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GENERATION OF TRANSGENIC, GPR64 TUMOR ANTIGEN-SPECIFIC T CELLS AND EVALUATION OF THEIR THERAPEUTIC EFFICACY IN PEDIATRIC SARCOMA

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Für meine Familie

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1 INTRODUCTION

1.1 Ewing Sarcoma

The Ewing Sarcoma (EwS) is a highly aggressive bone or soft tissue tumor mostly occurring in children and adolescents. In 1921 it was first described by the American pathologist James Ewing and characterized by an undifferentiated small blue round cell appearance (Ewing 1972). According to histopathological and molecular pathological characteristics the WHO classified the Ewing Sarcoma family tumors (ESFT), the peripheral primitive neuroectodermal tumor (PNET), the extraosseous Ewing Sarcoma (EES) and the Askin tumor uniformly as Ewing Sarcoma (Fletcher et al. 2013).

After osteosarcoma, EwS is the second most common primary sarcoma of bone in children and adolcents. The most common age at diagnosis is the second decade of life and male patients are a little more often diagnosed than female patients (1.2:1 ratio of males:females) (Bernstein et al. 2006). Furthermore the Caucasian population is more frequently affected than other populations implying a strong contribution of germline variation to Ewing sarcoma tumorigenesis (Worch et al. 2011). Possible causes of the epidemiologic difference are currently the subject of research. A previous study by Grunewald et al. describes the A risk allele, which might contribute to the variable susceptibility to Ewing sarcoma across populations. It is associated with significantly higher EGR2 expression in Ewing sarcoma tumors. EGR2 is a proliferation-enhancing gene whose suppression inhibits the proliferation of EwS cells and even led to tumor regression in a mouse model. A comparative study by the 1000 Genome Project yielded a significantly increased frequency of the described risk allele in the genetic material of Europeans compared to the genome of Africans (Grunewald et al. 2015). Genetically EwS is characterized by fusions between EWSR1 (Ewing sarcoma break point region 1) and members of the ETS (erythroblast transformation specific) gene family, in 85% of all cases FL1, in 10% ERG. The EWS-FLI1 fusion protein results from the chromosomal translocation t (11;22) (q24:q12) and encodes an aberrant transcription factor. It binds DNA at GGAA-microsatellites, which has either an activating or more frequent a suppressive effect on target gens (Riggi et al. 2014). Some of the proteins up-regulated by EWS-FLI1 are enhancer of zeste homolog 2 (EZH2), insulin like growth factor 1 (IGF1), six-

transmembrane epithelial antigen of prostate (STEAP1) and G protein-coupled receptor 64 (GPR64) contributing to proliferation, invasiveness and metastasis (Cironi et al. 2008, Richter et al. 2009, Grunewald et al. 2012, Richter et al. 2013).

There is an ongoing debate on the identification of the cell of origin of EwS, as EwS appears as an undifferentiated tumor (Ewing 1972). Numerous cells of origin have been suggested, such as mesenchymal stem cells (MSC) and neural crest cells (Staege et al. 2004, Kovar 2005, Riggi et al. 2008, Tanaka et al. 2014). Much of the difficulty in identifying the tissue of origin for EwS results from the toxicity of EWSR1-ETS fusion genes to most cell types (Kovar 2005). The present view is that the disease arises from some mesenchymal or neural crest derived stem or progenitor cell, yet the question of the cell of origin is not finally clarified (Kovar et al. 2016).

Main symptom of EwS is localized pain, followed by swelling and loss of function of the affected bone. EwS is often diagnosed late after first symptoms because they are misinterpreted for signs of growth or injury (Widhe et al. 2000). Most common localization is the pelvic bone (26%), followed by the diaphysis oft the long bones of the lower extremities (femur 20%) (Bernstein, Kovar et al. 2006). In adults EwS occurs less frequently, but the ratio of manifestation as soft tissue sarcoma increases with later age of onset (Potratz et al. 2012). About 20% to 30% of patients have metastases in lung, bone and/or bone marrow at the time of diagnosis, which is an unfavorable prognostic factor (Bernstein, Kovar et al. 2006, Thiel et al. 2016).

The treatment of EwS relies on a multidisciplinary approach that combines chemotherapy with local therapy consisting of surgery and radiation (Gaspar et al. 2015). Whenever possible, surgeries are performed aiming complete resection. But often bone replacement in growing children is still a challenge. Adjuvant as well as neoadjuvant chemotherapy is added using different protocols. An ongoing study is comparing the combination of vincristine, ifosfamide, doxorubicin and etoposide, which is recommended in Europe (Juergens et al. 2006), and the combination of ifosfamide and etoposide together with vincristine, doxorubicin and cyclophosphamide, which is recommended in North America (Grier et al. 2003, Felgenhauer et al. 2013). Radiation therapy is used neoadjuvant or post-surgery in cases of incomplete remission (Schuck et al. 2003). Allogeneic stem cell transplantation is an additional treatment option for patients with advanced EwS

showing early relapse or metastasis to multiple bones (Burdach et al. 2000). Patients with advanced Ewing sarcomas, which are defined by early relapse (<24 months after diagnosis) and/or by metastasis to multiple bones or bone marrow, have an overall survival of ≤10% after ten years (Burdach et al. 2010, Stahl et al. 2011). Although overall survival for patients with localized disease is with 65% to 75% a lot higher, these patients suffer from acute and long-term toxicities of the therapies (Gaspar, Hawkins et al. 2015). These poor future prospects demonstrate the urgent need for new therapy options. In this context, one promising new therapeutic treatment modality is the development of adoptive T cell therapy, such as engineered T-cell therapy.

1.2 CAR T cell therapy

Chimeric antigen receptor (CAR) T cell therapy is a type of adoptive T cell therapy using T cells transduced with a chimeric receptor specific to a tumor antigen. CAR T cell therapy uses gene transfer technology to reprogram a patient´s own T cells to express the CAR combining the high affinity binding of an antibody with the lytic potential of T cells. CAR T cells bind to the targeted antigen, get activated, proliferate and can perform potent anti-tumor effect. Eshhar and colleagues generated CARs for the first time in 1989. They combined an antibody and T cell receptor (TCR) subunits and expressed these fusion products on T cells to mediate major histocompatibility complex (MHC) independent T cell activation (first generation CARs) (Gross et al. 1989, Eshhar et al. 1993). As CARs do not require antigen processing or HLA expression to be recognized, they are broadly applicable to HLA-low expresser patient populations. They can still target tumor cells that have down regulated proteasomal antigen processing or HLA expression preventing tumor escape as seen in TCR T cell therapy, another approach in the field of adoptive T cell therapy that is heavily under investigation (Zhou et al. 2012). Another benefit of CAR T cell therapy is its low risk of graft-versus-host disease (Anwer et al. 2017). However, first generation CAR T cells showed limited efficacy in clinical trails, probably due to lack of long-term T cell expansion or activation induced cell death of the T cells (Lamers et al. 2006, Till et al. 2008, Jensen et al. 2010). But, over the last decades the structure of CARs has been intensely investigated to most commonly consisting in

the extracellular domain of a single-chain variable fragment (scFv) derived from a monoclonal antibody (mAb) and a spacer, typically the IgG_1 CH_2CH_3, and in the intracellular domain of a CD3ζ signaling chain providing the primary signal (referred to as first generation CAR). Combination with one (second generation CAR) or two (third generation CAR) costimulatory domains leaded to improved proliferation, cytotoxicity and persistence (**Figure 1**) (Maher et al. 2002, Finney et al. 2004, Imai et al. 2004, Milone et al. 2009). Despite the general finding that CARs that incorporate costimulatory signals are more potent, there is still research going on to find out if any particular costimulatory molecule is superior to another. CARs of the 4th generation, so-called TRUCKs or armored CARs, are engineered to secrete factors that improve anti-tumor activity even further, such as cytokines or costimulatory ligands (**Figure 1**) (Chmielewski et al. 2015).

Current CAR-modified T cells are limited to recognize cell surface antigens, but they can work as “living drugs” that may have both immediate and long-term effects. Redirecting immune reactivity toward a chosen target is not the only purpose of CAR T cells. CARs with different strengths and quality can modulate T cell expansion, persistence and the strength of T cell activation within the tumor microenvironment altering the efficacy and safety of tumor-targeted T cells. In this context, CAR transduced T cells have the potential of a broader range of functional effects than TCR transgenic T cells whose strength of signaling is mainly determined by the TCR´s affinity to the antigen (Sadelain et al. 2013).

In the growing field of tested CAR T cell therapies the ones using CD19, a B-cell activation receptor, specific CARs are the most advanced engineered T-cell therapies currently being under investigation. In patients with relapsed or refractory B-cell malignancies, including acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma, who were treated with CD19 CAR T cells expressing either a CD28 or a 4-1BB costimulatory domain, durable remissions have been induced in multiple clinical trails (Grupp et al. 2013, Davila et al. 2014, Maude et al. 2014, Kochenderfer et al. 2015). In August 2017 the US Food and Drug Administration (FDA) approved a gene therapy against cancer for the first time. Kymriah^{TM} is a suspension for intravenous infusion containing CD19 specific CAR T cells for the treatment of patients up to 25 years of age with B-cell precursor ALL that is refractory or in second or later relapse (Morrow 2017).

Even though these excellent results in CAR T cell therapy could be achieved, there are still two main safety concerns associated with the use of CARs. One of them is the immune-mediated rejection of normal tissues expressing the targeted antigen. This problem could be solved with truly tumor-specific cell surface molecules making the identification of suitable targets for CAR T cell therapy even more important. The most common and potentially severe toxicity that occurred in studies with CAR-modified T-cell therapy is the cytokine release syndrome (CRS), an inflammatory process related to T-cell proliferation resulting in highly elevated cytokine levels (Maus et al. 2014). This syndrome is closely associated with a systemic macrophage activation syndrome. CRS causes from mild flu-like symptoms to high fever, hypotension and in extreme cases life-threatening organ failure and shock. Due to high-dose steroids, vasopressors, ventilator support and supportive care most CRS toxicities are manageable. In most cases, developing CRS seems to correlate with efficacy of the antitumor activity, but there is not a strong correlation between the degree of CRS and the response to therapy (Davila, Riviere et al. 2014). Unlike the handling of many conventional drug-induced side effects, reduction of the CAR T cell dosage does not control the syndrome. As an increase in IL-6 is associated with severe CRS, the treatment with tocilizumab, an interleukin-6 receptor inhibitor, a treatment has been found to relieve CRS without affecting efficacy of CAR T cell treatment (Lee et al. 2014).

However, success with CAR T cell therapy so far is primary limited to hematologic malignancies and is rarely achieved in solid tumors. When applied to solid tumors CAR T cells have either shown limited clinical activity or toxic effects (Lamers, Sleijfer et al. 2006, Pule et al. 2008, Morgan et al. 2010). An exception is for example a series of pediatric patients with GD2-expressing neuroblastoma treated with GD2-specific CAR T cells resulting in tumor regression in 3 of 11 patients with active disease (Louis et al. 2011). Compared to hematologic malignancies solid tumors present a different set of challenges: to reach a solid tumor engineered T cells have to overcome barriers such as exiting the blood vessels and infiltrating in the tumor. Physical barriers from the surrounding stroma as well as abnormal vasculature complicate efficient infiltration into solid tumors. Within the tumor an altered metabolism with a multitude of immunosuppressive factors challenges T cells. Furthermore the selection of the target antigen plays an important role. In contrast to antigens presented on hematologic malignances, target antigens on solid tumors are

often heterogenous, differing not just within one tumor but also between both primary and metastatic tumors. In addition to these general problems targeting solid tumors with CAR-containing T cells have two main specific challenges to struggle with. First of all, CAR T cells seem to penetrate solid tumors less than TCR T cells. And furthermore, CAR T cells so far lack specific tumor targets because their target antigens are surface proteins often also expressed in normal tissue, which complicates their impact on solid tumors (Garber 2018).

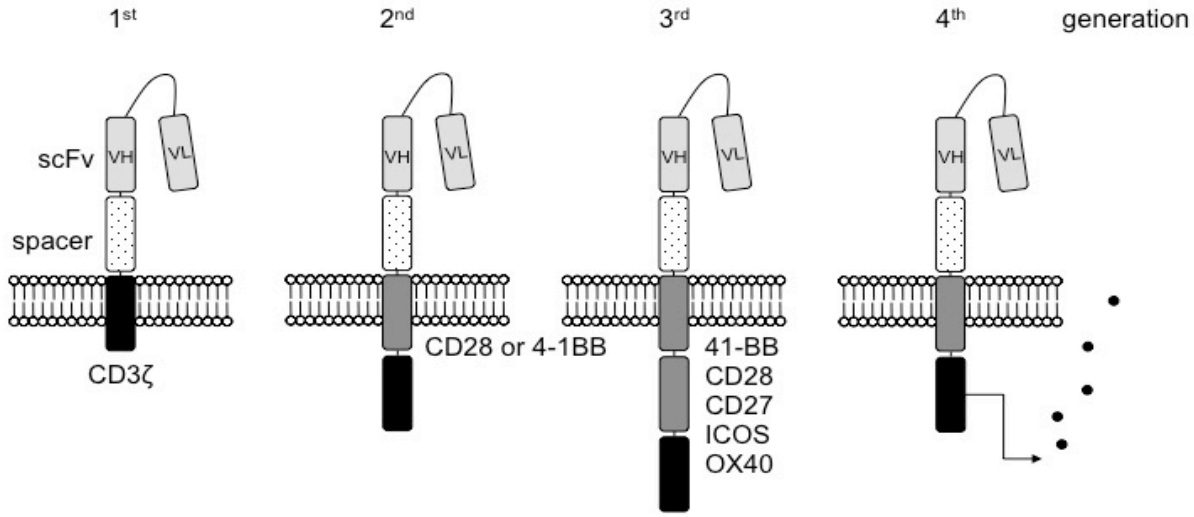

Figure 1: CAR structure. CARs contain an extracellular single-chain variable fragment (scFv), a spacer and intracellular signaling domains. The intracellular component includes the CD3ζ intracellular signaling domain of the T-cell receptor either alone (first generation) or in combination with one (second generation) or two (third generation) costimulatory domains. The 4th generation of CARs describes constructs with an inducible expression cassette for a secreted transgene.

That is why more research hast to be done to take the promising results of CAR-based therapy in hematologic malignancies on to solid tumors (Abken 2015).

1.3 GPR64

G protein-coupled receptor 64 (GPR64), also known as Adhesion G protein-coupled receptor G2 (ADGRG2) or human epididymis-specific protein 6 (HE6) is a heterodimeric membrane-bound protein encoded by the ADGRG2 gene (Hamann et al. 2015). It is an orphan member of the adhesion G-protein-coupled receptor (GPCR) family. GPR64 consists of two subunits: a large, hydrophilic and highly glycosylated ectosubunit and a hydrophobic endosubunit. It is characterized by a seven transmembrane (TM7) domain, that is linked to an extracellular region via a conserved GPCR proteolytic site (GPS) domain (**Figure 2**) (Kirchhoff et al. 2006). For the GPS motif cleavage has been shown (Obermann et al. 2003).

Under normal conditions GPR64 is highly expressed in the epithelia of ductuli efferentes and proximal epididymis, but not in any other adult tissue (Osterhoff et al. 1997). More specifically it is expressed in the non-ciliated principal cells of the proximal excurrent ducts, which are primarily implicated in sperm concentration and testicular fluid reabsorption (Kirchhoff et al. 2008). Mutant male mice with a targeted disruption of GPR64 showed sperm stasis and duct obstruction, resulting from dysregulation of fluid reabsorption, and complete loss of fertility. As hormonal levels in the knockout males were not significantly different from those in wild-type mice, it is supposed that GPR64 receptors exert their functions directly in a tissue or cell type of the male genital tract (Davies et al. 2004).

GPR64 was found to be highly up-regulated in EwS (Staege, Hutter et al. 2004). Richter et al. considered it to be a highly specific marker for EwS with a positivity/sensitivity of 94.7% and specificity of 89.8%. They also described its role in ES and showed that it promotes invasiveness and metastasis via up-regulation of PGF and MMP1. Apart from its high sensitivity and specificity for EwS among sarcomas, high levels of GPR64 expression were also found in prostate, kidney, non-small cell lung cancer and melanoma cell lines, while moderate to low levels of GPR64 expression were observed in brain, ovary, breast and colon cancer as well as in leukemia (Richter, Fasan et al. 2013).

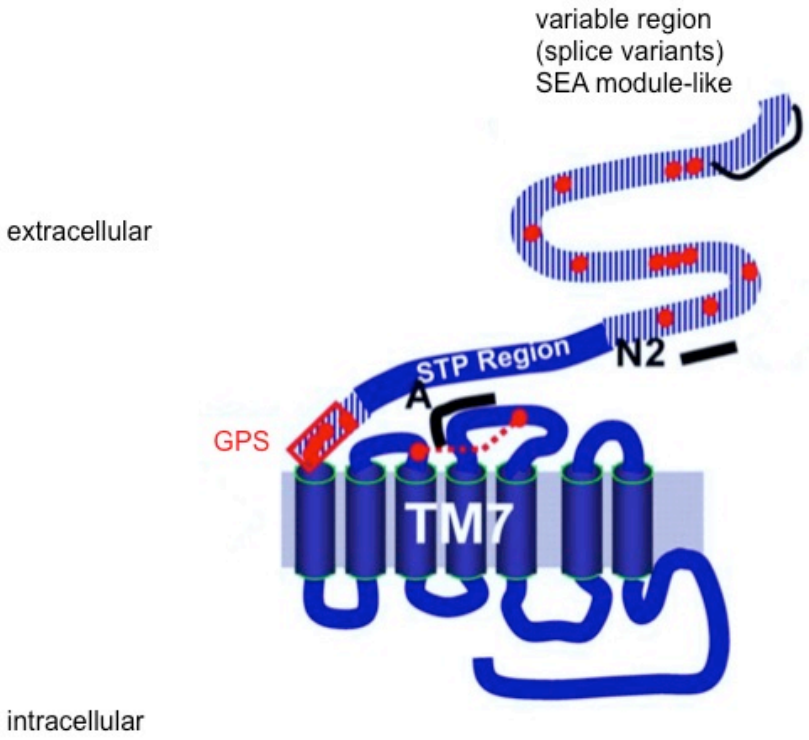

Figure 2: Scheme of GPR64 protein within the membrane. The seven transmembrane domain (TM7) is connected via the G-protein-coupled receptor proteolysis site (GPS) domain to the extracellular region containing the serine/threonine/proline-rich region (STP) and the sea urchin sperm protein, enterokinase and agrin (SEA) module-like region of variable length. A: epitope for antibody against endosubunit; N2: epitope for antibody against ectosubunit. Modified from Kirchhoff, Obermann et al. (2006).

2 Aim of this study

Based on the recent success in CAR T cell therapy, the aim of this study was to generate transgenic, GPR64 tumor antigen-specific T cells and to evaluate their therapeutic efficacy in Ewing sarcoma. Therefore, GPR64 had to be confirmed as a suitable target for CAR T cell therapy. Two different GPR64-specific monoclonal antibodies derived from rat hybridoma, namely 20F8 and 8F5, were sequenced and the identified specific binding portion of these antibodies were synthesized and cloned into retroviral vectors for the infection of human T cells to allow production of GPR64 targeting cells for cancer treatment. The transgenic T cells were tested for their target recognition and lytic capacity *in vitro* comparing the results of the two different CAR constructs to each other. Furthermore, their anti-tumor effect in an immunodeficient mouse model was evaluated.

3 MATERIAL

3.1 List of manufacturers

Manufacturer	Location
Abcam	Cambridge, UK
Abott	Wiesbaden, Germany
ACEA	San Diego, California, USA
Ambion	Austin, Texas, USA
Applied Biosystems	Darmstadt, Germany
ATCC	Rockyville, Maryland, USA
Autoimmun Diagnostika	Strassberg, Germany
B. Braun Biotech Int.	Melsungen, Germany
BD Bioscience Europe	Heidelberg, Germany
Beckman Coulter	Palo Alto, California, USA
Beckton Dickinson	Heidelberg, Germany
Berthold detection systems	Pforzheim, Germany
Biochrom	Berlin, Germany
BioRad	Richmond, California, USA
Biozym	Hessisch Oberdorf, Germany
Brand	Wertheim, Germany
Dako	Hamburg, Germany
DCS	Hamburg, Germany
Elma	Singen, Germany
Eppendorf	Hamburg, Germany
Falcon	Oxnard, California, USA
Fermentas	St. Leon-Rot, Germany
Cayman Chemical Company	Ann Arbor, Michigan, USA

GE Healthcare	Little Chalfont, USA
GeneArt	Regensburg, Germany
Genomed	St-Louis, Missouri, USA
Genescript	New Jersey, USA
Genzyme	Neu-Isenburg, Germany
GFL GmbH	Segnitz, Germany
GLW	Würzburg, Germany
Greiner Bio-One GmbH	Frickenhausen, Germany
Heraeus	Hanau, Germany
ImmunoTools	Friesoythe, Germany
Implen GmbH	München, Germany
Jackson ImmunoResearch	West Grove, USA
Kisker Biothech	Steinfurt, Germany
Leica	Wetzlar, Germany
LGC Standards GmbH	Wesel, Germany
Life Technologies	Carlsbad, California, USA
Lonza	Basel, Switzerland
Mabtech	Hamburg, Germany
Machery & Nagel	Düren, Germany
Merck Milipore	Darmstadt, Germany
Metabion	Planegg, Germany
Miltenyi	Bergisch Gladbach
Mirus	Madison, Wisconsin, USA
Molecular BioProducts	San Diego, California, USA
Nalgene	Rochester, New York, USA
New England BioLabs	Frankfurt am Main, Germany
Origene	Rockville, Maryland, USA

PAA	Cölbe, Germany
Peprotech	Rocky Hill, New Jersey
Peps 4 Life Sciences	Heidelberg, Germany
Perkin Elmer	Akron, Ohio, USA
R&D Systems	Mineapolis, Minnesota, USA
Roche	Penzberg, Germany
Roth	Karlsruhe, Germany
Sarstedt	Nümbrecht, Germany
Sartorius	Göttingen, Germany
Scientific Industries	Bohemia, New York, USA
Scotsman	Milan, Italy
Sequiserve	Vaterstetten, Germany
Sempermed	Wien, Austria
Sigma Aldrich	St. Louis, Missouri, USA
Stratagene	Cedar Creek, Texas, USA
Syngene	Cambridge, UK
Systec	Wettenberg, Germany
TaKaRa Bio Europe	Paris, France
Taylor-Wharton	Husum, Germany
Thermo Fisher Scientific	Ulm, Germany
Thermo Scientific	Braunschweig, Germany
TKA GmbH	Niederelbert, Germany
TPP	Trasadingen, Switzerland
VWR	Darmstadt, Germany

3.2 List of instruments and equipment

Device	Specification	Manufacturer
Autoclave	V95	Systec
Bacteria shaker	Certomat BS-T	Sartorius
Ice maker	AF 100	Scotsman
Cell counting chamber	Neubauer	Brand
Centrifuge	Multifuge 3 S-R	Heraeus
Centrifuge	Biofuge fresco	Heraeus
Controlled-freezing box	Mr. Frosty	Nalgene
Electroporator	Nucleofactor I	Amaxa biosytems
Electrophoresis chamber		BioRad
ELISA Reader	Multiskan Ascent	Thermo Scientific
ELISpot reader	AID-ELIRIFL04	Autoimmun Diagnostika
Flow cytometer	FACScalibur™	Becton Dickinson
Freezer (-80°C)	Hera freezer	Heraeus
Freezer (-20°C)	Cool vario	Siemens
Fridge (+4°C)	Cool vario	Siemens
Gel documentation	Gene Genius	Syngene
Heating block	Thermomixer comfort	Eppendorf
Incubator	Hera cell 150	Heraeus
Liquid nitrogen reservoir	L-240 K series	Taylor-Wharton
Luminometer	Sirius Luminometer	Berthold detection systems
Multichannel pipette	(10-100 µl)	Eppendorf
Micropipettes	(0.5–10 µl, 10–100 µl, 20–200 µl, 100–1000 µl)	Eppendorf
Microscope	DMIL	Leica
Nanophotometer		Implen

PCR cycler		Eppendorf
Pipetting assistant	Stripettor Plus	Falcon
Real-time PCR	7300 Real-Time PCR	Applied Biosytems
Rotator		GLW
Steril bench		Heraeus
Sonifier	S60H Elmasonic	Elma
Water bath		GFL GmbH
Vortexer	Vortex-Genie 2	Scientific Industries
Water purification system	TKA GenPure	TKA GmbH

3.3 List of consumables

Material	Manufacturer
6 well tissue culture plate	Falcon
24 well non- tissue culture plate	Falcon
96 well cell culture plate (round and flat bottom)	TPP
96 well cell culture plate (v bottom)	Greiner Bio-One
Cell culture flasks (25, 75, 175 cm^2)	Greiner Bio-One
Cell strainer 40 µm	Falcon
Columns (MACS, LS, MS)	Miltenyi
Cryovials 1.5 ml	Sarstedt
Cuvettes	Roth
E-plate 96	ACEA
Filters 0.45 µm	Sartorius
Gloves (nitril, latex)	Sempermed
MultiScreen-HA Filter Plates	Merck Milipore
Parafilm	Pechiney Plastics Packaging

Serological pipettes (5, 10, 25 ml)	VWR
Pipette filter tips (10, 100, 200, 1000 µl)	Thermo Scientific
Pipette tips (10, 100, 200, 1000 µl)	Molecular BioProducts
Plates for qRT-PCR (96 well)	Applied Biosystems
Reagent reservoir (50 ml)	Falcon
Reagent reservoir (for 12 channel pipette)	VWR
Tubes for cell culture (polypropylene, 15, 50 ml)	Greiner Bio-One
Tubes for cell culture (polystyrene, 15 ml)	Falcon
Tubes for flow cytometry (5 ml)	Sarstedt
Tubes for PCR	Sarstedt
Safelock tubes for molecular biology (1.5, 2 ml)	Roth
Syringes (5 ml)	B. Braun
Single-use hypodermic needles (29 G 0.33 mm x 12.7 mm)	B. Braun

3.4 List of chemical and biological reagents

Reagent	Manufacturer
Agar	Sigma
Agarose	Invitrogen
AIM-V Medium	Invitrogen
Ampicilin	Merck
Anti-FITC MicroBeads	MACS Miltenyi Biotec
Anti-PE MicroBeads	MACS Miltenyi Biotec
autoMACS™ Rinsing Solution	Miltenyi
Blue Juice Gel Loading Buffer	Invitrogen

CHAPS hydrate	Sigma
Dimethylformamide	Roth
dNTPs	Roche
Dimethyl sulfoxide (DMSO)	Merck
D-luciferin	Perkin Elmer
DMEM medium	Invitrogen
Dynabeads® Human T-Activator CD3/CD28	Thermo Fisher Scientific
eBio-GPR1 Peptide	Peps 4 Life Sciences
eBioscience™ Streptavidin PE	Invitrogen
Erythrocyte Lysis Buffer	Pharmazie, Klinikum rechts der Isar
Ethanol	Merck
Ethidium bromid (EtBr)	BioRad
Fetal calf serum (FCS)	Biochrome
Ficoll-Paque	GE Healthcare
Formaldehyd (37%)	Merck
Glycerol	Merck
Glycin	Merck
G418	PAA
Hank´s buffered salt solution (HBSS)	Invitrogen
HEPES	Sigma
Human IgG	Genzyme
Human male AB serum	Lonza
Hydrochloric acid (HCl)	Merck
Isofluran	Abott
Isopropanol	Sigma
L-glutamin	Invitrogen

MACS® BSA Stock Solution	Miltenyi
Magnesium chloride ($MgCl_2$)	Invitrogen
Maxima™ Probe/ROX qPCR Master Mix (2x)	Fermentas
Na-pyruvate	Invitrogen
Non-essential amino acids (100x, NEAA)	Invitrogen
NP-40 Surfact-Amps^{TM} Detergent Solution	Thermo Fisher Scientific
Paraformaldehyde (PFA)	Merck
PCR buffer (10x)	Invitrogen
Pepton	Invitrogen
Phosphate buffered saline (PBS) 10x	Invitrogen
Proleukin (recombinant human interleukin 2)	Novartis
Propidium iodid	Sigma
Prostaglandin E_2 (PGE_2)	Cayman Chemical Company
Protease Inhibitor Cocktail, EDTA-free	Sigma
Puromycin	PAA
Recombinant human interleukin 15	Peprotech
(rhIL-15)	
Retronectin	TaKaRa
RPMI 1649 medium	Invitrogen
Sodium azide (NaN_3)	Merck
Sodium hydroxide (NaOH)	Merck
Sodium Orthovanadate	New England BioLabs
Streptavidin-coated polysterene particles (SCPP)	Kisker Biotech
Streptavidin-Horse Radish Peroxidase	Mabtech
TransIT®-293	Mirus

Trypan blue	Sigma
Trypsin/EDTA	Invitrogen
TWEEN 20	Sigma
1-bromo-3-chloropane (BCP)	Sigma

3.5 List of commercial reagent kits

Name	Manufacturer
AccuPrime Taq DNA Polymerase System	Invitrogen
$CD8^+$ T cell Isolation Kit, human	Miltenyi
Cell line Nucleofector® Kit R	Lonza
High-Capacitiy cDNA Reverse Transcription Kit	Applied Biosystems
Human TNF-alpha Quantikine ELISA Kit	R&D Systems
Human INF-gamma Quantikine ELISA Kit	R&D Systems
Human IL-2 Quantikine ELISA Kit	R&D Systems
JETSTAR 2.0 Plasmid Maxiprep Kit	Genomed
MycoAlert™ Mycoplasma Detection Kit	Lonza
NucleoSpin® Plasmid Kit	Machery-Nagel
StrataPrep® DNA Gel Extraction Kit	Stratagene
TaqMan® Gene Expression Assays	Applied Biosystems
TRI Reagent RNA Isolation Kit	Ambion

3.6 Media, Buffer and Solutions

3.6.1 List of cell culture media and universal solutions

Name	Ingredients
Standard tumor medium	500 ml RPMI 1640 medium or DMEM medium, 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin
T cell medium (TCM)	500 ml AIM-V medium, 5% hAB serum, 100 U/ml penicillin and 100 µg/ml streptomycin
4% formaldehyde	4% Formalin, 55 mM Na_2HPO_4, 12 mM NaH_2PO_4-H_2O
4% paraformaldehyde	4% PFA in PBS, adjusted to pH 7.4
LB medium	10 g peptone, 5 g yeast extract, 10 g NaCl, in 1000 ml distilled water
LB agar medium	LB medium, 2% select agar
FACS staining buffer	0.5% BSA in PBS
Freezing medium (human cells)	hAb serum, 10% DMSO
Freezing medium (tumor cells)	FCS, 10% DMSO

3.6.2 List of buffer and gel for DNA electrophoresis

Name	Ingredients
TAE running buffer	50xTAE: 2 M Tris, 10% EDTA (0.5 M), 5.1% HCl
Electrophoresis gel	200 ml TAEbuffer (1x), 0.7-3% agarose, 3 µl EtBr

3.7 Antibodies

3.7.1 Antibody for crosslinking

Anti-Mouse IgG, Fcγ fragment specific (Jackson ImmunoResearch)

3.7.2 List of antibodies for flow cytometry

Name	Conjugation	Clone	Manufacturer
Anti-human CD8	FITC	RPA-T8	BD Biosciences
Anti-human CD8	APC	RPA-T8	BD Biosciences
Anti-human IgG (γ-chain specific),	FITC		Sigma Aldrich
Anti-human IgG1	APC	X40	BD Biosciences
Anti-human IgG Fc	PE		Abcam
Anti-human EGFR-biotinylated	PE		Merck/Erbitux (Institute of Virology, Technical University of Munich)
Anti-human EGF Receptor	PE	EGFR.1	BD Biosciences

3.7.3 List of antibodies for ELISpot assays

Name	Specification	Manufacturer
Anti-human IFNγ mAB	1-D1K, purified	Mabtech
Anti-human IFNγ mAB	7-B6-1, biotinylated	Mabtech

3.8 TaqMan® Gene Expression Assays for qRT-PCR

All TaqMan® Gene Expression Assays were obtained from Applied Biosystems.

Gene	Assay ID
ACTB	Hs99999903_m1
GAPDH	Hs99999905_m1
GPR64	Hs00971379_m1
PRDM1	Hs00153357_m1
TBX21	Hs00203436_m1

3.9 Bacterial strain for plasmid multiplication

The chemically competent One Shot® TOP 10 E. coli strain (Invitrogen) with the following genotype was used for the propagation of plasmids: F-mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG.

3.10 List of human cancer cell lines

Name	GPR64 status	Description
A673	positive	EwS cell line (type 1 translocation with additional p53 mutation, established from the primary tumor of a 15-year-old girl.
SaOs	negative	OS
SH-SY-5Y	negative	NB cell line which is a thrice-cloned sub-line of bone marrow biopsy-derived line SK-N-SH.
SIMA	negative	NB cell line established 1991 from a stage III tumor of a 20-month-old Caucasian boy.
SK-N-MC	positive	EwS cell line with a type 1 translocation established from the supraorbital metastases of a 14-year-old girl with Askin´s tumor (related to EwS).
TC-71	positive	EwS cell line with a type 1 translocation established on 1981 from a locally relapsed EwS of a 22-year-old man.

3.11 Vector

The retroviral vectors pMP71_20F8_CAR (**Figure 3**) and pMP71_8F5_CAR (**Figure 4**) were obtained from GeneArt.

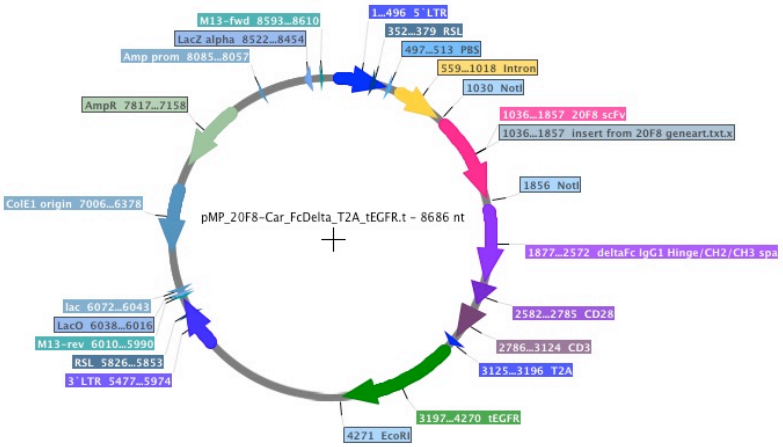

Figure 3: Vector map of the retroviral pMP-71 vector containing the 20F8 CAR construct.

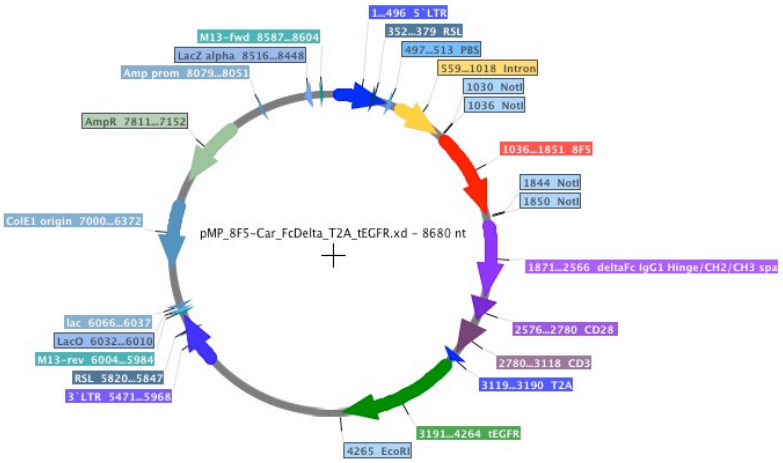

Figure 4: Vector map of the retroviral pMP-71 vector containing the 8F5 CAR construct.

3.12 Mouse model

In the experiments a mouse model on a BALB/c background, which has knockouts in the Rag2 as well as in the gamma(c) locus, was used. Deletion of the Rag2 locus leads to a complete loss of peripheral B-lymphocytes as well as thymus-derived T-lymphocytes. The common cytokine receptor gamma chain (γc) is a functional subunit of a variety of cytokine receptors including the IL-2, IL-7 and IL-15 receptor. Loss of this gene leads to an impaired development of natural killer cells (NK cells) and hampers survival of NK cells and T-lymphocytes. As a result the $Rag2^{-/-}\gamma c^{-/-}$ mouse model is completely abolished from NK cells as well as B- and T-lymphocytes and can be claimed as immunodeficient (Goldman et al. 1998). The animals were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and kept in the Zentrum für Präklinische Forschung (Klinikum rechts der Isar, Munich) under pathogen free conditions.

4 METHODS

4.1 Cell culture methods

4.1.1 Freezing and thawing of cells

For freezing, human T cells as well as transgenic T cells were cryopreserved in aliquots containing $3x10^5$ cells/ml in precooled human AB serum with 10 % DMSO, all other cells in concentrations between $1x10^6$ and $1x10^7$ cells/ml in precooled FCS with 10 % DMSO. After re-suspension of pelleted cells in the desired volume of the appropriate freezing medium, 1 ml aliquots of the cell suspension were transferred into pre-cooled cryovials, which were placed into controlled freezing boxes for 12 – 18 hours at -80°C. Afterwards they were transferred into a liquid nitrogen reservoir (-192°C) for long-term storage. For recovery of cryopreserved cells, cryovials were thawed at 37°C, then washed with appropriate medium to remove cytotoxic DMSO and transferred into T75 cell culture flasks.

4.1.2 Cultivation of cell lines

All Ewing Sarcoma and neuroblastoma cell lines were cultured in RPMI 1640 medium (Invitrogen) with 10% FCS (Biochrom), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). 293VecRD114 (RD114) packaging cell lines were cultured in DMEM (Invitrogen) containing 10% FCS, 1mM Na-pyruvate, 1mM non-essential amino acids and antibiotics. T cells were cultured in AIM-V medium (Invitrogen) with 5% human AB serum and antibiotics. All cells were cultivated in T25, T75 or T175 culture flasks (Greiner Bio One) at 37°C and 5% CO_2. Suspension cell lines were split every 3 - 4 days 1:2 to 1:10 according to their individual growth rate. Adherent cells received fresh medium every 3 - 4 days and were split 1:5 to 1:10 weekly. Therefore medium was aspirated completely and cells were washed with PBS. Then they were incubated with Trypsin/EDTA (Invitrogen) at 37°C. After detachment, cells were washed and an appropriate amount of cells was re-cultured in fresh cell culture flasks. All cell lines were tested regularly with the MycoAlert™ Mycoplasma Detection Kit according to the manufacturer´s instructions (Lonza) to detect mycoplasma contamination. In case of contamination with mycoplasms, cells were replaced by freshly thawed cell aliquots.

4.1.3 Cell counting

Cell numbers were determined with a Neubauer hemocytometer using Trypan-Blue (Sigma) exclusion method of dead cells.

4.2 Isolation of blood cells

4.2.1 Isolation of peripheral blood mononuclear cells

For the isolation of peripheral blood mononuclear cells (PBMCs), human peripheral blood samples provided by the DRK-Blutspendedienst Baden-Württemberg-Hessen, Ulm, Germany, were separated via density gradient centrifugation using Ficoll-Paque (GE Healthcare) according to the supplier's instructions. In detail, the blood-cell concentrate was diluted 1:2 with PBS and layered on top of Ficoll-Paque in a ratio of 2:3. After centrifugation for 30 minutes at 400 x g without active deceleration, the PBMC containing interlayer was collected and washed twice with PBS. In case of contamination with erythrocytes, the cell pellet was re-suspended in Erythrocyte Lysis Buffer and incubated for 5 minutes at 37°C before the reaction was stopped with PBS. Cells were counted and frozen or cultured.

4.2.2 Isolation of T cell subpopulations

To obtain an unlabeled $CD8^+$ T cell population, the $CD8^+$ T cell isolation kit (Miltenyi) was used according to the manufacturer´s instructions.

4.3 Mini- and maxipreparation of plasmid DNA

For Minipreparation of plasmid DNA the NucleoSpin® Plasmid Kit (Machery & Nagel) was used according to the manufacturers guidelines. To obtain large amounts of plasmid preparations were performed according to the supplier´s information using the JETSTAR 2.0 Plasmid Maxiprep Kit. Via spectrophotometry DNA content was measured. Plasmids were analyzed via restriction enzyme analysis and sequencing. For restriction analysis of plasmids, DNA was incubated for 1-2 hours at 37°C

together with EcoRI and NotI restriction enzymes and fragment size analyzed after agarose gelelectrophoresis.

4.4 Agarose gelelectrophoresis and gel extraction

Agarose gelelectrophoresis was performed to separate DNA fragments. Agarose was dissolved in 150ml TAE buffer, 4 µl EtBr were added and DNA was supplemented with 6x Blue Juice Gel Loading Buffer (Invitrogen). Gel pockets were filled with up to 20 µl DNA containing suspension. As a size standard the 1kb plus ladder (Invitrogen) was used. Electrophoresis was run at 5-10 V/cm gel for 45 minutes. For isolation of DNA from agarose gel, bands were cut out and purified using the StrataPrep® DNA Gel Extraction Kit according to the manufacturer´s instructions.

4.5 RNA isolation

RNA isolation was performed using TriReagent following the supplier´s instructions. For all nucleic acid measurements a Nanophotometer Pearl was used according to manufacturer´s information.

4.6 cDNA synthesis

To transcribe RNA into complementary DNA (cDNA), the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used following the manufacturer's instructions.

4.7 Quantitative Real Time PCR (qRT-PCR)

To perform qRt-PCRs MaximaTM Probe/ROX qPCR Master Mix (2x) was used together with specific TaqMan® Gene Expression Assays (Applied Biosystems), which consisted of two unlabeled PCR primers and a FAMTM dye-labeled TaqMan® MGB probe. Reaction mixes were prepared following the manufacturer´s instructions

(Fermentas PureExtremeTM Insert). Gene expression profiles were normalized to mRNA levels of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. They were calculated using the 2-ddCt method. The mean value and standard deviations of duplicates was displayed graphically using Microsoft Excel. Determination of the statistical significance was carried out by using the conventional t-test. Fluorescence measurement was performed using an AB 7300 Real-Time PCR system following a three-step cycling protocol: 1s 50°C; 10min 95°C; (15s 95°C; 1min 60°C) 40x (Applied Biosystems).

4.8 Design of retroviral CAR constructs

Two second generation CARs each containing the scFv fragments of one monoclonal antibody (mAb) directed against the extracellular region of GPR64 were synthesized and subcloned into pMP-71 retroviral backbones by GeneArt. The 20F8 and the 8F5 CAR were constructed by linking sequences from a signal peptide derived from the human immunoglobuline heavy chain to a GPR64-specific single chain variable fragment (scFv) derived from the 20F8 or the 8F5 antibody, followed by a spacer domain containing the CH_2CH_3 domains from human IgG_1. The two GPR64-specific CARs also include an intracellular CD28 costimulatory domain and a human CD3ζ signaling domain. Via a T2A domain they are connected to a truncated version of the EGF-receptor (EGFRt).

4.9 Transduction of PBMCs with CAR constructs

PBMCs were transduced using a retroviral infection system. On day $1.3x10^5$ RD114, which were used as packaging cell line, were seeded in 3 ml RD114 medium in a 6 well plate. On day 2, 200 µl supplement-free DMEM and 9 µl TansIT (Mirus) transfection reagent for each approach were incubated for 20 minutes at room temperature. Then 1 µg CAR encoding retroviral plasmid was added and incubated for another 30 minutes to enable complex formation between plasmid and transfection reagent. PBMCs were isolated the same day and activated either via 50 ng/ml OKT3 and 100 U/ml rhIL-2 or via anti-CD3/CD28 microbeads (bead: cell ratio 3:1) together with rhIL-7 and rhIL-15 (5 ng/ml each). On day 3, non-tissue culture 24

well plates were coated with 5 µg Retronectin/well to increase transduction efficacy by bringing virus and cells in close proximity. On day 4, spin transduction was performed. $1x10^6$ T cells were incubated with 1 ml virus supernatant, cytokines, 1% HEPES and 4 µg/ml protaminsulfate. Plates were centrifuged for 90 minutes at 32°C and then incubated at 37°C for 24 hours. On day 5, transduced cells were split 1:2 and the procedure of day 4 was repeated. Transduction efficacy was checked on day 10 via analysis in flow cytometry.

4.10 Enrichment of transduced T cell populations

4.10.1 Sorting via flow cytometry

Via the Flow Cytometry FACS Unit of the Institute of Medical Microbiology, Immunology and Hygiene (Technische Universität München) the CAR positive T cell population was sorted.

4.10.2 Sorting via magnetic beads

CAR expression after transduction of GPR64-specifc CAR constructs into PBMCs could be increased after CAR-based sorting using an anti-EGFR antibody (BD Biosciences) and anti-PE MicroBeads (Miltenyi).

4.11 Flow cytometry

Cells were washed with staining buffer (0.5% BSA in PBS) two times and re-suspended at a concentration of $4x10^6$ cells/ml staining buffer. Specific fluorochrome-labeled monoclonal antibodies (mAb) were added and the suspension was incubated for 30 minutes at 4°C. Two additional washing steps were performed before cells were re-suspended in 300 µl PBS and analyzed on a FACScalibur flow cytometer (Becton Dickinson) with at least 25000 events/sample recorded. Analysis was performed using the Cellquest software (Becton Dickinson).

4.12 Immobilization of biotinylated antigen to streptavidin-coated beads

To monitor the presence of functional GPR64-specific T cells transduced with a CAR the method published by Elkord et al. was used (Elkord et al. 2011). Biotinylated GPR64 peptide was prepared by Peps 4 Life Sciences and coated onto Streptavidin-coated polysterene particles (SCPP, Kisker Biotech) following the published instructions.

4.13 ELISpot assay

In IFNγ ELISpot assays, cells were used at an effector:target ratio of 1:20. All experiments were performed in triplicates. T cells without target served as a negative control.

On day 1, 96 well mixed cellulose ester plates (MultiScreen-HA Filter Plate) were coated overnight with 10 µg/ml IFNγ trapping mAB (1-D1K) per well. On day 2, plates were washed 4 times with 200 µl PBS at 4°C. Afterwards PBS was replaced by T cell medium (150 µl/well) for at least 1 hour at 37°C and 5% CO_2 to avoid unspecific binding. During blocking time, T cells were harvested and washed three times with TCM. They were put to rest for 30 minutes at 4°C. T cells were incubated without targets for 30 minutes at 37°C before adding tumor cells. To avoid dispersion of T cells, target cells were carefully dripped on top. Then plates were incubated for 20 hours at 37°C and 5% CO_2. On day 3, plates were washed 6 times with PBS/0.05% Tween and then incubated with 2 µg/ml 7-B6-1 (IFNγ) biotinylated mAb for 2 hours at 37°C and 5% CO_2. After incubation, plates were washed again six times with PBS/0.05% Tween and incubated with 200 µl Streptavidin-HRP (1:100 dilution) for 1 hour at RT, shielded from light. Then plates were washed three times with PBS/0.05% Tween and three times with normal PBS. 100 µl of development solution was allocated per well. Reaction was stopped after 5 to 10 minutes by washing the plates with cold water. After drying, Analysis was performed using the ELISpot reader.

4.14 xCelligence assay

The impedance based xCELLigence system allows the determination of specific inhibition of target cell growth by candidate T cell clones. The system detects cellular changes in real-time by measuring electrical impedance using golden microelectrodes. To allow target cell adhesion to the surface of the electrodes, cells were seeded 48 hours before adding T cells. For A673 cells 1 x 10^4 cells per well were used, for SK-N-MC, SaOs and SIMA 2.5 x 10^4 cells per well. Target specific T cells were added in titrated amounts ranging from an effector:target ratio of 20:1 – 0.3:1 on top of tumor cells and analysis performed over a total time period of 5 days with measurements every 15 minutes. Untreated target cells and medium alone served as controls. Experiments were executed in quintuplicates.

4.15 ELISA

To measure IFN-γ levels in cell culture supernates the Quantikine Human IFN-γ Immunoassay (RnDSystems) was utilized according to the manufacturer´s instructions. For detection of IL-2 levels in cell culture supernates the Quantikine Human IL-2 Immunoassay (R&DSystems) was used according to the producer´s briefings. To determine human TNF-α concentration in cell culture supernates the Quantikine Human TNF-α Immunoassay (R&DSystems) was used following the manufacturer´s instructions. All experiments were executed in duplicates. Reaction product was quantified by measuring the absorbance (450 nm) using a Multiscan Ascent ELISA reader (Thermo Scientific). Analysis was performed using elisaanalysis.com. The mean value and standard error of the mean are displayed graphically using Microsoft Excel. Determination of statistical significance was carried out by using the conventional t-test.

4.16 *In vivo* experiments

To analyze local tumor growth *in vivo*, Rag2$^{-/-}$γc$^{-/-}$ immunodeficient mice were inoculated with 2x10^6 A673 cells subcutaneously into the groin. After 3 days mice received a full body irradiation with 3.5 Gray to facilitate engraftment of human T

cells. One day later we injected intra peritoneally (i.p.) $3x10^5$ GPR64 specific 20F8 CAR transgenic T cells together with $5x10^5$ PBMCs or $3x10^5$ GPR64 specific 8F5 CAR transgenic T cells together with $5x10^5$ PBMCs or no cells at all. Additionally, cells were provided with hIL-15 by injecting $1.5x10^7$ hIL-15 producing NSO cells (NSO-IL15) i.p. twice a week. Half of the mice with 8F5 CAR T cell treatment did not receive NSO cells. The experiments were stopped when tumors of any treatment group reached 1 cm^3 or when mice suffered from any other disease. After 21 days tumors were weighted and analyzed via flow cytometry analysis.

4.17 Statistical analysis

Data are mean ± SEM as indicated. Differences were analyzed by unpaired two-tailed student's t-test as indicated using Excel (Microsoft), or Prism 5 (GraphPad Software); p values < 0.05 were considered statistically significant.

5 RESULTS

5.1 Identification of GPR64 as suitable target for adoptive T cell therapy in Ewing Sarcomas

To determine EwS-specific antigens, microarray analyses were performed as published by our group (Richter, Fasan et al. 2013), identifying GPR64 as a strongly up-regulated gene in EwS. As shown in **Figure 5**A GPR64 is highly overexpressed in EwS tissue, whereas it is only minimally expressed in normal tissue. The expression of GPR64 within EwS cell lines was further confirmed by qRT-PCR. Additionally, its expression in various osteosarcoma and neuroblastoma cell lines was assessed. Whereas GPR64 is strongly expressed in EwS cell lines, especially in A673 and TC-71, it is almost not detectable in pediatric bone tumor and neuroblastoma cell lines (**Figure 5**B).

Immunohistochemistry indicated a strong expression of GPR64 in Ewing Sarcoma tissue samples stained with GPR64 specific hybridoma 20F8 (**Figure 5**C). 212 tissue samples were analyzed by Prof. Dr. Ivo Leuschner (Institute of Pathology of the faculty of medicine, University of Kiel). Tumor samples were evaluated using an immunoreactive score (IRS) based on staining intensity and number of positive cells, which is shown in the Appendix. 74% percent of the samples showed expression of GPR64. Staining with GPR64 specific hybridoma 8F5 was negative (not shown).

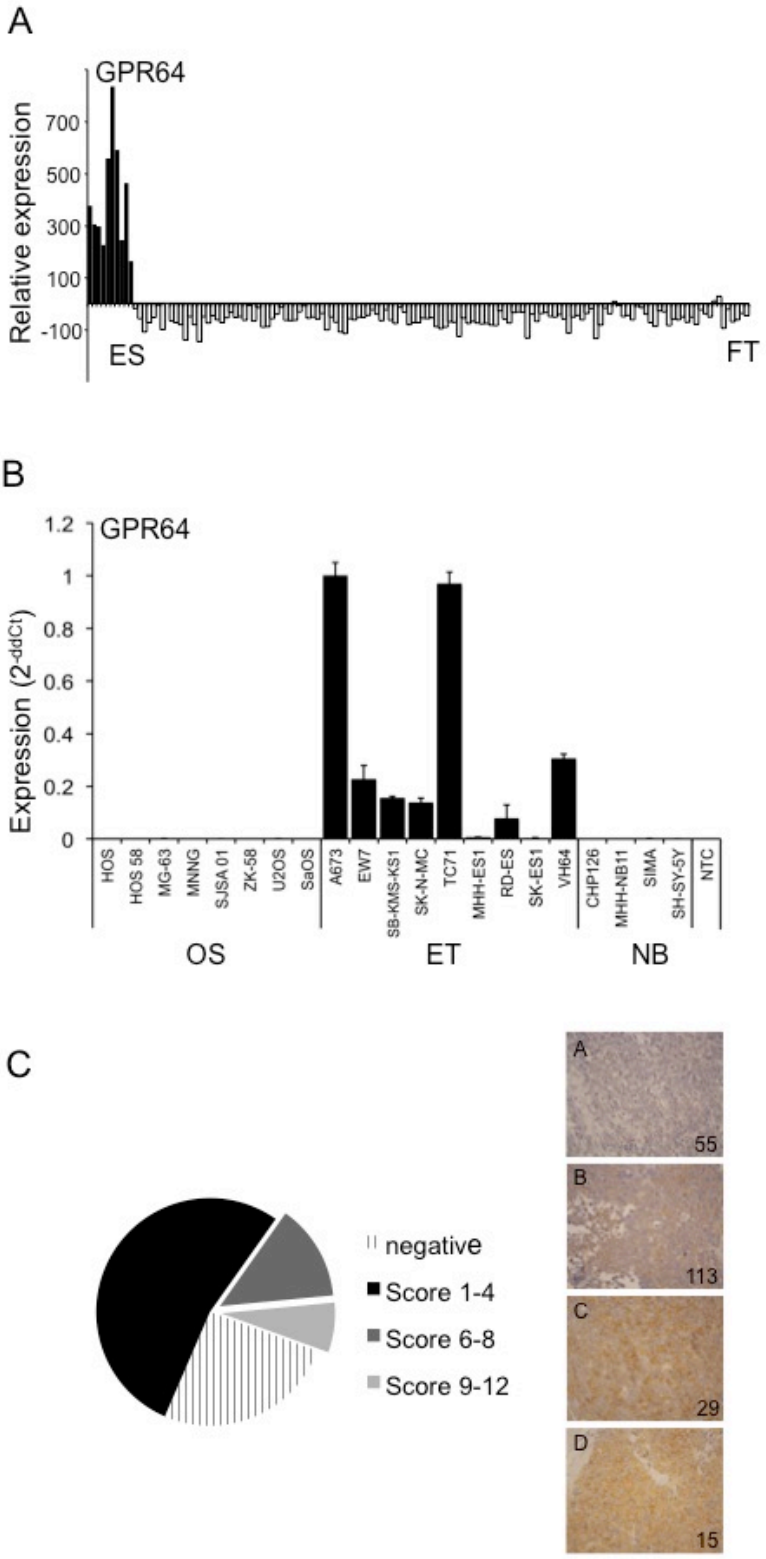

Figure 5: Expression profile of GPR64. In EwS GPR64 is highly up regulated. (A) Microarrays of 11 Ewing sarcomas (EwS) were compared with 133 samples of normal (black) and fetal tissue (FT) for GPR64 expression. (B) Quantification of GPR64 expression was analyzed by qRT-PCR of RNA in Ewing Sarcoma (EwS), Osteosarcoma (OS) and Neuroblastoma (NB) cell lines. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). NTC, non-template control. Figures taken from (Richter et al. 2013). (C) Tissue samples of

Ewing Sarcomas were stained with GPR64 specific hybridoma 20F8. (i) negative (ii) Score 1-4: weakly positive (iii) Score 6-8: moderately positive (iiii) Score 9-12: strongly positive. Stained by Prof. Dr. Ivo Leuschner (†), Institute of Pathology of the faculty of medicine, University of Kiel.

5.2 Characterization of monoclonal antibodies directed against GPR64

Two different monoclonal antibodies (mAb) directed against the extracellular region of GPR64 were generated and characterized. Antibodies specifically stained EwS cells as determined by flow cytometry (**Figure 6**). The signal intensity was reduced after RNAi mediated knock down of GPR64 in EwS cell lines A673 and SK-N-MC confirming specificity of the mAbs. Following sequence determination of these GPR64-specific Hybridomas two different CAR constructs, 20F8 CAR and 8F5 CAR, were designed by the help from a collaboration together with Elisabeth Kremmer (Helmholtz-Zentrum Munich).

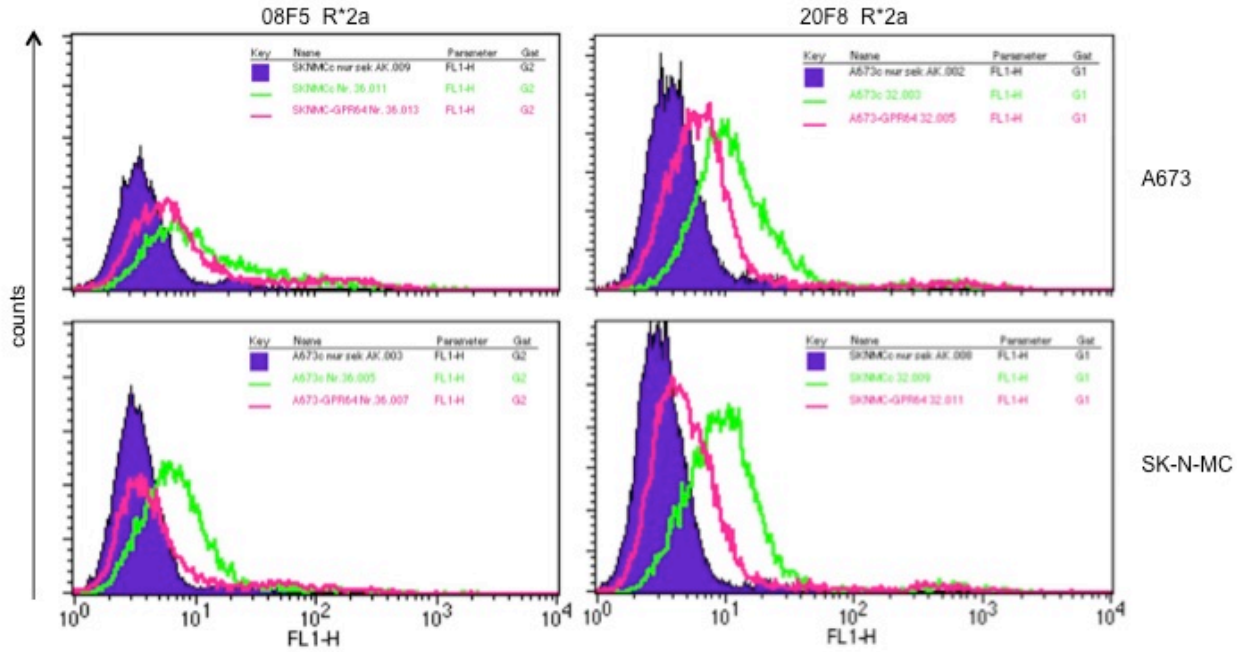

Figure 6: Characterization of GPR64 Hybridomas. Staining of EwS cell lines A673 and SK-N-MC with GPR64 specific hybridomas 8F5 and 20F8. Antibodies specifically stained EwS cells as determined by flow cytometry (green line). The signal intensity was reduced after RNAi mediated knock down of GPR64 in EwS cell lines (pink line) confirming specificity of mAbs. Staining with secondary Abs alone served as control.

5.3 Generation of GPR64 tumor antigen-specific CAR T cells

5.3.1 Design and features of the GPR64 specific 20F8 and 8F5 CAR T cells

The GPR64 specific 20F8 and 8F5 second generation CARs contain a human ΔIgG_1Fc and a CD28 trans membrane costimulatory domain complemented by a human CD3ζ signaling domain. These constructs have an additional (safety)-feature: they are connected to a truncated version of the EGF-receptor (EGFRt) via a T2A domain that enables purification of CAR-transgenic T cells or their *in vivo* depletion via cetuximab treatment.

Retroviral constructs containing second generation CARs together with the scFv fragments of the respective mAbs were designed by the help of Karin Wisskirchen and Felix Bohne of the Institute of Virology (Technical University of Munich).

5.3.2 Successful Transduction and enrichment of GPR64 specific CAR T cells

Retroviruses containing GPR64 specific 20F8 and 8F5 CAR constructs transduced primary lymphocytes with good efficiency. After infection of PBMCs infection rate of 20F8 CAR and 8F5 CAR was monitored using flow cytometry with EGFRt and IgG1 (**Figure 7**). Both antibodies for flow cytometry showed similar results.

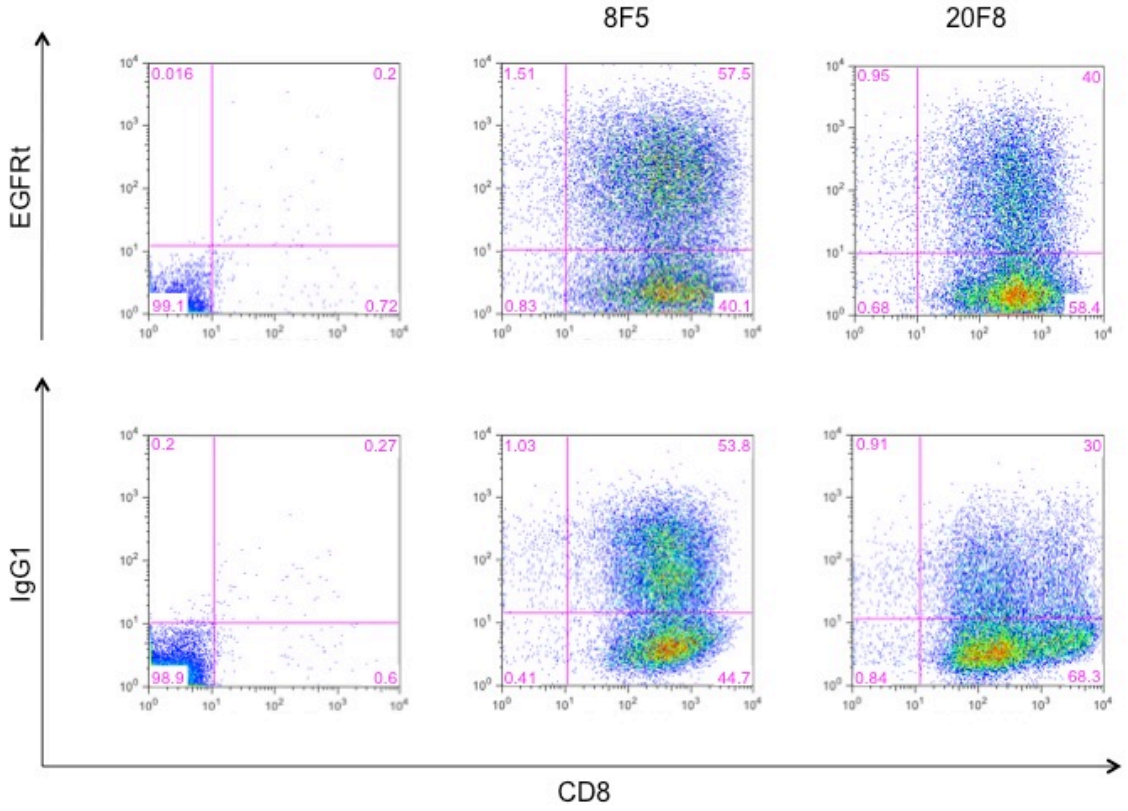

Figure 7: FACS staining after infection of peripheral blood mononuclear cells (PBMCs) with GPR64-CAR-positive retroviruses. After infection of PBMCs infection rate of both CARs, either 20F8 (right panel) or 8F5 (middle panel) is monitored via flow cytometry with EGFRt and IgG1 antibodies.

For further investigation the CAR transgenic T cells were enriched for $CD8^+$ CAR^+ cells via the Flow Cytometry FACS Unit of the Institute of Medical Microbiology, Immunology and Hygiene (Technical University of Munich) or via microbead isolation. Both ways of enrichment yielded rates of $CD8^+$ CAR^+ cells of over 95%. **Figure 8** shows an example of enrichment via magnetic beads.

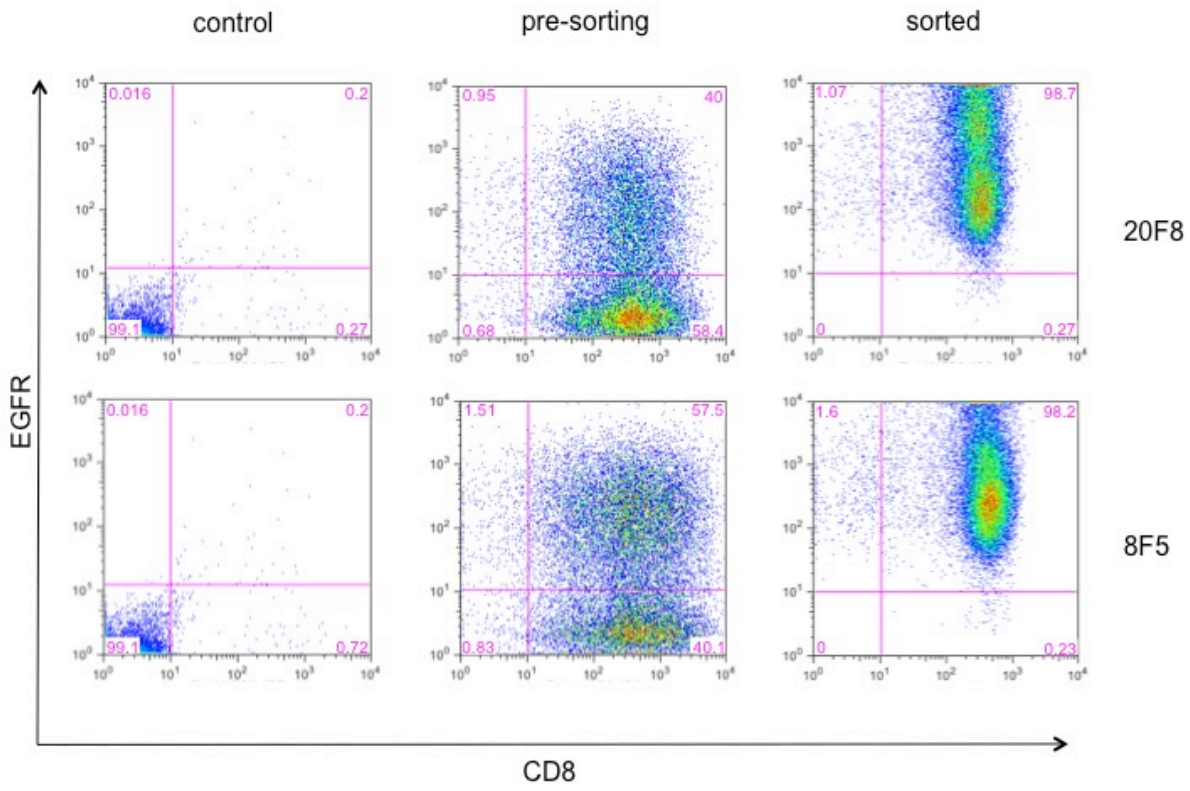

Figure 8: Enrichment of GPR64 specific CAR T cells. Infection rate of both CARs, either 20F8 (upper panel) or 8F5 (lower panel), is monitored via flow cytometry with EGFRt after infection of PBMCs and subsequent enrichment via magnetic beads.

5.3.3 Growth of GPR64 specific CAR T cells

The growth of transduced cells was evaluated over 15 days by counting cells (**Figure 9**). Until day 8 GPR64 specific 20F8 CAR T cells grow as fast as GPR64 specific 8F5 CAR T cells. After day 8 GPR64 specific 20F8 CAR T cells continue to grow, whereas the number of GPR64 specific 8F5 CAR T cells decreases.

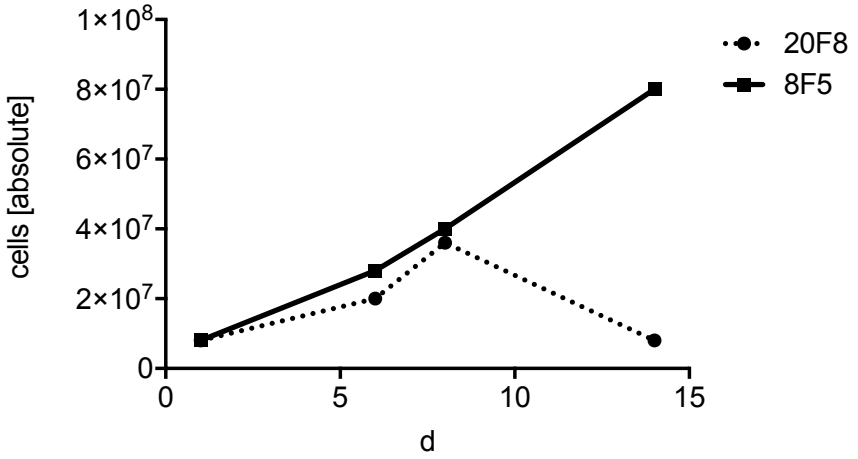

Figure 9: Growth curve of GPR64 specific CAR T cells. Growth of transduced cells was evaluated over 15 days by counting cells.

5.4 Functionality of GPR64 transgenic T cells

To test *in vitro* functionality of GPR64 specific CAR T cells human PBMCs of healthy donors were transduced with the 20F8 CAR and the 8F5 CAR construct and tested for specificity and lytic activity in ELISpot and xCelligence assays.

5.4.1 Peptide recognition of GPR64 specific CAR T cells

GPR64 specific CAR T cells were tested in ELISpot assays one to two weeks after transduction. After subsequent enrichment via magnetic beads the infection rate was monitored via flow cytometry with EGFRt (**Figure 10**A). Biotinylated GPR64-specific peptides were coated onto streptavidin-coated polystyrene particles (GPR64/SCPP) and used for determination of specific recognition by GPR64-CARs in ELISpot assay. Uncoated SCPP (PBS/SCPP) served as controls.

As shown in **Figure 10**B, upper panel, the GPR64 specific 8F5 CAR T cells recognized the appropriate antigen specifically and released IFNγ. Without presentation on SCPP they showed a very low release of IFNγ. 20F8 CAR T cells released high amounts of IFNy even without target presentation on SCPP (**Figure 10**B, upper panel).

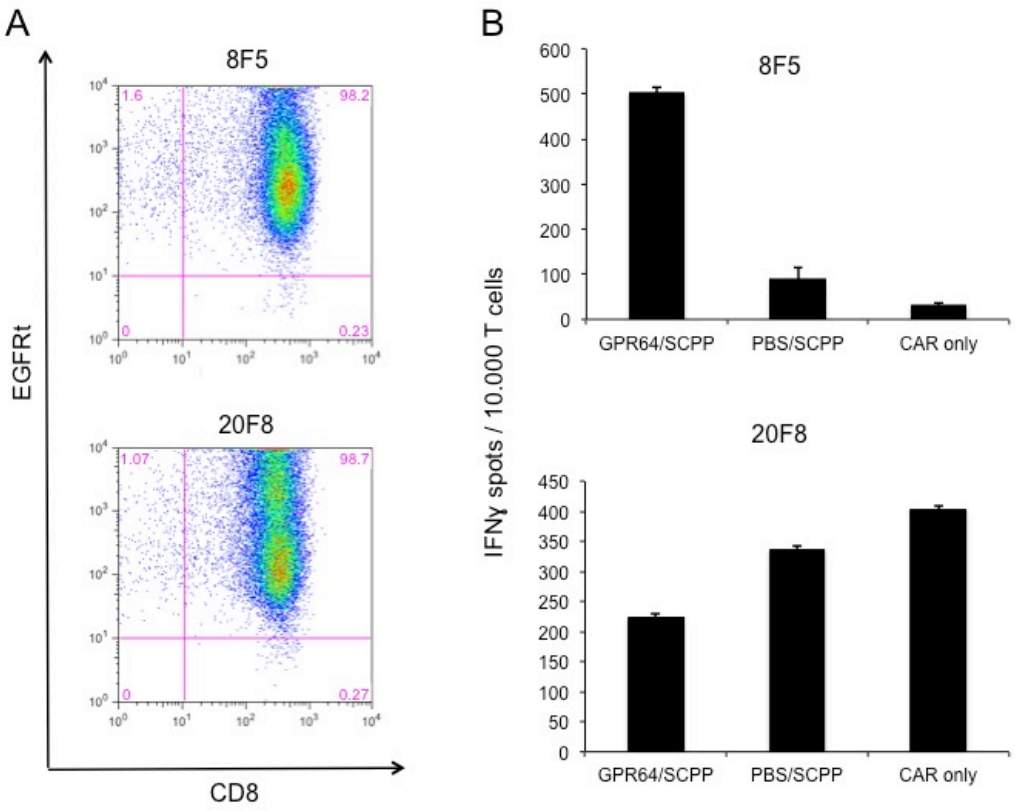

Figure 10: Peptide recognition of GPR64 specific CAR T cells. (A) Monitoring of infection rate of both CARs, either 8F5 (upper panel) or 20F8 (lower panel), after infection of PBMCs and enrichment via magnetic beads via flow cytometry with EGFRt. (B) IFNγ release of GPR64 specific CAR T cells during co-culture with biotinylated GPR64-specific peptides coated onto streptavidin-coated polystyrene particles (GPR64/SCPP) for determination of specificity. Uncoated SCPP (PBS/SCPP) served as controls.

5.4.2 Target recognition of GPR64 specific CAR T cells

One to two weeks after infection the GPR64 specific CAR T cells were tested in ELISpot assays. After enrichment using magnetic beads the infection rate was monitored via flow cytometry (**Figure 11**A). For further determination of specificity different GPR64-postive Ewing sarcoma cell lines (A673, TC-71, SK-N-MC) were compared to GPR64-negative cell lines (SIMA, SH-SY-5Y) in ELISpot assay. Also the IFNγ release of GPR64 specific CAR T cells without adding of any tumor cell line was measured.

As shown in **Figure 11**B, upper panel, GPR64 specific 8F5 CAR T cells were able to recognize GPR64-postive Ewing sarcoma cell lines, whereas GPR64-negtive cell

lines were not detected. GPR64 specific 8F5 CAR T cells without exposure to GPR64-positive or GPR64-negative cell lines present very low levels of IFNγ. However GPR64 specific 20F8 CAR T cells secret IFNγ in comparable levels when exposed to GPR64-postive or GPR64-negative cell lines as well as when they were cultivated alone (**Figure 11**B, lower panel).

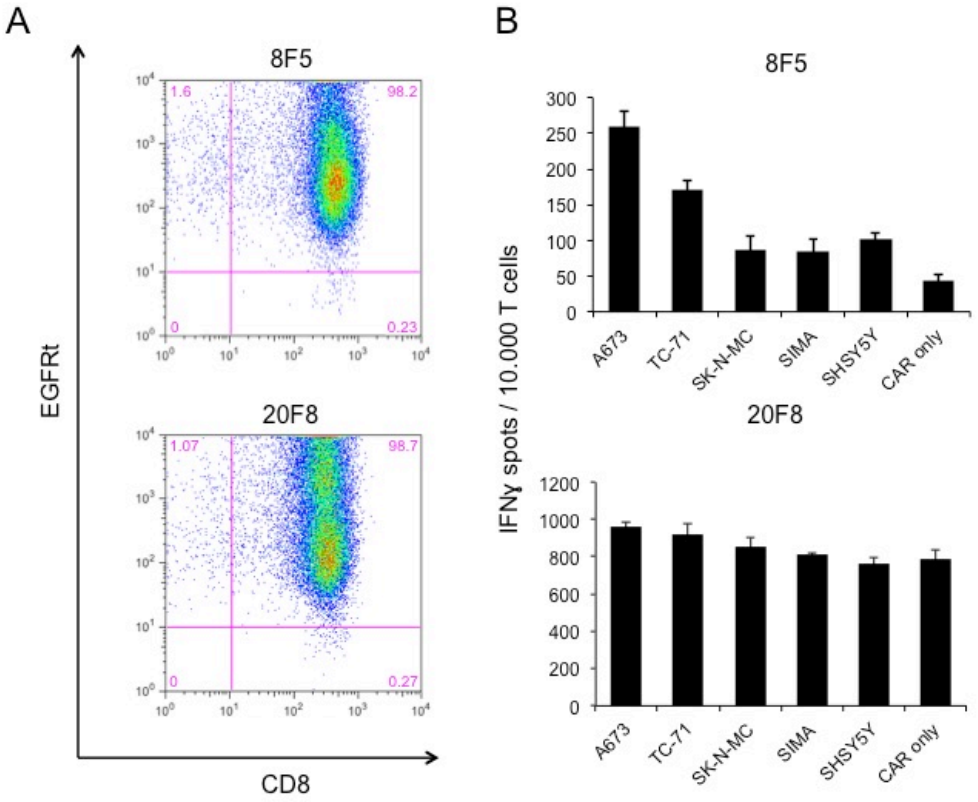

Figure 11: Target recognition of GPR64-specific CARs. (A) Monitoring of infection rate of both CARs, either 8F5 (upper panel) or 20F8 (lower panel), after infection of PBMCs and enrichment via magnetic beads via flow cytometry. (B) IFNγ release of GPR64 specific CAR T cells during co-culture with different GPR64-postive Ewing sarcoma cell lines (A673, SK-N-MC, TC-71) and GPR64-negative neuroblastoma cell lines (SIMA, SH-SY-5Y) for further determination of specificity.

5.4.3 Target-specific killing of GPR64 specific CAR T cells

Target-specific tumor cell lysis by GPR64 specific CAR T cells was detected via xCelligence assay one to two weeks after T cell transduction. GPR64 specific 8F5 as well as 20F8 CAR T cells were added to vital GPR64-postive cell line A673 and vital GPR64-negative cell lines SH-SY-5Y. The xCelligence assays showed the lytic ability of both CAR T cells as A673 cells were rapidly killed after administration of T cells, whereas the growth of SH-SY-5Y cells was not affected (**Figure 12**A and B).

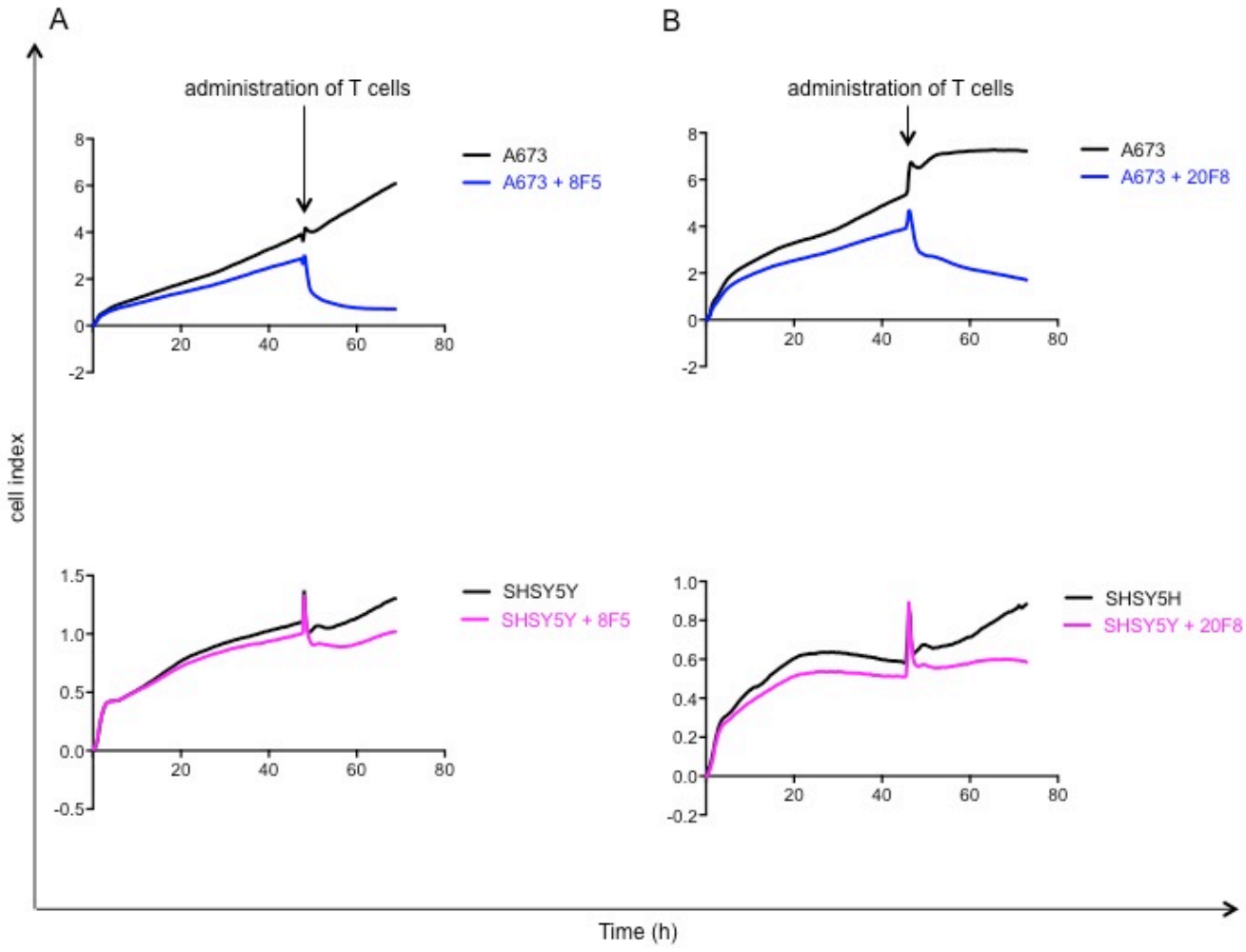

Figure 12: Target-specific killing of tumor cells by GPR64-specific CAR T cells. Contact dependent growth of Ewing sarcoma cells (A673) and neuroblastoma cells (SH-SY-5Y) was monitored in xCelligence assay. Specific recognition by GPR64-CARs 8F5 (A) or 20F8 (B).

5.5 Expression of exhaustion-associated transcription factors GPR64 specific CAR T cells

Expression of exhaustion-associated transcription factors PMDR1 (Blimp-1) and TBX21 (T-bet) of GPR64 specific 20F8 and 8F5 CAR T cells was measured via qRT-PCR at day 10 after infection (**Figure 13**). Gene expression of 20F8 and 8F5 CAR T cells was compared relative to the expression of PBMCs.

By day 10, GPR64 specific 20F8 CAR T cells showed a transcriptional profile consistent with exhaustion, including higher expression of exhaustion-associated transcription factors Blimp-1 and T-bet.

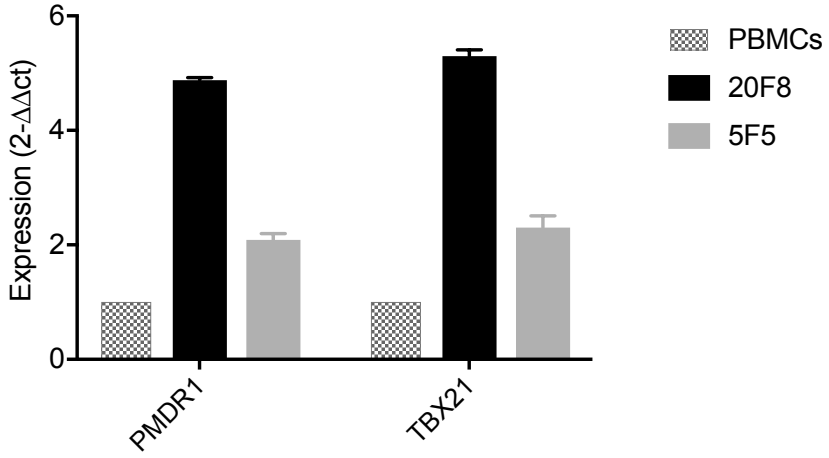

Figure 13: qRT-PCR expression levels of exhaustion-associated transcription factors PRDM1 and TBX21 of GPR64 specific 20F8 CAR and 8F5 CAR T cells. The measured expression levels were referred to the expression of PRDM1 and TBX21 of PBMCs. The measurements were taken at day 10 after infection. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

5.6 Cytokine secretion of GPR64 specific CAR T cells

As shown in **Figure 14** cytokine secretion of GPR64 specific 20F8 and 8F5 CAR T cells was examined via ELISA assays measuring IFNγ (A), IL-2 (B) and TNF-α (C) levels. Immunoassays were performed at days 7, 10, 13 and 17 after infection. IFNγ levels of 20F8 CAR T cells were higher than IFNγ levels of 8F5 CAR T cells at all measured points of time. Furthermore production of IFNγ of all CAR T cells was higher than the one of CAR negative cells. GPR64 specific 20F8 and 8F5 CAR T cell produce higher levels of IL-2 than CAR negative PBMCs. The TNF-α concentration of GPR64 specific 20F8 CAR T cells is higher compared to GPR64 specific 8F5 CAR T cells as well as compared to CAR negative cells. The production of TNF-α of GPR64 specific 8F5 CAR T cells is slightly lower than the one of CAR negative PBMCs at days 7, 10 and 13 after infection. At day 17 after infection the TNF-α concentration of GPR64 specific 8F5 CAR T cells is as high as the one of CAR negative cells.

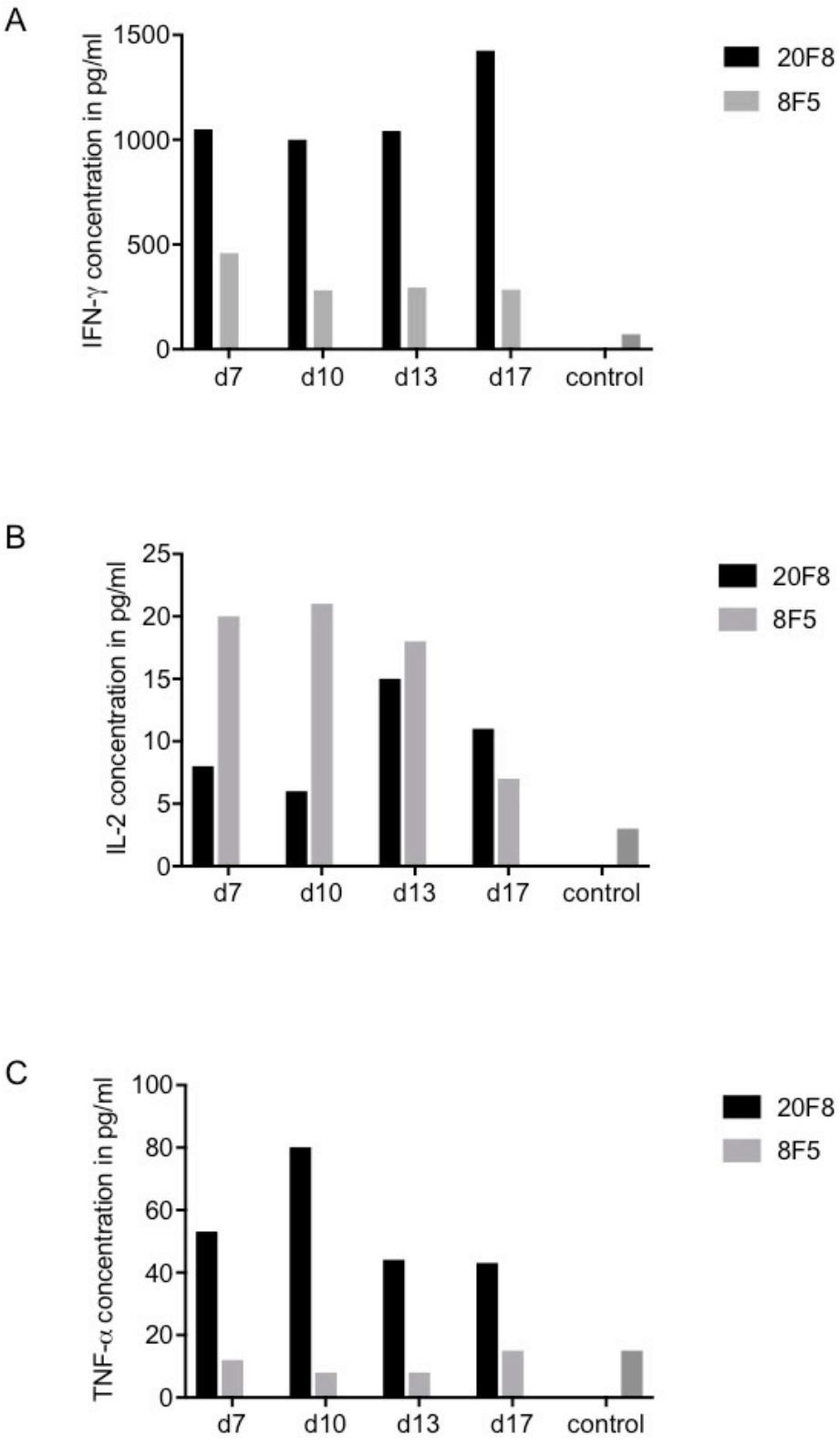

Figure 14: Cytokine secretion of GPR64 specific 20F8 and 8F5 CAR T cells at days 7, 10, 13 and 17 after infection and CAR negative PBMCs as control. Concentration of IFNγ (A), IL-2 (B) and TNF-α (C) measured via ELISA assays.

5.7 Fluorescence microscopy of 20F8 CAR T cells

Green fluorescent protein (GFP) fluoresces when stimulated with blue or ultraviolet light. To examine the spatial distribution of our 20F8 CAR T cells, especially possible clustering, we transfected PBMCs with a GFP tagged 20F8 CAR. In **Figure 15** the $20F8^+$ GFP^+ T cells are shown via fluorescence microscopy. It could be clearly observed that the CARs of 20F8 cluster without antigen contact.

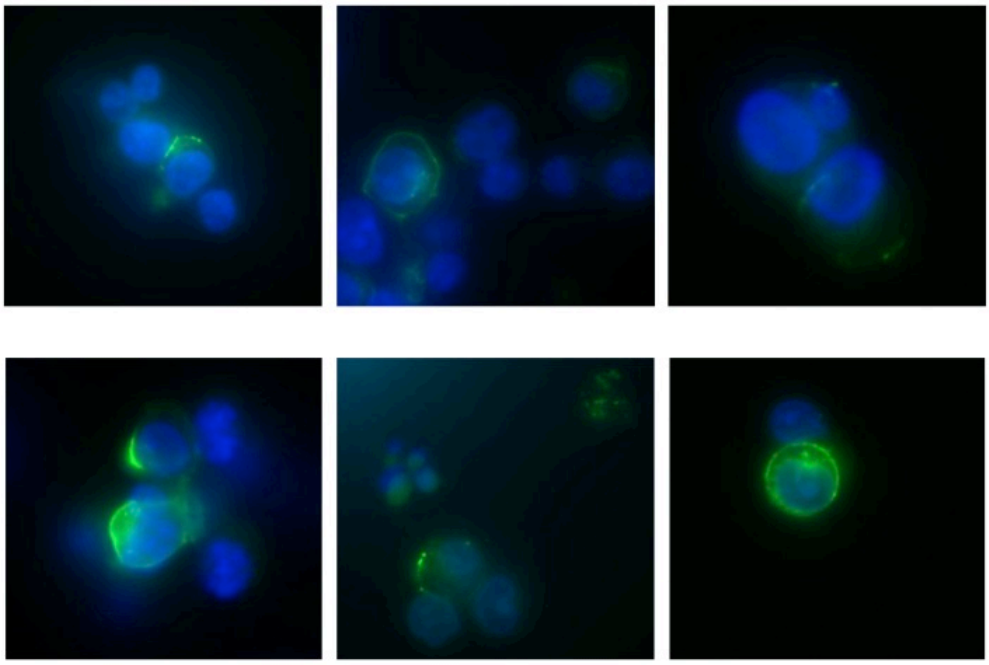

Figure 15: Fluorescence microscopy of $20F8^+$ GFP^+ CAR T cells. Clustering of PBMCs transfected with a GFP tagged 20F8 CAR.

5.8 *In vivo* reactivity of GPR64 specific CAR transgenic T cells in Rag2$^{-/-}$γc$^{-/-}$ mice

To demonstrate *in vivo* efficacy of GPR64 specific CAR T cells Rag2$^{-/-}$γc$^{-/-}$ mice were inoculated with EwS tumor cells and animals treated with CAR transgenic T cells 4 days later. To do this, 2 x 10^6 A673 cells were injected subcutaneously into the groin of immunodeficient Rag2$^{-/-}$γc$^{-/-}$ mice. After 3 days mice received full body irradiation with 3.5 Gray to facilitate engraftment of human T cells. One day later we injected intra peritoneally (i.p.) 3 x 10^5 GPR64 specific 20F8 CAR transgenic T cells together with 5 x 10^5 PBMCs or 3 x 10^5 GPR64 specific 8F5 CAR transgenic T cells together with 5 x 10^5 PBMCs or no cells at all. Additionally, cells were provided with hIL-15 by injecting 1.5 x 10^7 hIL-15 producing NSO cells (NSO-IL15) i.p. twice a week. Half of the mice with 8F5 CAR T cell treatment did not receive NSO cells. The experiments were stopped when tumors of any treatment group reached 1 cm^3.
In the course of the experiment some animals died unexpectedly. No Graft-versus-Host disease (GvHD) in any of the mice could be observed. 21 days after initial tumor cell injection the tumors of remaining mice were weighted and analyzed via flow cytometry analysis. In Figure 16 absolute tumor weights are compared. Tumors of mice treated with GPR64 specific 8F5 CAR T cells were smaller compared to tumors of animals treated with unspecific PBMCs. Data of the mice treated with 20F8 CAR T cells could not be evaluated because too many of the mice died probably because of infection. However, no T cells could be found in tumor or spleen except for PBMCs (results not shown.)

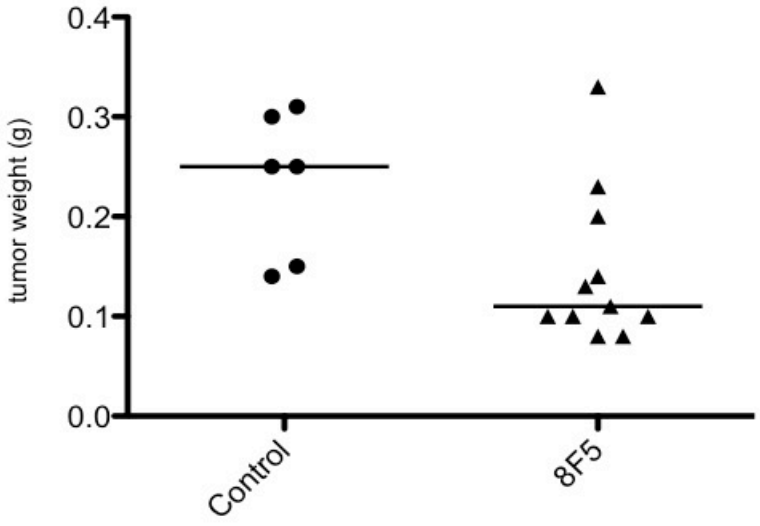

Figure 16: *In vivo* activity of GPR64 specific CAR T cells. Absolute tumor weight was analyzed 21 days after initial tumor cell injection.

6 DISCUSSION

So far therapy options for Ewing sarcoma (EwS) patients with recurrent disease are limited. Currently allogeneic stem cell transplantation (allo-SCT) is used as an established therapy for hematologic malignancies. Donor T cells induce a graft versus tumor effect leading to eradication of the hematologic malignancies (Kolb et al. 1995, Burdach, van Kaick et al. 2000). However, in EwS comparisons between the survival rates of patients that received different conditioning before allo-SCT implicate an absence of a graft versus Ewing sarcoma (GvES) effect (Thiel et al. 2011). Another recent study shows that AES patients with no bone marrow involvement may be cured whereas patients with bone marrow involvement had a fatal outcome despite high dose therapy and allo-SCT (Thiel, Wawer et al. 2016). In general stem cell transplantation may lead to a GvHD disease resulting in a life-threatening situation for the patient (Baird et al. 2010). Taking together an additional therapeutic strategy is required to direct T cell reactivity towards EwS rather than the host tissue (Thiel et al. 2011).

Immunotherapy, which allows the identification and isolation of tumor specific T cells, has had enormous success over the last couple of years making it an interesting opportunity also for EwS (Couzin-Frankel 2013, June et al. 2015, Rosenberg et al. 2015). Within immunotherapy two different types of adoptive T cell therapies have developed in the last decades: T cells expressing a T cell receptor (TCR) as well as T cells expressing a chimeric antigen receptor (CAR) (Gross et al. 1989, Gross, Waks et al. 1989). Especially CAR T cell therapy has already showed convincing clinical outcomes in the treatment of hematologic malignancies resulting in the approval of the first adoptive T cell therapy against cancer in the USA: Kymriah^{TM}, a CAR T cell therapy directed against CD19 (Morrow 2017). Following the success of CAR T cell therapy in the treatment of hematologic malignancies, many investigators are currently trying to obtain similar results in the treatment of solid tumors. Compared to hematologic malignancies solid tumors present a different set of challenges. They show various characteristics and are localized in a completely diverse compartment of the body. Challenging aspects of the transition into solid tumors include the careful selection of target antigen, the management of on-target off-tumor toxicity, and the modulation of the immunosuppressive tumor microenvironment.

To develop an efficient adoptive T cell therapy-approach the selection of a suitable target antigen for the cytotoxic T cells is essential. For CAR T cell therapy a tumor-selective target, a target that is exclusively expressed by cancer cells, would be optimal. Ideally the targeted antigen is required for the tumors survival and shows harboring mutations that are large enough to produce new epitopes that can be specifically recognized by the CAR (Gross et al. 2016). For the treatment of EwS a tumor specific antigen derived from the EWS-FL1 translocation, that typically characterizes EwS, would be an ideal target for T cell mediated therapy (Delattre et al. 1992). However, both proteins EWS and FLI1 are highly expressed in various cell types of healthy tissue making it a less suitable target as severe off-tumor toxicities are to be feared. As in many cases optimal targets are mostly unavailable so far targeted antigens are tumor-associated antigens being also expressed by healthy tissue. In the absence of an ideal tumor-selective target, however, antigens are preferred that are either topologically sequestered from T cells or co-expressed by non-essential tissues. In the first case, epithelial antigens can provide some tumor selectivity based on altered topology with polarized membrane distribution in healthy cells, while uniformly distributed on cancer cells, like carcinoembryonic antigen (CEA). Using DNA microarray 37 genes that are up-regulated in EwS were identified previously (Staege, Hutter et al. 2004). One of them is G-protein coupled receptor 64 (GPR64), an orphan member of the family of G-protein-coupled receptors (GPCRs), which have a history of being selected as a therapeutic target (Kirchhoff, Obermann et al. 2006). As current CAR-modified T cells are limited to recognize cell surface antigens the membrane-bound receptor GPR64 seems a potentially drugable protein. As shown in the results GPR64 is highly up-regulated in EwS. The two monoclonal antibodies 20F8 and 8F5 directed against the extracellular region of GPR64, that we generated and characterized, specifically stained EwS cells. Especially the 20F8 CAR shows great potential because of the strong expression of GPR64 in Ewing Sarcoma tissue samples stained with GPR64 specific hybridoma 20F8 shown via immunohistochemistry. Furthermore, GPR64 is normally expressed only in epididymis, which is considered to be a non-essential tissue. Therefore, it is a suitable target for adoptive T cell therapy in Ewing Sarcomas. As shown in the results GPR64 is specifically detected by our GPR64-specific 8F5 CAR T cells demonstrated via ELISpot assay.

In the past different CAR designs with respect to the extracellular domain, transmembrane domain and costimulatory moieties have already been tested (Si et al. 2018). For our experiments we used GPR64-specific 20F8 as well as 8F5 CAR T cells containing a CD28-CD3ζ signaling domain. As shown in the results the design of our CAR T cells is functional. We are able to transduce and purify the T cells with high efficacy as shown above. *In vitro* EwS cells are not only recognized by our GPR64-specific CAR T cells but are also killed as shown via xCelligence assay. In general many CAR modifications are merely empiric or were found functional in the particular context others still need systemic evaluation (Sadelain, Brentjens et al. 2013). New findings identified the choice of costimulatory domain among other things to be crucial for optimal T cell activation. Despite the general finding that CARs that incorporate costimulatory signals are more potent, there is research going on to find out if any particular costimulatory molecule is superior to another (Quintarelli et al. 2018). Comparing clinical trails with different costimulatory domains of CAR transgenic T cells directed against the same target there is no clear assertion that pleads for one combination of costimulatory domains. Especially *in vivo* studies show promising results with different costimulatory domains. Clinical investigations at the University of Pennsylvania with second generation CAR T cells directed against CD-19 containing a 4-1BB-CD3ζ achieved elimination of high tumor burdens and persistence of CAR T cells for at least 3 years, with retention of anti-tumor activity (Porter et al. 2015). Using anti-CD19 CAR T cells with a CD28-CD3ζ signaling domain, similar clinical results were achieved by the Memorial Sloan Kettering Cancer Institute (Brentjens et al. 2011) and the National Cancer Institute (Kochenderfer et al. 2012). In the long-term, patients treated with CD28-CD3ζ CAR T cells frequently underwent subsequent allogeneic stem cell transplantation, whereas most patients treated with 4-1BB-CD3ζ CAR T cells did not receive any further treatment (Holzinger et al. 2016). But it has to be mentioned that 4-1BB-CD3ζ CAR T cells have a prolonged persistence of up to 2 years compared to the one of about 30 days for CD28-CD3ζ CAR T cells. So in case of a treatment with CD28-CD3ζ CAR T cells doctors may tend to decide for a subsequent allogeneic stem cell transplantation because of the shorter persistence of CAR T cells. Despite the extensive use of CAR T cells in clinical studies, the attributes of CAR co-stimulatory domains that influence persistence and resistance to exhaustion of CAR T cells still remain largely undetermined. But *in vitro* studies comparing different costimulatory

domains recent results point to improved antitumor efficacy using 4-1BB signaling domains. It was recently reported that CD28 costimulation augments, whereas 4-1BB costimulation reduces, early exhaustion of CAR T cells explaining limited antitumor efficacy of some studies (Long et al. 2015). These results provide a possible explanation for the observations that CD19 CAR T cells incorporating the 4-1BB costimulatory domain are more persistent than those incorporating CD28 in clinical trials. Another recent study provided a mechanistic insight into the differential persistence of CAR-T cells expressing 4-1BB or CD28 signaling domains. CAR T cells with 4-1BB domains promoted the outgrowth of $CD8^+$ central memory T cells with significantly enhanced respiratory capacity, enhanced fatty acid oxidation and increased mitochondrial biogenesis. In contrast, inclusion of CD28 in the CAR architecture yielded effector memory cells with enhanced glycolytic metabolism (Kawalekar et al. 2016). These results suggest that one mechanism for the differential persistence of CAR T cells may be the metabolic reprogramming depending on the costimulatory domain. A clinical application of these findings is that with the choice of costimulatory domain the persistence of CAR T cells can be influenced. In case of targeting antigens where long-term CAR effects may not be tolerable due to extensive off-tumor toxicity short-lived CAR T cells should be preferred. In our case, targeting an antigen where concerns about off-tumor toxicity can be neglected the persistence of the GPR64 specific CAR T cells can be prolonged with the choice of costimulatory domain. Replacing the CD28 co-stimulatory domain with a 4-1BB domain in our GPR64 specific CAR T cells could ameliorate especially the results of our 20F8 CAR T cells and prolong their persistence as our 20F8 CAR T cells show shorter persistence then our 8F5 CAR T cells.

Another aspect to consider is that the T cell profile of a patient is individual suggesting that personalized immunotherapy and individualized T cell subset profiling ameliorates cancer treatment. Thus, as a potential approach to increase the persistence and efficacy of CAR T cells the modulation of interleukin cocktails affecting the memory functions of T cells can be used. For example, a combination of IL-12 plus IL-7 or IL-21 for 3 days with the withdrawal of IL-12 led to a up-regulation of stem cell markers and to a less differentiated T cell phenotype (Yang et al. 2013). Other studies showed that T memory cell function and anti-tumor activity of CAR T cells can be increased by pre-treatment of T cells with IL-15 or IL-15 and IL-7 or IL-

15 and IL-21 (Klebanoff et al. 2004, Lamers et al. 2014, Xu et al. 2014). The best effector activity targeting GD2 tumor antigen was reached culturing CAR T cells in the presence of IL-7 and IL-15 (Gargett et al. 2015). As the impact of T cell differentiation and its cytokine-directed regulation on the efficacy of CAR T cell therapy is very complex further investigations should be done to be able to ameliorate the persistence and the effects of our 20F8 CAR T cells.

In our studies we did not only test our CAR T cells for their persistence but also for their selectivity. The GPR64 specific 8F5 CAR T cells showed specific target recognition whereas the 20F8 CAR T cells showed low selectivity and shorter persistence. A possible explanation may be the occurrence of autoactivation leading to general activation and hence to early exhaustion. T cell exhaustion is defined as subsequent physical deletion and dysfunction of antigen-specific T cells arising during chronic infection and cancer (Gallimore et al. 1998, Zajac et al. 1998, Virgin et al. 2009). It has been described as leading to poor effector function, sustaining expression of inhibitory receptors and showing a unique state of T cell differentiation (Wherry et al. 2007). It prevents ideal control of infection or tumors (Wherry 2011). Investigating the cytokine secretion of our GPR64 specific CAR T cells it is apparent that the 20F8 CAR T cells secrete higher levels of IFNγ and TNF-α than our 8F5 CAR T cells. It was recently reported that antigen-independent clustering of CAR single-chain variable fragments triggers tonic CAR CD3ζ phosphorylation inducing autoactivation and early exhaustion of CAR T cells. Such activation limits antitumor efficacy and it can be found to varying degrees in many CARs studied, except the highly effective CD19 specific CAR (Long, Haso et al. 2015). As our 20F8 CAR T cells show higher cytokine levels and shorter persistence then our target specific 8F5 CAR T cells we tested them for early exhaustion via the measurement of the expression of exhaustion-associated transcription factors. Since some transcriptional pathways are used differently by exhausted T cells than by effector and memory T cells, analyzing the expression of exhaustion-associated transcription factors may provide insight into the potential presence of exhaustion (Wherry, Ha et al. 2007, Shin et al. 2009). For example, Blimp-1 (PMDR1) is a transcriptional repressor centrally involved in CD8+ T cell exhaustion (Shin, Blackburn et al. 2009). It regulates the terminal differentiation of cells in a variety of immunological settings and in large amounts it promotes the expression of inhibitory receptors and exhaustion (Martins et al. 2008, Bikoff et al. 2009). A similar role has Blimp-1, fostering the terminal

differentiation of T cells as well as the transcription factor T-bet (TBX21) when highly expressed (Joshi et al. 2008). Since the levels of the exhaustion-associated transcription factors Blimp-1 and T-bet of the 20F8 CAR T cells are strongly increased the theory of early exhaustion through autoactivation became even more probable (Long, Haso et al. 2015). Therefore, we further investigated clustering of the 20F8 CAR T cells via immunofluorescence in the absence of antigen and identified the existence of autoactivation as a possible explanation for limited effects of the 20F8 CAR T cells. Long et al. did not only discover the molecular pathways of early exhaustion of CAR T cells but also the influence of costimulatory domains on early exhaustion. 4-1BB endodomains ameliorate whereas CD28 costimulation augments exhaustion of CAR T cells providing another reason to replace the CD28 co-stimulatory domain with a 4-1BB domain in our GPR64 specific CAR T cells (Long, Haso et al. 2015). The identification that CAR ectodomains can influence CAR T cell exhaustion, and that antigen-independent activation and exhaustion occur to varying degrees in a variety of CARs, highlights the importance of optimizing CAR design and of further understanding of how receptor structure impacts function.

As low selectivity is a common problem, additional strategies to increase tumor selectivity concerning CAR design are currently being developed. For instance, co-expression of inhibitory CARs (iCARs) with PD-1 or CTLA-4 intracellular domains targeting an antigen exclusively expressed on healthy tissue can be included in CARs. The iCAR provides a dominant inhibitory signal when engaging healthy cells (Fedorov et al. 2013). Alternatively, split CAR systems are being developed. They consist of two CARs in the same T cell which both have to bind to their antigen to obtain T cell activation. Both of the CARs recognize different antigens by their individual scFvs, one CAR providing the CD3ζ signal, the other CAR the costimulatory signal. Combination with a suppressor signal to suppress T cell activity (inhibitory CAR), when binding to healthy tissue, is also possible (Kloss et al. 2013). Clinical exploration of these strategies is challenging since it requires the adjustment of CARs to the individual antigen levels in tumors and healthy tissues but they might contribute to increasing tumor selectivity.

Investigating *in vivo* reactivity of the GPR64 specific 20F8 and 8F5 CAR transgenic T cells no CAR transgenic T cells could be found in tumor or spleen. But tumors of mice treated with GPR64 specific 8F5 CAR T cells were smaller compared to tumors

of animals treated with unspecific PBMCs. Unfortunately, data of the mice treated with 20F8 CAR T cells could not be evaluated because too many of the mice died probably because of an unrelated infection. As the tumors of mice treated with 8F5 CAR T cells became smaller but did not contain CAR T cells 21 days after initial tumor cell injection we can assume that the CAR T cells infiltrated in the tumor but did not persist until their examination. *In vitro* the 8F5 CAR T cells demonstrated long persistence but *in vivo* there are other influencing factors. In case of accumulation at the tumor site, CAR T cell anti-tumor response can be repressed by various factors within in the tumor microenvironment. For instance, inhibitory immune-checkpoint signals present within the tumor microenvironment, such as those from programmed cell-death protein-1 (PD-1) ligands or cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) ligands, can limit function of CAR T cells (Pardoll 2012, Hingorani et al. 2015, McCaughan et al. 2016). These inhibitory receptor-ligand interactions can be blocked with monoclonal antibodies that prevent suppression of T cells. Although this approach has shown promising results in some malignancies, the success of these therapies clearly depends on the presence of a pre-existing endogenous tumor-specific T-cell response (Rizvi et al. 2015, Robert et al. 2015, Van Allen et al. 2015, Choudhury et al. 2016). As it has been proven that CAR T cells are susceptible to PD-1-mediated inhibition the combination of CAR T-cell therapy with immune-checkpoint inhibitors may be a promising approach to protect CAR-T-cell function within the tumor microenvironment (John et al. 2013). Furthermore, 4th generation, so called “armoured”, CAR T cells are modified to secrete pro-inflammatory cytokines to protect CAR T cells from the inhibitory tumor microenvironment. They showed resistance to immunosuppressive cells leading to improved antitumor efficacy, compared with T cells expressing the CAR alone. In several clinical studies armoured CAR T cells secreting IL-12 reached encouraging results (Chmielewski et al. 2011, Kerkar et al. 2011, Chinnasamy et al. 2012, Pegram et al. 2012).

Aside from the dynamic immunosuppressive tumor microenvironment also ineffective trafficking to the tumor or less in situ proliferation can hinder CAR T cell efficacy (Dotti et al. 2014). Not only to increase *in vivo* efficacy of CAR T cells but also to minimize the risk of “off-tumor” toxicities alternative routes for the delivery of T cells besides i.v. infusion are explored. For instance, CAR T cells were delivered via an endoscope to CEA positive metastases in the liver resulting in shrinking of metastases without systemic side effects (Katz et al. 2015). In another clinical study,

mesothelin-specific CAR T cells were administered directly in the pleura of mesothelioma patients (Beatty et al. 2014). Local application of the CAR T cells is assumed to have some advantages: It might include prompt antigen-induced activation to sustain robust T cell amplification. It could reduce the dose of CAR T cells required to induce efficacy when compared to systemic infusion. It is assumed to minimize systemic toxicities. And it might prolong persistence of CAR T cells in the tumor lesion.

Every therapy comes along with side effects that also have to be taken into account when working on new therapy approaches. Although adoptive CAR T cell therapy achieved spectacular results in the treatment of hematologic malignancies, significant toxicities occurred which need to be approached, as they can be life-threatening. Many toxic reactions in CAR T cell therapy are due to the expression of the cognate antigen on healthy tissue. So called “on-target off-tumor” targeting may result in life-threatening side effects, in particular, when the antigen is expressed on essential tissues such as lung, heart or liver. The death of a patient during the treatment of metastatic colon cancer with CAR T cells directed against HER2/neu (ErbB2) shows the severity of “on-target off-tumor” toxicity (Morgan, Yang et al. 2010). HER2/neu is not only highly expressed by the carcinoma cells but, although at lower levels, also by healthy lung epithelial cells which is speculated to be the death causing reason. This “on-target off tumor” toxicity of CAR T cells makes the choice of antigen even more important for the clinical use and underlines the suitability of GPR64 as a target for CAR T cell therapy as it is not expressed on essential tissues such as lung, heart or liver. But the most common and potentially severe toxicity occurred in studies with CAR-modified T-cell therapy is the cytokine release syndrome (CRS) that is also associated to the systemic macrophage activation syndrome. It is caused by extensive release of toxic levels of pro-inflammatory cytokines that accompanies CAR T cell activation. The extensive activation of CAR T cells, with high IFN-γ and TNF-α release, and the activation of monocytes or macrophages, which release IL-6, seem to play an important role in the pathomechanism of the toxic syndrome. In most cases, developing CRS seems to correlate with efficacy of the antitumor activity, but there is not a strong correlation between the degree of CRS and the response to therapy (Davila, Riviere et al. 2014). Unlike the handling of many conventional drug-induced side effects, reduction of the CAR T cell dosage does not control the syndrome. With tocilizumab®, an interleukin-6 receptor inhibitor, a treatment has

been found that improves CRS without affecting efficacy of the CAR T cell treatment (Lee, Gardner et al. 2014). As in our *in vivo* experiments some mice died unexpectedly we thought of possible side effects leading to their deaths. In addition to an infection leading to their deaths, but CRS is another possible cause of death and needs to be explored further.

Nevertheless, there are different safety features under investigation that are supposed to eliminate CAR transgenic T cells in cases of toxicity. Co-expression of inducible suicide genes represents a possibility to shut down cross-reactive T cells. For instance, herpes simplex virus thymidine kinase (HSV-tk), which phosphorylates the guanosine analog ganciclovir initiating the arrest of DNA replication, is a possible safety feature. Another inducible suicide gene under investigation is inducible caspase 9 (iCasp9), which encodes a modified truncated caspase 9 and initiates an apoptotic cascade upon drug-initiated dimerization (Hoyos et al. 2010, Budde et al. 2013). Another idea for a safety feature is the co-expression of a protein for which a depleting antibody is already in clinical use such as CD20 or EGFR. An elimination gene already used in the clinic is a nonfunctional, truncated version of EGFR that is co-expressed on the surface of CAR T cells. With the administration of cetuximab, an FDA-approved monoclonal antibody, these CAR T cells can be eliminated through activation of antibody-dependent cellular cytotoxity and/or the complement cascade (Wang et al. 2011). To be prepared for eventual toxicities also we incorporated a truncated version of EGF-receptor in our CAR designs. The GPR64 specific 20F8 as well as 8F5 second generation CAR constructs are connected to a truncated version of the EGFR via a T2A domain. We used an anti-EGFR monoclonal antibody together with anti-PE MicroBeads to purify our CAR-transgenic T cells. To show successful transduction and enrichment of GPR64 specific CAR T cells via flow cytometry we also applied EGFRt antibodies. In the future cetuximab could be used to eliminate the GPR64–specific 20F8 and 8F5 CAR T cells from the patient´s blood in any case of toxicities.

7 CONCLUSION AND FURTHER PERSPECTIVES

This study shows the generation and efficiency of transgenic, GPR64 specific CAR T cells. Those cells are able to specifically recognize and lyse EwS target cells *in vitro*. Since GPR64 is not only overexpressed in EwS, but also in a number of carcinomas derived from prostate, kidney or lung, GPR64-specific CARs may also be a future treatment option for other tumor entities.

Nonetheless, before using those cells in the clinic several characterizing steps have to be performed. Especially the effects of CAR design on anti-tumor efficacy have to be further investigated. As exhaustion through autoreactivity is incompletely analyzed so far, further verification has to be done.

A number of important biological questions, including defining ideal targets, optimizing CAR signaling, working out optimal combinatorial strategies and identifying the best process for subset selection and T cell manufacturing, remain to be addressed. Furthermore T cell mediated toxicities have to be reduced and further characterizing steps have to be performed to increase the safety and efficacy of CAR T cells.

As so far success of CAR T cell therapy is limited to treatment of hematologic malignancies more research has to be done to take the promising results on to solid tumors.

8 SUMMARY

Ewing Sarcoma (EwS) is the second most common bone malignancy in children and young adolescents with a high potential of dissemination into lung and bones. Patients with localized disease receiving current treatment, have an approximate long-term survival of >65%. Patients with disseminated disease into the bone have an approximate long-term survival rate of only 10%, compelling the search for new therapeutic treatment modalities like engineered T cell therapy. Here the therapeutic potential of chimeric antigen receptor (CAR) transgenic T cells directed against G-protein coupled receptor 64 (GPR64), an orphan receptor with normal expression restricted to human epididymis and significant overexpression in EwS, was examined. Therefore, two different monoclonal antibodies (mAb), namely 20F8 and 8F5, directed against the extracellular region of GPR64 were generated and characterized. Subsequently, retroviral constructs containing second generation CARs together with the scFv fragments of the respective mAbs were designed. Retroviruses containing either the 20F8 CAR or the 8F5 CAR transduced primary lymphocytes with good efficiency. The CAR transgenic T cells could be enriched for $CD8^+$ CAR^+ cells via microbead isolation and showed strong proliferative capacities *in vitro*. Furthermore, target structures were specifically recognized as determined by ELISpot and xCelligence assays. The significance of autoreactivity leading to less anti-tumor efficacy became evident in our 20F8 CAR T cells. First *in vivo* attempts show inhibition of tumor growth by 8F5 CAR T cells.

In summary, the cells generated in this study show strong specificity towards GPR64 and are able to control tumor cell growth *in vitro*. CAR transgenic T cells targeting GPR64 demonstrate a promising approach to transfer the success of CARs in hematological malignancies to solid tumors. Since GPR64 expression is not restricted to EwS but also up regulated in a number of carcinomas derived from prostate, kidney or lung, GPR64-specific CARs may also be a future treatment option for other tumor entities.

ZUSAMMENFASSUNG

Das Ewing Sarkom ist der zweithäufigste Knochentumor im Kindes- und Jugendalter und zeigt eine hohe Metastasierungsrate in Lunge und Knochen. Patienten mit lokalisiertem Befall haben derzeit unter leitliniengerechter Therapie ein durchschnittliches Langzeitüberleben von über 65%. Patienten mit metastasiertem Befund haben ein durchschnittliches Langzeitüberleben von nur 10%, was die Suche nach neuen Therapieoptionen wie CAR-T-Zell-Therapie zwingend nötig macht. In dieser Studie haben wir das therapeutische Potential von CAR transgenen T Zellen untersucht, die gegen GPR64, ein Orphan-Rezeptor mit normaler Expression beschränkt auf Nebenhoden und signifikanter Überexpression in Ewing Sarkomen, gerichtet sind. Hierfür wurden zwei verschiedene monoklonale Antikörper, namentlich 20F8 und 8F5, die gegen die extrazelluläre Region von GPR64 gerichtet sind, hergestellt und charakterisiert. Anschließend wurden retrovirale Konstrukte, die CAR Rezeptoren 2. Generation sowie die scFv Fragmente der jeweiligen monoklonalen Antikörper enthalten, designt. Retroviren, die entweder den 20F8 CAR oder den 8F5 CAR enthalten, transduzierten primäre Lymphozyten mit guter Effizienz. Die CAR transgenen T-Zellen konnten mittels magnetischer Beads auf $CD8^+$ CAR^+ Zellen aufgereinigt werden und zeigten gute Proliferationsfähigkeiten *in vitro*. Außerdem konnten wir durch ELISpot und xCelligence Untersuchungen demonstrieren, dass transgenen T-Zellen Zielstrukturen spezifisch wiedererkennen. Die Bedeutung der Autoreaktivität, die zu verminderter Anti-Tumor Wirksamkeit führt, wurde bei unseren 20F8 CAR T Zellen deutlich. Erste *in vivo* Versuche zeigten eine Hemmung des Tumorwachstums durch 8F5 CAR T Zellen.

Insgesamt zeigen die in dieser Studie generierten CAR T-Zellen eine hohe Spezifität gegenüber GPR64. Sie sind in der Lage, Tumorwachstum *in vitro* zu kontrollieren. Die gegen GPR64 gerichteten CAR transgenen T-Zellen stellen somit einen vielversprechenden Ansatz dar den Erfolg CAR transgener T-Zellen in der Therapie hämatologischer Erkrankungen auf die Behandlung solider Tumoren zu übertragen. Da die Expression von GPR64 nicht nur auf Ewing Sarkome beschränkt ist, sondern auch noch bei einer Vielzahl von Karzinomen der Prostata, Niere und Lunge beschrieben wurde, stellen GPR64 spezifische CAR T-Zellen auch eine zukünftige Therapieoption für andere Tumorentitäten dar.

9 APPENDIX

9.1 List of abbreviations

Ab	Antibody
ADGRG2	Adhesion G protein-coupled receptor G 2
ALL	Acute lymphoblastic leukemia
APC	Allophycocyanin
BCP	1-bromo-3-chloropane
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
cDNA	Complementary desoxyribonucleic acid
CEA	Carcinoembryonic antigen
CRS	Cytokine release syndrome
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
DMSO	Dimethylsulfoxid
EDTA	Ethylene diamine tetraacetic acid
EES	Extraosseous Ewing Sarcoma
EGFR	Epithelial growth factor receptor
ELISpot Assay	Enzyme Linked Immuno Spot Assay
EwS	Ewing sarcoma
ESFT	Ewing Sarcoma family tumors
EtBr	Ethidiumbromid
ETS	Erythroblast transformation specific
EWSR1	Ewing sarcoma break point region 1
EZH2	Enhancer of zeste homolog 2
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum

FDA	US Food and Drug Administration
FITC	Fluorescein isothiocyanate
FT	Fetal tissue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPR64	G protein-coupled receptor 64
GPS	G-protein-coupled receptor proteolysis site
GvHD	Graft-versus-Host disease
HBSS	Hank´s buffered salt solution
HCl	Hydrochloric acid
HE6	Human epididymis-specific protein 6
iCasp9	inducible caspase 9
IFN	Interferon
IGF1	Insulin like growth factor 1
IL	Interleukin
i.p.	Intra peritoneally
IRS	Immunoreactive score
mAb	Monoclonal antibody
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cell
NaN_3	Sodium azide
NaOH	Sodium azide
NB	Neuroblastoma
NEAA	Non-essential amino acids
NK cells	Natural killer cells
NTC	Non-template control
OS	Osteosarcoma
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell-death protein-1
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
PNET	Peripheral primitive neuroectodermal tumor
PSMA	prostate-specific membrane antigen
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
rhIL-2	Recombinant Human Interleukin-2
RNA	Ribonucleic acid
RNAi	RNA interference
scFv	Single chain variable fragment
SEA	Sea urchin sperm protein, enterokinase and agrin
STEAP	Six-transmembrane epithelial antigen of prostate
STP	Serine/threonine/proline-rich region
SV40	Simian virus 40
TCM	T cell medium
TCR	T cell receptor
TM7	Seven transmembrane
TNF	Tumor necrosis factor
TRUCK	T cells redirected for universal cytokine-mediated killing
γc	Gamma chain

9.2 Supplemental tables

Table 1: Immunoreactive score (IRS) to evaluate tissue samples of Ewing Sarcomas stained with GPR64 specific hybridomas. The values of the score are obtained by multiplying the staining intensity by the proportion of positive cells.

Staining intensity	Positive cells	IRS
0: no reaction	0: non	0: negative
1: weak reaction	1: less then 10%	1-4: weakly positive
2: medium reaction	2: between 10 and 50%	6-8: moderately positive
3: strong reaction	3: between 51 and 80%	9-12: strongly positive
	4: over 80%	

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