

## Novel Bispecific Antibodies Increase $\gamma\delta$ T-Cell Cytotoxicity against Pancreatic Cancer Cells

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### Abstract

The ability of human  $\gamma\delta$  T cells from healthy donors to kill pancreatic ductal adenocarcinoma (PDAC) *in vitro* and *in vivo* in immunocompromised mice requires the addition of  $\gamma\delta$  T-cell-stimulating antigens. In this study, we demonstrate that  $\gamma\delta$  T cells isolated from patients with PDAC tumor infiltrates lyse pancreatic tumor cells after selective stimulation with phosphorylated antigens. We determined the absolute numbers of  $\gamma\delta$  T-cell subsets in patient whole blood and applied a real-time cell analyzer to measure their cytotoxic effector function over prolonged time periods. Because phosphorylated antigens did not optimally enhance  $\gamma\delta$  T-cell cytotoxicity, we designed bispecific antibodies that bind CD3 or V $\gamma$ 9 on  $\gamma\delta$  T cells and Her2/neu (ERBB2) expressed by pancreatic tumor cells. Both antibodies enhanced  $\gamma\delta$  T-cell cytotoxicity with the Her2/V $\gamma$ 9 antibody also selectively enhancing release of granzyme B and perforin. Supporting these observations, adoptive transfer of  $\gamma\delta$  T cells with the Her2/V $\gamma$ 9 antibody reduced growth of pancreatic tumors grafted into SCID-Beige immunocompromised mice. Taken together, our results show how bispecific antibodies that selectively recruit  $\gamma\delta$  T cells to tumor antigens expressed by cancer cells illustrate the tractable use of endogenous  $\gamma\delta$  T cells for immunotherapy. *Cancer Res*; 74(5); 1349–60. ©2014 AACR.

### Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an extremely aggressive malignancy with poor prognosis. The overall 5-year survival rate is only <5%. A rapid disease progression and absence of specific symptoms often preclude early diagnosis and curative treatment. Approximately 80% of the patients have an advanced disease with distant metastases at diagnosis, and only 10% to 20% are accessible for surgical resection (1). Occasional resistance of PDAC to chemotherapy demanded alternative therapeutic approaches (2, 3). An increasing number of novel therapeutic strategies, including targeted therapy or immunotherapy alone or in combination with chemotherapy, have been

described but still failed to considerably improve survival times of the patients (4, 5).

Immunotherapy with unconventional T cells such as  $\gamma\delta$  T cells is of substantial interest based on their potent HLA-nonrestricted cytotoxicity against different tumor entities and their additional capacity to recognize and present antigens to  $\alpha\beta$  T cells (6–8). Several pilot studies reported a partial success in tumor reduction after *in vivo* activation of  $\gamma\delta$  T cells with nitrogen-containing bisphosphonates (n-BP) and rIL-2 or after adoptive transfer of *in vitro* activated  $\gamma\delta$  T cells (9–12). V $\gamma$ 9V $\delta$ 2  $\gamma\delta$  T cells recognize isopentenyl-pyrophosphate (IPP) of the eukaryotic mevalonate pathway, a phosphoantigen (PAG) whose production is enhanced in dysregulated transformed cells. The augmented production of IPP in tumor cells is further increased by treatment with n-BP such as zoledronic acid (13). Comparable to IPP, synthetic phosphoantigens (PAG) such as bromohydrin pyrophosphate (BrHPP) are also capable of inducing activation of V $\gamma$ 9V $\delta$ 2 T cells (14). We have previously shown the efficacy of human V $\gamma$ 9V $\delta$ 2 T cells from healthy donors against PDAC *in vitro* as well as *in vivo* after repetitive adoptive transfer of  $\gamma\delta$  T cells together with n-BPs and low-dose IL-2 into SCID mice (15). Although  $\gamma\delta$  T-cell-based immunotherapy delivered promising results, it is mandatory to optimize the cytotoxic activity of tumor-reactive  $\gamma\delta$  T cells in view of an observed exhaustion of  $\gamma\delta$  T cells, and to minimize the repetitive adoptive  $\gamma\delta$  T-cell transfer (7, 8, 15, 16).

To date, the most promising approach to enhance the cytotoxic potential and to recruit T cells to the tumor is based on the usage of single-chain bispecific antibody constructs of the bispecific T-cell engager (BiTE) class (17). BiTE antibodies

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

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such as blinatumomab with specificity for CD3 on T cells and for CD19 on lymphoma or leukemia cells have proved to be efficient for the treatment of patients with hematologic malignancies (18, 19).

In this study, we designed 2 novel bispecific antibodies with specificity for CD3 or V $\gamma$ 9TCR on  $\gamma\delta$  T cells and for receptor tyrosine kinase Her2/neu, which is overexpressed on 11% to 82% of tumor cells of patients with PDAC (20–23). We investigated the potential of these novel bispecific antibodies to enhance the cytotoxicity of  $\gamma\delta$  T cells from patients with PDAC measured *in vitro* with a real-time cell analyzer (RTCA) and *in vivo* upon adoptive transfer into PDAC bearing SCID-Beige mice. In addition, we established an immune monitoring system to determine the absolute cell number of  $\gamma\delta$  T-cell subsets of patients with PDAC *ex vivo*.

## Patients and Methods

### Ethic statement and patients information

Informed consent was obtained from all donors, and the research was approved by the relevant institutional review boards (code number: D 405/10). Twenty-one patients with histologically verified PDAC and stage pT3–4, pN0–1, L0–1, and V0–1 were enrolled. All patients had not been chemo- or radiotherapeutically treated before this investigation. Further information can be found in the Supplementary Materials and Methods.

### Immunomonitoring of $\gamma\delta$ T-cell numbers

Whole blood samples (50  $\mu$ L) from healthy donors or patients with PDAC were stained in BD TrueCount tubes with the following fluorochrome-conjugated monoclonal antibodies (mAb): CD45-PECy7 clone HI30, TCR $\gamma\delta$ -APC clone 11F2 (both from BD Biosciences), V $\delta$ 2-PE clone B6 (Beckman Coulter), V $\delta$ 1-FITC clone TS8.2 (Thermo Fisher Scientific). After lysing red blood cells with 200  $\mu$ L BD Lysing-solution, cells were directly analyzed using FACS Canto analyzer and FACS Diva software (BD Biosciences). Additional mAbs used for flow cytometry are listed in the Supplementary Materials and Methods.

### Isolation and culturing of lymphocyte populations

Peripheral blood mononuclear cells (PBMC) were isolated from the leukocyte concentrates or from heparinized blood by Ficoll-Hypaque (Biochrom) density gradient centrifugation. To separate freshly isolated  $\gamma\delta$  T or NK cells, we used negative selection kits (TCR $\gamma\delta^+$  T Cell Isolation Kit or NK Cell Isolation Kit, Miltenyi Biotec) according to the manufacturer's instructions.

To establish short-term  $\gamma\delta$  T-cell lines, PBMC were cultured in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 25 mmol/L HEPES, antibiotics, 10% FCS (complete medium), and stimulated either with 300 nmol/L of Pag BrHPP (kindly provided by Innate Pharma) or 5  $\mu$ mol/L of n-BP zoledronic acid (Novartis). Fifty U/mL rIL-2 (Novartis) was added every 2 days over a culture period of 14 to 21 days. After 2 to 3 weeks, most  $\gamma\delta$  T cells had a purity >95% V $\gamma$ 9V $\delta$ 2  $\gamma\delta$  T cells. CD8  $\alpha\beta$  T-cell lines were established from PBMC of patient with PDAC (PC 13) after stimulation with 0.5  $\mu$ g/mL phytohemagglutinin

(Thermo Fisher Scientific). CD8  $\alpha\beta$  T cells were purified by magnetic depletion of non-CD8 T cells and expanded by feeder cells and phytohemagglutinin (15). Further details are given in the Supplementary Materials and Methods.

### Tumor cell lines

PDAC cell lines PancTu-I, Colo357, and Panc89 were kindly provided by Prof. Kalthoff, Section of Molecular Oncology, Kiel, Germany. The genotype of PDAC cell lines was recently confirmed by short tandem repeats analysis. PDAC cell lines as well as Raji lymphoma cells (DSMZ) were cultured in complete medium. For removing adherent PDAC cells from flasks, cells were treated with 0.05% trypsin/0.2% EDTA.

### Construction of the recombinant bispecific antibody derivatives

The CD3 and CD19 single-chain fragment variables (scFv) were synthesized according to published sequences (24, 25). Appropriate restriction sites were introduced to allow directional cloning into a pSEC-Tag2-Hygro-CD20xCD16 vector allowing secretion of bispecific scFv (bsscFv; unpublished). The CD20-scFv was replaced by the CD19- or Her2-specific scFv described earlier (26). The final expression vectors pSEC-Tag2-Hygro-Her2xCD3, pSEC-Tag2-Hygro-CD19xCD3, and pSEC-Tag2-Hygro-CD20xCD3 were partially sequenced to confirm success of the cloning procedure.

The coding sequences for the variable regions of the V $\gamma$ 9  $\gamma\delta$  T-cell-specific antibody (clone 7A5) were isolated according to established procedures (27, 28). Cloning cassettes for the integration in expression vectors allowing generation of bispecific antibodies in the tribody format were *de novo* synthesized (Entelechon GmbH). Secretion leaders and appropriate restriction sites were introduced to allow directional cloning into expression vectors coding for a [(Her2) $_2$ xCD16] tribody described earlier (29). The CD16-binding variable regions were replaced by the 7A5 variable regions, resulting in two expression vectors: pSEC-Tag2-Her2-scFv-HC-V $\gamma$ 9-VH and pSEC-Tag2-Her2-scFv-LC-V $\gamma$ 9-VL coding the heavy chain and light chain derivative, respectively.

### Expression and purification of the antibody derivatives

Lenti-X 293T cells were transfected with the respective expression vectors coding for the tribody [(Her2) $_2$ xV $\gamma$ 9], and the bsscFv [CD19xCD3], [CD20xCD3], or [Her2xCD3] (29). The tribody was purified from supernatant as described (29, 30).

### <sup>51</sup>Cr-release assay

Cytotoxicity against PDAC cell lines was analyzed in a standard 4 hours <sup>51</sup>Cr-release assay as described (31). To investigate modulation of cytotoxic activity, effector T cells were preincubated for 1 hour before the assay as follows: with 300 nmol/L BrHPP, [Her2xCD3], or [CD19xCD3] bsscFv as a control construct. Cells were supplemented with 12.5 U/mL IL-2.

### Real-time cell analyzer

A total of 5,000 adherent PDAC cells/well were added to 96-well micro-E-plate to monitor the impedance of the cells every 15 minutes for up to 24 hours. After having reached the linear

growth time phase, [Her2xCD3] or [(Her2)<sub>2</sub>xVγ9], and corresponding control constructs were added in the previously titrated optimal concentrations for 1 hour. Thereafter, T-cell lines or freshly isolated γδ T cells together with 12.5 IU/mL IL-2 were added to the RTCA single-plate (SP) assay (Roche). Where indicated, Vγ9Vδ2 T-cell effector cells were stimulated with 300 nmol/L BrHPP. When effector cells induced lysis of the tumor cells, the loss of impedance of tumor cells was measured. The cells were monitored every minute for 6 hours and, thereafter, every 5 minutes for up to additional 20 hours.

#### ELISA and CD107a degranulation assay

These standard methods with commercial kits are described in Supplemental Materials and Methods.

#### Immunohistochemistry

Immunostaining with anti-CD3 mAb clone SP7 (dilution 1:100; NeoMarkers/LabVision) of serial paraffin-embedded tissue sections of 41 patients with PDAC (permission number of the Ethics Committee: A 110/99) was done with the fully automated Bond Max-System using the Bond Polymer Refine Detection Kit (Leica-Menarini). Automated antigen retrieval was performed in ER1 (citrate buffer Bond pH 6.0; Leica-Menarini). TCR γδ expression was detected from the same tissue sections after deparaffinization and treatment with antigen retrieval solution (pH 9.0; DAKO) by using 3 μg/mL anti-γδ TCR clone γ3.20 (Thermo Fisher Scientific) or mouse IgG1 isotype control mAbs. As second-step, antibody EnVision-mouse horseradish peroxidase (DAKO) was used. The substrate reaction was performed using the AEC substrate for peroxidase (DAKO). Finally, sections were stained with hemalaun and embedded in glycerine gelatine (Merck).

#### Xenograft tumor model

Female, 6-week-old SCID-Beige mice (Charles River) were housed under specific pathogen-free conditions of the Central Animal Facility of the University of Kiel. The research was approved by the institutional review boards (code number V241-72241.121-20 [108-7/13]). Mice were subcutaneously inoculated in the left-shaved flank with  $1.5 \times 10^6/50 \mu\text{L}$  PDAC cell-line PancTu-I. After 2 days, mice were randomized into 6 groups with 5 to 10 animals before subcutaneous injection of either 15 μg/kg ( $25 \times 10^4$  IU) IL-2 (Proleukin) with NaCl or 1.25 mg/kg [(Her2)<sub>2</sub>xVγ9] or 2.5 mg/kg zoledronic acid. Treatments were repeated weekly for a total of 4 weeks. Where indicated, mice received previously expanded Vγ9Vδ2 γδ T cells of one donor subcutaneously on day 2 ( $2.5 \times 10^6/\text{mouse}$ ), day 7 ( $8 \times 10^6/\text{mouse}$ ), day 14 ( $4.5 \times 10^6/\text{mouse}$ ), and day 23 ( $6 \times 10^6/\text{mouse}$ ). The total volume of IL-2, plus NaCl or [(Her2)<sub>2</sub>xVγ9] and NaCl or Vγ9Vδ2 T cells was 75 μL. To obtain short-term activated Vγ9Vδ2 T cells, 150 to  $250 \times 10^6$  PBMC freshly isolated at 4 time points from one donor were stimulated with 300 nmol/L BrHPP 14 to 19 days before application of the cells. The purity of the activated cells was >95%. To determine the tumor weight and size/volume (length × width × depth), all mice were sacrificed 29 days after tumor cell inoculation. Tumor take was 100% and all mice survived for the length of the experiment.

#### Statistical analysis

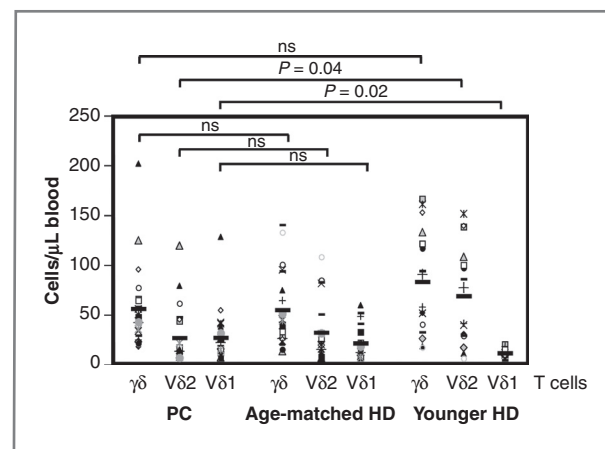
The statistical analysis of immune monitoring was assessed by Wilcoxon rang sum test using SPSS version 17.0. All statistical tests were 2-sided and the level of significance was set at 5%, not corrected for multiple testing.

Because no violation of normal distribution assumption was found (Shapiro–Wilk test), all statistical comparisons of the *in vivo* experiments were done parametrically by using *t* tests. The primary null hypothesis that there are no differences between mice, which received only tumor cells and tumor cells with tribody [(Her2)<sub>2</sub>xVγ9] or tumor cells with γδ T cells, respectively, was tested 2-sided at a 5% level of significance. For all other tested hypotheses, we did not adjust for multiple testing because of their explorative manner.

## Results and Discussion

#### γδ T-cell monitoring

The success of a γδ T-cell-based immunotherapy requires a profound knowledge about cell numbers and the functional capacity of patients' γδ T cells. We established an immune monitoring system that allows us to determine the absolute γδ T-cell numbers in small volumes of heparinized whole blood. In comparison to the absolute number of γδ T cells determined in the blood of younger healthy donors, Vδ2 T cells were decreased in patients with PDAC (Fig. 1). Several studies described a decrease of the absolute number of circulating γδ T cells in patients with cancer. However, the precise comparison with age-matched healthy donors clearly demonstrated that the reduction correlated with age and was not because of the cancer disease in patients with PDAC. Concomitantly with the age-related decrease of Vδ2 T cells, we observed an increase of Vδ1 T cells in elderly donors in comparison to the younger cohort (Fig. 1).



**Figure 1.** Quantification of γδ T-cell subsets in whole blood. The absolute cell number of γδ T cells, Vδ1- and Vδ2-γδ T-cell subsets were analyzed out of 50 μL blood samples from patients with PDAC (PC, *n* = 21; 9 females and 12 males; age:  $65.0 \pm 11.0$  years), from age-matched healthy donors (HD, *n* = 21; age:  $64.6 \pm 10.6$  years), and from younger HD (*n* = 16; age:  $36.4 \pm 9.4$  years). Each symbol represents the data of one donor, and thick bars represent the mean value of different experiments. Significances are represented as *P* value; n.s., nonsignificant.



In this study, we focused on numerically reduced V $\delta$ 2 T cells, which are exclusive targets for PAg applied in  $\gamma\delta$  T-cell–based immunotherapy. We established several short- and long-term cultured V $\delta$ 2 T-cell lines from patients with PDAC as well as from healthy donors (Supplementary Fig. S1A). In previous studies, we observed a limited cytotoxic capacity of V $\delta$ 2 T-cell lines from healthy donors against PDAC cell lines that, however, could be enhanced by the addition of PAg or n-BP (15). A sustained stimulation of V $\delta$ 2 T cells by PAg or n-BPs often leads to an exhaustion of the  $\gamma\delta$  T-cell pool, which requires novel or modified stimulation protocols (7).

Combining  $\gamma\delta$  T-cell–based immunotherapy with mAb provides novel perspectives. An enhanced efficacy against Her2<sup>+</sup> breast cancer cells has been described after adoptive transfer of expanded  $\gamma\delta$  T cells together with a humanized anti-Her2/neu mAb (trastuzumab) into human Her2<sup>+</sup> mammary carcinoma-bearing SCID mice (32). A recent review summarized the evidence that the combination of  $\gamma\delta$  T-cell–based immunotherapy with mAb in general exerts a considerable therapeutic potential for a variety of malignancies (33). In all these studies,  $\gamma\delta$  T cells bind to mAb-labeled tumor cells via FcR $\gamma$ III (CD16) and thereby exert antibody-dependent cell-mediated cytotoxicity. We observed a high donor-dependent variability of CD16 expression on  $\gamma\delta$  T cells from patients with PDAC (data not shown). This observation together with the observed expression of Her2/neu on the surface of all analyzed PDAC cell lines (Supplementary Fig. S1B) prompted us to design a bispecific antibody in a BiTE-like tandem scFv format by genetically fusing a scFv derived from the trastuzumab V-regions to a CD3-specific scFv via a flexible linker (Supplementary Fig. S2A and S2B).

#### [Her2xCD3] enhances $\gamma\delta$ T-cell–mediated lysis

The design of the [Her2xCD3] bsscFv in a BiTE-like format triggers full activation of T cells independent of other costimuli (34). [Her2xCD3] bsscFv purification was performed by IMAC chromatography and size exclusion chromatography (Supplementary Fig. S2C and S2D). The bsscFv retained the antigen specificities of the parental antibodies as evidenced by flow cytometry. [Her2xCD3] specifically bound to CD3-positive  $\gamma\delta$  T cells and Her2-positive PDAC cell lines. No binding was observed on Her2- and CD3-negative lymphoma cells (Raji) or NK cells (Supplementary Fig. S2E). As controls, similarly designed molecules targeting either CD19 or CD20, which both are not expressed on PDAC cell lines, were generated and demonstrated the expected binding patterns (not shown).

The comparison of  $\gamma\delta$  T-cell lines established from healthy donors or patients with PDAC showed a very similar cytotoxic activity against the indicated PDAC cell lines, as revealed in a <sup>51</sup>Cr-release assay (Fig. 2). In the absence of any additional stimulus, the  $\gamma\delta$  T-cell–mediated cytotoxicity against all PDAC cell lines was very weak (Fig. 2A and Supplementary Fig. S3A). We then examined several strategies to enhance the poor cytotoxic capacity of  $\gamma\delta$  T cells against the PDAC cells by comparing the enhancing effects of PAg and bispecific ab. As expected, the addition of PAg enhanced the cytotoxic activity of  $\gamma\delta$  T cells from healthy donors (Fig. 2B and Supplementary Fig. S3B). We obtained similar results using  $\gamma\delta$  T cells of

patients with PDAC instead of healthy donors (Fig. 2B). The addition of a previously titrated optimal concentration of [Her2xCD3] to the PAg-stimulated cells further increased cytotoxic activity compared with the PAg stimulation in combination with the control bsscFv (Fig. 2C and D and Supplementary Fig. S3C and S3D). Moreover, the [Her2xCD3] alone was sufficient to moderately enhance cytotoxicity of  $\gamma\delta$  T cells against PDAC cells in comparison to PAg alone or the combination of both (Fig. 2E and Supplementary Fig. S3E). Interestingly,  $\gamma\delta$  T-cell lines established from a given donor exerted similar cytotoxic activity independently of whether the cells were used after initial- or after restimulation (Fig. 2 and Supplementary Fig. S3; HD3/PC1 and data not shown).

#### Comparison of <sup>51</sup>Cr-release and RTCA-SP assay

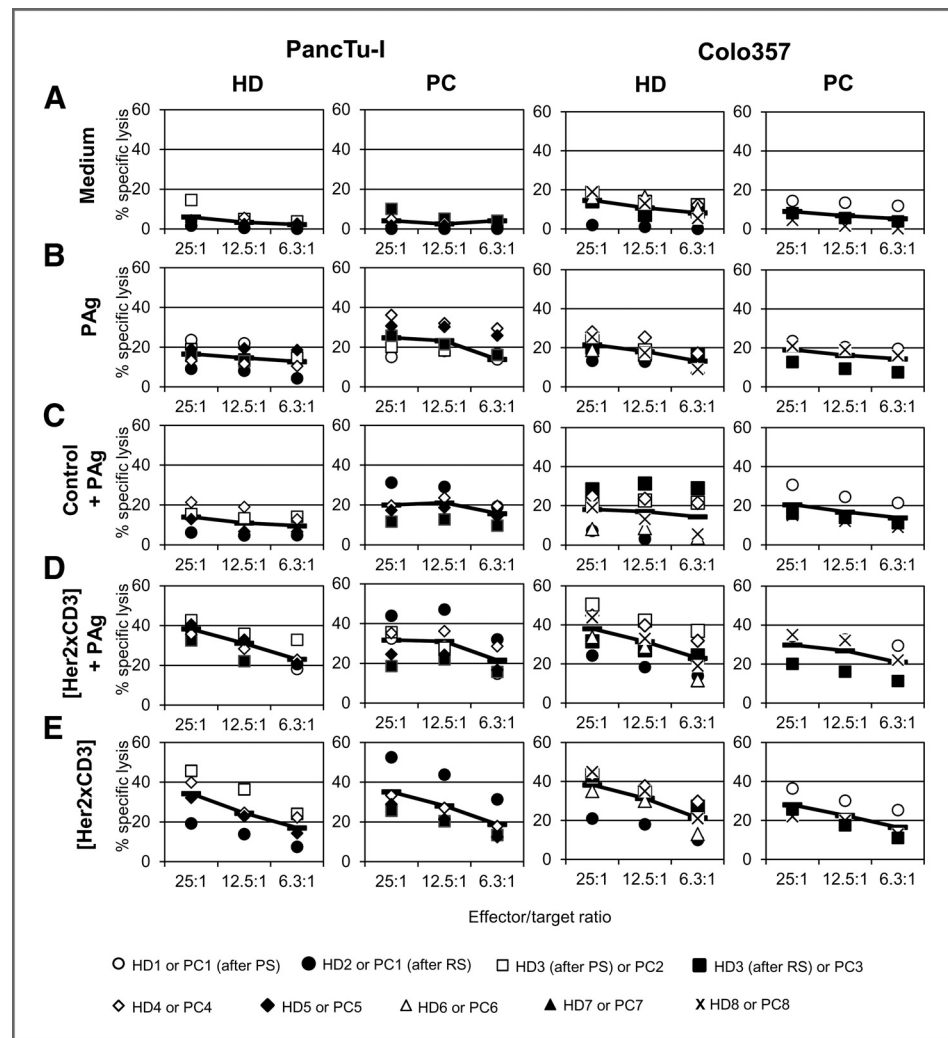
Bispecific antibodies engaging CD3 display enhanced cytotoxicity when it is measured for prolonged time intervals (35). The chromium release assay just gives a snap-shot of  $\gamma\delta$  T-cell cytotoxicity against tumor cells as it is usually measured after 4 hours of incubation. Therefore, we used in parallel the RTCA-SP assay, which monitors cellular events without the incorporation of labels in real time up to several days. Based on the extended time course, the RTCA-SP could also be used to determine whether a smaller number of effector T cells (as it probably occurs at the tumor site) completely lysed PDAC cells or if tumor cells can regenerate because of an incomplete lysis. Similar killing patterns were observed with the  $\gamma\delta$  T-cell line of one representative healthy donor against Panc89 or Colo357 determined by either <sup>51</sup>Cr-release assay or RTCA-SP (Fig. 3). Both PDAC cell lines were weakly lysed by  $\gamma\delta$  T cells without an additional stimulus. Over the extended time period of the RTCA-SP analysis, however, a more enhanced lysis of Panc89 cells than of Colo357 cells was evident compared with the <sup>51</sup>Cr-release assay (Fig. 3). Moreover, the comparison of a 50:1 with a 5:1 effector:target (E:T) ratio demonstrated that with the latter the difference between untreated and [Her2xCD3] treated cells was more prominent in the RTCA-SP assay than in the <sup>51</sup>Cr-release assay. Independently of the applied assay and the E:T ratio, [Her2xCD3] strongly enhanced  $\gamma\delta$  T-cell–mediated lysis of PDAC cells (Fig. 3). Similar results were obtained with  $\gamma\delta$  T-cell lines from 2 additional donors in 2 independent experiments (data not shown). A similarly designed control bsscFv resulted in a lysis comparable to that measured in the medium control (Fig. 3). The addition of the bispecific antibody constructs to the tumor cells in the absence of  $\gamma\delta$  T cells had no inhibitory/cytotoxic effect (data not shown).

Taken together, the [Her2xCD3] triggered the  $\gamma\delta$  T-cell lysis of largely resistant PDAC cell lines, which was particularly evident at lower E:T ratio in the RTCA-SP assay.

#### Generation and effect of a selective $\gamma\delta$ T-cell engager antibody construct

CD3-recruiting bsscFvs redirect all T cells, including immunosuppressive CD3/ $\alpha\beta$ -positive regulatory T cells (Treg), which often accumulate in the tumor environment (36). To selectively target  $\gamma\delta$  T cells, we further developed a new antibody construct. Antibody constructs enabling bivalent tumor targeting have been reported to enhance the avidity to

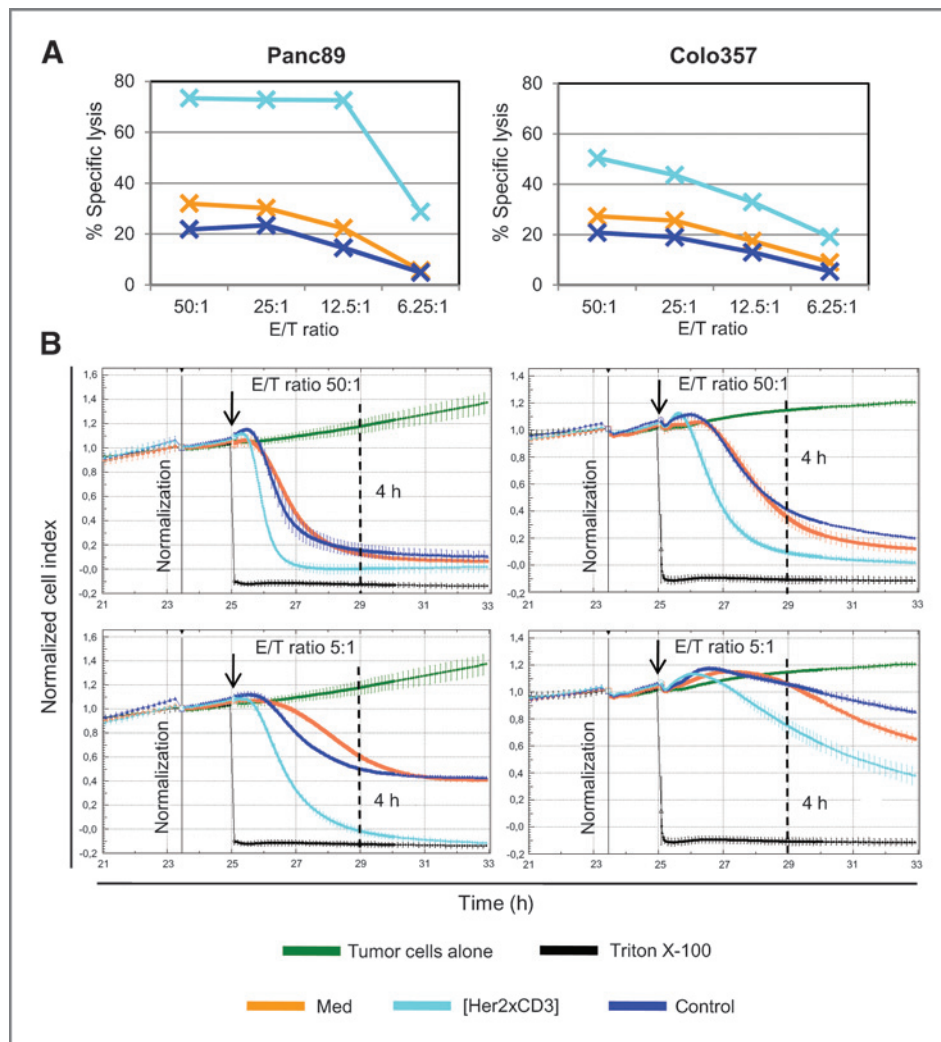
**Figure 2.** Enhancement of PDAC lysis by [(Her2xCD3)] bsscFv. Vγ9Vδ2 γδ T-cell lines of healthy donors (HD) or patients with PDAC (PC) were cultured with medium (A), 300 nmol/L PAg BrHPP (B), 300 nmol/L BrHPP plus 1 μg/mL control bsscFv (C), 300 nmol/L BrHPP plus 1 μg/mL [(Her2xCD3)] bsscFv (D), or 1 μg/mL [(Her2xCD3)] bsscFv (E) before the addition of the indicated <sup>51</sup>Cr-labeled PDAC cell lines PancTu-I or Colo357. Each symbol represents the mean value of triplicate assays of one donor (SD < 10%). Representative results from 6 different donors are shown in the three left-hand columns (PancTu-I/HD and PC, Colo357/HD), whereas results from three donors are included in the right-hand column (Colo357/PC). PC1 and HD3 were tested at two different time points after culturing, after primary stimulation (PS), and after restimulation (RS).



the tumor cells and increase the cytolytic activity of bispecific antibodies (37). Thus, we generated a bispecific antibody in the so-called tribody format, allowing bivalent Her2-targeting and monovalent binding to γδ T cells (Fig. 4A). To this end, the V-regions from the Vγ9-specific hybridoma 7A5 (28) were cloned and used for the generation of the tribody [(Her2)<sub>2</sub>xVγ9]. [(Her2)<sub>2</sub>xVγ9] was transiently expressed in HEK293T cells and purified by 2-step affinity chromatography and size exclusion chromatography (Supplementary Fig. S4A and S4B). The tribody retained the antigen specificities of the parental antibodies as evidenced by flow cytometry. [(Her2)<sub>2</sub>xVγ9] specifically bound to Vγ9 expressing γδ T cells and to Her2-positive PDAC cell lines. No binding to antigen-negative cells (Raji or NK cells) was observed (Supplementary Fig. S4C).

Next, the PDAC cell line PancTu-I was cultured alone (green line) or together with different numbers of γδ T cells for a representative patient with PDAC (PC; Fig. 4). A reduced impedance of PancTu-I cells was dependent on the number of added γδ T cells in the RTCA-SP (Fig. 4B). The addition of PAg as well as [(Her2)<sub>2</sub>xVγ9] dose dependently enhanced the γδ T-cell-mediated cytotoxicity at an E:T ratio of 12.5:1 using

the γδ T cells of the same donor (Fig. 4C and D). Focussing on the first 2 hours of lysis measured with the RTCA-SP assay, we noted that the higher concentrations of [(Her2)<sub>2</sub>xVγ9] induced a stronger γδ T-cell-mediated lysis than the highest concentration of PAg (Fig. 4C and D). This observation was more pronounced with the γδ T-cell line of an additional patient with PDAC, which did not lyse PancTu-I cells at an E:T ratio of 3:1 (Fig. 4E). However, the addition of 1 μg/mL [(Her2)<sub>2</sub>xVγ9] increased γδ T-cell-mediated lysis more effectively than the addition of 300 nmol/L PAg, whereas the combination of [(Her2)<sub>2</sub>xVγ9] together with PAg did not further enhance the cytotoxic activity of the γδ T-cell line (Fig. 4E). As shown in Fig. 4F, γδ T cells of another donor were not able to lyse PancTu-I cells at an E:T ratio of 12.5:1 unless 1 μg/mL of [(Her2)<sub>2</sub>xVγ9] was added, whereas the cytotoxicity of an autologous CD8 T-cell line against PancTu-I cells was not enhanced with [(Her2)<sub>2</sub>xVγ9], but with [Her2xCD3], which demonstrates the specificity of [(Her2)<sub>2</sub>xVγ9] in activating only γδ T cells (Fig. 4F and G). Similar results were obtained when 0.1 μg/mL of [(Her2)<sub>2</sub>xVγ9] were used instead of 1 μg/mL (Fig. 4G). In addition, the specificity of [(Her2)<sub>2</sub>xVγ9] and [Her2xCD3] was



**Figure 3.** Comparative analysis of PDAC lysis by RTCA versus  $^{51}\text{Cr}$ -release. Lysis of Panc89 or Colo357 cells is shown in response to graded numbers of  $\gamma\delta$  T cells of one representative donor (HD8) as indicated in a  $^{51}\text{Cr}$ -release assay after 4 hours (A) or in a RTCA-SP assay over 8 hours (B). PDAC cells were labeled for 1 hour with  $^{51}\text{Cr}$  (A) or cultured for approximately 24 hours on an E-plate before addition of medium (orange line), 1  $\mu\text{g}/\text{mL}$  [Her2xCD3] bsscfv (light blue line) or 1  $\mu\text{g}/\text{mL}$  control construct (dark blue line; B). A, results of  $^{51}\text{Cr}$ -release assay are presented as % specific lysis. The mean value of triplicate assays was analyzed (SD < 10%). B, the cell index was determined every 5 minutes over the course of the experiment and normalized to 1 at the time point of addition of bispecific antibody constructs as shown by the vertical black thin line. The addition of  $\gamma\delta$  T cells or a final concentration of 1% Triton X-100 (for the induction of maximal lysis) is illustrated as an arrow. The green line is derived from tumor cells in the absence of  $\gamma\delta$  T cells (tumor cells alone), whereas the black line represents lysis because of the presence of Triton X-100. The average of triplicates with the SD is shown.

confirmed by the observation that these bispecific antibodies enhanced the  $\gamma\delta$  T-cell mediated lysis of Her2-positive PancTu-I cells, but not of Her2-negative cells (Supplementary Fig. S5).

To further substantiate these results, we analyzed the cytotoxic activity of additional  $\gamma\delta$  T-cell lines established from healthy donors and patients with PDAC against the 3 different PDAC cell lines in the absence or presence of PAg, [Her2xCD3], or [(Her2) $_2$ xV $\gamma$ 9] at a low E:T ratio of 12.5:1 (Fig. 5 and Supplementary Fig. S6). Again,  $\gamma\delta$  T-cell lines from healthy donors as well as from patients with PDAC only weakly lysed the tumor cells in the absence of an additional stimulus. The addition of PAg or the [Her2xCD3] enhanced the  $\gamma\delta$  T-cell-mediated lysis of PancTu-I cells and more prominently of Panc89 cells in a donor-dependent fashion, but generally not the lysis of Colo357 cells at this low E:T ratio of 12.5:1.

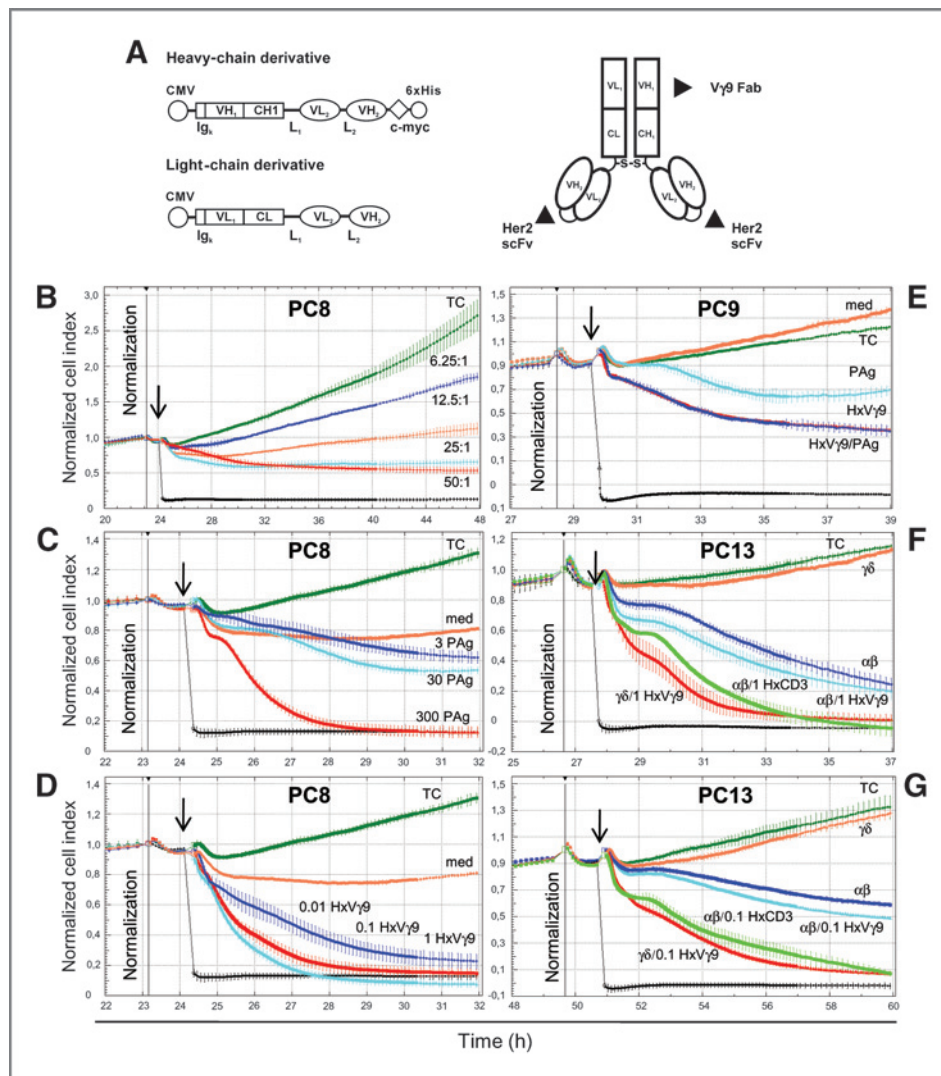
In contrast, [(Her2) $_2$ xV $\gamma$ 9] triggered in all experiments  $\gamma\delta$  T-cell-mediated cytotoxicity against all PDAC cell lines, however, more pronounced in PancTu-I and Colo357 cells, which are almost resistant against  $\gamma\delta$  T-cell-mediated lysis (Fig. 5 and Supplementary Fig. S6). The superior activity of [(Her2) $_2$ xV $\gamma$ 9] in comparison to the [Her2xCD3] might be because of the

bivalent targeting of the tumor cell or due to a qualitative different signalling via CD3 compared with  $\gamma\delta$  T-cell receptor triggering.

#### [(Her2) $_2$ xV $\gamma$ 9] enhances the release of cytolytic granules

The enhanced  $\gamma\delta$  T-cell cytotoxicity mediated by [(Her2) $_2$ xV $\gamma$ 9] prompted us to examine the mediators of this cytotoxic activity. The cytotoxic capacity of  $\gamma\delta$  T cells can be mediated by CD95–CD178 interaction, but also through the release of cytolytic granules. As shown previously, PDAC cells are almost resistant to CD95-induced cell death (15, 38). Therefore, we examined the exocytosis with the CD107a degranulation assay. Coculturing Colo357 cells with  $\gamma\delta$  T-cell lines of 2 healthy donors resulted in a weak cytotoxic activity of  $\gamma\delta$  T cells as determined by RTCA-SP assay, which could be correlated with a weak induction of CD107a at the cell surface (Fig. 6A and B). The addition of PAg enhanced  $\gamma\delta$  T-cell cytotoxicity and also the surface expression of CD107a, both of which could be further increased by adding [(Her2) $_2$ xV $\gamma$ 9] instead of PAg (Fig. 6A and B). The release of granzyme B and perforin was measured in parallel by ELISA (Fig. 6C and D). By





**Figure 4.** Tribody [(Her2)<sub>2</sub>xVγ9] selectively enhances γδ T-cell-mediated lysis of PancTu-I cells. **A**, scheme of the expression cassette for the tribody molecule and of the assembled tribody protein. CMV, cytomegalovirus immediate early promoter; Ig<sub>κ</sub>, murine Ig κ secretion leader; VH<sub>1</sub> and VL<sub>1</sub>, cDNA sequence coding for the immunoglobulin heavy and light chain variable regions from the antibody 7A5, respectively; CH1 and CL, cDNA sequence coding for the human immunoglobulin heavy chain constant region 1 and κ light chain constant regions, respectively; VH<sub>2</sub> and VL<sub>2</sub>, cDNA sequences coding for the variable heavy and light chain regions building a scFv with specificity for Her2; L<sub>1</sub> and L<sub>2</sub>, cDNA sequence coding for a 15 amino acid flexible linker (G<sub>4</sub>S)<sub>3</sub> and a 20 amino acid flexible linker (G<sub>4</sub>S)<sub>4</sub>, respectively; c-myc and 6xHis, cDNA sequence coding for the c-myc epitope and a hexahistidine tag, respectively. S-S, disulfide bond (**B–G**) PancTu-I cells had been cultured overnight before they were left either untreated (SL, green line) or were treated as follows: **B**, with different numbers of effector γδ T cells of one patient with PDAC PC8; **C** and **D**, with medium (orange line); **C**, with different concentrations of PAg BrHPP; **D**, with tribody [(Her2)<sub>2</sub>xVγ9] (HxVγ9) as indicated at an E:T ratio of 12.5:1, with γδ T cells of patient PC8; **E**, with either medium, 300 nmol/L BrHPP, 1 μg/mL tribody [(Her2)<sub>2</sub>xVγ9], or the combination of PAg and tribody [(Her2)<sub>2</sub>xVγ9] together with γδ T cells of patient PC9 at an E:T ratio of 3:1; **F** and **G**, with untreated or tribody [(Her2)<sub>2</sub>xVγ9] treated γδ T cells (orange or red line, respectively) or CD8 αβ T-cell line (dark or light blue line, respectively) of patient PC13. In **F**, 1 μg/mL of the tribody [(Her2)<sub>2</sub>xVγ9] or [(Her2)<sub>2</sub>xCD3] (light green line) was applied, and in **G**, 0.1 μg/mL. The black line is derived from PancTu-I treated with Triton X-100 for determination of maximal lysis. The cell index was measured every 5 minutes over the course of the experiment and normalized to 1 at the time point of addition of the different substances as presented by the vertical black line. The addition of γδ T cells or of 1% Triton X-100 (final concentration) is represented as an arrow. The average of three replicates with the SD is represented.

using PancTu-I cells instead of Colo357 cells as a target, we also observed an enhanced CD107a expression on the surface of γδ T cells after addition of PAg or [(Her2)<sub>2</sub>xVγ9] compared with the medium control in 6 additional donors, which was well in line with the cytotoxic activity of their γδ T cells as shown in Supplementary Fig. S7A. Similar patterns of enhanced CD107a expression after addition of [(Her2)<sub>2</sub>xVγ9] were determined by

using Panc89 cells as target cells instead of PancTu-I cells (data not shown).

This study demonstrates that the addition of [(Her2)<sub>2</sub>xVγ9] induced a different cytotoxic strength in comparison to PAg, which further enhanced the release of perforin and granzyme B, and thereby the cytotoxic potential of γδ T cells, even against almost resistant PDAC cells such as Colo357 cells.

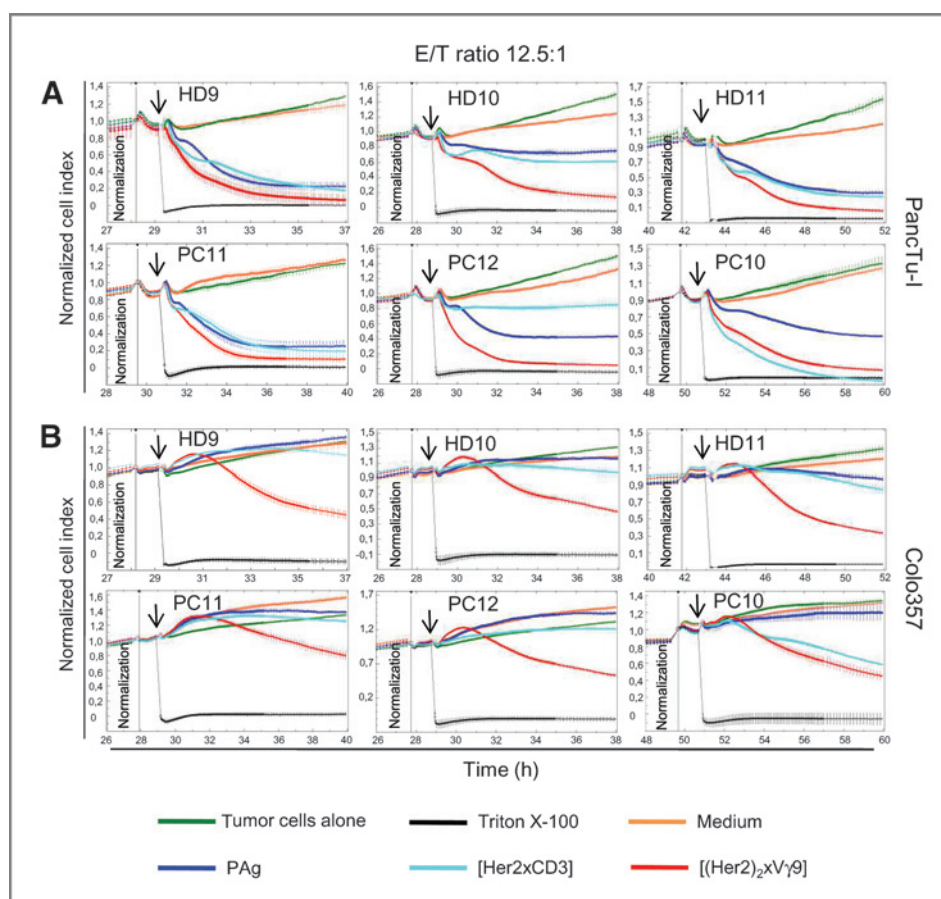


Figure 5. Enhancement of  $\gamma\delta$  T-cell cytotoxicity against different PDAC cells by tribody [(Her2)<sub>2</sub>xV $\gamma$ 9]. A, PancTu-I; B, Colo357 cells had been cultured overnight before they were left untreated (green line) or were treated with medium (orange line), 300 nmol/L PAg BrHPP (dark blue line), 1  $\mu$ g/mL [Her2xCD3] bsscFv (light blue line), or 1  $\mu$ g/mL [(Her2)<sub>2</sub>xV $\gamma$ 9] tribody (red line) as indicated in the presence of  $\gamma\delta$  T cells. Cell index values were normalized at the time of addition of the various substances. The arrow indicates the addition of  $\gamma\delta$  T cells of different healthy donors (HD) or patients with PDAC (PC) with an E:T ratio of 12.5:1. Normalized cell index values were analyzed in 5 minute increments as the average of triplicates with SD. The black line represents the usage of 1% Triton X-100 (final concentration) to determine maximal lysis.

#### Detection of tumor infiltrating $\gamma\delta$ T cells and effect of [(Her2)<sub>2</sub>xV $\gamma$ 9] on freshly isolated $\gamma\delta$ T cells *in vitro* and preactivated $\gamma\delta$ T cells *in vivo*

We asked whether [(Her2)<sub>2</sub>xV $\gamma$ 9] could also enhance the cytotoxicity of freshly isolated blood  $\gamma\delta$  T cells. It is obvious that  $\gamma\delta$  T cells need to be activated before exerting their cytotoxic activity, however, there are no data available defining the initial time point when cytotoxic activity of  $\gamma\delta$  T cells starts. The data in Fig. 7A illustrate that the RTCA-SP assay allows the determination of this starting point. Negatively isolated  $\gamma\delta$  T cells required 12 to 20 hours before their cytotoxic activity was measurable. As expected,  $\gamma\delta$  T-cell cytotoxicity against PancTu-I and Panc89 cells was not induced in the absence of a stimulus. However, triggering of  $\gamma\delta$  T-cell cytotoxicity was observed in the presence of PAg, and enhanced cytotoxic activity in the presence of [(Her2)<sub>2</sub>xV $\gamma$ 9] (Fig. 7A).

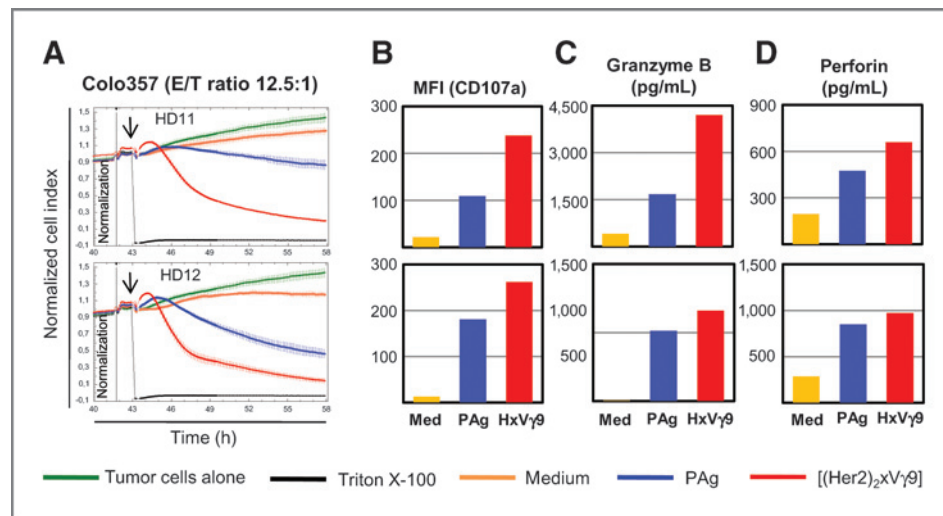
Although [(Her2)<sub>2</sub>xV $\gamma$ 9] can be used to retarget  $\gamma\delta$  T cells to Her2/neu-positive tumor cells, an important prerequisite for an effective  $\gamma\delta$  T-cell-based immunotherapy would be the migration and infiltration of  $\gamma\delta$  T-cell from the circulation into the tumor tissue. Therefore, we analyzed infiltration of  $\gamma\delta$  T cells in tumor tissues from a cohort of 41 patients with PDAC.  $\gamma\delta$  T cells were mainly localized in the stroma or adjacent to or within the ductal epithelium in 44% of the patients with PDAC (Supplementary Table S1). Staining of CD3 in the consecutive sections revealed that most of the

CD3-positive cells in the ductal epithelium were  $\gamma\delta$  T cells as shown for one representative paraffin-embedded serial tissue section (Fig. 7B). The  $\gamma\delta$  T-cell infiltration might be because of the high CXCL12 production of cancer-associated fibroblasts described elsewhere (39). Viey and colleagues reported about the CXCL12-mediated regulation of  $\gamma\delta$  T-cell migration to renal carcinoma (40). We reported previously that the CXCL12-specific receptor CXCR4 is highly expressed on  $\gamma\delta$  T cells (41).

In contrast to the ductal epithelium, more CD3-positive  $\gamma\delta$  TCR-negative T cells were localized in the stroma of the patients with PDAC (Supplementary Table S1). The localization of  $\gamma\delta$  T cells, but not of  $\alpha\beta$  T cells adjacent to or within the ductal epithelium suggest that  $\gamma\delta$  T cells bridging innate and adaptive immunity function here as a first-line defence.

To evaluate the antitumor activity of  $\gamma\delta$  T cells in the presence of the novel tribody [(Her2)<sub>2</sub>xV $\gamma$ 9] *in vivo*, a SCID-Beige xenograft tumor model with subcutaneous PancTu-I tumors was used. Mice treated with NaCl/IL-2 only developed tumors with an average weight of 533 mg and a size/volume of 876 mm<sup>3</sup> (NaCl; Fig. 7C and Supplementary Fig. S7B). Additional repetitive subcutaneous application (4-time intervals) of [(Her2)<sub>2</sub>xV $\gamma$ 9] or low number of short-term-activated V $\gamma$ 9V $\delta$ 2  $\gamma\delta$  T cells ( $\gamma\delta$ ) alone (Supplementary Fig. S7D) did not influence tumor growth (Fig. 7D and Supplementary Fig. S7B). In contrast, tumor growth was significantly reduced when





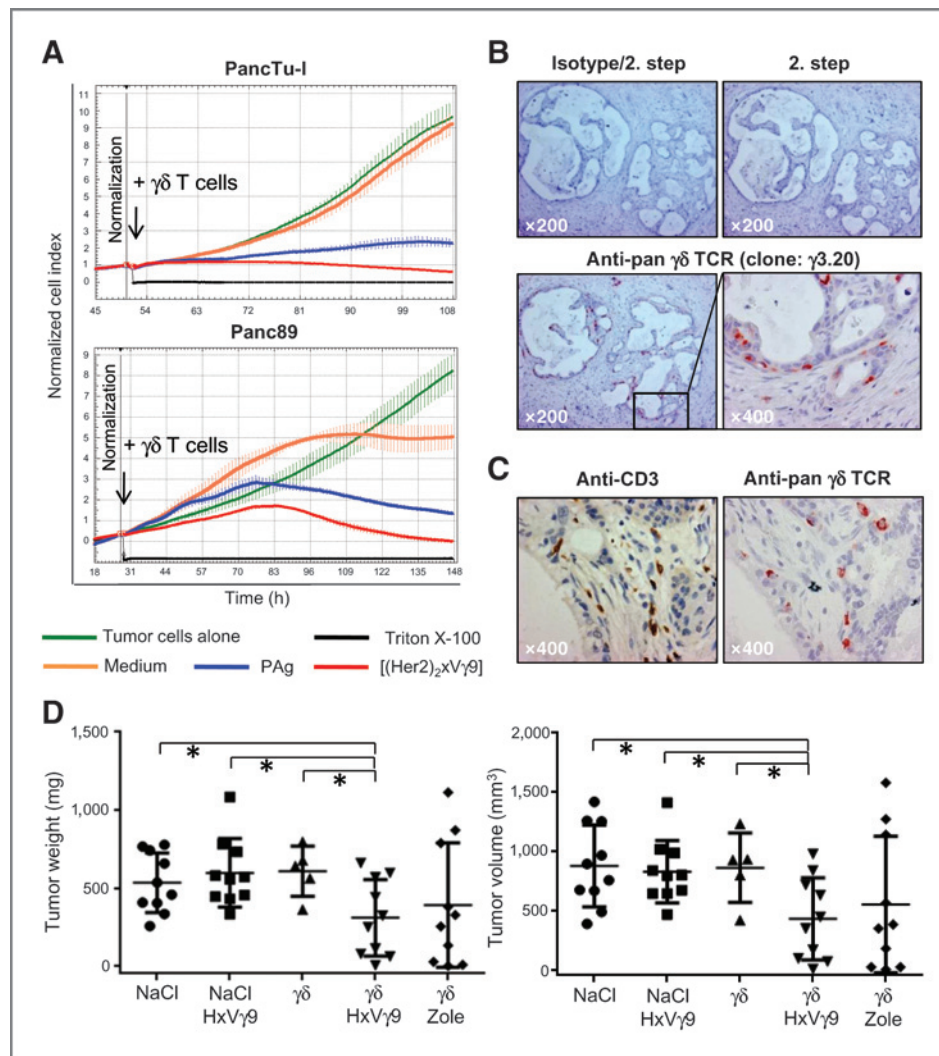
**Figure 6.** Tribody [(Her2)<sub>2</sub>xVγ9] induces an enhanced release of granzyme B and perforin. **A**, after culturing Colo357 cells overnight, cells were treated with medium (orange line), 300 nmol/L PAG BrHPP (blue line), 1 μg/mL [(Her2)<sub>2</sub>xVγ9] tribody (red line) or Triton X-100 (black line), or left untreated (green line, tumor cells alone). The arrow indicates the addition of γδ T cells of different healthy donors (HD) with an E:T ratio of 12.5:1. Cell index values were measured in 5 minute steps and were normalized at the time of treatment of Colo357 cells. The average of triplicates with SD is shown. The black line represents the addition of a final concentration of 1% Triton X-100. **B–D**, the same effector γδ T-cell lines as shown in **A** were cocultured in a parallel assay with Colo357 cells (E:T ratio of 12.5:1) in medium, 300 nmol/L PAG BrHPP, or 1 μg/mL of the tribody [(Her2)<sub>2</sub>xVγ9] and degranulation was analyzed by staining γδ T cells with anti-Vδ2 TCR and anti-CD107a mAbs (**A**), and then determining the expression of CD107a on Vδ2 TCR-expressing γδ T cells using flow cytometry or by measuring the release of granzyme B (**B**) or perforin (**C**) in the supernatants using specific ELISAs after 4 hours of coculturing. Representative results of 2 donors of 4 are shown.

[(Her2)<sub>2</sub>xVγ9] with IL-2 was given together with short-term activated γδ T cells. As shown in Fig. 7D and Supplementary Fig. S7B, 4 of 10 mice receiving repetitively [(Her2)<sub>2</sub>xVγ9] with IL-2 together with γδ T cells showed a very strong decline of tumor growth (S7B, right side), whereas 2 mice showed strongly reduced tumor growth (S7B, middle) and 4 a slightly reduced tumor growth (S7B, left side). Mice receiving n-BP zoledronic acid instead of [(Her2)<sub>2</sub>xVγ9] together with IL-2 and γδ T cells were initially considered as a positive control group. However, this group showed tumor reduction that was not significantly different from the control group, because of the heterogeneous outcome in this group (Fig. 7D and Supplementary Fig. S7B). Taken together, our results demonstrate a clear therapeutic effect of Vγ9Vδ2 γδ T cells in combination with the novel tribody [(Her2)<sub>2</sub>xVγ9] *in vivo* being more efficient than in combination with zoledronic acid.

## Conclusions

Although numerically reduced, the Vδ2 T cells of patients with PDAC still have the capacity to exert cytotoxicity after stimulation with PAG, n-BP, or [(Her2)<sub>2</sub>xVγ9]. In on-going experiments, we observed that an absolute number of >25 Vδ2 T cells/μL blood is required to induce cytotoxic activity of Vδ2 T cells within PBMC by [(Her2)<sub>2</sub>xVγ9] (not shown). We observed that γδ T cells of patients with <25 Vδ2 T cells/μL blood could exert strong cytotoxic activity after preactivation with PAG or n-BP for 7 to 14 days followed by stimulation with [(Her2)<sub>2</sub>xVγ9]. Therefore, we suggest that an *in vivo* activation initially with n-BP or PAG and IL-2 followed by several applications of [(Her2)<sub>2</sub>xVγ9] might overcome exhaustion of γδ T cells. However, the use of [(Her2)<sub>2</sub>xVγ9] could be limited by the

suppressive microenvironment of PDAC. One of the defining characteristics of PDAC is the presence of a dense desmoplastic stroma, which comprises of mesenchymal cells such as fibroblasts and pancreatic stellate cells as well as myeloid-derived suppressor cells (MDSC) or suppressive tumor-associated macrophages (TAM) preventing the penetration of cytotoxic T-lymphocytes to the tumor site (42–44). Preliminary experiments demonstrate that enhancement of the cytotoxic potential of γδ T cells could overcome suppression by TAMs (unpublished). Treatment of patients with PDAC with gemcitabine, the standard therapy for PDAC, can inhibit MDSC, while enhancing cross-presentation of tumor-associated antigens by dendritic cell (45, 46). An exact mechanism of MDSC or gemcitabine on γδ T cells has not been examined yet. However, an increased γδ T-cell cytotoxicity after treatment with other chemotherapeutic agents in combination with n-BP has been described (47). Moreover, gemcitabine resistance as well as a relapse based on residual population of cancer stem cells is frequently reported (48, 49). γδ T cells have been shown to lyse human colon cancer stem cells, which might provide an additional advantage for γδ T-cell-based immunotherapy (50). Meraviglia and colleagues demonstrated a considerable therapeutic potential for γδ T cells in treatment of Her2-expressing breast cancer as well as B-cell malignancies by co-application of therapeutic mAb alone or in combination with chemotherapy (33). Because bispecific antibodies have demonstrated a higher cytolytic potential than mAbs (29), even better responses might be achieved by using bispecific antibodies engaging CD3 or the γδ TCR instead of mAb in combination with a γδ T-cell-based immunotherapy, possibly further supporting chemotherapy.



**Figure 7.** Detection of tumor-infiltrating  $\gamma\delta$  T cells and enhancement of cytotoxicity of freshly isolated  $\gamma\delta$  T cells *in vitro* and preactivated  $\gamma\delta$  T cells *in vivo*. **A**, PDAC cells were cultured overnight and, thereafter, cells were left untreated (green line) or were cocultured with freshly isolated  $\gamma\delta$  T cells at an E:T ratio of 25:1 in the presence of medium (orange line), 300 nmol/L PAg BrHPP (blue line), or 1  $\mu$ g/mL [(Her2)<sub>2</sub>xVγ9] tribody (red line). The addition of the different stimuli assigned the time point for normalization of the cell index (vertical black line), which was determined every 5 minutes during the whole time course. The black line is derived from PDAC cells treated with Triton X-100. The addition of  $\gamma\delta$  T cells or of 1% Triton X-100 (final concentration) is represented as an arrow. The average of three replicates with the SD is represented. **B**, serial paraffin-embedded tissue stained with isotype control plus second step Ab, with second step alone or with anti- $\gamma\delta$  TCR mAb clone γ3.20 as indicated. Immunohistochemical staining was performed as described in Materials and Methods. Data of the tissue of one representative PDAC donor are shown. **C**, serial paraffin-embedded tissue stained with anti-CD3 mAb clone SP7 or anti- $\gamma\delta$  TCR mAb clone γ3.20 is shown for another representative PDAC donor. **D**, SCID-Beige mice inoculated subcutaneously with  $1.5 \times 10^6$  PancTu-1 cells were weekly treated subcutaneously with sodium chloride or Vγ9Vδ2  $\gamma\delta$  T cells. All mice received 15  $\mu$ g/kg IL-2, and, as indicated, 1.25 mg/kg [(Her2)<sub>2</sub>xVγ9] or 500  $\mu$ g/kg zoledronic acid. Tumor weight (left) and tumor size/volume (right) of each mouse were determined. Each symbol represents the data of one mouse, and thick bars represent the mean value of the group. Significances are represented as P value: \*,  $P < 0.05$ .

Taken together, the tribody [(Her2)<sub>2</sub>xVγ9] gives us a tool to further increase  $\gamma\delta$  T-cell cytotoxicity *in vitro* as well as *in vivo*, where PAg failed because of exhaustion, anergy, or depletion of these cells. Despite the urgent need of an improved understanding of possible suppressive effects of the PDAC micro-environment on  $\gamma\delta$  T-cell effector function,  $\gamma\delta$  T cells seems to be a promising therapeutic tool for the treatment of patients with PDAC as they infiltrate adjacent to or within the pancreatic ductal epithelium and they combine both innate and adaptive immune responses.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Disclaimer

This work forms part of the diploma thesis of S. Krause and of the MD thesis of D. Petrick.

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** H.-H. Oberg, M. Peipp, C. Kellner, D. Petrick, S. Adam-Klages, C. Röcken, S. Freitag-Wolf, M. Gramatzki, D. Kabelitz, D. Wesch

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**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** H.-H. Oberg, M. Peipp, D. Wesch

**Study supervision:** H.-H. Oberg, M. Gramatzki, D. Wesch

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