and combination with EpCAMxCD3 potentiates the effect as analyzed in vitro and in vivo. From our results we conclude that EpCAMxCD3 guided TRAIL-lymphocytes to the tumor microenvironment and increased exposure time of TRAIL overexpressed by lymphocytes to its receptors expressed by tumor cells. Although we did not directly detect that EpCAMxCD3 increases the exposure time of TRAIL and its receptors by linking tumor cells and TRAIL-lymphocytes in vivo, there are strong indirect hints that this was indeed the case. First we know that EpCAMxCD3 increases duration of the contact between lymphocytes and carcinoma cells without affecting lymphocyte migration in vitro (15). Second, we found that intraperitoneal injected EpCAMxCD3 increased cytotoxicity of TRAIL-lymphocytes, which were co-transplanted with tumor cells in mice. In contrast, parental antibody and irrelevant bsAb control antibodies did not increase the anti-tumor effect of TRAIL-lymphocytes. Importantly, we did not observe toxic side effects of this gene-immunotherapeutic approach, such as a decrease of body weight, liver toxicity or metastasis in mice.

The mechanisms by which the two independent stimuli, TRAIL-lymphocytes and EpCAMxCD3, each for its own induces cell death are already known and published. TRAIL induces apoptosis in sensitive tumor cells expressing TRAIL receptors by death receptor-mediated apoptosis signaling (4). EpCAMxCD3 brings T cells and tumor cells in close contact by redirecting T cells to kill carcinoma cells (27). By binding to CD3 molecules on lymphocytes with one arm and to EpCAM antigen on tumor cells with the other arm, EpCAMxCD3 activates lymphocytes in an antigen-dependent manner. This lymphocyte activation leads to the production of effector cytokines and cytolytic substances such as IFN-γ, TNF-α, granzyme B and perforin (15). Furthermore, Wimberger et al. (31) showed that
tumor resident T cells following treatment with EpCAMxCD3 efficiently lysed malignant cells *ex vivo*. Therefore in the present study it may be assumed that TRAIL-lymphocytes combined with EpCAMxCD3 eliminated tumor cells by a combination of TRAIL receptor-induced apoptosis and activation of lymphocytes, which in turn produced effector cytokines and cytolytic substances and eliminated the cells by the granzyme B and perforin death system. Another advantage of our strategy is that delivering of mTRAIL by lymphocytes may increase the half-life of TRAIL *in vivo* and thus potentiate the cytotoxic effect, since fast dilution and loss of efficacy has been described for directly delivered sTRAIL (32). Our data are confirmed by a recent study in which cell-mediated mTRAIL delivery has been successfully investigated for neural and mesenchymal stem cells in solid tumors (7, 33). In related publications describing targeting of lymphocytes by bsAb *in vitro*, tumor cells were eradicated by pore-forming, pro-apoptotic components of cytotoxic T cells involving perforin and granzyme B (34, 35). These mechanisms of general T cell cytotoxicity may also be involved in the anti-tumor effects observed in our experiments, due to EpCAMxCD3-induced expression of perforin and granzyme B (15). Importantly, the combined cytotoxicity of TRAIL-lymphocytes and EpCAMxCD3 even reduced the tumor-initiating potential of advanced pancreatic cancer cells, which exhibit resistance toward gemcitabine chemotherapy. This result is underlined by a recent report (33), where delivery of TRAIL by mesenchymal stem cells was able to target tumor initiating cells of squamous and lung cancer. Quite similarly, Herrmann et al. (36) demonstrated that EpCAMxCD3-mediated engaging of human T cells eliminates colorectal tumor-initiating cells.

We also observed inhibition of tumor vessel density in xenograft tumors with a most significant effect for combination treatment. These results are in line with recent publications demonstrating that TRAIL induces apoptosis in cultured endothelial cells (37, 38). Anti-angiogenic effects of TRAIL were also observed in xenografts of glioblastoma, multiple
myeloma and pancreatic cancer transplanted to immunodeficient mice (26, 39). More recently, intravenous injection of mTRAIL-expressing CD34+ cells exerted a potent antitumor activity in NOD/SCID mice bearing systemic multiple myeloma xenografts, which was characterized by early vascular disruption, resulting in hemorrhagic necrosis and tumor destruction (8). In the present study, EpCAMxCD3-induced release of IFN-γ and TNF-α from activated lymphocytes may have contributed to the observed anti-angiogenic effect, since these molecules induce expression of chemokines CXCL-9 (MIG), CXCL-10 (IP-10) and CXCL-1 (I-TAC), which inhibit chemotaxis of endothelial cells (40). On the other side we detected enhanced VEGF mRNA expression in xenograft tissue upon treatment with TRAIL-lymphocytes alone or in combination with EpCAMxCD3. This observation is in line with data from Secchiero et al. 2004, who reports that soluble recombinant TRAIL acts proangiogenic by inducing endothelial cell migration and vessel tube formation (41). Besides VEGF expression, TRAIL-lymphocytes alone and to a stronger extend in combination with EpCAMxCD3 led to enhanced mRNA expression of NF-κB, a factor associated with inflammation and survival. Survival signaling in parallel to apoptosis signaling is a well known matter upon treatment with TRAIL (42). This was reflected in our experiments by induction of for example FADD and SMAC/DIABLO by TRAIL-lymphocytes in 3D constructs suggesting upregulated apoptosis signaling. At the same time enhanced HIF1α and later on also enhanced VEGF occurred and reflects anti-apoptotic signaling. However, since we did not detect enhanced CD31+ vessels but reduction after combined treatment with TRAIL-lymphocytes and bsAB, induction of hypoxia and enhanced angiogenesis by the observed expression of HIF1α and VEGF may be excluded. We suggest that the cellular balance of signaling pathways upon treatment with TRAIL-lymphocytes is shifted toward cell death, since membrane TRAIL may have stronger effects than soluble TRAIL and lymphocytes enhance TRAIL-induced cytotoxicity, especially when EpCAMxCD3 comes into play, which act as an additional enhancer.
In the present study we observed cyst formation in xenografts of mice, which were injected with TRAIL-lymphocytes alone or in combination with EpCAMxCD3. Since we did not observe cyst formation in xenografts of mice injected with EpCAMxCD3 alone (15), TRAIL-lymphocytes may be the reason for cyst formation. The reason for cysts is not entirely clear, however, as tumor cells die, e.g. upon induction of apoptosis by TRAIL-lymphocytes, they release several factors that attract phagocytes, in particular macrophages (43) as observed in our study. EpCAMxCD3 may have contributed by induction of secretion of multiple chemokines in lymphocytes (15) resulting in combination with the effect of TRAIL-lymphocytes in pronounced inflammation sufficient for induction of liquid containing cysts within the tumor tissue. This hypothesis is supported by our observation of enhanced NF-κB mRNA expression upon TRAIL-lymphocytes alone or combined with EpCAMxCD3, since NF-κB is a key player of inflammation. In line with our results pancreatic cyst formation is frequently observed in inflamed pancreas of patients (44). Examinations of the fluid inside the cysts of patients suggest that it contains liquefied pancreatic tissue and cells of inflammation. In addition to inflammation, anti-angiogenic effects mediated by our gene-immunotherapeutic approach may have increased cyst formation by inducing leaky blood vessels. Accordingly, Lavazza et al. showed that CD34+ cells engineered to express mTRAIl induced hemorrhagic necrosis in multiple myeloma xenografts (8). Thus cyst formation by TRAIL-lymphocytes and combination with EpCAMxCD3 may be a result of inflammation, induction of host response and vasculature disruption together.

In our cell lines we see that 50% of the population are TRAIL resistant and do not die upon TRAIL treatment. This matches with the observation that TRAIL resistance is frequently observed in around 50% of tumor cell lines. Upon treatment with TRAIL-lymphocytes, only a small cell population of the gemcitabine resistant cancer stem-like BxPc-3 cells has active caspase-3 and showed less tumorigenicity in mice. This suggests that
TRAIL-lymphocytes alone do not completely target cancer stem-like cells. However, combination with EpCAMxCD3 strongly increased therapeutic efficacy as shown by treatment of mice with tumor xenografts, by tumor take experiments in mice transplanted with pre-treated cells and by in vitro experiments of caspase-3 analysis. We also performed 3D experiments but saw no additional effect upon combination of TRAIL-lymphocytes with bsAb compared to TRAIL lymphocytes alone. This may be due to the fact that bsAb does only weakly induce apoptosis signaling, but rather acts by the perforine/granzyme system (15).

Regarding translational relevance of local injection of TRAIL-lymphocytes and EpCAMxCD3, our model does not clearly mimic human T cell-based adoptive cancer immunotherapy, where tumor-reactive/specific T lymphocytes are usually systemically administered to patients. We used a direct injection model since systemic administration of lymphocytes results in high organ distribution i.e. in lungs and liver and removal from circulation. In addition, pre-activated lymphocytes have a short half-life due to activation-induced cell death. Our model rather reflects a proof of concept study similar to experimental approaches performed by Schlereth et al. (45), or Brischwein et al. (46). Even in a more patient-related metastatic model with systemic injection we assume that TRAIL-lymphocytes combined with EpCAMxCD3 may affect tumor cells stronger than EpCAMxCD3 or agonistic TRAIL receptor antibodies, which are already in clinical use. The advantage of our approach is that two different cell death systems act together, which potentiates the effect. Even highly therapy-resistant cells with cancer stem cell characteristics may be targeted since EpCAMxCD3 sensitizes tumor cells for TRAIL-induced apoptosis and vice versa. Regarding recent concerns about the safety of older retroviral vectors in patients, previous work has focused attention on self-inactivating (SIN) lentiviral vectors as potential alternative delivery platforms. In our study we used these improved lentiviral vectors for TRAIL-transfer to
lymphocytes. Lentiviral vectors limit the risk of viral LTR enhancer mutagenesis and permit the use of lineage-specific expression cassettes (47). However, we are aware that before our gene-immunotherapy may be applicable to treatment in patients further experimental studies in a model closely resembling the situation in patients are necessary.

In conclusion, we demonstrate that TRAIL-lymphocytes combined with EpCAMxCD3 induces a strong antitumor response in our experimental system. This therapeutic approach was even effective in tumorigenic cells with cancer stem cell characteristics, which do not respond to conventional chemotherapy. An important positive effect of our method may be the "opening" of the tumor microenvironment for conventional cytotoxic therapy due to induction of an advanced inflammatory response, which may sensitize tumor cells for cytotoxic therapy or innate immune responses. The clinical feasibility of such a systemic gene-immunotherapy may be understood as an initial aid to support innate immune responses and to enhance induction of cell death by cytotoxic tumor therapy.
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Conflict of Interest

The authors declare that no conflicts of interests or disclosures exist.
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apoptosis-inducing ligand and/or interleukin-2 gene in human renal cell carcinoma.


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Figure Legends

Figure 1 Induction of apoptosis in BxPc-3 pancreatic cancer cells by TRAIL-lymphocytes in vitro. (A) BxPc-3 were left untreated (CO) or were co-cultured with TRAIL-lymphocytes (TRAIL-L) or MOCK-lymphocytes (MOCK-L) for 24 h. Representative photographs of morphology or after staining for active caspase-3 (act casp 3) are shown under 100 x magnification. (B) BxPc-3 tumor cells were mixed with TRAIL- or MOCK-lymphocytes at ratios indicated and incubated for 4 h. The percentage of PI-positive tumor cells (% PI^+ cells) was detected by FACS analysis. Tumor cells were gated based on MHC class I positivity.

Figure 2 EpCAMxCD3 enhances tumor retardation by TRAIL-lymphocytes. (A) BxPc-3 or PC-3 cells were s.c. engrafted in the presence of lymphocytes to NOD/SCID mice (n = 6 per group) and treated three days later with i.p. injections of EpCAMxCD3 in doses indicated as described in Materials & Methods. (L) Non-transduced lymphocytes. Tumor size was measured every third day. Tumor volume is depicted as mean with standard deviation. (B) Weight of tumors at the end-point of the experiment at day 23. *p < 0.05.

Figure 3 Reduction of proliferation and blood vessel density and induction of apoptosis in vivo. BxPc-3 tumor xenografts as described in Figure 2B were harvested at day 23 after treatment. Ki-67 expression indicating proliferation was detected in frozen tissue sections by immunohistochemistry and quantified in 10 vision fields under 400 x magnification. Blood vessel density was detected by staining with CD31/PECAM-1 and quantified under 100 x magnification. Apoptosis was detected by staining of active caspase-3 and TUNEL assay and quantified in 10 vision fields under 400 x magnification. *p < 0.05.

Figure 4 Gene-immunotherapy reduces tumor mass of advanced xenografts by cyst...
formation. BxPc-3 xenografts (n = 5 per group) were engrafted in mice. Twelve days later treatment was initiated by s.c. injections around the tumor. PBS (CO), EpCAMxCD3 (bsAb) or (TRAIL-L + bsAb) TRAIL-L preloaded with EpCAMxCD3 1 h in vitro prior to injection as described in Materials & Methods. (A) At day 23 after treatment mice were sacrificed and xenografts were resected. Tumor morphology was evaluated by microscopy following H&E staining of frozen tissue. Representative mouse xenografts were photographed and images of are shown at 5 x and 100 x magnification. (B) Tumor growth is shown as the increase of tumor size starting from day 12 after tumor cell implantation. (C) Blood vessel density was analyzed by staining of CD31 and evaluation of CD31-positive structures under 100 x magnification (n = 4 per group). *p < 0.05.

**Figure 5** EpCAMxCD3 and/or TRAIL-lymphocytes induce macrophage infiltration of xenograft tumors. Tumor tissue from mice transplanted with BxPc-3 cells and treated as described in Figure 2B was examined at day 23. Mouse macrophages were detected by immunohistochemistry for CD68 and F4/80 antigens in frozen tumor sections.

**Figure 6** Inhibition of tumor take, proliferation and apoptosis resistance of tumor cells with cancer stem features by TRAIL-lymphocytes and EpCAMxCD3. (A) Viability was measured in BxPc-3 and gemcitabine-resistant GEM-BxPc-3 cells 72 h after treatment with 50 nM gemcitabine (GEM) or recombinant sTRAIL at concentrations indicated. (B) For in vivo experiments GEM-BxPc-3 cells were left untreated (CO) or were co-incubated with TRAIL-L alone or TRAIL-L preloaded with EpCAMxCD3. Two hours later, surviving cells were counted and 6 x 10^3 viable cells were subcutaneously transplanted to mice (n=4/group). (C) Tumor engraftment (tumor take) was monitored for 35 days. (D) Frozen tissue sections of grown GEM-BxPc-3 tumors were analyzed for proliferation by immunohistochemistry. Ki-67-positive tumor cells were quantified in 10 vision fields under 400 x magnification.
Apoptosis was detected by staining for active caspase-3 and positive tumor cells were quantified in 10 vision fields under 400 x magnification. *p < 0.05.
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