Pappalysin-1 T cell receptor transgenic allo-restricted T cells kill Ewing sarcoma in vitro and in vivo

Andreas Kirschner, Melanie Thiede, Thomas G. P. Grünewald, Rebeca Alba Rubio, Günther H. S. Richter, Thomas Kirchner, Dirk H. Busch, Stefan Burda, and Uwe Thiel

Laboratory for Functional Genomics and Transplantation Biology, Department of Pediatrics and Children’s Cancer Research Center, Klinikum rechts der Isar, Technische Universität München, München, Germany

ABSTRACT
Pregnancy-associated plasma protein-A (PAPPA), also known as pappalysin, is a member of the insulin-like growth factor (IGF) family. PAPPA acts as a protease, cleaving IGF inhibitors, i.e., IGF binding proteins (IGFBPs), thereby setting free IGFs. The insulin/IGF-axis is involved in cancer in general and in Ewing sarcoma (ES) in particular. ES is a highly malignant bone tumor characterized by early metastatic spread. PAPPA is associated with various cancers. It is overexpressed and required for proliferation in ES. PAPPA also stimulates normal bone growth. We isolated HLA-A*02:01/peptide-restricted T cells from A*02:01–healthy donors directed against PAPPA, generated by priming with A*02:01–PAPPA peptide loaded dendritic cells. After TCR identification, retrovirally TCR transduced CD8+ T cells were assessed for their in vitro specificity and in vivo efficacy in human ES bearing Rag22−/−;γc−/− mice. Engraftment in mice and tumor infiltration of TCR transgenic T cells in the mice was evaluated. The TCR transgenic T cell clone PAPPA-2G6 demonstrated specific reactivity toward HLA-A*02:01/PAPPA+ ES cell lines. We furthermore detected circulating TCR transgenic T cells in the blood in Rag22−/−;γc−/− mice and in vivo engraftment in bone marrow. Tumor growth in mice with xenografted ES was significantly reduced after treatment with PAPPA-2G6 TCR transgenic T cells in contrast to controls. Tumors of treated mice revealed tumor-infiltrating PAPPA-2G6 TCR transgenic T cells. In summary, we demonstrate that PAPPA is a first-rate target for TCR-based immunotherapy of ES.

ARTICLE HISTORY
Received 4 November 2016
Revised 9 December 2016
Accepted 10 December 2016

KEYWORDS
Allogeneic; Ewing sarcoma; PAPPA; Pappalysin; T cell therapy; transgenic TCR

CONTACT Andreas Kirschner andreasmanfred.kirschner@tum.de Laboratory for Functional Genomics and Transplantation Biology, Department of Pediatrics and Children’s Cancer Research Center, Koelner Platz 1, D-80804 Munich, Germany.

Introduction
Insulin and insulin-like growth factor (IGF) pathways are involved in cancer of both children and adults. In adults, insulin signaling represents a key mechanisms linking obesity and cancer, whereas in childhood the IGF axis is involved in the nexus between birth weight and cancer. Moreover, pregnancy-associated glycosaminoglycan-mediated mechanisms of invasion and growth have recently gained attention as potent therapeutic targets in cancer. Pregnancy-associated plasma protein-A (PAPPA) also known as pappalysin was first described as a circulating protein during pregnancy and has been associated with breast, ovarian, renal, gastric and lung cancer, as well as pleural mesothelioma. More than a decade ago, we had identified PAPPA as being overexpressed in primary Ewing sarcoma (ES) and metastases—to our knowledge the first description of PAPPA in association with a mesenchymal neoplasm. PAPPA functions as a highly specific metalloproteinase cleaving IGF binding proteins (IGFBPs) −2, −4 and −5, thereby activating IGFs. It is tightly bound to membrane anchored glycosaminoglycans present on the cell surface. Cleavage of IGFBPs occurs in close proximity to the IGF1 receptor (IGF1R), increasing the IGF concentration at its binding site. IGF1 is one of the most prominent growth factors deposited in the bone matrix and bound to IGFBP4 it represents a key player in bone modeling. PAPP knockout mice are reduced in size by 40% as early as newborn. Apart from its role in bone metabolism, IGF is important for growth, differentiation and development in many if not all tissues. IGF1R is deregulated in many cancers types making it a promising therapeutic target. In ES, the IGF-1 pathway has been identified as an important growth factor and IGF1R monoclonal antibody therapy is clinically evaluated. In ES, deregulation of IGF1R expression is caused by the ES-specific EWS-FLI1 fusion oncogene. Upon IGF binding the PI3K/AKT/mTOR and MEK/ERK/MAPK pathways are mediating cell growth and tumorogenesis.
ES are highly malignant tumors. They are characterized by early metastases. ES were originally described by Ewing in 1921 as endothelioma of the bone. In 1985, we described a neuroectodermal histogenesis for ES and confirmed both the endothelial and the neuroectodermal signature by microarray analysis in 2004. A11 ES are molecularly defined by chromosomal translocations leading to EWS-ETS gene fusions. The translocation-derived chimeric transcription factors yield transactivation, transformation, and the malignant phenotype. A11, A31, A32 At diagnosis about 20% to 30% of patients have overt metastases in lung, bone, and/or bone marrow. A53 Patients who have been diagnosed with bone marrow metastases have a fatal outcome irrespective of therapy. A74

T cell receptor (TCR) affinity enhanced T cells have shown some efficacy in sarcomas. A35 However, TCR affinity enhancement may increase TCR cross reactivity with the risk of severe and even lethal T cell attack on critical organ function. A36-38 TCR allo-restriction circumvents the risks of TCR affinity enhancement in T-cell-based cancer immunotherapy. A39 In addition, there is evidence for a graft vs. tumor effect in ES, providing an additional rationale for the utilization of allo-restricted T cells. A40, A41, A42 However, allogeneic donor lymphocyte infusion (DLI) lacks specificity. A43, A44

Here, we report on the generation of TCR transgenic T cells directed against the tumor-associated antigen (TAA) PAPPA overexpressed in ES.

Material and methods

Cell lines

SK-N-MC and TC-71 (both ES cell lines) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). A673 (ES cells) were obtained from ATCC (LGC Standards GmbH, Wesel, Germany). The EW7 ES cell line was obtained from Olivier Delattre, Institut Curie, Paris. The TAP-deicient HLA-A*02:01 T2 cell line (somatic cell hybrid) was obtained from P. Cresswell (Yale University School of Medicine, New Haven, CT, USA). The HLA-A*02:01-erythroid leukemia cell line K562 was a gift from A. Knuth and E. Jäger (Krankenhaus Nordwest, Frankfurt, Germany). All cell lines were routinely tested for purity and mycoplasma contamination. Tumor cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (all from Life Technologies). RPMI 1640 medium for LCL and T2 cells was supplemented with 1 mM sodium pyruvate and non-essential amino acids, additionally.

Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from human peripheral blood samples of healthy donors (obtained with IRB approval and informed consent from the DRK-Blutspendedienst Baden-Württemberg-Hessen in Ulm, Germany) by centrifugation over Ficoll-Paque (GE Healthcare, Freiburg, Germany) according to the supplier’s recommendations.

Generation of dendritic cells (DCs)

CD14+ cells were isolated from PBMCs with anti-human CD14 magnetic particles (BD Biosciences, Heidelberg, Germany) according to the manufacturer’s instructions. Purity of cells was confirmed by flow cytometry on a FACS Calibur (BD Bioscience). Culture and maturation of CD14+ cells was done as described previously. A45

Isolation of CD8+ T cells

CD8+ T cells were isolated from human HLA-A*02:01- PBMCs by negative isolation using a cocktail of biotin-conjugated non-CD8+monoclonal antibodies and anti-biotin micro beads followed by column depletion according to manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of isolated CD8+ T cells was confirmed by flow cytometry.

In vitro priming of HLA-A*02:01/PAPPA1434 allo-restricted T cells

Matute DCs were re-suspended in T cell medium (AIM-V supplemented with 5% human AB serum, 2 mM L-glutamine, and 50 μL/mL gentamycin) and pulsed with selected peptides at a concentration of 30–50 μM in the presence of 20 μg/mL β1MG (Sigma, Taufkirchen, Germany) for 4 h at 37°C and 5% CO2. Pulsed cells were than washed and used for T cell priming as described previously. A45

Multimer-staining and cell sorting

Two weeks after in vitro priming activated T cells were pooled and stained with specific peptide/HLA-A*02:01-multimer-PE (PAPPA1434, IILPMNVTV) and CD8+FITC (BD Bioscience) for cell sorting. An unspecified peptide/HLA-A*02:01-multimer-PE directed against LIPI (Lipase member I, LLNEEDMNV) served as a negative control. A46 Cell sorting was done on a FACS Aria (BD Bioscience).

Limiting dilution

After FACS sorting, multimer-PE-specific T cells were expanded using limiting dilution. Expansion was conducted in round-bottom 96-well plates in 200 μL T cell medium supplemented with anti-CD3 (30 ng/mL), rhIL-2 (100 U/mL), rhIL-15 (2 ng/mL); irradiated LCL (1x10⁵ per well) and irradiated PBMCs pooled from three different donors (5 x 10⁴ per well) were used as feeder as previously described. A45 Cytokines and 100 μL medium/well were replaced after 1 week. Expanded T cells were further characterized in ELISpot assays.

Vβ analysis of T cell receptor repertoire

To determine T cell clonality and Vβ expression, the IOTest® Beta Mark Kit (Beckman Coulter, Brea, CA, USA) was used according to the manufacturer’s protocol. This kit is designed for flow cytometric determination of the T cell repertoire (TCR) and covers about 70% of the normal human TCR Vβ repertoire.
**ELISpot assay**

96-well mixed cellulose ester plates (MultiScreen-HA Filter Plate, 0.45 μm Millipore, Eschborn, Germany) and capture-antibody solutions (all Mabtech, Hamburg, Germany) were used for IFNγ and granzyme B ELISpot assays as described previously.45 Spots in plates were counted on an AID-ELIRIFL04 ELISpot reader (Autoimmun Diagnostika, Strassberg, Germany). All experiments were performed in triplicates with exception of the initial screening ELISpot.

**xCELLigence proliferation assay**

Cell proliferation was measured with an impedance-based instrument system (xCELLigence, Roche/ACEA Biosciences) enabling label-free real-time analysis. Briefly, 1 × 10⁴ to 2.5 × 10⁴ targets cells were seeded in 200 μL medium. During the exponential growth phase 100 μL was replaced by a 100 μL T cell suspension. Cellular impedance was measured periodically every 15 min after T cell addition.

**Identification of TCR sequence**

Primers for the identification of the TCR were used according to Schuster et al.47 RNA from T cell clones was isolated via TRI Reagent Solution (Invitrogen). For cDNA synthesis, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used according to manufacturers protocol. TCR PCR was carried out using the AccuPrime™ Taq DNA Polymerase System (Invitrogen) and an Eppendorf Master Cycler. PCR reaction was done in twin.tec real-time PCR plate 96 (Eppendorf). Primers, PCR composition, and cycler settings were used as described previously.48 PCR samples were loaded onto 1.5% agarose gels and run –30 mA for 3 h. PCR products at the expected sizes (370–500 bp for alpha chain and 190–290 bp for beta chain) were isolated from Buffy coats and stimulated with 50 ng/mL OKT-3 and 100 U/mL rhIL-2 48 h prior to spin infection. The day before transduction non-treated 24-well plates were coated with 400 μL Retronectin® in PBS at a concentration of 12.5 μg/mL and stored at 4°C. Directly before transduction the supernatant was removed. Wells were blocked with 2% BSA in PBS for 30 min at 37°C and washed twice with 2.5% HEPES in HBSS. Stimulated PBMCs were collected and set to a concentration of 1×10⁶ /mL in TCM. 1 mL of each Virus and PBMCs were added into coated 24-well plates plus additional Protamine-sulfate (Cend = 4 μg/mL), HEPES (Cend = 0.5%), and IL-2 (Cend = 100 U/mL). Plates were centrifuged for 90 min at 820 g in 32°C preheated centrifuge and stored at 37°C, 5% CO₂ overnight. The next day cells were harvested and split 1:1. Cells were again placed on coated 24-well plates with fresh virus plus additives and centrifuged at 820 g/90 min/32°C. Medium was replaced after 48 h and transduction efficiency was checked after 72 h via FACS multimer staining. TCR transgenic T cells were isolated via magnetic anti-PE microbeads according to manufacturers manual (Miltenyi). Isolated cells were then cultured using irradiated mixed PBMCs and LCLs as feeder cells.

**In vivo validation of TCR transgenic T cell efficacy**

Immune deficient Rag2<sup>−/−</sup>/γ<sup>−/−</sup> mice on a BALB/c background were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and maintained in our animal facility under pathogen-free conditions in accordance with the institutional guidelines and approval by local authorities. Experiments were performed in 6–16-week-old mice. To analyze local tumor growth in vivo, 2 × 10⁶ A673 cells were re-suspended in a final volume of 0.2 mL PBS/0.2% FCS. 2 × 10⁶ A673 tumor cells were inoculated subcutaneously at the lower back of immune deficient Rag2<sup>−/−</sup>/γ<sup>−/−</sup> mice. After 3 d, mice received a full body irradiation with 3.5 Gy to enable engraftment of human T cells. 5 × 10⁶ TCR transgenic T cells together with 5 × 10⁶ CD8<sup>+</sup> depleted PBMCs were injected i.p. the following day. Control groups were untreated, or received either CD8<sup>+</sup> depleted PBMCs, or CD8<sup>+</sup> depleted PBMCs plus 5 × 10⁶ unspecific T cells. 1.5 × 10⁶ IL-15 secreting NSO cells (previously irradiated with 80 Gy) were injected i.p. twice per week. Mice were sacrificed after 17 d of tumor growth or at a maximum tumor size of > 10 mm for reason of animal protection. Tumor weight was determined. Also blood, bone marrow, and tumor samples were collected. Mice organs were stained for human T cells using CD8<sup>+</sup>-APC, CD4<sup>+</sup>-FITC (both BD), and multimter-PE antibodies and measured using a BD FACSCalibur™.

**Immunohistochemistry**

Histological analyses were performed on formalin fixed, parafin-embedded samples. All tissue slides were collected at the...
Department of Pathology of the Ludwig-Maximilians University München. The following primary antibodies were used: CD8 (1:100, SP16, DCS) and PAPPA (1:50, HPA001667, Sigma Aldrich)

**Statistical analysis**

Descriptive statistics were used to determine mean and standard deviation of the mean (SD). Differences were analyzed by unpaired two-tailed Student’s t-test using either Excel (Microsoft) or Prism 5 (GraphPad Software); p values < 0.05 were considered statistically significant (’p < 0.05; **p < 0.005; ***p < 0.0005).

**Results**

**Identification of PAPPA-derived peptides for allogeneic T cell priming**

PAPPA is highly overexpressed in ES in contrast to normal tissue in RNA microarrays and in relation to other tumor entities (Figs. S1A and B). Suitable peptides for in vitro priming were selected after SYFPEITHY in silico prediction. The six peptides with the highest predicted binding affinities were loaded onto tap deficient T2 cells and measured for MHC I stabilization (Fig. S1C). In titrations, the PAPPA1434 peptide was identified as the most potent peptide and therefore chosen for further T cell in vitro priming (Fig. 1A).
ES specificity of PAPPA-2G6 T cells

The isolated T cell clone PAPPA-2G6 specifically recognizes PAPPA\(^{1434}\) peptide (T2+) when loaded on T2 cells in contrast to influenza (T2−) negative control (Fig. 1B). Reactivity was concentration dependent as shown in the T2 titration assay (Fig. 1C). Furthermore, specific reactivity toward A673, TC-71, and EW7 (all HLA-A*02:01\(^{+}\)) was observed, whereas the ES HLA-A*02:01\(^{−}\) cell line SK-N-MC was not recognized. The MHC\(^{−}\) cell line K562 served as a NK cell control and was also not recognized (Fig. 1D). Specific lysis of A673 target cells was shown for PAPPA-2G6 T cells in xCELLigence assay. SK-N-MC cells (A2\(^{−}\)) served as a negative control and were not affected, whereas A673 ES cells were effectively lysed (Fig. 1E).

Identification of the PAPPA-2G6 TCR sequence

To identify the V\(\beta\)-chain of the TCR-clone the IOTest\({\textregistered}\) Beta Mark Kit was used and indicated the expression of V\(\beta7.2\) (Fig. S2A). Flow cytometry results were further confirmed via PCR (Fig. S2B). The PCR product was sequenced, analyzed via IMGT/V-Quest research, and confirmed the sequence of the TRBV4-2\(^{01F}\) V\(\beta\)-chain.

For the screening of the V\(\alpha\)-chain the PCR revealed initially three different PCR products, which were further sequenced (Fig. S2C). However, IMGT/V-Quest analysis predicted only the TRVA5\(^{01F}\) as the native V\(\alpha\)-chain sequence. New specific primers directed against both identified variable chains were used to amplify the whole V\(\alpha/\beta\)-chains (Fig. 2) and for further construction of the retroviral vector after sequencing.

ES reactivity of PAPPA-2G6 TCR transgenic T cells

After retroviral transduction PAPPA-2G6 TCR transgenic T cells were isolated via multimer labeling and magnetic bead separation and cultured for further analysis (Fig. 3A). In IFN\(\gamma\) ELISpot assays specificity of the TCR transgenic T cells toward T2 cells pulsed with the PAPPA\(^{1434}\) peptide was maintained in contrast to the influenza control peptide (Fig. 3B). Also, in T2 peptide titrations the sensitivity of the TCR toward the peptide pulsed T2 cells remained comparable to the original T cell clone (Fig. 3C). In addition, HLA-A*02:01\(^{+}\) ES cell lines A673, TC-71, and EW7 were similarly recognized in contrast to the HLA-A2\(^{−}\) ES cell lines SK-N-MC and SB-KMS-KS1 (Fig. 3D). Furthermore, PAPPA-specific T cells specifically lysed A673 cell lines in xCELLigence assay in contrast to SK-N-MC controls (Fig. 3E).

Reduced tumor burden after application of PAPPA-2G6 TCR transgenic T cells

Prior to adoptive transfer T cell were checked for phenotypic markers. T cells showed a CD45RO\(^{−}\), CD62L\(^{+}\), CCR7\(^{\dim}\), and CD45RA\(^{+}\) phenotype with features of central memory (T\(\text{CM}\)) as well as effector memory (T\(\text{EM}\)) T cells (Fig. 4A).

Mice in all groups received s.c. inoculated A673 cells and a total body irradiation (3.5 Gy) on day 3. Additionally, 1.5\(\times\)10\(^{7}\) irradiated IL15-secreting NSO cells were injected twice per week i.p. T cells were applied on day 4. The control groups were either untreated (n = 6), received 5\(\times\)10\(^{6}\) CD8\(^{+}\) depleted PBMCs (n = 6) or 5\(\times\)10\(^6\) CD8\(^{+}\) depleted PBMCs substituted with 5\(\times\)10\(^6\) unspecific T cells (n = 5). The study group received 5\(\times\)10\(^6\) CD8\(^{+}\) depleted PBMCs substituted with 5\(\times\)10\(^6\) specific PAPPA-2G6 TCR transgenic T cells (n = 14). Only the study group that was treated with the TCR transgenic T cells showed a significant weight reduction in contrast to the controls whereas the control groups showed no reduction in tumor growth (Fig. 4B).

Detection of TCR transgenic T cells in blood, bone marrow, and tumor samples

Samples from blood, bone marrow, and tumors of sacrificed mice were stained by CD8\(^{+}\) mAb and MHC-multimers to detect PAPPA-specific T cells (mice 1–3, Fig. 5). Via flow cytometry T cells were detectable in all three types of tissue. Yet, T cell infiltration into the tumor site was greater in the study group treated with the TCR transgenic T cells in comparison to the control group treated with unspecific T cells (mice 4–6, Fig. S3).

Figure 2. Identification of the PAPPA-2G6 TCR sequence. Full TCR PCR with specific primers for TRAV5 and TRBV4-4. PCR products (green boxes) of expected sizes were extracted and sequenced.
Immunohistochemistry reveals tumor-infiltrating T cells and PAPPA positivity in A673 xenografts

Tumor-infiltrating T cells were detected in A673 tumors with a specific CD8^+ antibody (Fig. 6A). Unspecific T cells of the control group were less frequently detected at the tumor site in contrast to the PAPPA-2G6-treated mice. Furthermore, we showed PAPPA expression in A673 xenografts (Fig. 6B) in contrast to adjacent normal murine tissue. Placental tissue served as a positive control.

Discussion

Insulin and IGF pathways represent widely investigated mechanisms and targets in cancer. The IGF-axis plays an important role in pediatric cancer in general and ES in...
particular. Pre-clinical and clinical studies addressing the IGF axis revealed IGF1R pathway inhibition as a promising treatment strategy. Variations of this strategy remain to be investigated, in particular those that may be capable of overcoming resistance against IGF1R antibodies. One of these could be to decrease IGF concentration by targeting PAPPA to prevent IGF from binding to alternate receptors, e.g., the insulin receptor. In addition, utilization of antigens present in pregnancy privileged sites as therapeutic targets in cancer have recently gained renewed interest.

PAPPA expression has been associated with various epithelial cancers. We previously showed its overexpression in ES. PAPPA functions as a highly specific metalloproteinase cleaving IGF binding protein-4 (IGFBP-4) thereby activating IGFs. Of note for bone cancer, it is critically involved in bone growth.

PAPPA is an important factor for growth in ES as recently shown by knockout assays in ES cells. In vitro ES growth was hampered and in vivo survival of ES-bearing mice was prolonged. Targeting the IGF pathway via PAPPA may thus represent a novel option for PAPPA positive bone sarcoma, in particular for advanced ES patients.

The recent breakthroughs using by T cell checkpoint inhibitors and CARs has renewed the interest in immunotherapy of cancer. In our study, we first isolated T cells specific for the PAPPA peptide (IILPMNVTV) and introduced its TCR retrovirally into random T cells. To ensure specific pairing of the transgenic TCR, we performed minimal murinization in addition to codon optimization for increased homologous pairing and expression in human T cells. Next, we showed ES specificity of this TCR in vitro. Then, we demonstrated comparable peptide affinities of wild type and transgenic TCR as well as ES specificity. Finally, we demonstrated in vivo efficacy against
human ES in Rag2<sup>−/−</sup>γc<sup>−/−</sup> mice. PAPPA-2G6 TCR transgenic T cells proved to be effective and no in vivo adverse effects were observed. Of interest, we observed a larger amount of infiltrating T cells into the tumor site in mice treated with the PAPPA-2G6 TCR transgenic T cells as compared to the unspecific T cell control group. In vivo efficacy may further be improved by applying certain T cell subtypes, e.g., T<sub>CM</sub> or T<sub>SCM</sub> or by using a more efficient mouse strand for T cell engraftment. 58-61 Yet in our studies, we could demonstrate T cell engraftment in treated mice. Functionality of <i>ex vivo</i> T cells remains to be shown in future studies. Given our recent finding that, irrespective of treatment, bone marrow involvement determines fatal outcome in ES patients, our finding of ES-specific T cell infiltration into the bone marrow is of particular interest. It may open new treatment options for these dismal patients. 62

To the best of our knowledge, this is the first study upon PAPPA in ES and the first successful attempt to target PAPPA via TCR transgenic T cells. In contrast to chimeric antigen receptor (CAR) bearing T cells, TCR-based T cell therapy is not restricted to surface molecules. Rather, TCR employing T cells can target peptides derived from all proteins required for malignancy and metastasis. TCR-based recognition is restricted to MHC, whereas CAR T cell action is MHC independent, making this approach easily accessible to a wider range of patients. 63 However, selection of a target that is not dispensable for malignancy is critical for avoidance of resistance evolution and success of targeted therapies. This applies in particular to oligo-mutated malignancies such as ES, where checkpoint inhibitors are not efficacious.

In other tumor entities, feasibility of targeting PAPPA via antibodies already has been demonstrated. 64 However, PAPPA is not internalized into the tumor cell and may rather be cleaved on the cell surface resulting in free-floating PAPPA/antibody complexes in the blood stream. The immunological synapse between TCR and MHC is about 15 nm wide. 65 PAPPA in its active form is covalently bound to glycosaminoglycans on the cell surface. 14,15 This type of binding is giving PAPPA a spacer function possibly advantageous for its function, i.e., capturing IGFBP-4/IGF complexes for cleavage and thereby activating IGFs. While the distance between a CAR and its target is not clearly defined, the spacer PAPPA will increase this distance and may reduce CAR efficacy, 14,66 making a TCR-based approach more attractive. Furthermore, circulating inactive PAPPA bound to the pro-form of eosinophil major basic protein (proMBP) in the blood may non-specifically activate CAR T cells causing a cytokine release syndrome. 67,68 A drawback of TCR-based T cell therapy is potential cross reactivity of the TCR with unknown target structures. While CARs are not tumor specific, their cross reactivity with normal cells is defined. In contrast, a TCR may have cross reactivity with an unknown target. This risk of cross reactivity is greatly enhanced by TCR affinity enhancement, an approach utilized by expert groups in the TCR-based immunotherapy field to enhance affinity and avidity of the TCR against the tumor target. 36-38

Figure 6. Immunohistochemistry staining confirms tumor infiltration by transgenic T cells and target gene expression. (A) Tumor slides were stained with a specific antibody against CD8<sup>+</sup> in immunohistochemistry. Infiltration by T cells could be shown in PAPPA-2G6-treated mice (top). CD8 positivity upon mice treated with unspecific T cells was less frequent (bottom). (B) Immunohistochemistry further showed strong immunoreactivity in trophoblast layers of placental villi (left; positive control) and xenografted A673 (right).
Distinct from TCR affinity enhancement, we addressed the challenge of reconciling efficacy with specificity by generating allo-restricted TCRs against a tumor-specific peptide.49,69

Taken together, these considerations in conjunction with the data reported here may render TCR transgenic T cells a promising approach to target PAPP-A expressing malignancies, in particular ES.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Anna Hochholzer and Lynette Henkel for expert technical assistance. Wolfgang Uckert and Matthias Leisegang are acknowledged for supplying the MP-71 vector and for valuable scientific advice and Angela Krackhardt and Richard Klar for advice in the identification and construction of the TCR. This work contains part of the doctoral thesis of A. Kirschner.

Funding
This work was supported by grants to GR and SB from the Wilhelm Sander-Stiftung (2006.109.3), Else Kröner-Fresenius-Stiftung (GR and SB; P31/08/A123/07), BMBF (GR and SB; TransNet FK01GM0870) and the Deutsche Kinderkrebsstiftung (GR and SB; DKS 2010.07) and to SB, GR and UT from the BMBF (TransNet FK01GM1104B); UT is supported by a grant from the Curaplaica Children’s Cancer Research Foundation.

T.G.P.G. is supported by a grant from “Verein zur Förderung der Wissenschaft und Forschung an der Medizinischen Fakultät der LMU München (WiFoMed)”, the Daimler and Benz Foundation in cooperation with the Reinhard-Frank Foundation, the ”Mehr LEBEN für krebskranke Kinder—Betinia-Brau-Stiftung”, the Walter Schulz Foundation, the Friedrich-Baur Foundation, the Fritz-Thyssen Foundation (FTF-Mehr LEBEN f.€ Mennach), the Daimler and Benz Foundation in cooperation with the Reinhard-Frank Foundation, the ”Mehr LEBEN für krebskranke Kinder—Betinia-Brau-Stiftung”, the Walter Schulz Foundation, the Friedrich-Baur Foundation, the Fritz-Thyssen Foundation (FTF-40.15.0.030MN), and by the German Cancer Aid (DKH-70112257).

Author contributions
A.K., U.T., and S.B. designed the research. A.K., U.T., and S.B. wrote the manuscript. A.K., T.G.P.G., R.A.R., and M.T. performed experiments. A.K., U.T., T.G.P.G., M.T., D.B., G.H.S.R., and S.B. developed methodology. A.K., U.T., T.G.P.G., T.K., and S.B. analyzed and interpreted data.

ORCID
Thomas G. P. Grünewald  http://orcid.org/0000-0003-0920-7377

References
1. Klil-Drori AJ, Azoulay L, Pollak MN. Cancer, obesity, diabetes, and antidiabetic drugs: is the fog clearing? Nat Rev Clin Oncol 2016; PMID:27502359; http://dx.doi.org/10.1038/nrclinonc.2016.120
2. Zunnkeller W, Burdach S. The insulin-like growth factor system in normal and malignant hematopoietic cells. Blood 1999; 94(11):3653-7; PMID:10572076
3. Salanti A, Clausen TM, Agerbak MØ, Al Nakouzi N, Dahlbäck M, Oo HZ, Lee S, Gustavsson T, Rich JR, Hedberg BJ et al. Targeting human cancer by a glycosaminoglycan binding malaria protein. Cancer Cell 2015; 28 (4):500-14; PMID:26461094; http://dx.doi.org/10.1016/j.ccell.2015.09.003
4. Lin TM, Galbert SP, Kiefert D, Spellacy WN, Gall S. Characterization of four human pregnancy-associated plasma proteins. Am J Obstet Gynecol 1974; 118(2):223-36; PMID:4129888; http://dx.doi.org/10.1016/0002-9378(74)90553-5
5. Boldt HB, Conover CA. Overexpression of pregnancy-associated plasma protein-A in ovarian cancer cells promotes tumor growth in vivo. Endocrinology 2011; 152(4):1470-8; PMID:21303951; http://dx.doi.org/10.1210/en.2010-1095
6. Dalgin GS, Holloway DT, Liu LS, DeLisi C. Identification and characterization of renal cell carcinoma gene markers. Cancer Inform 2007; 3:65-92; PMID:19455236
7. Loddo M, Andryszkiewicz J, Rodriguez-Acebes S, Stoebker K, Jones A, Dafou D, Apostolidou S, Wollenschlaeger A, Widschwendter M, Sainsbury R et al. Pregnancy-associated plasma protein A regulates mitosis and is epigenetically silenced in breast cancer. J Pathol 2014; 233(4):344-56; PMID:24931331; http://dx.doi.org/10.1002/path.4393
8. Bulit I, Coskun A, Ciftci A, Cetinkaya E, Altalay G, Caglar T, Gulcan E. Relationship between pregnancy-associated plasma protein-A and lung cancer. Am J Med Sci 2009; 337(4):241-4; PMID:19363517; http://dx.doi.org/10.1097/MAJ.0b013e31819867a3
9. Nagarajan N, Bertrand D, Hilmer AM, Zang ZJ, Yao F, Jacques PÉ, Teo AS, Cutcutache I, Zhang Z, Lee WH et al. Whole-genome reconstruction and mutational signatures in gastric cancer. Genome Biol 2012; 13(12): R115; PMID:22327666; http://dx.doi.org/10.1186/gb-2012-13-12-r115
10. Huang J, Tabata S, Kakiuchi S, The Van T, Goto H, Hanibuchi M, Nishioka Y. Identification of pregnancy-associated plasma protein A as a migration-promoting gene in malignant pleural mesothelioma cells: a potential therapeutic target. Oncotarget 2013; 4(8):1172-84; PMID:23896451; http://dx.doi.org/10.18632/oncotarget.11126
11. Staeger MS, Hutter C, Neumann I, Foja S, Hattenhorst UE, Hansen G, Afar D, Burdach SE et al. DNA microarrays reveal relationship of Ewing family tumors to both endothelial and fetal neural crest-derived cells and define novel targets. Cancer Res 2004; 64(22):8213-21; PMID:15548687; http://dx.doi.org/10.1158/0008-5472.CAN-03-4009
12. Conover CA, Oxvig C, Overgaard MT, Christiansen M, Giudice LC. Evidence that the insulin-like growth factor binding protein-4 protease in human ovarian follicular fluid is pregnancy associated plasma-protein-A. J Clin Endocrinol Metab 1999; 84(12):4742-5; PMID:10599745; http://dx.doi.org/10.1210/jcem.84.12.6342
13. Monget P, Oxvig C, P.A.P.-A and the IGF system. Ann Endocrinol (Paris) 2016; 77(2):90-6; PMID:27155776; http://dx.doi.org/10.1016/j.annde.2016.04.015
14. Laursen LS, Overgaard MT, Weyer K, Boldt HB, Ebbesen P, Christiansen M, Sottrup-Jensen L, Giudice LC, Oxvig C et al. Substrate specificity of the metalloproteinase pregnancy-associated plasma protein-A (PAPP-A) assessed by mutagenesis and analysis of synthetic peptides: substrate residues distant from the scissile bond are critical for proteolysis. Biochem J 2002; 367( Pt 1):31-40; PMID:12421545; http://dx.doi.org/10.1042/bj20020831
15. Canalis E, Pash J, Gambitas B, Rydziel S, Varghese S. Growth factors regulate the synthesis of insulin-like growth factor-I in bone cell cultures. Endocrinology 1993; 133(1):33-8; PMID:8319580; http://dx.doi.org/10.1210/en.133.1.8319580
16. Pfleischfeder J, Lautkuh F, Muller-Bekmann B, Blum WF, Pfister T, Ziegler R. Parathyroid hormone increases the concentration of insulin-like growth factor-I and transforming growth factor beta 1 in rat bone. J Clin Invest 1995; 96(2):767-74; PMID:7635970; http://dx.doi.org/10.1172/JCI118121
17. Mohan S, Nakao Y, Honda Y, Landale E, Leser U, Dony C, Lang K, Baylink DJ. Studies on the mechanisms by which insulin-like growth factor (IGF) binding protein-4 (IGFBP-4) and IGFBP-5 modulate IGF actions in bone cells. J Biol Chem 1995; 270(35):20424-31; PMID:75544787; http://dx.doi.org/10.1074/jbc.270.35.20424
18. Conover CA, Bale MK, Overgaard MT, Johnstone EW, Laursen UH, Fuchtbauer EM, Oxvig C, van Deursen J. Metalloproteinase...
pregnancy-associated plasma protein A is a critical growth regulatory factor during fetal development. Development 2004; 131(5):1187-94; PMID:14973274; http://dx.doi.org/10.1242/dev.009977.

30. Pollak M. The insulin receptor/insulin-like growth factor receptor family as a therapeutic target in oncology. Clin Cancer Res 2012; 18(1):40-50; PMID:22215905; http://dx.doi.org/10.1158/1078-0432.CCR-11-0998.

31. Pappo AS, Patel SR, Crowley J, Reineke DK, Kuenkele KP, Chawla SP, Toner GC, Maki RG, Meyers PA, Chugh R et al. R1507, a monoclonal antibody to the insulin-like growth factor 1 receptor, in patients with recurrent or refractory Ewing sarcoma family of tumors: results of a phase II Sarcoma Alliance for Research through Collaboration study. J Clin Oncol 2011; 29(34):4541-7; PMID:22025149; http://dx.doi.org/10.1200/JCO.2010.34.0000.

32. Richter GH, Plehm S, Rassner A, Reineke DK, Kuenkele KP, Chawla SP, Toner GC, Maki RG, Meyers PA, Chugh R et al. R1507, a monoclonal antibody to the insulin-like growth factor 1 receptor (IGF-1R), in patients with recurrent or refractory rhabdomyosarcoma, osteosarcoma, synovial sarcoma, and other soft tissue sarcomas: results of a Sarcoma Alliance for Research Through Collaboration study. Cancer 2014; 120(16):2448-56; PMID:24777726; http://dx.doi.org/10.1002/cncr.287292.

33. Bernstein M, Kovar H, Paulussen M, Randall RL, Schuck A, Teot LA, Juegersen H. Ewing’s sarcoma family of tumors: current management. Oncologist 2006; 11(5):503-19; PMID:16720851; http://dx.doi.org/10.1634/theoncologist.11-5-503.

34. Uwe Thiel AW, von Luetichau I, Bender H-U, Blaschke F, Grunwald TGP, Steinborn M, Rüper B, Bonig H, Klingebiel T, Bader P et al. Bone marrow involvement identifies a subgroup of advanced Ewing sarcoma patients with fatal outcome irrespective of therapy in contrast to curable patients. J Clin Oncol 1999; 17(14):4394-401; PMID:10514962; http://dx.doi.org/10.1002/1097-0432(19990715)17:14%3c4394::AID-JCO10.1634/jclon.199910522.
57. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. Nature 2006; 439(7077):911-21; PMID:16795967; http://dx.doi.org/10.1038/nature04479

58. Klebanoff CA, Gattinoni L, Restifo NP. Sorting through subsets: which T-cell populations mediate highly effective adoptive immunotherapy? J Immunother 2012; 35(9):651-60; PMID:23090074; http://dx.doi.org/10.1097/CJI.0b013e31827806e6

59. Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, Finkelstein SE, Theoret MR, Rosenberg SA, Restifo NP. Acquisition of full effector function in vitro paradoxically impairs the in vivo anti-tumor efficacy of adoptively transferred CD8+ T cells. J Clin Invest 2005; 115(6):1616-26; PMID:15931392; http://dx.doi.org/10.1172/JCI24480

60. Huang J, Khong HT, Dudley ME, El-Gamil M, Li YF, Rosenberg SA, Robbins PF. Survival, persistence, and progressive differentiation of adoptively transferred tumor-reactive T cells associated with tumor regression. J Immunother 2005; 28(3):258-67; PMID:15838833; http://dx.doi.org/10.1016/j.jimmed.2004.12.004

61. Zhou Q, Facchione J, Jin M, Shen Q, Lin Q. Humanized NOD-SCID II.2rg−/− mice as a preclinical model for cancer research and its potential use for individualized cancer therapies. Cancer Lett 2014; 341(1-3):93-100; PMID:24515265; http://dx.doi.org/10.1016/j.canlet.2013.10.015

62. Thiel U, Wauer A, Wolf P, Badoglio M, Santucci A, Klingebiel T, Basu O, Borkhardt A, Laws HJ, Kodera Y et al. No improvement of survival with reduced- versus high-intensity conditioning for allogeneic stem cell transplants in Ewing tumor patients. Ann Oncol 2011; 22(7):1614-21; PMID:21245159; http://dx.doi.org/10.1093/annonc/mdq703

63. Kershaw MH, Westwood JA, Darcy PK. Gene-engineered T cells for cancer therapy. Nat Rev Cancer 2013; 13(8):525-41; PMID:23880905; http://dx.doi.org/10.1038/nrc3565

64. Becker MA, Haluska P Jr, Bale LK, Oxvig C, Conover CA. A novel neutralizing antibody targeting pregnancy-associated plasma protein-a inhibits ovarian cancer growth and ascites accumulation in patient mouse tumourgrafts. Mol Cancer Ther 2015; 14(4):973-81; PMID:25695953; http://dx.doi.org/10.1158/1535-7163.MCT-14-0880

65. Dustin ML, Chakraborty AK, Shaw AS. Understanding the structure and function of the immunological synapse. Cold Spring Harb Perspect Biol 2010; 2(10):a002311; PMID:20843980; http://dx.doi.org/10.1101/cshperspect.a002311

66. Srivastava S, Riddell SR. Engineering CAR-T cells: Design concepts. Trends Immunol 2015; 36(8):494-502; PMID:26169254; http://dx.doi.org/10.1016/j.it.2015.06.004

67. Davila ML, Riviere I, Wang X, Bartido S, Park J, Curran K, Chung SS, Stefanski J, Borquez-Ojeda O, Olszewski M et al. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. Sci Transl Med 2014; 6(224):224ra25; PMID:24553386; http://dx.doi.org/10.1126/scitranslmed.3008226

68. Lee DW, Gardner R, Porter DL, Louis CU, Ahmed N, Jensen M, Grupp SA, Mackall CL. Current concepts in the diagnosis and management of cytokine release syndrome. Blood 2014; 124(2):188-95; PMID:24876563; http://dx.doi.org/10.1182/blood-2014-05-552729

69. Felix NJ, Allen PM. Specificity of T-cell alloreactivity. Nat Rev Immunol 2007; 7(12):942-53; PMID:18007679; http://dx.doi.org/10.1038/nri2200