Novel bispecific antibodies increase γδ T-cell cytotoxicity against pancreatic
cancer cells

Hans-Heinrich Oberg1*, Matthias Peipp2*, Christian Kellner2, Susanne Sebens3, Sarah
Krause1, Domantas Petrick1, Sabine Adam-Klages1, Christoph Röcken4, Thomas Becker5, Ilka
Vogel6, Dietrich Weisner7, Sandra Freitag-Wolf8, Martin Gramatzki2, Dieter Kabelitz1# and
Daniela Wesch1§

1Institute of Immunology; 2Division of Stem Cell Transplantation and Immunotherapy,
3Institute for Experimental Medicine, 4Institute of Pathology, 5Clinic of General and Thoracic
Surgery, 1-5,8Christian-Albrechts-University Kiel, Germany; 6Municipal Hospital, Dep. of
Surgery, 7Clinic of Gynaecology and Obstetrics, Kiel, Germany; 8Institute for Medical
Informatics and Statistic

*shared first authorship

#shared senior authorship

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**Correspondence address:**
Daniela Wesch, PhD
Institute of Immunology, Christian-Albrechts-University Kiel
Arnold-Heller Strasse 3, Haus 17
D-24105 Kiel, Germany
phone: +49-431-5973379; fax:+49-431-5973335
e-mail: wesch@immunologie.uni-kiel.de

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Abstract

The ability of human γδ T-cells from healthy donors to kill pancreatic ductal adenocarcinoma (PDAC) \textit{in vitro} and \textit{in vivo} in immunocompromised mice requires the addition of γδ T-cell-stimulating antigens. In this study, we demonstrate that γδ 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 γδ T-cell subsets in patient whole blood, and applied a Real-Time Cell Analyzer to measure their cytotoxic effector function over prolonged time periods. Since phosphorylated antigens did not optimally enhance γδ T-cell cytotoxicity, we designed bispecific antibodies that bind CD3 or Vγ9 on γδ T-cells and Her2/neu (ERBB2) expressed by pancreatic tumor cells. Both antibodies enhanced γδ T-cell cytotoxicity with the Her2/Vγ9 antibody also selectively enhancing release of granzyme B and perforin. Supporting these observations, adoptive transfer of γδ T-cells with the Her2/Vγ9 antibody reduced growth of pancreatic tumors grafted into SCID-Beige immunocompromised mice. Taken together, our results show how bispecific antibodies that selectively recruit γδ T-cells to tumor antigens expressed by cancer cells illustrate the tractable use of endogenous γδ T-cells for immunotherapy.
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 10-20 % 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 γδ T-cells is of substantial interest based on their potent HLA-non-restricted cytotoxicity against different tumor entities and their additional capacity to recognize and present antigens to αβ T-cells (6-8). Several pilot studies reported a partial success in tumor reduction after in vivo activation of γδ T-cells with nitrogen-containing-bisphosphonates (n-BP) and rIL-2 or after adoptive transfer of in vitro activated γδ T-cells (9-12). Vγ9Vδ2 γδ 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 Vγ9Vδ2 T-cells (14). We have previously shown the efficacy of human Vγ9Vδ2 T-cells from healthy donors against PDAC in vitro as well as in vivo after repetitive adoptive transfer of γδ T-cells together with n-BPs and low dose IL-2 into SCID mice (15). Although γδ T-cell-based immunotherapy delivered promising results, it is mandatory to
optimize the cytotoxic activity of tumor-reactive γδ T-cells in view of an observed exhaustion of γδ T-cells, and to minimize the repetitive adoptive γδ 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 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 hematological malignancies (18, 19).

In this study, we designed two novel bispecific antibodies with specificity for CD3 or Vγ9TCR on γδ T-cells and for receptor tyrosine kinase Her2/neu, which is over-expressed on 11 to 82% of tumor cells of PDAC-patients (20-23). We investigated the potential of these novel bispecific antibodies to enhance the cytotoxicity of γδ T-cells from PDAC-patients 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 γδ T-cell subsets of PDAC-patients ex vivo.
MATERIALS 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: D405/10). 21 patients with histologically verified PDAC and stage pT3-4, pN0-1, L0-1, V0-1 were enrolled. All patients had not been chemo- or radiotherapeutically treated prior to this investigation. Further information can be found in the supplemental Material and Methods.

Immunomonitoring of γδ T-cell numbers. Whole blood samples (50 μl) from healthy donors or PDAC-patients were stained in BD TrueCount™ tubes with the following fluorochrome-conjugated monoclonal antibodies (mAb): CD45-PECy7 clone HI30, TCRγδ-APC clone 11F2 (both from BD Biosciences, Heidelberg), Vδ2-PE clone B6 (Beckman Coulter, Krefeld), Vδ1-FITC clone TS8.2 (Thermo Fisher Scientific, Karlsruhe). After lysing red blood cells with 200 μl 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 supplemental Material and Methods.

Isolation and culturing of lymphocyte populations. PBMC were isolated from the leukocyte concentrates or from heparinized blood by Ficoll-Hypaque (Biochrom, Berlin) density gradient centrifugation. To separate freshly isolated γδ T-cells or NK-cells, we used a negative selection kits (TCRγδ+ T Cell Isolation Kit or NK cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach), according to the manufacturer´s instructions.

To establish short-term γδ T-cell lines, PBMC were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 25 mM HEPES, antibiotics, 10% FCS (complete medium), and stimulated either with 300 nM of PAg BrHPP (kindly provided by Innate Pharma, Marseille, France) or 5 μM of n-BP zoledronic acid (Novartis, Basel, Swiss). 50 U/ml rIL-2 (Novartis)
was added every two days over a culture period of 14 to 21 days. After 2-3 weeks, most γδ T-cells had a purity > 95% Vγ9Vδ2 γδ T-cells. CD8 αβ T-cell lines were established from PBMC of PDAC-patient 13 after stimulation with 0.5 μg/ml phytohemagglutinin (Thermo Fisher Scientific). CD8 αβ 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 supplemental Material and Methods.

**Tumor cell lines.** PDAC-cell lines PancTu-I, Colo357 and Panc89 were kindly provided by Prof. Kalthoff, Section of Molecular Oncology, Kiel. The genotype of PDAC-cell lines was recently confirmed by Short Tandem Repeats Analysis. PDAC-cell lines as well as Raji lymphoma cells (DSMZ, Braunschweig) 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 Vγ9 γδ 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, Regensburg). Secretion leaders and appropriate restriction sites were introduced to allow directional cloning into expression
vectors coding for a [(Her2)₂xCD16] 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-Vγ9-VH and pSEC-Tag2-Her2-scFv-LC-Vγ9-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)₂xVγ9], and the bsscFv [CD19xCD3], [CD20xCD3] or [Her2xCD3] (29). The tribody was purified from supernatant as described (29, 30).

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

Real Time Cell Analyzer. 5000 adherent PDAC-cells/well were added to 96-well micro-E-plate to monitor the impedance of the cells every 15 min for up to 24 h. After having reached the linear growth time phase, [Her2xCD3] or [(Her2)₂xVγ9], and corresponding control constructs were added in the previously titrated optimal concentrations for 1 h. Thereafter, T-cell lines or freshly isolated γδ T-cells together with 12.5 IU/ml IL-2 were added to the Real Time Cell Analyzer (RTCA)-Single plate (SP) assay (Roche, Mannheim). Where indicated, Vγ9Vδ2 T-cell effector cells were stimulated with 300 nM 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 h and, thereafter, every 5 min for up to additional 20 h.
**ELISA and CD107a-degranulation assay.** These standard methods with commercial kits are described in the supplemental Material and Methods.

**Immunohistochemistry.** Immunostaining with anti-CD3 mAb clone SP7 (dilution 1:100; NeoMarkers/LabVision, Fremont, CA, USA) of serial paraffin embedded tissue sections of 41 PDAC-patients (permission number of the ethics committee: A 110/99) was done with the fully automated BondTM Max-System using the BondTM Polymer Refine Detection Kit (Leica-Menarini, Berlin). Automated antigen retrieval was performed in ER1 (citrate buffer Bond pH 6.0; Leica-Menarini). TCR \( \gamma \delta \) expression was detected from the same tissue sections after deparaffinization and treatment with antigen retrieval solution (pH 9.0) (DAKO, Hamburg) by using 3 \( \mu \)g/ml anti-\( \gamma \delta \) TCR clone \( \gamma3.20 \) (Thermo Fisher Scientific) or mouse IgG1 isotype control mAbs. As second-step antibody EnVision-mouse HRP (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, Darmstadt).

**Xenograft tumor model**

Female, 6 week old SCID-Beige mice (Charles River, Sulzfeld) 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 s.c. inoculated in the left shaved flank with 1.5x10^6/50 \( \mu \)l PDAC cell-line PancTu-I. After two days, mice were randomized into 6 groups with 5-10 animals before s.c. injection of either 15 \( \mu \)g/kg (25x10^4 IU) IL-2 (Proleukin®) with NaCl or 1,25 mg/kg \([(\text{Her2})_2 \times V\gamma9]\) or 2,5 mg/kg zoledronic acid. Treatments were repeated weekly for a total of 4 weeks. Where indicated, mice received previously expanded \( V\gamma9V\delta2 \) \( \gamma \delta \) T-cells of one donor s.c. on day 2 (2.5x10^6/mouse), day 7 (8x10^6/mouse), day 14 (4.5x10^6/mouse) and day 23 (6x10^6/mouse). The total volume of IL-2, plus NaCl or \([(\text{Her2})_2 \times V\gamma9]\) and NaCl or \( V\gamma9V\delta2 \)
T-cells was 75μl. To obtain short-term activated Vγ9Vδ2 T-cells, 150-250x10^6 PBMC freshly isolated at 4 time points from one donor were stimulated with 300 nM BrHPP 14-19 days before application of the cells. The purity of the activated cells was >95%. To determine the tumor weight and size/volume (length x width x depth), all mice were sacrificed 29 days after tumor cells 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 two-sided and the level of significance was set at 5%, not corrected for multiple testing.

Since 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)_2xVγ9] or tumor cells with γδ T-cells, respectively, was tested two-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 PDAC-patients (Fig. 1). Several studies described a decrease of the absolute number of circulating γδ T-cells in cancer patients. However, the precise comparison with age-matched healthy donors clearly demonstrated that the reduction correlated with age and was not due to the cancer disease in PDAC-patients. 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).

We focused in this study on numerically reduced Vδ2 T-cells, which are exclusive targets for PAg applied in γδ T-cell-based immunotherapy. We established several short- and long-termed-cultured Vδ2 T-cell lines from PDAC-patients as well as from healthy donors (Fig. S1A). In previous studies, we observed a limited cytotoxic capacity of Vδ2 T-cell lines from healthy donors against PDAC-cell lines which, however, could be enhanced by the addition of PAg or n-BP (15). A sustained stimulation of Vδ2 T-cells by PAg or n-BPs often leads to an exhaustion of the γδ T-cell pool, which requires novel or modified stimulation protocols (7).

Combining γδ T-cell-based immunotherapy with mAb provides novel perspectives. An enhanced efficacy against Her2+ breast cancer cells has been described after adoptive transfer of expanded γδ T-cells together with a humanized anti-Her2/neu mAb (trastuzumab) into human Her2+ mammary carcinoma-bearing SCID mice (32). A recent review summarized the evidence that the combination of γδ 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 PDAC-patients (data not shown). This observation together with the observed expression of Her2/neu on the surface of all analyzed PDAC-cell lines (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 (Fig. S2A/B).

**[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 co-stimuli (34). [Her2xCD3] bsscFv purification was performed by IMAC chromatography and size exclusion chromatography (Fig. S2C/D). 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 (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 PDAC-patients showed a very similar cytotoxic activity against the indicated PDAC-cell lines, as revealed in a \(^{51}\)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, 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, S3B). We obtained similar results using $\gamma\delta$ T-cells of PDAC-patients instead of healthy donors (Fig. 2B). The addition of a previously titrated optimal concentrations of [Her2xCD3] to the PAg-stimulated cells further increased cytotoxic activity compared to the PAg stimulation in combination with the control bsscFv (Fig. 2C/D, S3C/D). 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, 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 re-stimulation (Fig. 2, S3, HD3/PC1 and data not shown).

**Comparison of $^{51}$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 h 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 be also 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 due to an incomplete lysis tumor cells can regenerate. Similar killing patterns were observed with the $\gamma\delta$ T-cell line of one representative healthy donor against Panc89 or Colo357 determined by either $^{51}$Cr-release assay or RTCA-SP was used (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 to the $^{51}$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 $^{51}$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 two additional donors in two 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 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 $\gamma\delta$ T-cells (Fig. 4A). To this end, the V-regions from the $V_{\gamma9}$-specific hybridoma 7A5 (28) were cloned and used for the generation of the tribody [(Her2)$_2$x$V_{\gamma9}$]. [(Her2)$_2$x$V_{\gamma9}$] was transiently expressed in HEK293T-cells and purified by two-step affinity chromatography and size exclusion chromatography (Fig. S4A/B). The tribody retained the antigen specificities of the parental antibodies as evidenced by flow cytometry. [(Her2)$_2$x$V_{\gamma9}$] specifically bound to $V_{\gamma9}$ expressing $\gamma\delta$ T-cells and Her2-positive
PDAC to cell lines. No binding to antigen negative cells (Raji or NK-cells) was observed (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 PDAC-patient (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)2xVγ9] 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/D). Focussing on the first two hours of lysis measured with the RTCA-SP assay, we noted that the higher concentrations of [(Her2)2xVγ9] induced a stronger γδ T-cell mediated lysis than the highest concentration of PAg (Fig. 4C/D). This observation was more pronounced with the γδ T-cell line of an additional PDAC-patient, 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)2xVγ9] increased γδ T-cell-mediated lysis more effectively than the addition of 300 nM PAg, whereas the combination of [(Her2)2xVγ9] 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)2xVγ9] was added, whereas the cytotoxicity of an autologous CD8 T-cell line against PancTu-I cells was not enhanced with [(Her2)2xVγ9], but with [Her2xCD3], which demonstrate the specificity of [(Her2)2xVγ9] in activating only γδ T-cells (Fig. 4F/G). Similar results were obtained when 0.1 μg/ml of [(Her2)2xVγ9] were used instead of 1 μg/ml (Fig. 4G). Additionally, the specificity of [(Her2)2xVγ9] and [Her2xCD3] was confirmed by the observation that these bispecific antibodies enhanced the γδ T-cell mediated lysis of Her2-positive PancTu-I cells, but not of Her2-negative cells (Fig. S5).

To further substantiate these results, we analyzed the cytotoxic activity of additional γδ T-cell lines established from healthy donors and PDAC-patients against the three different PDAC-
cell lines in the absence or presence of PAg, [Her2xCD3] or [(Her2)xVγγ9] at a low E/T ratio of 12.5:1 (Fig. 5, S6). Again, γδ T-cell lines from healthy donors as well as from PDAC-patients only weakly lysed the tumor cells in the absence of an additional stimulus. The addition of PAg or the [Her2xCD3] enhanced the γδ 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)xVγγ9] triggered in all experiments γδ T-cell-mediated cytotoxicity against all PDAC-cell lines, however, more pronounced in PancTu-I and Colo357 cells which are almost resistant against γδ T-cell-mediated lysis (Fig. 5, S6). The superior activity of [(Her2)xVγγ9] in comparison to the [Her2xCD3] might be due to the bivalent targeting of the tumor cell or to a qualitative different signalling via CD3 compared to γδ T-cell receptor triggering.

**[(Her2)xVγγ9] enhances the release of cytolytic granules**

The enhanced γδ T-cell cytotoxicity mediated by [(Her2)xVγγ9] prompted us to examine the mediators of this cytotoxic activity. The cytotoxic capacity of γδ 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. Co-culturing of Colo357 cells with γδ T-cell lines of two healthy donors resulted in a weak cytotoxic activity of γδ T-cells as determined by RTCA-SP assay, which could be correlated with a weak induction of CD107a at the cell surface (Fig. 6A/B). The addition of PAg enhanced γδ T-cell cytotoxicity and also the surface expression of CD107a, both of which could be further increased by adding [(Her2)xVγγ9] instead of PAg (Fig. 6A/B). The release of granzyme B and perforin was measured in parallel by ELISA (Fig. 6C/D). By 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)xVγ9] compared to the medium control in six additional donors, which was well in line with the cytotoxic activity of their γδ T-cells as shown in Fig. S7A. Similar patterns of enhanced CD107a expression after addition of [(Her2)xVγ9] were determined by using Panc89 cells as target cell instead of PancTu-I cells (data not shown).

This study demonstrates that the addition of [(Her2)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.

**Detection of tumor infiltrating γδ T-cells and effect of [(Her2)xVγ9] on freshly isolated γδ T-cells in vitro and pre-activated γδ T cells in vivo**

We asked whether [(Her2)xVγ9] could also enhance the cytotoxicity of freshly isolated blood γδ T-cells. It is obvious that γδ 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 γδ T-cells starts. The data in Fig. 7A illustrate that the RTCA-SP assay allows the determination of this starting point. Negatively isolated γδ T-cells required 12 to 20 h before their cytotoxic activity was measurable. As expected, γδ T-cell cytotoxicity against PancTu-I and Panc89 cells was not induced in the absence of a stimulus. However, triggering of γδ T-cell cytotoxicity was observed in the presence of PAg, and enhanced cytotoxic activity in the presence of [(Her2)xVγ9] (Fig. 7A).

Although [(Her2)xVγ9] can be used to retarget γδ T-cells to Her2/neu-positive tumor cells, an important prerequisite for an effective γδ T-cell-based immunotherapy would be the migration and infiltration of γδ T-cell from the circulation into the tumor tissue. Therefore, we analysed infiltration of γδ T-cells in tumor tissues from a cohort of 41 PDAC-patients. γδ T-
cells were mainly localized in the stroma or adjacent to or within the ductal epithelium in 44% of the PDAC-patients (supplemental table 1). Staining of CD3 in the consecutive sections revealed that most of the CD3-positive cells in the ductal epithelium were γδ T-cells as shown for one representative paraffin-embedded serial tissue section (Fig. 7B). The γδ T-cell infiltration might be due to the high CXCL12 production of cancer-associated fibroblasts described elsewhere (39). Viey et al. reported about the CXCL12-mediated regulation of γδ T-cell migration to renal carcinoma (40). We reported previously that the CXCL12-specific receptor CXCR4 is highly expressed on γδ T-cells (41).

In contrast to the ductal epithelium, more CD3-positive γδ TCR-negative T-cells were localized in the stroma of the PDAC-patients (supplemental table 1). The localisation of γδ T-cells, but not of αβ T-cells adjacent to or within the ductal epithelium suggest that γδ T-cells bridging innate and adaptive immunity function here as a first-line defense.

To evaluate the antitumor activity of γδ T-cells in the presence of the novel tribody ((Her2)2xVγ9) 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 mm3 (NaCl, Fig. 7C, S7B). Additional repetitive s.c. application (4-time intervals) of ((Her2)2xVγ9) or low number of short-term activated Vγ9Vδ2 γδ T-cells (γδ) alone (Fig. S7D) did not influence tumor growth (Fig. 7D, S7B). In contrast, tumor growth was significantly reduced when ((Her2)2xVγ9) with IL-2 was given together with short-term activated γδ T-cells. As shown in Fig. 7D and S7B, four of ten mice receiving repetitively ((Her2)2xVγ9) with IL-2 together with γδ T-cells showed a very strong decline of tumor growth (S7B, right side), whereas two mice showed strongly reduced tumor growth (S7B, middle) and four a slightly reduced tumor growth (S7B, left side). Mice receiving n-BP zoledronic acid instead of ((Her2)2xVγ9) together with IL-2 and γδ T-cells were initially considered as a positive control group. However, this group showed tumor
reduction which was not significantly different from the control group, due to the heterogeneous outcome in this group (Fig. 7D, S7B). Taken together, our results demonstrate a clear therapeutic effect of Vγ9Vδ2 γδ T-cells in combination with the novel tribody [(Her2)xVγ9] in vivo being more efficient than in combination with zoledronic acid.

CONCLUDING REMARKS

Although, the numerically reduced Vδ2 T-cells of PDAC-patients still have the capacity to exert cytotoxicity after stimulation with PAg, n-BP or [(Her2)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)xVγ9] (not shown). We observed that γδ T-cells of patients with < 25 Vδ2 T-cells/μl blood could exert strong cytotoxic activity after pre-activation with PAg or n-BP for 7-14 days followed by stimulation with [(Her2)xVγ9]. Therefore, we suggest that an in vivo activation initially with n-BP or PAg and IL-2 followed by several applications of [(Her2)xVγ9] might overcome exhaustion of γδ T-cells. However, the use of [(Her2)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 PDAC-patients with gemcitabine, the standard therapy for PDAC, can inhibit MDSC, while enhancing cross-presentation of tumor-associated antigens by DC (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 co-workers 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). Since 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.

Taken together, the tribody [(Her2)2xVγ9] gives us a tool to further increase γδ T-cell cytotoxicity in vitro as well as in vivo where PAg failed due to exhaustion, anergy or depletion of these cells. Despite the urgent need of an improved understanding of possible suppressive effects of the PDAC microenvironment on γδ T-cell effector function, γδ T-cells appear to be a promising therapeutic tool for the treatment of PDAC-patients as they infiltrate adjacent to or within the pancreatic ductal epithelium and they combine both innate and adaptive immune responses.
Acknowledgments

This work forms part of the diploma thesis of S.K. and of the MD thesis of D.P.

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Abbreviations

BiTE, bispecific T-cell engager; BrHPP, bromohydrin-pyrophosphate; bsscFv, bispecific single chain fragment variable; IPP, isopentenyl-pyrophosphate; n-BP, nitrogen-containing bisphosphonates; PAg, phosphoantigen; PDAC, pancreatic ductal adenocarcinoma; RTCA, real time cell analyzer;
Legends to Figures:

**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 PDAC-patients (PC, n = 21; 9 females and 12 males; age: 65.0 ± 11.0 years), from age-matched healthy donors (HD, n = 21; age: 64.6 ± 10.6 years), and from younger HD (n = 16; age: 36.4 ± 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. (non-significant).

**Figure 2. Enhancement of PDAC lysis by [Her2xCD3] bsscFv.** Vγ9Vδ2 γδ T-cell lines of healthy donors (HD) or PDAC-patients (PC) were cultured with (A) medium, (B) 300 nM PAg BrHPP (C) 300 nM BrHPP plus 1 µg/ml control bsscFv (D) 300 nM BrHPP plus 1 µg/ml [Her2xCD3] bsscFv or (E) 1 µg/ml [Her2xCD3] bsscFv before the addition of the indicated 51Cr-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 six 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 re-stimulation (RS).

**Figure 3. Comparative analysis of PDAC lysis by RTCA versus 51Cr-release.** Lysis of Panc89 or Colo357 cells is shown in response to graded numbers of γδ T-cells of one representative donor (HD8) as indicated in (A) a 51Cr-release assay after 4 h or (B) in a
RTCA-SP assay over 8 h. PDAC-cells were labeled for (A) 1 h with $^{51}$Cr or (B) cultured for approximately 24 h on an E-plate before addition of medium (orange line), 1 μg/ml [Her2xCD3] bsscFv (light blue line) or 1 μg/ml control construct (dark blue line). (A) Results of $^{51}$Cr-release assay are presented as % specific lysis. The mean value of triplicate assays were 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 due to the presence of Triton X-100. The average of triplicates with the SD is shown.

**Figure 4. Tribody [(Her2)$_2$V$\gamma9$] selectively enhances $\gamma\delta$ 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; Igk = murine Ig kappa secretion leader; VH$_1$, VL$_1$ = cDNA sequence coding for the immunoglobulin heavy and light chain variable regions from the antibody 7A5, respectively; CH$_1$, CL = cDNA sequence coding for the human immunoglobulin heavy chain constant region 1 and kappa light chain constant regions, respectively; VH$_2$, VL$_2$ = cDNA sequences coding for the variable heavy and light chain regions building a scFv with specificity for Her2; L$_1$, L$_2$ = cDNA sequence coding for a 15 amino acid flexible linker (G$_4$S)$_3$ and a 20 amino acid flexible linker (G$_4$S)$_4$, respectively; c-myc, 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 $\gamma\delta$ T-cells of one PDAC-patient PC8, (B/D) with
medium (orange line) or in (C) with different concentrations of PAg BrHPP or (D) with tribody [(Her2)$_2$V$_{9}$] (HxV$_{9}$) as indicated at an E/T ratio of 12.5:1, with $\gamma\delta$ T-cells of patient PC8, (E) with either medium, 300 nM BrHPP, 1 $\mu$g/ml tribody [(Her2)$_2$V$_{9}$] or the combination of PAg and tribody [(Her2)$_2$V$_{9}$] together with $\gamma\delta$ T-cells of patient PC9 at an E/T ratio of 3:1, (F/G) with untreated or tribody [(Her2)$_2$V$_{9}$ treated] $\gamma\delta$ T-cells (orange or red line, respectively) or CD8 $\alpha\beta$ T-cell line (dark or light blue line, respectively) of patient PC13. In (F) 1 $\mu$g/ml of the tribody [(Her2)$_2$V$_{9}$] was applied, and in (G) 0.1 $\mu$g/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 $\gamma\delta$ 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.

Figure 5. Enhancement of $\gamma\delta$ T-cell cytotoxicity against different PDAC-cells by tribody [(Her2)$_2$xV$_{9}$]. (A) PancTu-I and (B) Colo357 cells had been cultured overnight before they were left untreated (green line) or were treated with medium (orange line), 300 nM PAg BrHPP (dark blue line), 1 $\mu$g/ml [Her2xCD3] bsscFv (light blue line) or 1 $\mu$g/ml [(Her2)$_2$V$_{9}$] 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 PDAC-patients (PC) with an E/T ratio of 12.5:1. Normalized cell index values were analyzed in 5 min increments as the average of triplicates with SD. The black line represents the usage of 1% TritonX-100 (final concentration) to determine maximal lysis.
Figure 6. Tribody [(Her2)2xVγ9] induces an enhanced release of granzyme B and perforin. (A) After culturing Colo357 cells overnight, cells were treated with medium (orange line), 300 nM PAg BrHPP (blue line), 1 μg/ml [(Her2)2Vγ9] tribody (red line) or Triton X-100 (black line) or left untreated (green line, tumor cells alone). The arrow indicated 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 min 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 co-cultured in a parallel assay with Colo357 cells (E/T ratio of 12.5:1) in medium, 300 nM PAg BrHPP or 1 μg/ml of the tribody [(Her2)2Vγ9] and degranulation was analyzed by (A) staining γδ T-cells with anti-Vδ2 TCR and anti-CD107a mAbs and then determining the expression of CD107a on Vδ2 TCR-expressing γδ T-cells using flow cytometry or by (B) measuring the release of granzyme B or (C) perforin in the supernatants using specific ELISAs after 4 h of co-culturing. Representative results of two donors out of four are shown.

Figure 7. Detection of tumor-infiltrating γδ T-cells and enhancement of cytotoxicity of freshly isolated γδ T-cells in vitro and pre-activated γδ T-cells in vivo. (A) PDAC-cells were cultured overnight and, thereafter, cells were left untreated (green line) or were co-cultured with freshly isolated γδ T-cells at an E/T ratio of 25:1 in the presence of medium (orange line), 300 nM PAg BrHPP (blue line) or 1 μg/ml [(Her2)2Vγ9] 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 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. (B) Serial paraffin embedded tissue stained with isotype control plus second step Ab, with second step alone or with anti-γδ TCR mAb clone γ3.20 as indicated. Immunohistochemical staining was performed as described in Material & Methods. Data of the tissue of one representative PDAC donor is shown. (C) Serial paraffin embedded tissue stained with anti-CD3 mAb clone SP7 or anti-γδ TCR mAb clone γ3.20 is shown for another representative PDAC donor. (D) SCID-Beige mice inoculated s.c. with 1.5x10⁶ PancTu-I cells were weekly treated s.c. with sodium chloride (NaCl) or Vγ9Vδ2 γδ T-cells. All mice received 15 μg/kg IL-2, and, as indicated, 1.25 mg/kg [(Her2)2Vγ9] or 500 μg/kg zoledronic acid. Tumor weight (left panel) and tumor size/volume (right panel) 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.

Reference List


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Figure 1

[Graph showing cell counts per microliter of blood for different categories: PC, age-matched HD, and younger HD. The graph includes annotations for p-values and statistical significance (ns).]
Figure 2

PancTu-I
HD
PC

Colo357
HD
PC

A
medium
% specific lysis

B
PAG
% specific lysis

C
control + PAG
% specific lysis

D
[Her2xCD3] + PAG
% specific lysis

E
[Her2xCD3]
% specific lysis

25:1  12.5:1  6.3:1

effector/ target ratio

○ HD1 or PC1 (after PS)  ● HD2 or PC1 (after RS)  □ HD3 (after PS) or PC2  ■ HD3 (after RS) or PC3

◊ HD4 or PC4  ▲ HD5 or PC5  △ HD6 or PC6  ▲ HD7 or PC7  ◎ HD8 or PC8
Figure 3
Figure 5

E/T ratio 12.5:1

A

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B

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Time (hrs)
- tumor cells alone
- Triton X-100
- medium
- PAg
- [Her2xCD3]
- [(Her2)₂xVγ9]
Figure 6

A

Colo357 (E/T ratio 12.5:1)

Normalized cell index

Time (hrs)

HD11

HD12

B

MFI (CD107a)

C

granzyme B (pg/ml)

D

perforin (pg/ml)

med PAg HxVγ9

med PAg HxVγ9

med PAg HxVγ9

[Her2]xVγ9

tumor cells alone

Triton X-100

medium

PAg
Novel bispecific antibodies increase γδ T-cell cytotoxicity against pancreatic cancer cells


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