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Dissertation
zum Erwerb des Doctor of Philosophy (Ph.D.)
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität München

Characterization of the influence of antibody valency on murine synthetic agonistic receptor-transduced T cell activation and action

vorgelegt von
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Direktor: Prof. Dr. med. Martin Reincke

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1 Introduction

1.1 Cancer development – an interplay between tumor cells and the immune system

1.1.1 Hallmarks of cancer

Cancer is one of the major causes of death world-wide with a prognosis for a total of 9.6 million deaths in 2018 according to the World Health Organization (WHO). Six major hallmarks of cancer have been postulated to define the capabilities of previously normal cells acquired during the multistep development of malignant tumors (Hanahan et al., 2011). Key factors underlying these hallmarks are both inherited and acquired genetic mutations, leading to genome instability and promoting tumor growth and survival. For example, overexpression and somatic mutations in receptor tyrosine kinases (RTKs) can foster a sustained mitogenic signaling resulting in an uncontrolled expansion of cancer cells. Here, alterations and oncogenic mutations in the EGFR-ERBB and FGFR RTKs families were revealed in a wide range of tumor entities (Eswarakumar et al., 2005, Lee et al., 2006, Slamon et al., 1989). Therefore, RTKs are important drug targets for small-molecule inhibitors and monoclonal antibodies to block and down-regulate RTK activation (Arnould et al., 2006, Shawver et al., 2002). Another hallmark of cancer is the invasion of neighboring tissue and the ability to form distant metastases which is mainly associated with the alteration and inactivation of cell-to-cell adhesion molecules such as E-cadherin and N-cadherin (Berx et al., 2009, Cavallaro et al., 2004, Johnson et al., 2007).

1.1.2 The impact of the tumor microenvironment on cancer progression

The complexity and development of a tumor can only be fully understood when the tumor microenvironment (TME) constructed during tumorigenesis is not neglected (Hanahan et al., 2011). Tumors are no longer seen as a homogenous accumulation of cancer cells, they are more complex tissues encircled by an extracellular matrix (ECM), stromal cells and a repertoire of recruited immune cells that together form the TME (Wang et al., 2017). In many cases among the variety of carcinomas, fibroblasts are the preponderant cell population within the tumor stroma and are often designated as cancer-associated fibroblasts (CAFs). These CAFs remain perpetually activated within the tumor tissue and thus are substantially involved in cancer progression, angiogenesis, invasion and metastasis formation by the release of growth factors, cytokines and mesenchymal-epithelial cell interactions (Kalluri et al., 2006). Furthermore, the pervasion of the neoplastic lesion with a blood and lymphatic vascular network sustaining the oxygen and sustenance supply is essential for the

implantation and survival of the tumor. Thus, the angiogenic switch, the formation of new blood vessels by activation of quiescent endothelial cells, remains active during the entire process of tumorigenesis (Hanahan et al., 1996). In addition, the broad variety of recruited immune cells, including cytotoxic T lymphocytes, natural killer cells (NK cells) as well as macrophages and neutrophils mediate conflicting signals for both immune escape and immune surveillance of the tumor (DeNardo et al., 2010). Because of the complexity of the different cellular components and their synergistic pathways involved in tumorigenesis, targeting of not only the malignant cancer cells but also of multiple cell types within the TME is of critical importance for the development of highly efficient anti-tumor therapies.

1.1.3 Immunosurveillance and immunoediting in carcinogenesis

For a long time, it has been predicted, that the immune system can repress the outgrowth of carcinomas and effectively control and eliminate the formation of cancer. Recently, several studies reinvigorated and validated the cancer immunosurveillance concept and elucidated the contribution of both, innate and adaptive immunity in this hypothesis (Dunn et al., 2004). However, certain tumor entities are capable of escaping immune recognition and destruction and create an equilibrium phase between tumor and immune cells. This equilibrium phase indicates the initial stage of tumor progression, followed by tumor immune escape. This constant interaction between cancer and immune cells is also referred to immunoediting where cancer cells shape the immune system and vice-versa. In the state of equilibrium, the elimination of certain immunogenic alterations but also the outgrowth of clones with immunosuppressive or non-immunogenic manifestations may give rise to new populations of tumor cells selected for limited immunogenicity. Accordingly, this dysfunctional immunological control may lead to an outbreak of the disease in the escape phase. Based on the principle effectiveness of immunosurveillance, immunotherapeutic approaches aim to generate or to restore an effective anti-tumor immune response.

1.2 Immunotherapy of malignant diseases

The main pillars of cancer treatment are chemotherapy, surgery and irradiation. Failure to respond to treatment and disease relapse occur frequently, urgently requiring novel treatment approaches and therapeutic agents to improve the therapeutic success in cancer patients. A promising approach

is utilizing the body's natural defense mechanism, the immune system, to specifically attack cancer cells.

The aim of immunotherapy is to utilize, activate and – or stimulate the endogenous immune system to redirect immune effector cells towards the tumor and achieve an effective elimination of cancer cells. Simultaneously, a long-lasting and antigen-specific immunological memory should be induced. In principle, there are several types of immunotherapy including vaccine treatments or the administration of immune stimulatory compounds such as cytokines but only antibody and cell-based therapies have clinical relevance.

1.3 Antibody-based immunotherapy

1.3.1 Antibodies are humoral constituents of the adaptive immune system

Antibodies are capable to discern distinct molecular epitopes on cell surfaces and are crucial for the formation of a long-lasting immunological memory. This unique function can be achieved by somatic recombination of gene regions coding for the variable region of antibodies. As a result, each antibody is different from its companions leading to a broad diversity with more than 10^{12} different molecules (Rajewsky, 1996). The re-arrangement of immunoglobulin genes is initiated in the bone marrow at the pro-B cell stage, engenders a pre-B cell receptor (BCR) and finally a mature BCR is expressed on the cell surface. From this stage on, B cells undergo several selection processes in order to prevent the formation of self-reactive, autoimmune antibodies. Further differentiation into an antibody-secreting plasma cell requires a sequential activation of the B cell by the encounter of the complementary antigen and the interaction with $CD4^+$ T cells in secondary lymphoid organs. Once activated, plasma cells produce antibodies for a short time period but also migrate to the bone marrow. There, long-lived plasma cells persist and constantly secrete immunoglobulins into the bloodstream (Hoffman et al., 2016).

Antibodies are Y-shaped glycoproteins consisting of four polypeptide subunits: two identical light chains and two identical heavy chains named according to their different molecular weights. These chains are connected by covalent disulfide bonds. Furthermore, individual parts of the antibody can be classified based on their functional behavior. The antigen binding fragment is located at the amino terminal end of both arms of the molecule and is composed of both, the heavy and light chain. Here, the complementarity-determining regions are of major importance for specific antigen recognition.

The fragment crystallizable (Fc) part, assembled only by the two heavy chains, determines the possible effector functions of the antibody (Hoffman et al., 2016). When encountering the complementary antigen, antibodies bind to the surface molecules and thereby opsonize the target cells. This can result in antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent cellular phagocytosis (ADCP) mediated by immune effector cells (Ayyar et al., 2016).

1.3.2 Monoclonal antibodies for cancer immunotherapy

Monoclonal antibodies (mAbs) targeting a specific tumor-associated surface protein overexpressed by the tumor represent the most established and widely used immunotherapy for cancer treatment (Byrd et al., 2001, Goldenberg, 1999). The underlying mode of action includes direct interactions like the blockade of essential signaling pathways and neutralization of receptor ligands leading to cancer cell death or the classical route via opsonization and induction of ADCC, CDC or ADCP.

One of the first approved therapeutic mAb is rituximab which targets the CD20 surface antigen on B cell lymphomas. Rituximab has proven effectivity both in combination with chemotherapy and as maintenance monotherapy in treating chronic lymphocytic leukemia (Byrd et al., 2005, Schulz et al., 2002) and diffuse large B cell lymphomas (Coiffier et al., 2010, Pfreundschuh et al., 2006).

Most target structures for mAbs are altered and overexpressed growth factor receptors such as the epidermal growth factor receptors HER2/neu and EGFR. Trastuzumab, a mAb directed against the HER2 antigen significantly ameliorated outcome of breast cancer patients with HER2-positive tumors (Slamon et al., 2001). Another example is cetuximab, a mAb targeting EGFR which increases response rates and prolong progression-free survival in patients with metastatic colon cancer in combination with chemotherapy (Van Cutsem et al., 2009).

Despite the striking results of antibody-based immunotherapy, there are still challenges and limitations leading to an incomplete elimination and escape of tumors. In particular, for ubiquitously expressed target antigens, severe on-target-off-tumor effects can cause organ damage, excessive cytokine secretion by activated immune cells and life threatening side effects for the patients, even when occurring in low densities on healthy tissue (Suntharalingam et al., 2006, Winkler et al., 1999). Furthermore, solid tumors are insufficiently penetrated by mAbs. The limitation is on one hand due

to the size and half-life of the molecules and on the other hand caused by the tumor structure including the ECM and the poor vascularization of the lesion (Beckman et al., 2006).

1.3.3 Enhancing antibody-based therapy with bispecific antibodies

To further increase the therapeutic efficacy of antibody-based immunotherapy, antibodies simultaneously targeting multiple antigens were developed. These bispecific antibodies (BiAbs) could either increase the treatment specificity by addressing two surface markers on the tumor cell at once or recruit T cells to the lesion by engagement of the pan-T cell marker CD3.

Catumaxomab, an anti-EpCAM-anti-CD3 BiAb was the first molecule studied in patients with non-small cell lung cancer (Sebastian et al., 2007) or malignant ascites (Jäger et al., 2012) and was approved for clinical application. However, in several clinical studies, catumaxomab induced an unspecific and exuberant immune response by crosslinking T effector cells with accessory immune cells via the Fc part of the BiAb (Eissler et al., 2013, Mau-Sørensen et al., 2015, Sebastian et al., 2007). Catumaxomab was finally withdrawn from the market by the provider based on limited implementation of this treatment modality (Viardot et al., 2018).

Therefore, molecules devoid of the Fc portion were constructed by assembling only the antigen recognition parts, the single-chain variable fragments (scFvs) of two antibodies. These bispecific T cell engagers (BiTEs) can still recruit T cells by an incorporation of an anti-CD3 scFv but lack the interaction with bystander immune cells via the Fc part. Furthermore, their diminished size enables enhanced penetration of the tumor tissue, albeit with a shortened half-life of the agent. This drawback was tackled with the continuous infusion of the BiTE through an infusion pump system (Lee et al., 2016).

Evidence for the success of these molecules had been previously shown in *in vitro* studies which used low BiTE concentrations and high target to effector ratios to induce remarkable cytotoxic activity (Dreier et al., 2002, Mack et al., 1995). These findings suggest a serial lysis of several target cells mediated by the BiTE (Hoffmann et al., 2005). Upon synchronous engagement with the tumor cell, T cells are activated and induce target cell lysis (Brischwein et al., 2007, Dreier et al., 2002). Several clinical studies using the anti-CD19-anti-CD3 BiTE blinatumomab were conducted in patients with various types of B cell non-Hodgkin lymphoma and leukemia (Bargou et al., 2008). Based on initial

promising results, studies in patients with persistent or relapsed minimal residual disease-positive B-cell acute lymphatic leukemia were successfully initiated, resulting in the clinical approval of blinatumomab (Jen et al., 2018, Topp et al., 2011). However, concerns have been raised about long-lasting T cell activation during BiTE treatment accompanied by uncontrolled immune activation (Teachey et al., 2013), calling for further improvements of the constructs with respect to enhanced safety profiles.

1.3.4 Switching off T cells' natural breaks with checkpoint blocking antibodies

The most successful breakthrough for cancer immunotherapy was achieved by mAbs blocking inhibitory checkpoint molecules on immune effector cells. These molecules target inhibitory receptors on the surface of T cells and thereby prevent the interaction with the respective ligands expressed by immunosuppressive leukocytes and tumor cells. Consequently, an ameliorated and prolonged T cell response as well as less tumor-induced immunosuppression can be achieved resulting in prolongation of patient survival and enhanced anti-tumor responses. So far, cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) have proven to be excellent target structures for checkpoint blocking antibodies in a broad variety of malignancies. Clinical trials using ipilimumab, an anti-CTLA-4 antibody, demonstrated prolonged overall survival in patients with metastatic melanoma (Hodi et al., 2010). Also nivolumab and pembrolizumab, antibodies blocking the PD-1-PD-L1 axis, showed major clinical success in various tumor entities (Borghaei et al., 2015, Motzer et al., 2015, Robert et al., 2014). In addition, the combination therapy of both checkpoint inhibitors provided a superior benefit when compared to monotherapy (Hellmann et al., 2018, Motzer et al., 2018).

Although antibody monotherapy has already marked an entirely new level of cancer treatment, the combination of checkpoint blocking antibodies with tumor-specific T cells exceed all expectations regarding clinical efficacy. Therefore, cytotoxic T lymphocytes are the most relevant immune effector cell population for cancer immunotherapy and must be further explored, to enhance the tumor recognition and the anti-tumor potential of these powerful cells.

1.4 Cell-based immunotherapy

The fundamental principle of cellular immunotherapy is to induce and strengthen the ability of tumor antigen-specific immune cells to recognize and eliminate tumor cells. A common approach currently used in clinics and clinical studies is the adoptive cell transfer (ACT) of mainly T cells. In this approach, tumor-infiltrating lymphocytes (TILs) are isolated from tumor biopsies. After *ex vivo* enrichment and expansion, tumor-specific effector cells can be reinfused into the patient.

To further enhance the specificity of this technique, immune cells extracted from peripheral blood can be engineering *ex vivo* to achieve a specific redirection against tumor-associated antigens (TAAs). Here, TAA-specific T cells can be generated by stimulation with autologous, TAA-pre-loaded dendritic cells. Recent clinical trials with *in vitro* generated antigen-specific cytotoxic T lymphocytes recognizing a melanoma target antigen showed beneficial therapy responses of metastatic melanoma patients upon treatment (Dudley et al., 2002, Mackensen et al., 2006). Another approach to conceive tumor-specific T cells is the equipment with a cloned and transduced T cell receptor (TCR). The integration of additional artificial TCR cDNA into the T cell genome achieved tumor regression after ACT in melanoma (Johnson et al., 2009), colorectal cancer (Parkhurst et al., 2011) and synovial sarcoma (Morgan et al., 2013).

However, the majority of the TCR T cell clinical trials was accompanied by severe on- or off-target cytotoxicity since most tumor-associated proteins lack specificity and are co-expressed on healthy tissue (Linette et al., 2013, Morgan et al., 2013, Parkhurst et al., 2011). In addition, the activation of TCR-engineered T cells depends on a major histocompatibility complex (MHC)-mediated presentation of the TAA by tumor cells. Thus, a frequently observed escape mechanism by tumors is the loss of antigen expression through down-regulation of MHC (Angell et al., 2014, Aptsiauri et al., 2007). To bypass this limitation, a new class of recombinant chimeric antigen receptors was developed which enables T cells to recognize any surface antigen of interest unrestricted to MHC presentation.

1.4.1 Chimeric antigen receptors

In general, chimeric antigen receptors (CARs) are synthetic fusion proteins which are constructed through assembling domains from different proteins, each of which enables the CAR to carry out specific functions.

The most common structure of CARs consists of an extracellular domain, mediating MHC-independent antigen recognition, a transmembrane region and an intracellular signaling part (Eshhar et al., 1993). The antigen-binding moiety typically derives from a scFv of an antibody to ensure MHC-independent specificity for the TAA. The scFv molecule is linked to the transmembrane domain via a flexible spacer to increase the distance from the plasma membrane. Although the extracellular spacer domain is not involved in the signaling pathway, it is decisive for the function of the CAR due to the orientation of the scFv in different directions to facilitate antigen recognition (Hudecek et al., 2015). The most commonly used hinge regions originate from CD8 or IgG4 molecules. Upon antigen recognition, the intracellular domain is crucial for transmitting an activation signal into the cell, leading to cytokine production and cytotoxicity. First-generation CARs mimicked endogenous TCR activation through the fusion of the cytoplasmic signaling domain CD3ζ to the transmembrane region (Figure 1a).

In initial studies with these molecules, CAR T cells targeting HER2/neu were used for *in vitro* functional validation. As expected, T cells transduced with an anti-HER2-specific CAR showed activation, cytokine release and target cell lysis in response to co-culture with HER2-positive tumor cells (Stancovski et al., 1993). Subsequent studies with CAR T cells targeting a broad variety of TAAs confirmed the anti-tumoral activity *in vitro* and in several mouse models (Geiger et al., 1999, Haynes et al., 2002). Surprisingly, a translation into human clinical trials could not be managed due to poor persistence and loss of function after transfer (Walker et al., 2000). These findings suggest that in first generation CARs the signals from ITAM-bearing receptors alone are not sufficient to induce polyclonal expansion and sustain anti-tumoral activity *in vivo*. Pursuant to the two-signal model required for lymphocyte activation, the addition of a co-stimulatory signal should rescue the effector phenotype of the engineered T cells.

Indeed, the incorporation of an additional intracellular signaling domain from various co-stimulatory domains as CD28 (Krause et al., 1998), CD137 (4-1BB) (Finney et al., 2004) or ICOS (Shen et al., 2013) to the cytoplasmic part of the CAR, led to an increased function and extended lifespan of the T cells (Figure 1b). The advantages of these second generation CARs could be confirmed *in vitro* with superior T cell activity measured by IFN-γ and IL-2 secretion (Hombach et al., 2001) and *in vivo* studies with leukemia xenograft models (Brentjens et al., 2007, Kowolik et al., 2006). Recently, third generation CARs combining multiple signaling domains (e.g. CD3ζ-CD28-CD134 (OX40) or

CD3ζ-CD28-41BB) to further augment *in vivo* persistence, anti-tumor activity and tumor trafficking were developed (Enblad et al., 2018, Guedan et al., 2018)(Figure 1c).

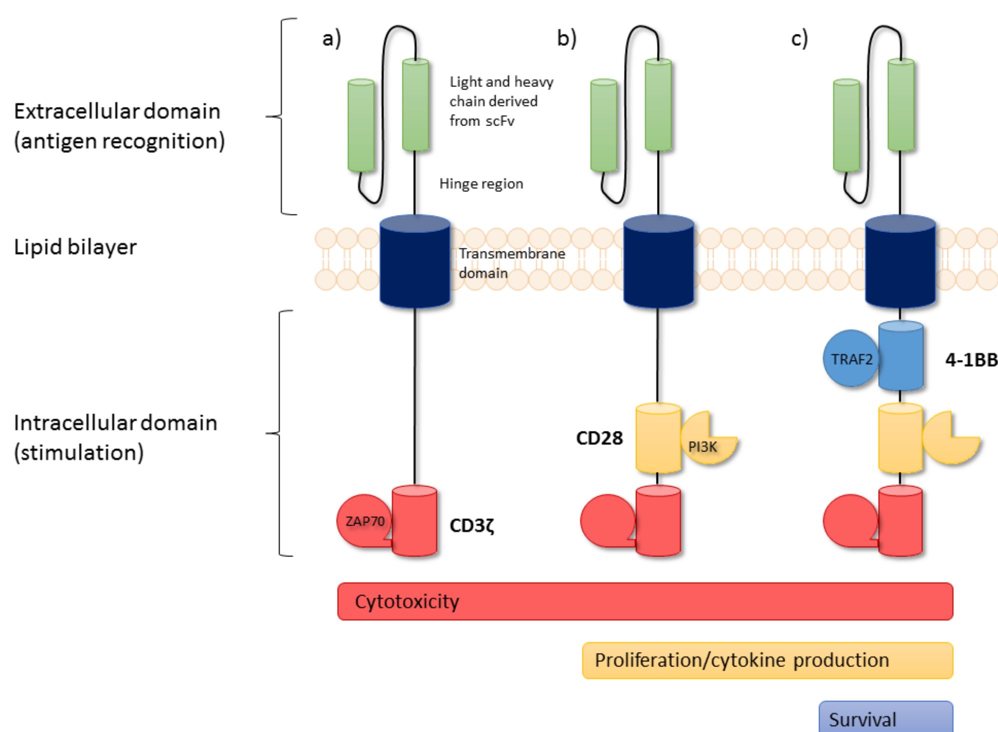

Figure 1 | Design and development of chimeric antigen receptors (CARs).

a | First-generation chimeric antigen receptors consist only of the CD3ζ chain from the endogenous TCR. **b** | Second-generation chimeric antigen receptors incorporate a co-stimulatory signaling domain which leads to an increased function and proliferation. **c** | Third-generation chimeric antigen receptors use multiple signaling domains to further augment potency (adapted from Casucci et. al., 2011)**.**

Second and third generation CAR T cells targeting the B cell antigen CD19 showed strong response rates in clinical studies with B cell lymphoma. These findings led to the U.S. Food and Drug Administration approval of two CAR T cell therapies for patients with relapsed or refractory acute lymphoblastic leukemia and refractory B cell lymphoma in 2017 (Zheng et al., 2018).

As seen for T cell-activating antibody-based therapies, CAR T cell therapy is also accompanied by severe side effects due to the ubiquitous expression of the target antigen on healthy tissue as well and therefore related on-target-off-tumor toxicities (Kochenderfer et al., 2012). Cytokine release syndrome (CRS) frequently occurs during CAR T cell therapy but seems to correlate with a beneficial outcome for patients (Davila et al., 2014).

To further implement CAR T cells in the therapy of malignant diseases, unwanted and unpredictable side effects must be controllable and reversible for the safety of patients. Along these lines, incorporation of safety switches, which allow a fast and durable shut down of T cell activity or even the total elimination of the engineered cells, has come into focus. Suicide genes, allowing the selective elimination of the transferred cells upon the administration of a non-toxic prodrug, have already been tested in CAR T cells in preclinical experiments (Di Stasi et al., 2011). Indeed, the activation of a suicide gene within the engineered T cells leads to the total ablation of the CAR T cells. An alternative approach is the co-expression of non-signaling marker antigens such as CD20 (Philip et al., 2014) or truncated EGFR (Paszkiewicz et al., 2016). Here, CAR T cells can be targeted with the corresponding antibodies rituximab or cetuximab and thereby eliminated by ADCC or CDC. Again, this strategy depletes the total CAR T cell population which results in the abrogation of the therapeutic potential. So far, none of these approaches for a safer application of CAR T cell therapy have been tested in the settings of clinical toxicities. The clinical impact therefore remains elusive.

Overall, CAR T cells are a breakthrough innovation in cancer immunotherapy of the last decade and surpassed the expectations of a new era in cancer treatment. Unfortunately, CAR T cell therapy is accompanied by unpredictable and life-threatening side effects which are so far hardly manageable. Moreover, the adaptation of CARs to newly identified TAAs is a time-consuming process were design, generation and validation of the respective constructs must be performed prior to *in vivo* application. Time that many cancer patients no longer have. Improving the safety profile while at the same time increasing flexibility and modularity are therefore two essential aspects that must be taken into account in the further development of CAR T cells.

2 Aim of the project

2.1 Synthetic agonistic receptors for T cell activation

To address and improve the major drawbacks of CARs, two doctoral students from our laboratory (Mathias Kurzay and Moritz Schmidbauer) have designed a new class of T cell-activating receptors comprising protein structures with an inert extracellular part fused to T cell-activating domains such as CD28, CD137 and CD3ζ. These synthetic agonistic receptors (SARs) can trigger specific T cell activation upon binding of a BiAb which is linked to a surface antigen of a malignant tumor cell.

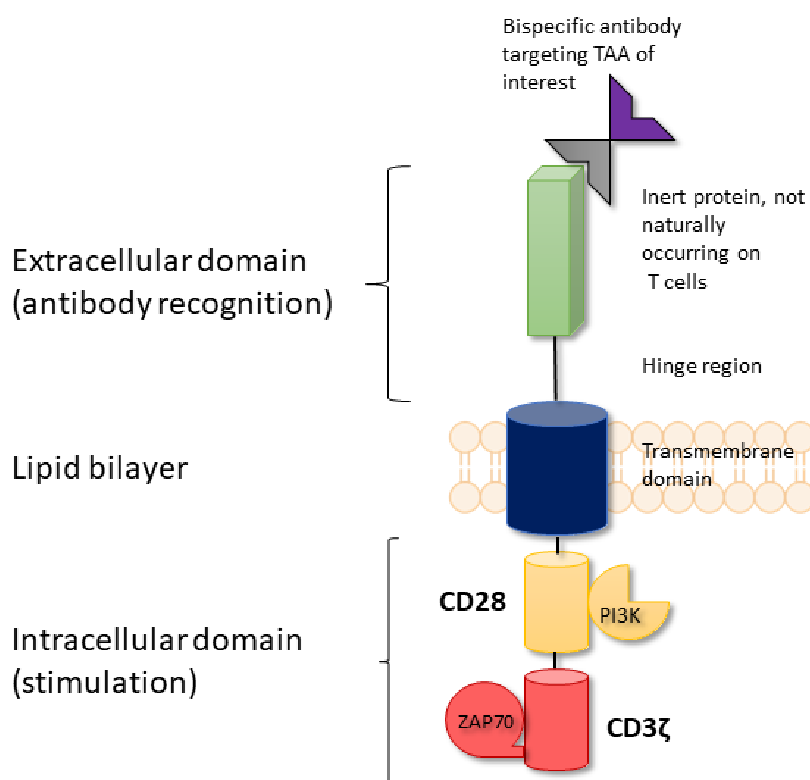

Figure 2 | Design and mode of action of synthetic agonistic receptors.
Binding of fusion receptor to a bispecific antibody for recruiting and activation of the T cells at the tumor site

2.2 Combining SAR T cells with BiAb

To elucidate the functional relevance of SAR T cells in combination with BiAb, previous doctoral students in the laboratory (Mathias Kurzay and Moritz Schmidbauer) designed and cloned two different SAR constructs. These consist of an extracellular domain, not naturally occurring on T cells,

derived from the epithelial growth factor receptor transcript variant III (EGFRv3) or cryptic antigen 1 (Cripto) fused to two intracellular domains for T cell activation (CD28 and CD3ζ). The receptors are further referred to as E3 or C3. *In vitro* functionality, measured by cytokine production and target cell lysis, was demonstrated by using an anti-EpCAM x anti-EGFRv3 or anti-EpCAM x anti-Cripto BiAb respectively combined with EpCAM-expressing tumor cells. Furthermore, a SAR T cell differentiation towards an effector memory phenotype upon engagement with target cells was shown (Schmidbauer, 2018). The BiAb used for this and the previous study were designed and manufactured by collaboration partners from the Roche Innovation Center Zurich. Initial molecules were bivalent for both the TAA and the SAR. Thus, an unspecific activation of SAR T cells due to potential crosslinking by the BiAb itself could be observed. To further improve the specificity of the approach and to reduce off-target toxicities, an improved BiAb format monovalent for the SAR was developed in cooperation with the Roche Innovation Center Zurich. Furthermore, SAR T cells showed an increased granzyme B gene transcription after coculture with $EpCAM^+$ tumor cells and BiAb. This finding indicated a tumor cell lysis due to the release of lytic vesicles by SAR T cells. In contrast, SAR T cells deficient for perforin showed no limitation of their cytotoxic potential *in vitro* (Schmidbauer, 2018).

Based on the previously collected data from Mathias Kurzay and Moritz Schmidbauer, this thesis aims to elucidate the following aspects:

The first aim of this thesis will be to characterize the new BiAb format in terms of T cell activation and target cell lysis. To evaluate the enhanced specificity of the trivalent BiAb, a head-to-head comparison of the different antibody formats for $EpCAM^-$ target cell killing and unspecific cytokine release will be conducted.

The second aim of this project will be to further investigate the killing mechanism underlying the elimination of tumor cells by SAR T cells. Therefore, different apoptosis-inducing molecules expressed by T cells should be scrutinized for their involvement in cytotoxicity mediated by SAR T cells.

The third aim of the study is to evaluate the function of the potential safety switches implied in the approach. Since CAR T cell therapy is often accompanied by severe side effects including cytokine release syndrome, the incorporation of safety switches to the engineered T cells is of major interest

for the translation into clinics. For the combination of SAR T cells with BiAb, the limited half-life of the BiAb can be used to control excessive cytokine production by the SAR T cells. This should be addressed *in vitro* by deprivation of the BiAb from a coculture of target cells with SAR T cells. Furthermore, the E3 SAR utilizing the EGFRv3 extracellular domain could potentially be targeted with the clinical grade antibody cetuximab to selectively deplete the transferred T cells *in vivo*.

3 Material

3.1 Technical equipment

Analytical balance	CPA1003S	Sartorius Laboratory, Göttingen, DE
Autoclave Varioklav	500E HP	Medizintechnik, München, DE
Cell culture incubator	BBD 6220	Heraeus, Hanau, DE
Centrifuges	3L-R	Multifuge Heraeus, Hanau, DE
	Centrifuge 5318R	Eppendorf, Hamburg, DE
	Rotina 420R	Hettich GmbH, Tuttlingen, DE
FACS	Canto II	BD Biosciences, Franklin Lakes, USA
	LRSFortessa	BD Biosciences, Franklin Lakes, USA
Heating block	Thermomixer 5436	Eppendorf, Hamburg, DE
Incubator (cell culture)	BD 6220	Heraeus Instruments, Hanau, DE
Laminar flow hoods	HeraSAFE KS	Heraeus, Hanau, DE
LightCycler®	480 II	Roche, Mannheim, DE
Microscopes	Axiovert 40C	Zeiss, Jena, DE
	Axiovert HAL 100	Zeiss, Jena, DE
Multilabel plate reader	Mithras LB 940	Berthold, Bad Wildbad, DE
Photometer	NanoDrop 2000c	Thermo Fisher, Waltham, USA
pH-Meter	inoLab pH 720	WTW GmbH, Weilheim, DE
Vortex mixer	RS-VA 10	Phoenix, Garbsen, DE
Water bath	Unitherm-HB	UniEquip, München, DE
iCELLigence RTCA Systems	iCELLigence	ACEA Biosciences, San Diego, USA

3.2 Materials

Cell culture flasks (T25 to T175)	Costar Corning, New York, USA
Cell culture plates (6- to 96-well)	BD Medical, Franklin Lakes, USA
Cryotubes	Greiner Bio One, Frickenhausen, DE
Disposable scalpels (No. 10)	FEATHER, Osaka, JP
ELISA microplates (96-well)	Costar Corning, New York, USA
Eppendorf tubes (0.5 ml; 1.5 ml; 2.0 ml)	Sarstedt, Nürnbrecht, DE
FACS tubes	BD Biosciences, Franklin Lakes, USA

Nylon filter SmartStrainers (100 µm, 30 µm)	Miltenyi Biotec, Bergisch Gladbach, DE
Petri dishes	BD Medical, Franklin Lakes, USA
Pipetboy	Hirschmann Laborgeräte, Eberstadt, DE
Pipettes	Eppendorf, Hamburg, DE
Polypropylene round bottom tubes	BD Biosciences, Franklin Lakes, USA
Serological pipettes	Costar Corning, New York, USA
Syringes (2 ml, 10 ml)	BD Medical, Franklin Lakes, USA

3.3 Chemicals and reagents

β-Mercaptoethanol	Roth, Karlsruhe, DE
Agarose	Biozym, Oldendorf, DE
Albumin Fraction V (BSA)	Sigma-Aldrich, Steinheim , DE
Calcium chloride	Merck, Darmstadt, DE
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, DE
DNA loading dye	Thermo Scientific, Rockford, USA
DNA Ladder (100 bp)	Thermo Scientific, Rockford, USA
Dithiothreitol (DTT)	Sigma-Aldrich, Steinheim, DE
Ethanol 96 - 100 % (puriss.)	Sigma-Aldrich, Steinheim, DE
FACSFlow	BD Biosciences, Franklin Lakes, USA
Isopropanol p.A.	AppliChem, Darmstadt, DE
Kapa Probe Universal Master Mix	VWR, Darmstadt, DE
Methanol	Merck, Darmstadt, DE
Sulfuric acid (2 N)	Pharmacy of LMU, München, DE
Tris (Tris(hydroxymethyl)aminomethane)	Roth, Karlsruhe, DE
Trypan blue	Sigma-Aldrich, Steinheim, DE
Trypsin	PAA, Pasching, AT
Tween-20	Sigma-Aldrich, Steinheim, DE

3.4 Commercial assay kits

CytoTox 96® Non-Radioactive Cytotoxicity Assay	Promega, Mannheim, DE
GzmB ELISA (murine)	R & D Systems, Minneapolis, USA

IFN-γ ELISA (murine)	BD Biosciences, Franklin Lakes, USA
InviTrap Spin Cell RNA Mini Kit	Stratec Molecular GmbH, Berlin, DE
SuperScript™ II Reverse Transcriptase Kit	Invitrogen, Carlsbad, USA
Universal Probe Library	Roche, Mannheim, DE
Fixable Viability Dye eFluor™ 780	Thermo Scientific, Rockford, USA
Cell Proliferation Dye eFluor™ 450	Thermo Scientific, Rockford, USA
Cell Proliferation Dye eFluor™ 670	Thermo Scientific, Rockford, USA

3.5 Cytokines and stimulants

Recombinant Human IL-2	PeproTech, Rocky Hill, USA
Recombinant Human IL-15	PeproTech, Rocky Hill, USA
Dynabeads Mouse T-Activator CD3-CD28	Life technologies, Munich, DE

3.6 Antibodies

Table 1 | FACS antibodies

Antibody	Reactivity	Clone	Concentration	Manufacturer
Anti-CD3ε-FITC	Murine	500-A2	5 µg/ml	BioLegend
Anti-CD3ε-PE	Murine	145-2C11	5 µg/ml	BioLegend
Anti-CD8a-FITC	Murine	53-6.7	5 µg/ml	BioLegend
Anti-CD8a-Pacific Blue	Murine	53-6.7	5 µg/ml	BioLegend
Anti-EGFR-APC	Human	AY13	20 µg/ml	BioLegend
Anti-IgG-Cy2	Murine	Poly		Jackson Immuno-Research
Anti-IgG-FITC	Human	Poly		Jackson Immuno-Research

Table 2 | Bispecific antibodies

Antibody	Reactivity	Manufacturer
2 + 2 Anti-EpCAM x anti-Cripto	Murine, Human	Roche Innovation Center, Schlieren, CH
2 + 2 Anti-EpCAM x anti-EGFRv3	Murine, Human	Roche Innovation Center, Schlieren, CH
2 + 1 Anti-EpCAM x anti-EGFRv3	Murine, Human	Roche Innovation Center, Schlieren, CH

Table 3 | Unconjugated antibodies

Antibody	Reactivity	Clone	Manufacturer
Anti-CD3ε	Murine	145-2C1	eBioscience, San Diego, USA
Anti-CD28	Murine	37.51	eBioscience, San Diego, USA
Anti-CD178	Murine	MFL3	eBioscience, San Diego, USA
Anti-CD253	Murine	N2B2	eBioscience, San Diego, USA
Anti-EGFR (Cetuximab)	Human	Hu1	Merck KGaA, Darmstadt, DE

3.7 Cell lines, supplements and medium

3.7.1 Cell lines

Table 4 | Cell lines used in this study

Name	Cell type	Species	Medium	Reference
PlatinumE	Human embryonic kidney	Human	PlatE culture medium	Morita, et al. 2000
Panc-OVA-EpCAM	Mouse pancreatic ductal adenocarcinoma (C57BL/6) transduced with murine EpCAM cDNA	Murine	$DMEM^{+++}$	Corbett, et al. 1984
B16-F10-EpCAM	Mouse melanoma (C57BL/6) transduced with murine EpCAM cDNA	Murine	$DMEM^{+++}$	Fidler, 1975
4T1	Malignant neoplasms of the mouse mammary gland (BALB/c)	Murine	$DMEM^{+++}$	Dexter, et al. 1978

3.7.2 Supplements

Blasticidin	Sigma-Aldrich, Steinheim, DE
Dulbecco's modified Eagle's medium (DMEM)	Lonza, Basel, CH
Fetal bovine serum (FBS) - heat inactivated	Gibco, Carlsbad, USA
HEPES (1 M)	Sigma-Aldrich, Steinheim, DE
L-Glutamine (200 mM)	PAA, Pasching, AT
Non-essential amino acids (NEAA)	Gibco, Carlsbad, USA

Penicillin/Streptomycin (100 x)	PAA, Pasching, AT
Phosphate-buffered Saline (PBS)	Lonza, Basel, CH
Puromycin	Sigma-Aldrich, Steinheim, DE
Roswell Park Memory Institute (RPMI) 1640	Lonza, Basel, CH
Sodium pyruvate (100 mM)	Biochrom AG, Berlin, DE

3.7.3 Cell culture media

Complete DMEM medium (DMEM^{+++})

DMEM full medium	500 ml
FBS	10 % (v/v)
Penicillin/Streptomycin (100 x)	1 % (v/v)
L-Glutamine (200 mM)	1 % (v/v)

Complete RPMI medium (RPMI^{+++})

RPMI 1640 full medium	500 ml
FBS	10 % (v/v)
Penicillin/Streptomycin (100 x)	1 % (v/v)
L-Glutamine (200 mM)	1 % (v/v)

T cell medium (TCM)

RPMI^{+++}	500 ml
Sodium pyruvate (10 mM)	1 % (v/v)
HEPES (1 M)	0.1 % (v/v)

PlatE culture medium

DMEM^{+++}	500 ml
Blasticidin	10 µg/ml
Puromycin	1 µg/ml

Cytotoxicity medium

RPMI1640 w/o phenol red	500 ml
FBS	1 % (v/v)
Penicillin/Streptomycin (100 x)	1 % (v/v)
L-Glutamine (200 mM)	1 % (v/v)
Sodium pyruvate (10 mM)	1 % (v/v)
HEPES (1 M)	0.1 % (v/v)

Cryopreservation medium

FBS with cells	900 µl
DMSO	100 µl

3.8 Buffer solutions

Lysis buffer

Ammonium chloride	8.92 g
Potassium bicarbonate	29 mg
EDTA	1 g

fill up to 1000 ml with ddH_2O, adjust pH to 7.2 with HCl (1 M) or NaOH (1 M)

Transfection buffer

Sodium chloride	1.6 g
Potassium chloride	74 mg
Disodium phosphate	50 mg
HEPES	1 mg

fill up to 100 ml with ddH_2O, adjust pH to 7.1 with HCl (1 M) or NaOH (1 M)

FACS buffer

PBS	500 ml
BSA	2 % (v/v)

3.9 Software

Adobe Creative Suite	Adobe Systems, San Jose, USA
BD FACSDiva	BD Biosciences, Franklin Lakes, USA
EndNote X6	Thomson Reuters, Carlsbad, USA
Flow Jo 7.6.2 and 10	Tree Star, Ashland, USA
GraphPad PRISM® Version 5.0 and 7.0	GraphPad Software, La Jolla, USA
LightCycler® 480 SW 1.5	Roche, Mannheim, DE
Microsoft Office 2016	Microsoft, Redmond, USA
RTCA Data Analysis	ACEA Biosciences, San Diego, USA
SnapGene 3.3.4	GSL Biotech LLC, Chicago, USA

4 Methods

4.1 Cell biological methods

4.1.1 General cell culture conditions

All cell lines were cultured in incubators at 37 °C, 5 % CO_2 and 95 % humidity. Cell manipulations were performed under sterile cell culture conditions under a laminar flow hood. To detach adherent cell lines, cells were washed with PBS and incubated with a trypsin-EDTA solution for 5 min at 37 °C. Detached cells were spun down at 400x g for 5 min and resuspended in full growth medium. All cell lines were monitored regularly for their confluency under a light microscope and were divided at 80 % confluence. Neubauer hemocytometers were used to determine number and viability of trypan blue stained cells. Total cell numbers were calculated by using the following formula:

$$\text{Total cell number} = \left(\frac{\text{total cells counted}}{\text{number of squares}}\right) * D_T * V_M * 10^4$$

D_T: Dilution trypan blue

V_M: Volume media

4.1.2 Isolation of murine splenocytes

Wild-type C57BL/6 or BALB/c mice were sacrificed by cervical dislocation under isoflurane anesthesia, spleens were taken into 2 ml Eppendorf tubes containing TCM and kept on ice. For generating single cell suspension of splenocytes, the whole organ was mashed through a 100 µm and 30 µm cell strainer by using the plunger end of a syringe under sterile conditions. The filters were washed with 8 ml TCM and the cell suspension was centrifuged at 400x g for 5 min. Red blood cell lysis was performed by incubating the cell pellet in 2 ml of the described lysis buffer for 2 min. To stop the lysis, 8 ml TCM were added. Cells were centrifuged, resuspended in fresh TCM and the total cell number was adjusted to 2 x 10^6 cells/ml. For T cell differentiation, 10 IU/ml human IL-2, 50 µM ß-mercaptoethanol, 1 µg/ml anti-mouse CD3 and 0.1 µg/ml anti-mouse CD28 were added and cells were cultured in 6-well plates overnight.

4.1.3 Transduction of primary murine T cells

Retroviral transduction is a common technique to genetically modify cells by stable introduction of foreign DNA to the host genome via a retroviral vector. The protocol described here corresponds to a modified version of already published methods (Leisegang et al., 2008).

0.8-1.5 x 10^6 cells of the retroviral producer cell line Platinum-E (PlatE) were cultured in 6-well tissue culture plates in 3 ml $DMEM^{+++}$ 24 h before the transfection to yield a confluence of about 70 %. On the following day, a plasmid solution (18 µg plasmid DNA, 15 µl $CaCl_2$ 2.5 M, filled up to 150 µl with ddH_2O) was prepared and added drop by drop to 150 µl transfection buffer. Strong vortexing of the polystyrene tube containing the transfection buffer lead to enhanced formation of the calcium precipitates and enhanced the transfection efficiency. After 30 min incubation at RT, 300 µl plasmid solution was added to each well of PlatE cells. 5 h later the medium was replaced by fresh, pre-warmed $DMEM^{+++}$ for the virus production. The virus-containing supernatants were collected 43 h later, filtered through a 0.45 µm filter to remove residual cell debris and could be used immediately for the first transduction. 3 ml of fresh TCM were added to the PlatE for further virus production.

To enhance the colocalization of the retrovirus and target cells, tissue culture 24-well plates were coated with 400 µl/well of 6.25 µg/ml RetroNectin solution and incubated overnight at 4°C.
After the RetroNectin-coated wells were blocked with 2 % BSA in ddH_2O for 30 min and washed once with 25 mM HEPES in 1 ml PBS, 1 ml viral supernatant was added to each well. The virus particles were attached to the RetroNectin by centrifugation at 3000x g for 2 h at 4 °C. 10^6 primary murine T cells in 1 ml TCM supplemented with 10 U/ml recombinant human IL-2, 50 µM ß-mercaptoethanol and 4 x 10^5 sodium acid-free mouse T cell activator beads/ml were co-incubated with the virus overnight. A second transduction hit was performed on the next day. The transduction efficiency was determined by quantification of the vector-encoded surface proteins by flow cytometry using the appropriate FACS antibodies.

T cells were adjusted to 10^6 cells/ml in fresh TCM containing 50 ng/ml human IL-15 and 50 µM ß-mercaptoethanol for expansion and re-cultured every 48 h. Transduction efficiencies of > 20 % and > 30 % were used for *in vitro* and *in vivo* assays, respectively.

4.2 Molecular biological methods

4.2.1 Generation of synthetic agonistic receptors by overlap extension polymerase chain reaction

The extension of overlapping gene segments by PCR is an efficient and reliable method to generate chimeric genes or site-directed mutagenesis.

The murine EGFRv3 – CD28 –CD3ζ molecule (E3) consists of human EGFRv3 (Uniprot Entry P00533 AA 1-29, 298-646), murine CD28 (Uniprot Entry P31041 AA 151-218) and murine CD3ζ (Uniprot Entry P24161 AA 52-164). The Cripto 1-CD28-CD3ζ construct (C3) consists of human Cripto 1 (Uniprot Entry P13385 amino acids (AA) 31-150), murine CD8a (Uniprot Entry P01731 AA 153-196), murine CD28 (AA 178-218) and murine CD3ζ (AA 52-164). Both SAR constructs were generated and provided by Mathias Kurzay.

For the E3-GFP construct, E3 was coupled through a self-cleaving 2A peptide (Ryan et al., 1991) to GFP (Uniprot Entry P42212). The E3 variants were generated from E3 by point mutations as follows: mutation of YMNM (AA 189-192) to FMNM (E3-FMNM), mutation of PYAP (AA 206-209) to AYAA (E3-AYAA) and the double mutant E3-FMNM-AYAA.

All constructs were cloned into the retroviral vector pMP71 (Engels et al., 2003).

4.2.2 Quantitative real-time PCR (qRT PCR)

Total RNA was extracted from stimulated cells using the InviTrap Spin Universal RNA extraction kit and cDNA was synthesized from 2-5 µg of RNA using the Superscript II kit according to the manufacturer's instructions. PCR primers were designed automatically from the NCBI GenBank sequence NM_001146708.1 (murine CD95) in the assay design center from the Roche Universal Probe Library. The oligonucleotides used as PCR primers were: CD95 (left) 5'-GCA GAC ATG CTG TGG ATC TG-3' and CD95 (right) 5'-TCG GAG ATG CTA TTA GTA CCT TGA G-3'. Real-time PCR was performed using the Kapa Probe Universal Master Mix and probe 34 for CD95 in a LightCycler 480 instrument.

4.3 Immunological methods

4.3.1 Enzyme-linked immunosorbent assay (ELISA)

To determine the T cell activation and degranulation, several murine ELISA kits were used to measure the cytokine concentrations in the cell supernatant. Samples were collected from culture plates and stored at -20 °C prior to analysis. ELISA and buffer preparation were performed according to the manufacturer's protocol except for using only half of the indicated volumes.
Absorbance at 450 nm was measured using a microplate reader (Berthold Mithras LB940). A four-parameter logistic (4PL) regression was used to calculate protein concentrations from the standard curve.

4.3.2 LDH release assay

The LDH release assay is a standard method to measure effector cell-mediated target cell killing. Upon cell lysis, high levels of lactate dehydrogenase (LDH) are released into the cell supernatant which can be measured in a coupled enzymatic assay.

T cells were co-cultured with target cells and indicated antibodies for 8-16 h in cytotoxicity medium without phenol red. Maximum release of LDH was determined by complete target cell lysis with the manufacture's lysis solution. Supernatants were collected, centrifuged and analyzed immediately according to manufacturer's protocol. Absorbance at 490 nm was measured using a microplate reader (Berthold Mithras LB940). Specific cell lysis was calculated by the following formula:

$$(LDH^{\text{of interest}} - LDH^{\text{effector only}}) / (LDH^{\text{total lysis}} - LDH^{\text{minimal lysis}}) \times 100\ \%$$

Overall negative lysis values indicate a superior tumor cell outgrowth compared to the tumor cell maximum control.

4.3.3 Real-time killing assay

In contrast to the LDH release endpoint assay, target cell killing over time was measured with the impedance-based iCELLigence device. Here, the iCELLigence system detects the cellular events in real-time by measuring electrical impedance using microelectrodes at the bottom of each cell culture

plate well. This relative change in the electrical impedance at a defined frequency (ƒn) is termed the Cell Index (CI).

Target cells were seeded in an 8-well plate and monitored over the time frame of 20 h every 20 min. When reaching a CI between 0.8 and 1.5, effector cells and indicated antibodies were added and target cell lysis was quantified for 20 h every 6 min, followed by intervals of 15 min over a period of 45 h. All the described calculations are based on the RTCA Software version 1.

4.3.4 Flow cytometry (FACS)

Flow cytometry allows a quantification of cell according to their size, granularity and expression of surface and intracellular proteins based on staining with appropriate fluorophore-conjugated antibodies. For saturation curve analysis T cells were incubated with increasing concentrations of either the tetra- or trivalent BiAb for 30 min at 37 °C. Cells were washed with PBS and stained with the corresponding secondary antibody for 30 min at 4 °C. After washing, cells were resuspended in FACS buffer and analyzed. For proliferation analysis, cells were stained with Fixable Viability Dye and fluorophore-conjugated antibodies for CD8a, CD3ε and EGFR for 30 min at 4 °C. Cells were washed and resuspended in FACS buffer containing 1.5 µl count bright absolute counting beads. Samples were analyzed with a BD FACS Canto II or LRSFortessa, for data analysis FlowJo 7.6.2 or 10 was used.

4.4 Animal experiments

4.4.1 Care of laboratory animals

For animal experiments, four weeks old wild type C57BL/6 and BALB/c mice were purchased from Janvier (St. Bertevin, France) or Charles River (Sulzfeld, Germany). The spleens from the transgenic mouse strain B6Smn.C3-Fasl$^{<gld>}$/J were purchased from The Jackson Laboratory (Bar Harbor, USA). The perforin deficient spleens from C57BL/6-Prf1^{tm1Sdz} mice were provided by J. vom Berg (Zurich, Switzerland) and the granzyme B knock-out splenocytes were a gift from J. Pardo (Zaragoza, Spain). All mice were kept under germ-free conditions in the animal facility “Zentrale Versuchstierhaltung Innenstadt” at least one week prior to the experiment. All animal experiments were approved by the local regulatory agency (Regierung von Oberbayern, reference number: 55.2.1.54-2532-90-12, 36-14, 183-12 and 135-17) and adhered to the NIH guide for the care and use of laboratory animals.

4.4.2 *In vivo* subcutaneous co-injection of tumor cells and T cells

For co-injection E3 SAR T cells, BiAb and 2 x 10^6 Panc02-OVA-EpCAM, 5 x 10^4 B16-EpCAM or 1.25 x 10^5 4T1 tumor cells were preincubated in an effector to target ratio of 10:1 with 1 µg/ml BiAb for 30 min. C57BL/6 or BALB/c female mice (6 – 10 week old) were injected subcutaneously into the right flank. For re-challenge experiments, mice were injected subcutaneously with corresponding numbers of tumor cells in the right flank. Tumor size was measured blinded to the treatment groups three times a week and was defined as area in mm^2 (length in mm x width in mm). Scoring every other day included also tumor morphology, body weight, body condition and general behavior. Animals reaching endpoint criteria were anesthetized with CO_2 inhalation and sacrificed by cervical dislocation.

4.4.3 *In vivo* depletion of transferred T cells

C57BL/6 female mice were injected intravenously with 10^7 E3-GFP SAR T cells and were randomized after injection into two groups (n = 5). After 6 h, animals received either 1 mg cetuximab in 100 µl of PBS or vehicle solution intravenously. 24 h after T cell transfer, mice were anesthetized by CO_2 inhalation and sacrificed via cervical dislocation. Blood, spleens, lymph nodes and lungs were harvested, processed and analyzed for remaining transferred T cells via flow cytometry.

4.5 Statistical analysis

Statistical analysis was performed by using GraphPad Prism software 5.0 or 7.0. Data is shown as arithmetic mean values and standard error of the mean of at least three biological replicates or independent experiments, unless otherwise indicated. Differences between experimental conditions were analyzed using the unpaired two-tailed Student's t test. In all *in vivo* experiments, two-way ANOVA with correction for multiple testing by the Bonferroni method was used to analyze differences between the groups. Survival analysis was performed using the Log-Rank test. P-values < 0.05 were considered to be significant.

5 Results

5.1 Specific antigen-dependent activation of SAR T cells is dependent on the BiAb valency

The E3 SAR, composed of the extracellular portion of human epidermal growth factor receptor variant III (EGFRv3) and the transmembrane and intracellular domains of murine CD28 and CD3ζ was generated by overlap extension PCR and kindly provided by Mathias Kurzay. A control E3^{del} SAR, lacking the intracellular T cell activating domains, was also created. The receptors were transduced into primary murine T cells by retroviral transduction (Figure 3a and 3b).

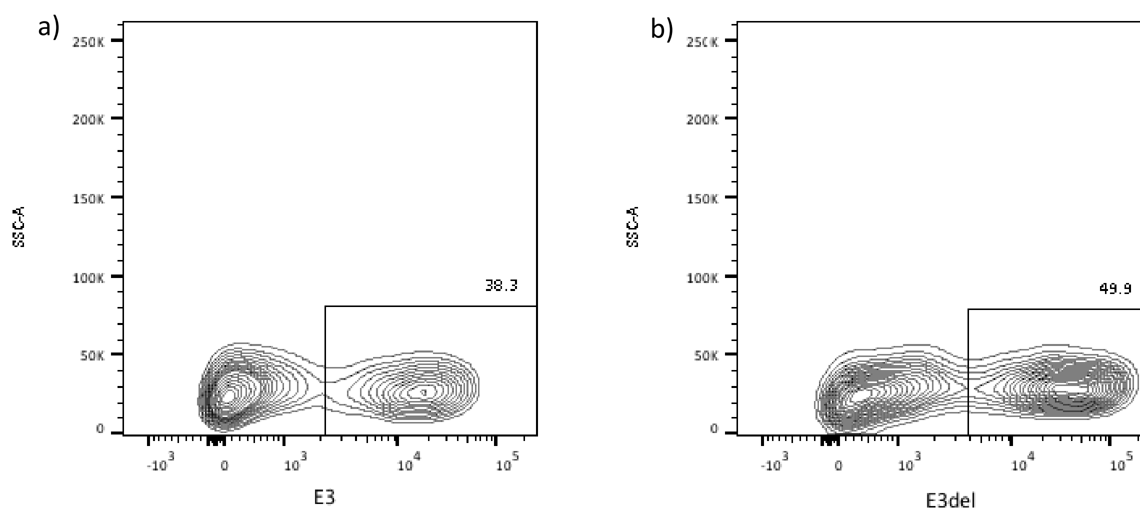

Figure 3│ Retroviral transduction of murine CD8^{+} T cells.

Primary murine T cells were differentiated from isolated splenocytes and genetically modified using retroviral transduction. T cells were engineered with **a**│ the E3 SAR or **b**│ the non-signaling form E3^{del}. Transduction efficiencies were examined by flow cytometry analysis after surface staining with an anti-hEGFR-APC antibody. One representative plot of over fifty independent transductions is depicted.

Previous data from our group have shown that E3 SAR T cells can be specifically activated through engagement of an immobilized BiAb, either plate-bound or linked to epithelial cell adhesion molecule (EpCAM) expressing tumor cells (Schmidbauer, 2018). However, the BiAb used was bivalent for both the SAR and the target antigen murine EpCAM and could therefore induce unspecific activation of the E3 SAR T cells by crosslinking the receptor itself in the absence of tumor cells (Schmidbauer, 2018). To overcome off-target T cell activation, a trivalent BiAb was generated with only one binding moiety for EGFRv3. These two different BiAb formats were compared head-to-head for their specific and antigen dependent SAR T cell activation (Figure 4a).

Both BiAbs induced secretion of the immunostimulatory cytokine IFN-γ when immobilized on the plate, indicating strong T cell activation through the E3 SAR. When added in soluble form to E3 SAR T cells, only the BiAb bivalent for the SAR could trigger T cell activation whereas the monovalent BiAb did not (Figure 4b and 4c). This clearly indicates that, unlike the format monovalent for SAR, the SAR-bivalent BiAb is able to crosslink receptors on T cells on its own and thereby induce unspecific E3 SAR T cell activation.

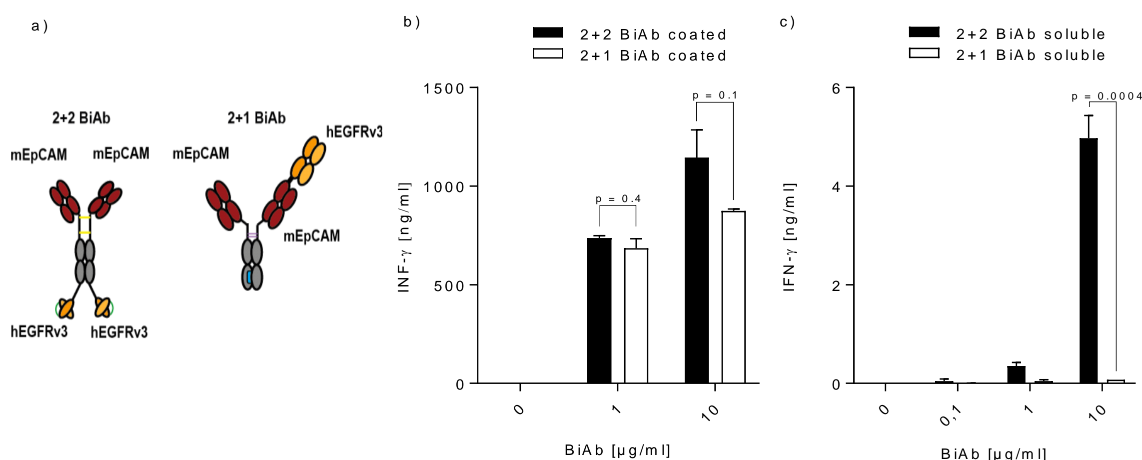

Figure 4│ Specific SAR T cell activation is dependent on bispecific antibody valency.

a│ Schematic presentation of both 2 + 2 and 2 + 1 bispecific antibodies. **b│** Tissue culture plates were pre-coated with indicated concentrations of either 2 + 2 BiAb or 2 + 1 BiAb at 4 °C overnight. 1.25 x 10^5 E3 SAR T cells were added to each condition. **c│** In the control experiment, the same concentrations of soluble antibodies were added prior to culture. Supernatants were analyzed for IFN-γ after 48 h of incubation to determine T cell activation. Unspecific E3 SAR T cell activation by soluble antibodies occurred only with the 2 + 2 BiAb whereas soluble 2 + 1 BiAb could not trigger IFN-γ due to the loss of one binding site for the SAR. All graphs show mean values of experiments performed at least in triplicates. One representative of three independent experiments is depicted. p-values by two-sided unpaired t-test are indicated. $p < 0.05$ was considered statistically significant.

Similarly, in a co-culture with target antigen-negative ($EpCAM^-$) tumor cells, unspecific off-target lysis (Figure 5a) occurred only with the bivalent BiAb. In contrast, in a co-culture with target antigen positive ($EpCAM^+$) tumor cells, on-target lysis of $EpCAM^+$ tumor cells (Figure 5b) was not altered by the type of BiAb used.

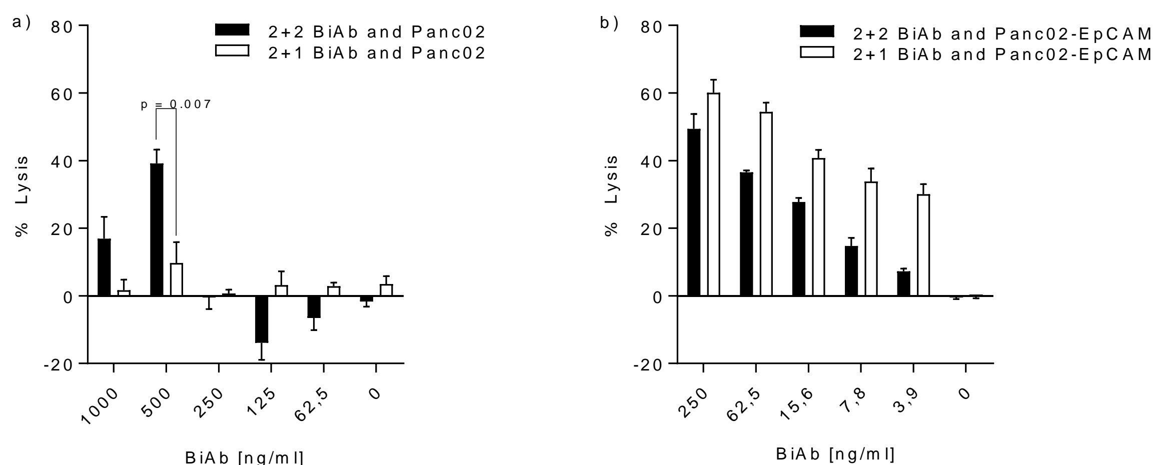

Figure 5 | 2 + 2 BiAb can mediate unspecific lysis of antigen negative tumor cells.

a | 2 x 10^4 Panc02 or **b** | Panc02-EpCAM tumor cells were cocultured with 10^5 E3 SAR T cells. T cells were incubated with decreasing amounts of either 2 + 2 or 2 + 1 BiAb as indicated. After 10-14 h supernatants were collected and LDH levels were measured. Antigen independent lysis of target cells was only achieved when E3 SAR T cells were preloaded with the 2 + 2 BiAb. Both antibody formats showed dose-dependent killing of target antigen-positive tumor cells with similar efficiency. All graphs show mean values of experiments performed at least in triplicates. One representative of three independent experiments is depicted. p-values by two-sided unpaired t-test are indicated. $p < 0.05$ was considered statistically significant.

There findings were comparable for two additionally tested EpCAM-positive cell lines (Figure 6a and 6b).

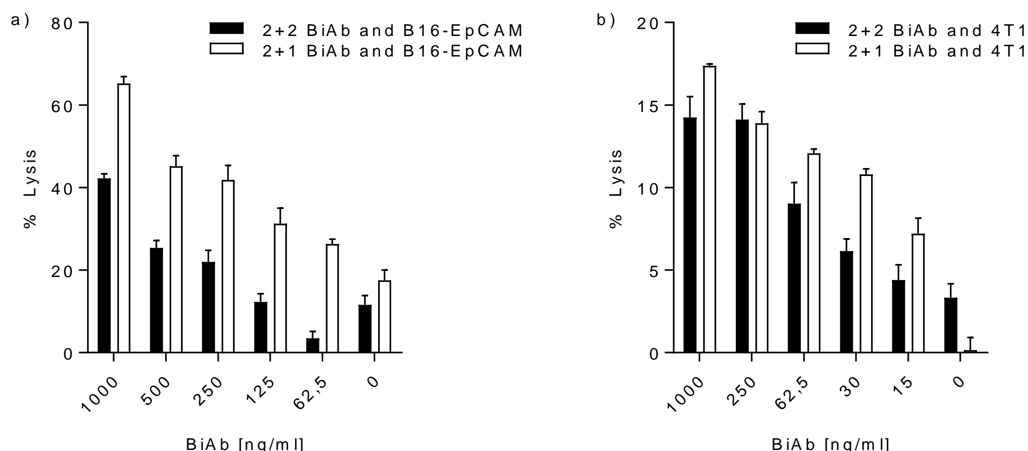

Figure 6 | 2 + 2 and 2 + 1 BiAb can mediate equivalent specific lysis of antigen-expressing tumor cells.

a | 2 x 10^4 B16-EpCAM or **b** | 4T1 EpCAM-expressing tumor cells were cocultured with 10^5 E3 SAR T cells. T cells were incubated with decreasing amounts of either 2 + 2 or 2 + 1 BiAb as indicated. After 10-14 h supernatants were collected and LDH levels were measured. Both antibody formats showed dose-dependent killing of target antigen-positive tumor cells with similar efficiency. All graphs show mean values of experiments performed at least in triplicates. One representative of three independent experiments is depicted.

Furthermore, the levels of T cell degranulation, measured by GzmB release, correlated with the off-target lysis when using the bivalent BiAb on $EpCAM^-$ target cells (Figure 7a) while degranulation was similar for both antibody formats on $EpCAM^+$ target cells (Figure 7b). Therefore, in all following experiments, 2 + 1 BiAb was used unless otherwise indicated.

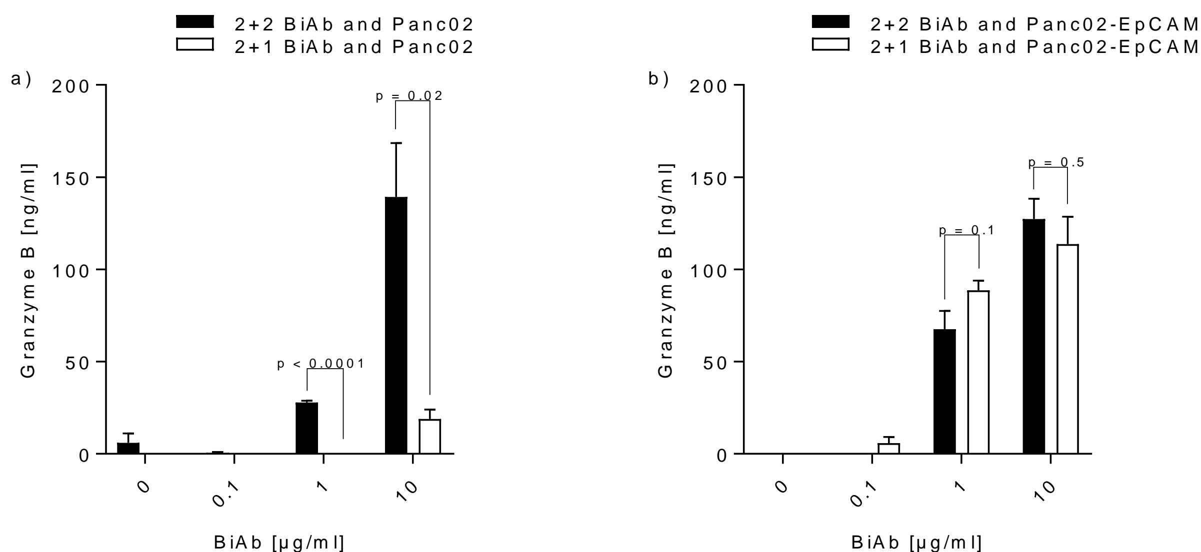

Figure 7│ Antibody bivalent for SAR can trigger unspecific T cell degranulation.

a│ 2 x 10^4 Panc02 or **b**│ Panc02-EpCAM tumor cells were cocultured with 10^5 E3 SAR T cells. T cells were incubated with decreasing amounts of either 2 + 2 or 2 + 1 BiAb as indicated. Only T cells incubated with the 2 + 2 BiAb showed unspecific granzyme B release in coculture with antigen-negative tumor cells while both antibody formats showed similar degranulation with antigen-positive tumor cells. All graphs show mean values of experiments performed at least in triplicates. One representative of **a**│ three or **b**│ two independent experiments is depicted. p-values by two-sided unpaired t-test are indicated. $p < 0.05$ was considered statistically significant.

5.2 Tumor cell killing by SAR T cells is predominantly mediated by Fas-FasL interactions

Next, we investigated the mode of action underlying the killing of antigen-positive tumor cells by SAR T cells triggered through the 2 + 1 BiAb. Our group previously showed that E3 SAR T cells deficient for perforin are still capable of inducing complete target cell lysis similar to wild-type E3 SAR T cells (Schmidbauer, 2018). To test whether target cell lysis was also independent of granzyme B release by the E3 SAR T cells, a cytotoxicity assay was performed with granzyme B knock-out T cells. Unexpectedly, $GzmB^{-/-}$ E3 SAR T cells also revealed a complete and durable lysis of target cells only in combination with BiAb (Figure 8a and 8b).

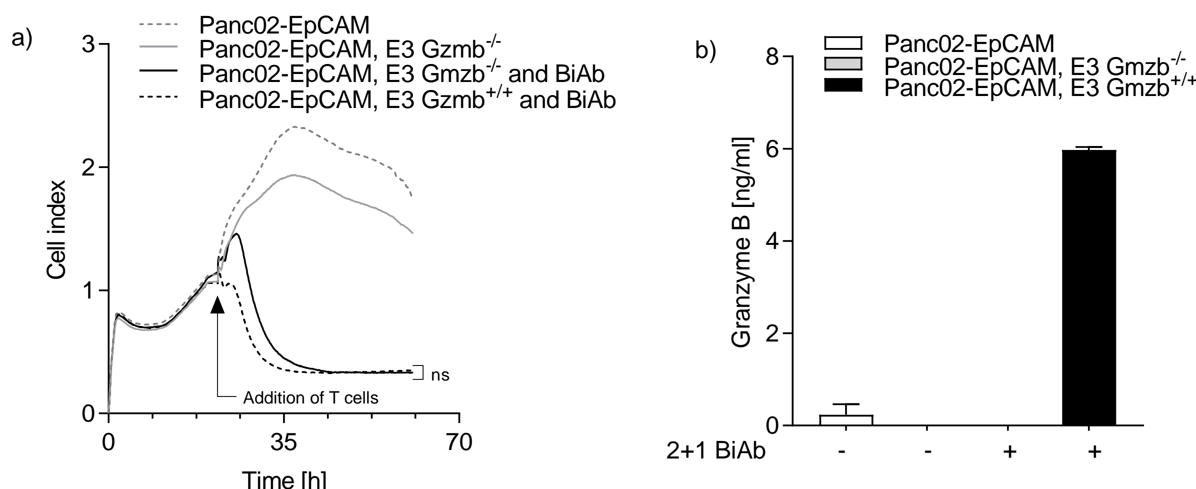

Figure 8 | Tumor cell lysis by E3 SAR T cells is independent of granzyme B release.

a | Panc02-EpCAM tumor cells were seeded in an impedance-quantifying device (iCELLigence) at a density of 5 x 10^4 cells per well and 5 x 10^5 $GzmB^{+/+}$ or $GzmB^{-/-}$ E3 SAR T cells preloaded with 1 µg/ml 2 + 1 BiAb were added as indicated. Target cells were only killed in the presence of E3 SAR T cells and BiAb and independently of granzyme B expression. **b** | Granzyme B competence of T cell was verified by Elisa. T cells deficient for granzyme B showed no release of the serine protease. All graphs show mean values of experiments performed at least in triplicates. Impedance-based cytotoxicity assays were performed in duplicates for technical reasons. One representative of three independent experiments is depicted. p-values by two-sided unpaired t-test are indicated. For impedance-based cytotoxicity assays the total curve over time was compared. $p < 0.05$ was considered statistically significant.

This indicates a minor contribution of perforin (Schmidbauer, 2018) and granzyme B in the target cell lysis by SAR T cells. To further dissect the mechanism of effective tumor cell killing by SAR T cells, we assessed the role of other molecules expressed by T cells which can potentially induce tumor cell lysis: Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL).

Upon addition of blocking antibodies specific for these surface molecules to a co-culture with antigen-positive tumor cells and E3 SAR T cells in the presence of 2 + 1 BiAb, we identified FasL to predominantly mediate tumor cell lysis (Figure 9a, 9b and 9c).

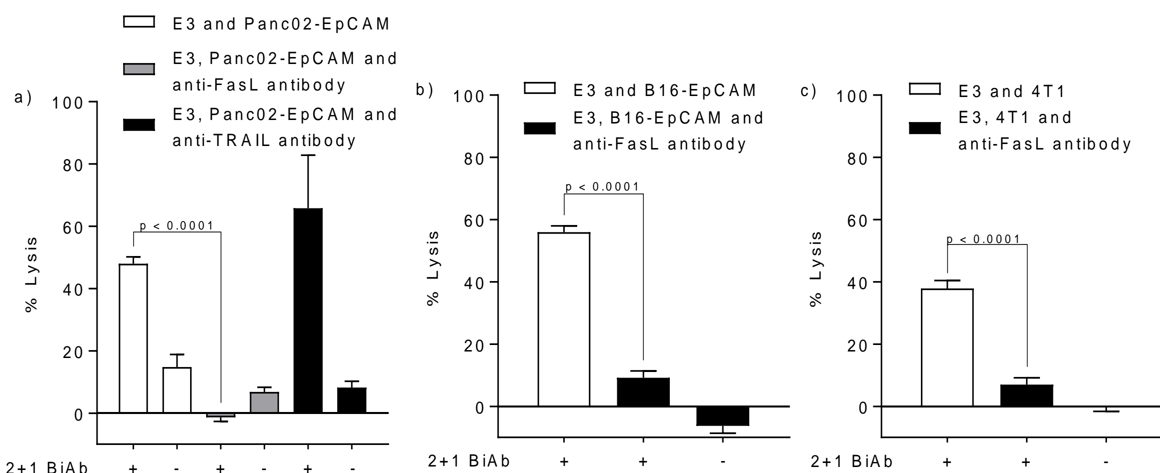

Figure 9│ Target cell lysis by E3 SAR T cells is predominantly mediated by Fas-FasL interactions.

E3 SAR T cells were co-cultured with **a│** Panc02-EpCAM tumor cells in addition of antibodies to block T cell-induced tumor cell apoptosis (anti-CD178 [anti-FasL] or anti-CD253 [anti-TRAIL]; each at 10 µg/ml). T cells were incubated with 1 µg/ml 2 + 1 BiAb prior to culture. Blocking of FasL significantly reduced lysis of tumor cells to comparable values of the control condition without BiAb measured by LDH release. In contrast, blocking of TRAIL did not alter tumor cell killing by E3 SAR T cells. Dependency on FasL-Fas interaction in tumor cell killing by SAR T cells were verified in two other tumor cell models: **b│** B16-EpCAM and **c│** 4T1. All graphs show mean values of experiments performed at least in triplicates. One representative of **a**│, **b│** three or **c│** two independent experiments is depicted. p-values by two-sided unpaired t-test are indicated. $p < 0.05$ was considered statistically significant.

To verify if the impact of FasL on tumor cell killing is shared by the therapeutic platform of SAR T cells, a previously generated SAR composed of the human Cripto-1 protein fused to the murine CD28 and CD3ζ domains (C3) with the respective BiAb was used. As observed for the E3 SAR T cells the lytic capacity of C3 SAR T cell was blocked by the addition of anti-FasL antibodies (Figure 10).

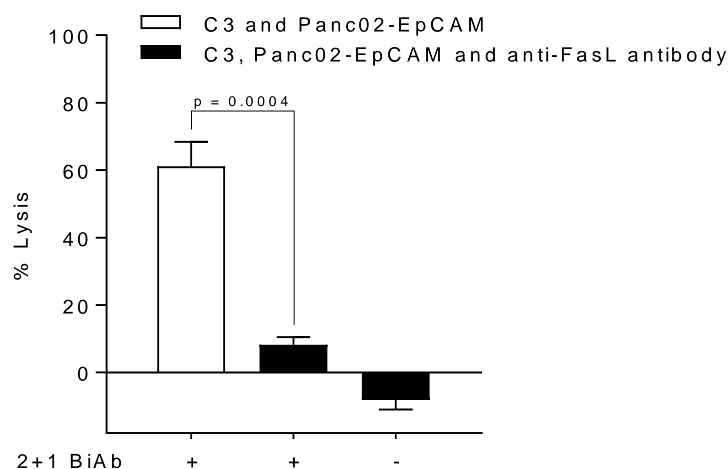

Figure 10│ Impact of FasL-dependent tumor cell killing is shared by the therapeutic platform of SAR T cells.

C3 SAR T cells were co-cultured with Panc02-EpCAM tumor cells in the presence of 10 µg/ml anti-FasL antibody. T cells were incubated with 1 µg/ml of the respective 2 + 2 BiAb prior to culture. C3 SAR T cell-mediated killing was measured by LDH release. As observed before, blocking of FasL significantly reduced lysis of tumor cells to baseline values in absence of BiAb. Graph shows mean values of experiments performed in quadruplicates. One representative of three independent experiments is depicted. p-values by two-sided unpaired t-test are indicated. $p < 0.05$ was considered statistically significant.

In contrast to these findings, T cells transduced with an EpCAM-specific CAR with identical intracellular signaling domains mainly utilized perforin-dependent degranulation for effective target cell lysis (Figure 11a). However, inhibition of the Fas-FasL axis did not alter the cytolytic function of CAR T cells (Figure 11b).

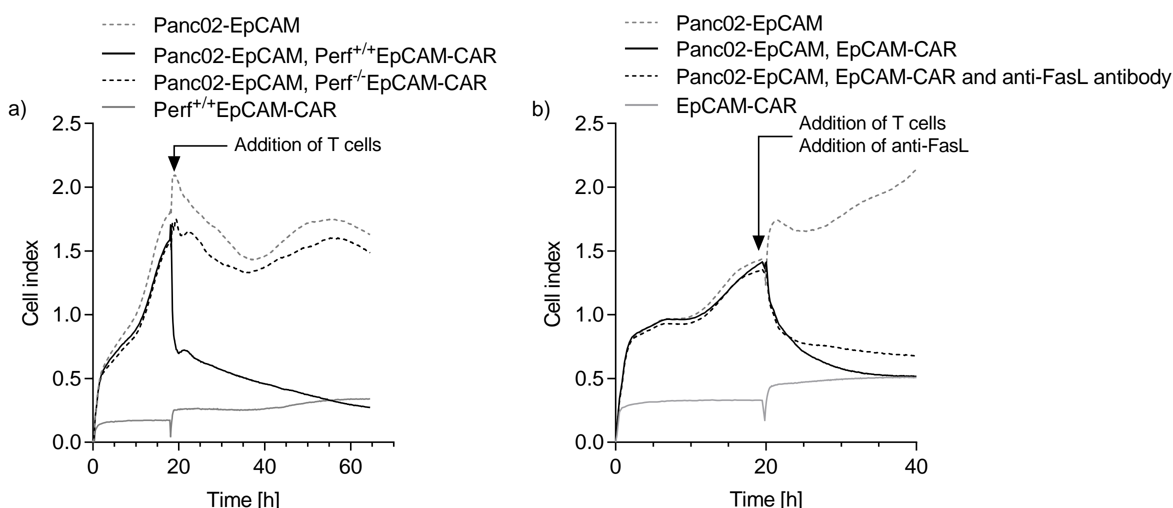

Figure 11 | EpCAM-specific CAR T cells rely on the release of perforin for efficient killing.

Panc02-EpCAM tumor cells were seeded in an impedance-quantifying device (iCELLigence) at a density of 5 x 10^4 cells per well and 5 x 10^5 Prf1$^{+/+}$ or Prf1$^{-/-}$ anti-EpCAM CAR T cells were added as indicated. **a**| Wild-type anti-EpCAM CAR T cells mediated immediate and complete tumor cell lysis whereas killing by perforin-deficient anti-EpCAM CAR T cells was abolished. **b**| Addition of anti-FasL antibody to a co-culture of Panc02-EpCAM tumor cells with anti-EpCAM CAR T cells did not affect target cell lysis compared to the non – blocking condition. All graphs show mean values of experiment performed in duplicates for technical reasons. One representative of **a**| two or **b**| three independent experiments is depicted.

In addition, an upregulation of Fas expression upon IFN-γ stimulation could be detected on all tumor cell lines tested indicating a high susceptibility of the tumor cells to SAR T cell treatment (Figure 12).

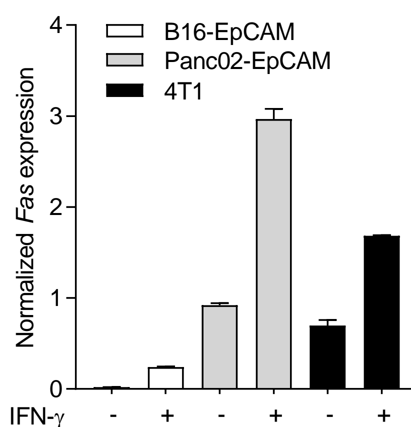

Figure 12| Transcriptional regulation of Fas on tumor cells under inflammatory conditions.

Panc02-EpCAM, B16-EpCAM and 4T1 tumor cells were seeded to a density of 2 x 10^4 and stimulated with 100 U/ml recombinant murine IFN-γ. Gene expression levels of CD95 (*Fas*) were analyzed by qRT PCR and normalized to the expression of the housekeeping gene *Hprt*. Upon stimulation with IFN-γ, all tumor cell lines show an upregulation of *Fas* expression. All graphs show mean values of experiment performed in duplicates for technical reasons. One representative of three independent experiments is depicted.

5.3 The co-stimulatory domain CD28 does not influence the mode of action but enhances T cell activation and proliferation

We further investigated the necessity of the co-stimulatory domain CD28. For this purpose, SAR using only CD3ζ for signaling (referred from now on as E2) and the respective first generation anti-EpCAM CAR were generated. The loss of the CD28 domain did not influence the function and the FasL-dependent killing of the E2 SAR (Figure 13a and 13b). Target cell killing was comparable for E3 SAR and E2 SAR T cells but significantly reduced upon adding anti-FasL antibody. The lytic capacity of the 1st generation and 2nd generation anti-EpCAM CAR was not altered under blocking conditions.

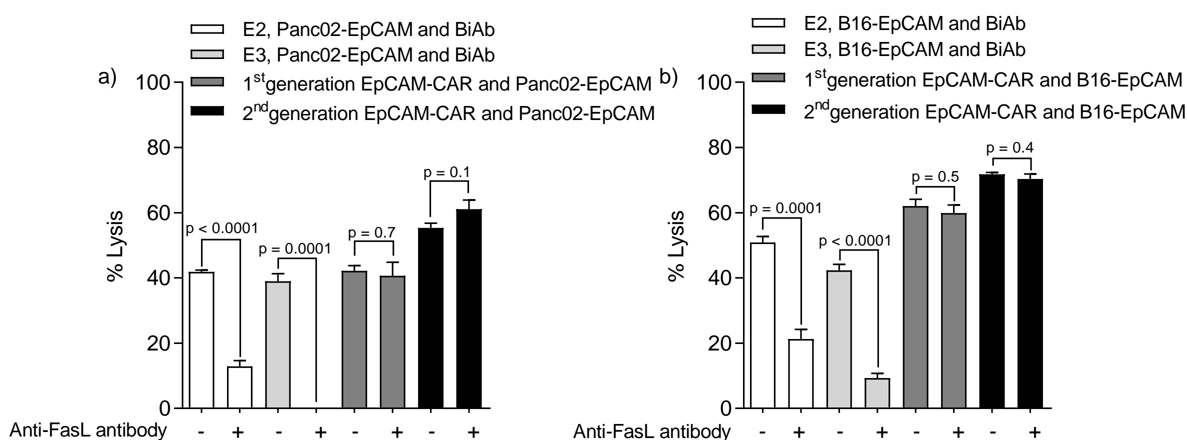

Figure 13│FasL-mediated target cell killing by SAR T cells does not require the co-stimulatory CD28 domain.

a│ Panc02-EpCAM or **b│** B16-EpCAM tumor cells were cocultured with either E3 SAR, E2 SAR, 1st generation anti-EpCAM CAR or 2nd generation anti-EpCAM CAR T cells for 14 h in an effector to target ratio of 5 : 1. Inhibition of FasL reduced the target cell lysis by E2 SAR or E3 SAR T cells whereas the function of the T cells transduced with either of the two CAR constructs was not altered. Cytotoxicity was determined by LDH release. All graphs show mean values of experiments performed at least in triplicates. One representative of three independent experiments is depicted. p-values by two-sided unpaired t-test are indicated. $p < 0.05$ was considered statistically significant.

To demonstrate the impact of the CD28 co-stimulatory domain, which is associated with higher T cell activation and proliferation, we generated two distinct point mutations within the intracellular signaling part of CD28 in the E3 SAR. Here, the YMNM motif is needed for cytokine secretion upon activation. The second motif PYAP is required for both cytokine production and cell proliferation (Boomer et al., 2010).

The single mutant receptors E3-FMNM and E3-AYAA, as well as the double mutant construct E3-FMNM-AYAA showed significant reduction in both T cell activation (Figure 14a) and T cell proliferation (Figure 14b) compared to the unmutated E3 SAR when stimulated with immobilized anti-EGFR antibody. These results highlight the synergistic function of the CD28 domain in transduced T cells.

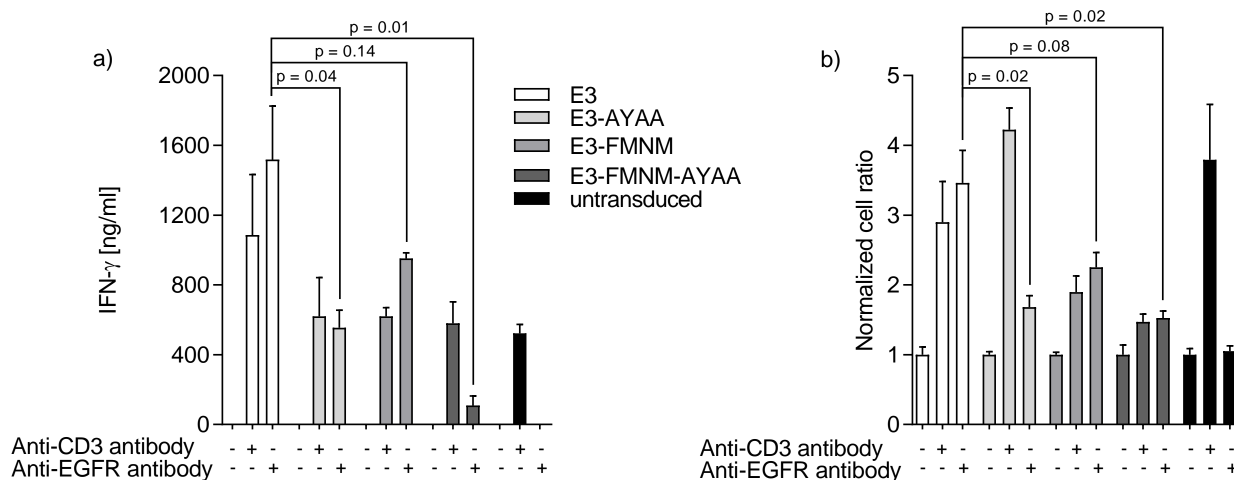

Figure 14│ Functional comparison of different E3 SAR with different CD28 signaling moieties.

E3 SAR, E3-tyrosine-mutated SAR (E3-FMNM), E3-proline-mutated SAR (E3-AYAA) and E3-tyrosine- and proline-mutated SAR (E3-FMNM-AYAA) or untransduced T cells were cultured on plates pre-coated with anti-CD3 antibody or with the anti-EGFR antibody cetuximab for 48 h. **a│** T cell activation was determined by IFN-γ secretion measured by ELISA. T cells with fully functional E3 SAR secreted higher levels of IFN-γ than any of the mutated versions or the untransduced control. **b│** T cell proliferation was determined by cell counting. The number of viable cells normalized to counting beads showed the highest proliferation capacity of E3 SAR T cells compared to the mutated constructs and to untransduced T cells. All graphs show mean values of experiments performed at least in triplicates. One representative of three independent experiments is depicted. p-values by two-sided unpaired t-test are indicated. $p < 0.05$ was considered statistically significant.

5.4 SAR T cells co-administered with BiAb inhibit tumor cell outgrowth *in vivo* and induce an immunological memory

To determine the *in vivo* efficacy of E3 SAR T cells in combination with BiAb C57BL/6 mice were co-injected with Panc02-EpCAM or B16-EpCAM tumor cells and either E3 SAR T cells or BiAb or both. In both tumor models, only the combination of E3 SAR T cells with BiAb prevented tumor outgrowth and prolonged survival of the cohort (Schmidbauer, 2018). To demonstrate that this effect is also relevant for an endogenous EpCAM-expressing cell line, BALB/c mice were injected with 4T1 in combination with E3 SAR T cells and BiAb or treated with monotherapy lacking either the SAR T cells

or BiAb. Again, tumor outgrowth was significantly delayed (Figure 15a) and the full treatment cohort showed a survival benefit and enhanced tumor elimination (Figure 15b).

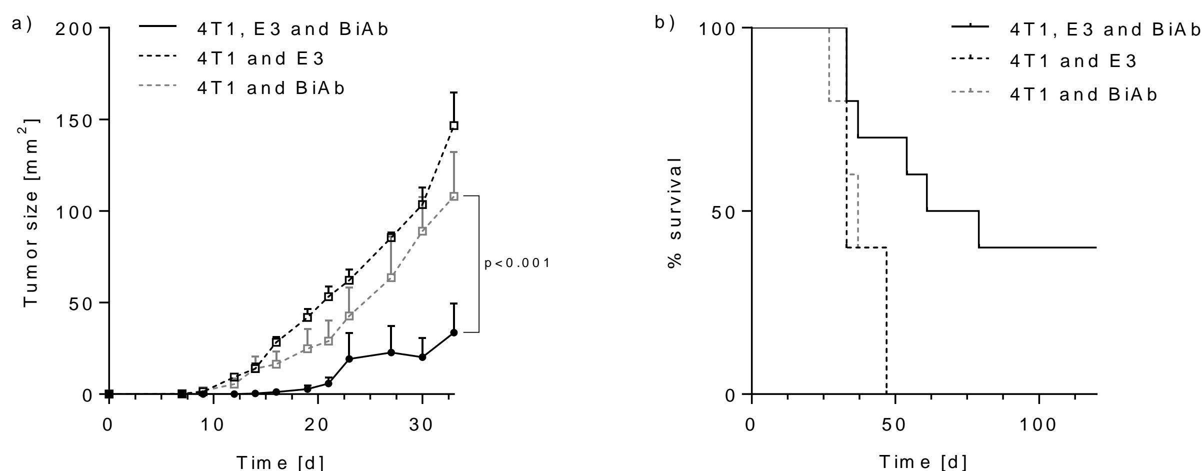

Figure 15│ Combinational therapy of E3 SAR T cells and bispecific antibody prevented mice from tumor outgrowth and prolonged survival in 4T1 tumor model.

1.25 x 10^5 4T1 tumor cells were injected in the right flank of BALB/c mice. Five mice received a co-injection with E3 SAR T cells (effector to target ratio of 10:1), 5 mice received a co-injection with 2 + 2 BiAb (1 µg) and 10 mice received a combination of both. Only the combination therapy of SAR T cells and 2 + 2 BiAb **a**│ delayed or prevented tumor growth (mean values and SEM of tumor sizes are shown) and **b**│ prolonged survival**.** The experiment shown is representative of two independent experiments. Analysis of differences between groups for the tumor growth curves were performed using two-way ANOVA with correction for multiple testing by the Bonferroni method. $p < 0.05$ was considered as statistically significant.

To study the impact of the therapy on the induction of an immunological memory, the co-injection *in vivo* studies with either B16-EpCAM or Panc02-EpCAM tumor cells, already performed by Moritz Schmidbauer, were repeated and the obtained results could be verified. After 60 days of tumor free survival, these mice were re-challenged with a single injection of corresponding tumor cells at the same time as untreated control mice. Here, the combination therapy of E3 SAR T cells and BiAb induced an antigen-specific immunological memory, which protected the majority of the re-challenged mice from tumor recurrence (Figure 16a and 16b).

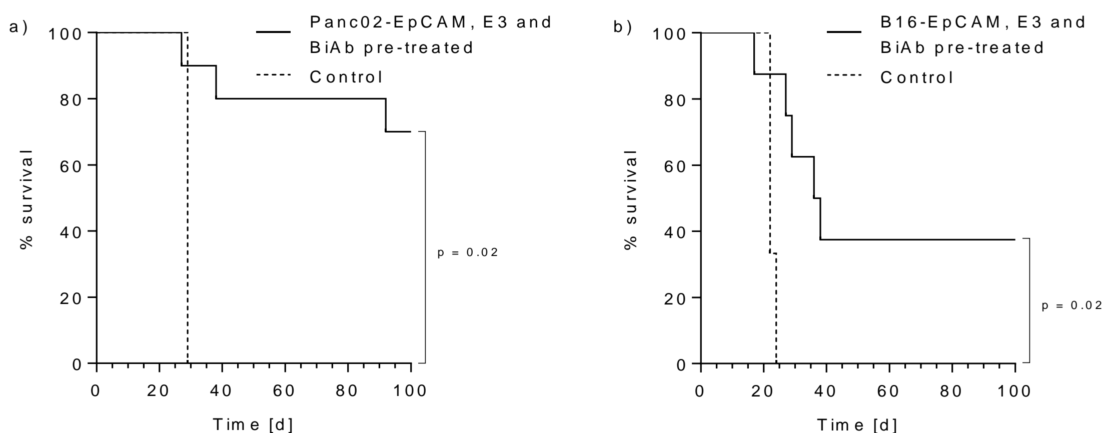

Figure 16│ Combinational therapy of E3 SAR T cells and BiAb-induced immunological memory in pre-treated mice.

Tumor free mice from previous the experiment were re-challenged with **a**│ 2 x 10^6 Panc02-EpCAM (n=10, control n = 2) or **b**│ 2 x 10^4 B16-EpCAM (n=8, control n = 3) by subcutaneous injection into the right flank. Animals, pre-treated with the combination therapy, showed prolonged survival compared to untreated control mice, indicating an immunological memory**.** The experiment shown is representative of **a**│ two or **b**│ one independent experiments. Statistical evaluation of the survival analysis was performed using the log-rank test. $p < 0.05$ was considered as statistically significant.

5.5 Selective and reversible activation of E3 SAR T cells depends on the abundance of BiAb

For translation into clinical studies, a validated safety profile of the therapy is crucial to avoid potentially lethal side effects. Upon occurrence of therapeutic-related toxicities, it is preferred to tune down T cell activity rather than depleting the transferred cells. In contrast to CAR T cell therapy, the combination of SAR T cells with BiAb incorporates already an intrinsic safety switch as T cell activity should resolve with the elimination of the BiAb. To further investigate this aspect, SAR T cells were repetitively co-cultured with tumor cells without readjustment of the BiAb concentration. IFN-γ levels decreased rapidly after BiAb dilution following several wash steps. In contrast, redosing of BiAb could rescue the anti-tumoral activity of E3 SAR T cells comparable to the level of anti-EpCAM CAR T cell (Figure 17). Therefore, the half-life of the BiAb provides a fast and durable shut down of T cell activity if needed.

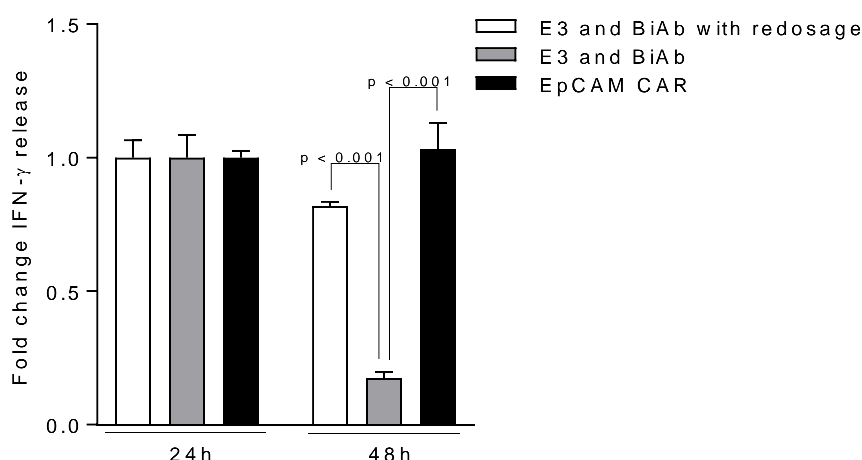

Figure 17 | Activation of E3 SAR T cells is dependent on presence of the BiAb.

2 x 10^4 Panc02-EpCAM tumor cells were cocultured with either 2 x 10^5 anti-EpCAM CAR or E3 T SAR cells and direct addition of 5 µg/ml BiAb. After 24 h supernatants were collected, T cells were washed with medium and transferred into a new plate of seeded tumor cells. For the redosage of the BiAb, 5 µg/ml BiAb was added to the respective condition. Supernatants were harvested after an additional 24 h and remaining T cell activation was determined by IFN-γ release. Readjustment of BiAb concentration sustained E3 SAR T cell activation comparable to anti-EpCAM CAR T cells whereas T cell activation was reversible when the BiAb was washed off. Graph shows mean values of experiments performed at in quadruplicates. One representative of two independent experiments is depicted. p-values by two-sided unpaired t-test are indicated. $p < 0.05$ was considered statistically significant.

5.6 *In vivo* depletion of E3 SAR T cells by targeting the SAR EGFRv3 domain

In addition, if the BiAb decline is not sufficient to ensure rapid reversibility of occurring side effects, SAR T cells bear the advantage of the targetable EGFRv3 ectodomain of the E3 SAR. Thus, we made use of the clinically approved anti-EGFR antibody cetuximab, which can effectively detect the E3 SAR due to the presence of the conserved cetuximab-binding epitope in EGFRv3, to achieve immediate and complete depletion of T cells *in vivo*. A single dose of 1 mg cetuximab per mouse was enough to significantly reduce the number of transferred cells after 24 h in all main secondary lymphoid organs (Figure 18).

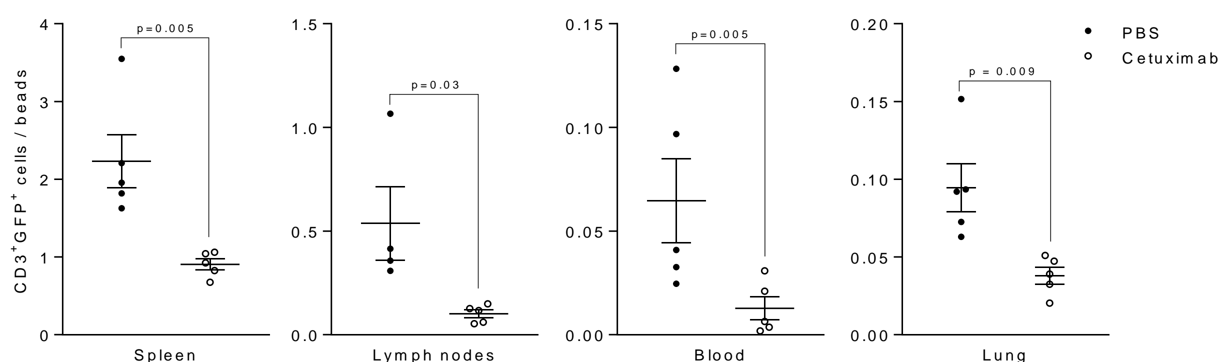

Figure 18│ *In vivo* depletion of transferred E3 SAR T cells by administration of anti-EGFR antibody cetuximab.

C57BL/6 mice were injected intravenously with 10^7 E3-GFP SAR T cells. 6 h after injection, mice were randomized in two groups and received a single dose of 1 mg cetuximab per mouse or vehicle solution intravenously (n = 5 mice per group). 24 h after T cell transfer, mice were sacrificed and spleens, lymph nodes, blood and lungs were harvested. Single cell suspensions were analyzed for remaining E3-GFP SAR T cell via flow cytometry. In all organs, the number of $CD3^+$ GFP^+ cells were reduced when mice were infused with cetuximab. The experiment shown is representative of two independent experiments. p-values by two-sided unpaired t-test are indicated. $p < 0.05$ was considered statistically significant.

A major part of the data presented here, in particular Figures 4, 5, 6, 8, 9, 10, 11, 17 and 18, is currently in the publication process.

6 Discussion

The presented study sought to characterize and evaluate the application of SAR T cells in combination with BiAb and to provide evidence for further translation in studies in a human system. A critical prerequisite for SAR T cell-based treatment strategies is the restriction of specific T cell activation and degranulation solely in the tumor tissue expressing the target antigen. This was achieved by using a BiAb monovalent for the SAR compared to the bivalent BiAb used for previous projects. Mechanistically, SAR T cells mediate anti-tumoral activity mainly by expression and induction of FasL while CAR T cells rely on degranulation for functionality. Beneficial for this finding is the upregulation of the Fas receptor on tumor cells under inflammatory conditions leading to a potentially higher susceptibility to SAR T cell therapy. *In vivo*, co-application of the combination therapy with tumor cells resulted in delayed tumor outgrowth and induced an immunological memory preventing mice from tumor recurrence. To further support translational applicability of SAR T cells combined with BiAb, the proposed safety switches demonstrated rapid and effective reversibility of uncontrolled T cell activation. SAR T cell activity vanishes with the half-life of the BiAb and furthermore, transferred T cells can be depleted by targeting the SAR ectodomain with a depleting clinical grade antibody.

6.1 The composition of the SAR is crucial for the translation of the platform

The objective of genetically modified T cells for cancer immunotherapy is the reconditioning of a tumor-directed cellular immune response (Sadelain et al., 2017). The basis of the approach used for adoptive T cell therapy is the physiological antigen recognition by the immunological synapse, which is characterized by the interaction of TCR, adhesion molecules and co-stimulatory receptors (Finetti et al., 2018). Crucial for the antigen-mediated redirection of the modified T cells is the extracellular portion of the transgene-encoded synthetic receptor. This can differ from synthetic TCR$\alpha\beta$ chains (Dembić et al., 1986), antigen specific scFv (G. Gross et al., 1989) or ligands for binding to respective complementary receptors (Altenschmidt et al., 1996; Kahlon et al., 2004) and multivalent antibodies (Kobold et al., 2015b). Here, the fusion protein on the T cells was selected to be restricted to the transduced T cells to avoid infiltration of T cells into normal tissues.

The SAR used in this study utilizes the ectodomain of the membrane protein human epidermal growth factor receptor variant III (EGFRv3) for BiAb binding, which is exclusively expressed on a small

subset of certain tumor entities in humans and therefore fulfills the main requirements of restricted expression (Li et al., 2008, O'Rourke et al., 2017). EGFRv3 results from the in-frame deletion of exons 2 to 7 accompanied by the generation of a novel glycine residue at the junction of exons 1 and 8. Although the postulated binding site for the natural ligand EGF in the L2 region (domain 3) remains in the splicing variant (Woltjer et al., 1992), the novel extracellular domain architecture is unable to bind EGF (Gong et al., 2014, Huang et al., 1997). These reports are consistent with previous studies on E3 SAR T cells showing that soluble EGF could not trigger a specific T cells response (Schmidbauer, 2018). In contrast, the EGFR-specific antibodies panitumumab and cetuximab exhibit comparable binding affinities for both EGFR and EGFRv3 (Gong et al., 2014, Patel et al., 2007) which unveils further prospects to target and track E3 SAR T cells. However, the ligand-independent formation of transient dimers by the EGFRv3 must be carefully considered as the SAR consists of the ectodomain of EGFRv3 (Chu et al., 1997). Aggregation of two or multiple SAR molecules can potentially lead to unspecific T cell activation. One possible approach to prevent aggregation could be the insertion of the CD28 transmembrane domain or to further truncate the EGFRv3 while the antibody-binding epitope remains intact.

The choice of the intracellular domains was mainly based both on previous experience from our group and published literature on CARs and CAR-like approaches demonstrating that the CD3ζ chain of the TCR is able to induce sufficient signals to mediate immune effector functions (Irving et al., 1991, Letourneur et al., 1992, Lim et al., 2017). As shown for CAR T cells, an integration of cytoplasmic domains from co-stimulatory proteins such as CD28 (Krause et al., 1998), 4-1-BB (Finney et al., 2004) or OX40 (Pulè et al., 2005) results in a superior function in T cell proliferation and secretion of immune stimulatory cytokines IL-2 and IFN-γ (Hombach et al., 2001, Maher et al., 2002). Furthermore, we could demonstrate the importance of the CD28 signal for synergistic activity to the CD3ζ since a SAR with mutations in the signaling motifs of the CD28 domains led to altered T cell function and proliferation (Boomer et al., 2010).

Recent studies have shown that the selection of the CAR signaling domains can have a significant impact on the T cell metabolism and subsequently, on the fate of the T cells. The use of CD28 as a co-stimulus for T cells activation can trigger a higher Glut1 surface expression associated with an increased reliance on aerobic glycolysis (Frauwirth et al., 2002). This may drive T cell differentiation into short-lived T effector cells which can be beneficial to reduce off target toxicities due to the insufficient persistence of transduced cells. Congruently, SAR T cells utilizing the CD28 co-stimulatory

domain appeared to expand into a large effector memory cohort after specific activation with the BiAb (Schmidbauer, 2018).

In contrast, CAR T cells utilizing 4-1BB co-stimulatory domains showed an enhanced fatty acid oxidation which is characteristic for memory T cells. This findings match the superior proliferation and persistence of 4-1BB over CD28 CAR T cells as memory T cells are known to be long-lived T cells (Porter et al., 2015). Furthermore, the incorporation of the 4-1BB domain resulted in an increased capacity to generate mitochondrial mass providing a survival advantage within the tumor microenvironment (Kawalekar et al., 2016). Based on this data, future studies should aim to elucidate whether a mixture of 4-1BB and CD28 CAR T cells might have a superior outcome to a single CAR T cell population. Here, the endogenous immune response with an early dominance of T effector cells and the later appearance and persistence of memory T cells would be more mimicked. For SAR T cells it remains to be proven, whether the addition or change of co-stimulatory domains to 4-1BB could result in further or even synergistic activity (Wilkie et al., 2008) and a phenotypically differentiation towards a memory T cell population.

6.2 BiAb can facilitate specific T cell activation and broaden the spectrum of tumor targets

Similar to the high demands on SAR, the design and composition of the BiAb must be carefully scrutinized.

Early studies on T cell activation with antibodies binding to parts of the TCR such as anti-CD3ε are the rationale for the engineering of the BiAb (Minguet et al., 2007). In this setting, bi- or multivalent anti-CD3ε antibodies such as OKT3 were used to activate T cells by TCR clustering thereby inducing conformational changes, a property which seems to be lost for monovalent antibodies. Similarly, the BiAb bivalent for the SAR was able to induce spontaneous SAR T cell activation to some degree by crosslinking two receptors. This ability was lost for the monovalent BiAb and could only be restored by immobilizing the tumor-associated binding moiety of the antibody either by attachment to a plastic surface or to antigen presentation by tumor cells.

Another decisive advantage of the presented SAR platform is selective activation solely of the transferred SAR T cells by the marker antigen EGFRv3. In contrast, other approaches utilizing bispecific antibody formats such as BiTE use the pan-T cell marker CD3 as the T cell recruiting

domain, leading to a tremendous and unspecific activation of the entire T cell population. This frequently induces severe CRS in treated patients (Teachey et al., 2013). Further clinical studies including BiTE reveled that also the number of immunosuppressive regulatory T cells (Tregs), recruited and activated by the BiTE, increased and which consequently serves as a negative predictive factor (Duell et al., 2017).

As the BiAb is indispensable for reliable SAR T cell activation it provides a highly adjustable element in the system. The modularity of the whole platform is driven by a flexible exchange of the targeted tumor antigen which can be achieved by the rapid replacement of the respective BiAb. Also the BiAb itself can be further engineered to target more than one TAA simultaneously as it is already shown for a triplebody targeting CD19, CD16 and CD33 at once (Schubert et al., 2011). This can enhance the specificity of the approach while reducing the risk of tumor immune escape due to TAA down regulation. In addition, T cell stimulating cytokines such as IL-2 or IL-12 can be fused to the BiAb to further stimulate and attract T cells within the tumor microenvironment (Rudman et al., 2011). Also conceivable is the application of a BiAb mixture, targeting a variety of predicted antigens restricted to a certain tumor entity. Here, the likelihood to eradicate not only the primary lesion, but also metastasis with diverging gene signatures and biomarker expression patterns (Vignot et al., 2012) might be ameliorated compared to single targeted therapies. To elucidate this synergistic potential of different BiAb, further tumor specific target antigens must be identified and validated. Given that the model antigen used in this study EpCAM is widely expressed on a variety of healthy epithelial tissues, including pancreas, lung, bile ducts and intestine (Amann et al., 2008) it is likely that the BiAb is retained by these organs and therefore cannot reach the tumor leading to an insufficient anti-tumoral response by the SAR T cells.

Furthermore, the affinities of both, the EGFRv3 and the TAA binder must be carefully considered. The affinity of a ligand to its receptor or the respective antibody can be described by the binding constants K_D, where low values indicate a high affinity. It has been shown that antibodies with lower affinities might be able to discriminate between normal cells and tumor cells over-expressing the target antigen (Garrido et al., 2011). A high affinity towards the tumor cells can also result in an almost exclusive binding of the BiAb to the target cells, creating a T cell-activating matrix on their surface. This is in favor to facilitate serial killing mediated by the effector cells. In addition, lower binding affinities of the BiAb to the T cells foster the disengagement of target and effector cells to ensure movement of T cells (Hoffmann et al., 2005). The BiAb used in this study is based on already

published antibody clones with a K_D (anti-EGFRv3; MR1.1) of 1,5 x 10^{-9} for the SAR and K_D (anti-EpCAM; G8.8) of 6,1 x 10^{-8} for the target antigen (Farr et al., 1991, Kuan et al., 2000). Here, the higher binding affinity for the SAR indicates a preferentially engagement of the BiAb with the T cells which might induce extensive T cell-target cell clustering and thereby hamper serial killing.

Thus, the design and composition of both, SAR and BiAb, must be precisely evaluated to ensure a focused interplay of both therapeutic components and to reduce potential off-target effects.

6.3 Modular approaches combining transduced T cells with nanobodies or antibodies

The rapid development of monoclonal antibodies, BiAbs and also nanobodies for cancer immunotherapy conceded a new field of strategies to possibly tune down the intrinsic potency of genetically engineered T cells. With titratable recombinant proteins, the immunological synapse formation between target and transferred effector cells can be precisely controlled and monitored. One of the most advanced products is the “universal CAR” (UniCAR), a CAR specifically redirected against a short peptide motif of 10 amino acids derived from a human nuclear protein. The separately applied target module compromises a tumor-specific binding domain fused to the peptide motif recognized by the UniCAR (Cartellieri et al., 2015). This approach showed already great success in preliminary *in vitro* and *in vivo* studies, but the short half-life of the nanobody requires continuous infusion which can potentially limit the clinical application of the therapy. Further examples are Fc-specific CAR T cells, utilizing the CD16 receptor to elicit anti-tumoral activity in combination with TAA-specific monoclonal antibodies (Kudo et al., 2014) or anti-FITC CAR T cells engaging with FITC-tagged small molecules (Kim et al., 2015). Overall, these switchable CAR T-cell dosing regimens bear the advantages of being adaptable to a broader range of target antigens and are furthermore accompanied by lower levels of cytokine secretion compared to conventional CAR T cells. However, the use of synthetic molecules, not naturally occurring in the human body, might lead to immunogenicity issues, thus limiting the translation potential of the approaches. In particular, for the CD16-CAR, the unspecific activation by circulating or tissue-bound antibodies has to be further elucidated.

The concept of SAR T cells presented in this study effectively bypass these circumstances by availing only endogenously expressed molecules, which are however restricted to malignant tissue, thus ensuring no occurrence of immunogenicity. Furthermore, the platform includes a switch to modulate

T cell activity but also provides the opportunity to target and deplete the T cells as a last resort. Hence, SAR T cells approximate the efficiency of CAR T cells combined with the high flexibility of other modular approaches, but display a superior safety profile, providing evidence for clinical translation.

6.4 SAR T cells utilize predominantly the Fas-FasL axis to mediate target cell lysis

For the effective lysis of infected or malignant cells by cytotoxic T lymphocytes, two major mechanisms have been described. The first mode of action relies on the expression of membrane bound tumor necrosis factor (TNF) family ligands which are capable to induce apoptosis through engagement of the respective death receptor. Here, TRAIL and FasL (CD95L) have been proven to have an essential role in this interaction (Griffith et al., 1995, Wang et al., 2003). Another mechanism of redirected target cell killing is the release of secretory granules containing lytic proteins such as perforin and serine proteases of the granzyme family (Darcy et al., 2000, Isaaz et al., 1995). It has been shown that the interaction of TNF receptors and their ligands play a major role in the immune cell maturation (Strasser et al., 1995) and homeostasis (Takahashi et al., 1994) whereas the secretion of perforin and granzyme eradicates malignant cell populations (Kägi et al., 1994, Smyth et al., 2000).

Consistent with these reports and the present study, CAR T cells mainly utilize the perforin–granzyme axis to mediate target cell lysis (Darcy et al., 2000, Davenport et al., 2015) and were inefficient when perforin-deficient T cells were used. Although the same mode of action through the identical signaling moieties was expected, SAR T cells maintain their lytic capacity in the absence of perforin and granzyme B. Here, FasL was identified to be predominantly involved in tumor cell killing. Remarkably, this unexpected finding for E3 SAR T cells could be verified in all tumor models tested and was found to be transferable to the whole platform when tested with a different SAR and the respective BiAb.

Fas and FasL play a major role in modulation of immune function and are the main perpetrators for activation-induced cell death (AICD). Upon TCR stimulation, T cells shown an induction in FasL expression. The engagement with its receptor Fas has been reported to be critical for the elimination of activated T cells during termination of acute immune responses (Nguyen et al., 2001). The suicide and fratricide among the activated cytotoxic T lymphocytes can be beneficial for protection from unwanted immune responses but also leads to hampered proliferation and removal of T cells.

Therefore, FasL as the predominate mediator of target cell lysis by SAR T cells can potentially favor the induction of AICD and fratricide, which so far has not been observed for *in vitro* cell culture. Furthermore, the impact of the co-stimulatory domains CD28 or 4-1BB on AICD is controversially discussed for the design and construction of CAR T cells (Boussiotis et al., 1997, Kerstan et al., 2004, Mamonkin et al., 2016). Exchange of the CD28 with the 4-1BB co-stimulatory domain might lead to an increased surface expression of the apoptotic mediator FasL on SAR T cells (Gomes-Silva et al., 2017), reveling further aspects to strengthen SAR T cell effectivity. In addition, SAR lacking the CD28 co-stimulatory domain were not altered in their killing mechanism, pointing towards a sufficient endogenous expression of the ligand. Consequently, it is assumed that the unique mode of action for SAR T cells in combination with BiAb is more driven by the influence of the BiAb rather by the receptor itself.

Another important aspect is the ability of tumor cells to evade immunological detection by the expression of FasL. Various cancer entities show a high surface expression of FasL, thus inducing apoptosis in infiltrating T cells expressing Fas (Bennett et al., 1998, Koyama et al., 2001, Motz et al., 2014). Tumors can therefore counterattack Fas-bearing immune cells to overcome rejection and in addition participate in the destruction of the immune system. Also, the expression of FasL on the tumor vasculature provides a selective immune barrier to effectively prevent T cell infiltration. Consequently, the combination of ACT and FasL blockade in mice resulted in a synergistic treatment effect with enhanced T cell homing and prolonged overall survival of the cohort (Motz et al., 2014). As FasL is essential for anti-tumoral activity of SAR T cells, this approach cannot be taken into consideration for the therapeutic platform. Furthermore, Fas-FasL interaction has been reported to be also involved in promoting malignant tumor growth (Ceppi et al., 2014, Chen et al., 2010). Thus, tumor response must be carefully monitored during SAR T cell therapy to immediately interrupt treatment if any evidence for a tumor-promoting impact occurs.

The underlying molecular processes for these mutually exclusive modes of action for SAR T cells in contrast to CAR T cells have not been fully investigated and require future studies to give more insights in the distinct mechanisms. So far, this study clearly demonstrates a separation of the SAR T cell approach from other engineered T cell-based therapeutic strategies.

6.5 SAR T cells include two incorporated safety switches to ensure rapid reversibility of T cell activation

A prerequisite for ACT with genetically engineered CAR T cells is the expression of distinct TAA on the tumor tissue that serve as targets. The lack of antigens solely restricted to the tumor bears the risk of severe and lethal side effects such as CRS (Beatty et al., 2014a, Lee et al., 2014) or the damage of healthy tissue expressing the TAA (Morgan et al., 2010, Wang et al., 2014). Patients suffering from such accompanying symptoms of ACT commonly require intensive care often including systemic treatment with high doses of corticosteroids (Lee et al., 2015). The concurrent ablation of the transferred CAR T cells results in a decreased anti-tumoral response (Davila et al., 2014) and could potentially lead to further disease relapses. An alternative strategy is the application of the IL-6 receptor blocking antibody tocilizumab to reverse the symptoms of CRS (Maude et al., 2014). Further safety switches such as the incorporation of suicide genes (Casucci et al., 2013, Jensen et al., 2010) or the use of depleting argents such as antibodies (Paszkiewicz et al., 2016) are already in clinical development. The drawback of these strategies is the total elimination of the CAR T cell population, depleting the active anti-tumoral compound.

One of the most used strategy in clinical settings to ensure the rapid removal of uncontrolled CAR T cells utilizes the expression of the herpes simplex thymidine kinase (HSV-TK) as a suicide gene in the engineered T cells. Upon administration of the antiviral prodrug ganciclovir, T cell activity should vanish immediately. But the mechanism of action involves interference with DNA synthesis, leading to an incomplete and delayed T cell elimination with prolonged progression of side effects. Moreover, the HSV-TK derives from a virus and is therefore potentially immunogenic itself (Bordignon et al., 1995, Ciceri et al., 2009, Jones et al., 2014). Considering the challenges, the use of viral enzymes might not be acceptable in the acute occurrences of toxicities and live threatening events for the patient.

A very advanced and effective approach is the use of an inducible caspase 9 (iCasp9) suicide system, which couples caspase 9 to a drug-sensitive binding protein. Administration of the synthetic molecule AP1903 induces dimerization of the fusion construct resulting in T cell apoptosis. T cells expressing the iCasp9 were systemically eliminated 30 min after infusion of AP1903 accompanied by the abrogation of all clinical symptoms (Zhou et al., 2015). Unfortunately, the depletion of the transferred cells rescinds their therapeutic potential and might result in a disease relapse. Therefore,

approaches to modulate the activation of either the switch or the T cells themselves are potential strategies to overcome this issue.

In contrast to T cell ablation, this study illustrates that the dependency of SAR T cell activity is restricted to the half-life of the BiAb and vanishes rapidly with BiAb clearance. Hence, SAR T cells would remain inactive in the patient but anti-tumoral activity could be restored upon re-dosing of the BiAb after resolution of potential side effects. Furthermore, SAR T cells utilize a variant of an endogenous, non-immunogenic surface protein for the specific activation of T cells and thus cannot trigger an immunogenic rejection of the therapy. Also, the BiAb with an inert human Fc part, should not interfere with the patients' immune system and additionally can be engineered with a half-life according to therapeutic schedule.

Nevertheless, if a situation occurs in which it is necessary to completely ablate the SAR T cells, the clinical grade anti-EGFR antibody cetuximab can be applied systemically to deplete E3 SAR T cells by targeting the SAR ectodomain EGFRv3. Upon engagement of the SAR, cetuximab immediately prevents the binding of the BiAb and induces antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytolysis (CDC) of SAR T cells (Kimura et al., 2007). As demonstrated here, the numbers of transferred SAR T cells were reduced significantly in less than 24 h after cetuximab administration.

6.6 Co-administration of SAR T cells and BiAb delays tumor outgrowth and induces an immunological memory in mice

Previous studies from our group already showed increased survival when tumor cells over-expressing EpCAM are co-administrated with SAR T cells and an EpCAM- and SAR T cells-engaging BiAb (Schmidbauer, 2018). These findings were confirmed with endogenous EpCAM-expressing tumor cells to rule out any genetic alteration in the tumor cells occurring from retroviral transduction. The main reason for selecting this co-application model was to substantiate the *in vivo* tolerance of the gene-modified SAR T cells and to determine potential off-target toxicities. Thus, the therapeutic success indicates that the engineered T cells in an immunocompetent experimental animal are not subject to premature elimination due to high immunogenicity. Furthermore, this efficacy test demonstrates that modification, expansion and re-infusion with these artificial constructs is technically feasible without observing systemic or local toxicity.

Interestingly, cured mice were protected from tumor recurrence even when inoculated again with the respective tumor cells subcutaneously. As no additional SAR T cell transfer was conducted, the first treatment induced an immune response against antigen-bearing tumor cells resulted from the release of secondary tumor antigens by dying target cells. These antigen spread can initiate a subsequent immune response including secretion of antibodies specific for cancer-related proteins. The occurrence of immunotherapy-induced antigen spread is already reported for various clinical trials (Chapuis et al., 2016, Hardwick et al., 2011) and is associated with a beneficial outcome of the therapy. In contrast to chemo- and radiotherapy, where the tumor burden decreases quickly after treatment initiation, immunotherapy often results in a slowdown of diseases progression. However, effects from conventional tumor therapy are known to be short-lived after treatment cessation. Therefore, even with delayed treatment responses, immunotherapy bears a tremendous advantage due to the induction of a new protective immunological memory.

This study could demonstrate that SAR T cell therapy is accompanied by antigen spreading and the establishment of a robust, durable and adaptable immune responses over a long period of time. Future studies might aim to identify new specific tumor antigens involved in the spread. Furthermore, the kinetics of tumor clearance by immunotherapy strongly encourage an earlier treatment initiation as patients with lower tumor burden already showed better clinical response (Kwon et al., 2014).

6.7 Limitations and drawbacks of the applied model for *in vivo* efficiency evaluation

Overall, the experimental setup of the co-injection model raises some issues which do not allow any final assessment of the *in vivo* efficacy for the treatment of established tumors with SAR T cells. In this setting, neither the migratory capacity of SAR T cells towards the tumor nor the resistance to the immunosuppressive microenvironment can be addressed.

The most advanced T cell-based therapies have limited activity against solid tumors which mainly results from an impaired migration and infiltration of the T cells to the tumor tissue (Bernhard et al., 2008, Fesnak et al., 2016). Whether SAR T cells can overcome these difficulties could not be elucidated in this *in vivo* treatment model and remains still unanswered. A potential strategy to enhance the recognition of malignant tissue and to guide transferred T cells specifically to the

tumor site involves additional engineering of T cells with selected chemokine receptors (Rapp et al., 2016). Therefore, prospective studies could include the combination of SAR T cells with chemokine receptors to improve the migration towards solid tumors.

Another drawback of cellular immunotherapy is the immunosuppressive microenvironment of most solid tumor entities. The inhibition of an anti-tumoral immune response is either mediated by cell-cell interactions or the secretion of inhibitory cytokines (Beatty et al., 2014b) which leads to T cell anergy or exhaustion. Several strategies to face these challenges are already in clinical or pre-clinical development. The co-expression of a dominant-negative transforming growth factor-β (TGFβ) receptor type II shields the modified T cells from this tumor-derived, suppressive cytokine (Foster et al., 2008). A key axis of immune cell suppression is the engagement of the programmed cell death protein 1 (PD-1) on the effector cell with its ligand (PD-L1) expressed by the target cell. The thereby mediated inhibitory signals for the T cells can limit the duration and strength of the adaptive immune response and lead to a potential immune evasion by the tumors (Blank et al., 2004, Dong et al., 2002). Thus, the addition of a PD-1-CD28 hybrid receptor could convert T cell inhibitory signals into a co-stimulus to further boost the immune response (Kobold et al., 2015a). Furthermore, the application of anti-PD-1- or anti-PD-L1-blocking antibodies achieved a marked increase of treatment response in clinical trials (Brahmer et al., 2012, John et al., 2013, Topalian et al., 2012) and is thus approved by the U.S. Food and Drug Administration for various cancer entities such as metastatic melanoma (Beaver et al., 2017), non-small cell lung cancer (Kazandjian et al., 2016, Sul et al., 2016) or Hodgkin's lymphoma (Kasamon et al., 2017).

This highlights the diversity of prospects to further arm and engineer SAR T cells to create more powerful and adaptable strategies for directed cancer immunotherapy.

6.8 Conclusion and perspectives

In summary, this thesis describes a novel therapeutic platform for ACT for targeting a broad variety of solid tumor entities as well as hematological diseases. The combination of SAR T cells with the respective BiAb allows a fast and flexible exchange of the targeted TAA independent of MHC presentation without substitution of the transferred modified T cells. In this context, the co-application of two or more BiAb specific for several TAAs expressed by the tumor should be taken into account to reduce the risk of tumor escape by TAA downregulation. This therapeutic concept

could be beneficial for targeting primary tumors as well as secondary lesions and metastasis with diverging gene signatures and biomarker expression patterns (Vignot et al., 2012) and should be evaluated in further studies.

A critical prerequisite for T cell-based treatment strategies is the tolerance, efficiency and safety of genetically engineered T cells in patients. The *in vivo* co-application of SAR T cells and BiAb in immunocompetent mice demonstrates, that these requirements are fulfilled. However, the treatment of established and vascularized tumors remains elusive. In order to enhance the *in vivo* efficacy of SAR T cells for solid tumors, more BiAb for truly cancer-specific antigens must be validated for their applicability to the platform. Furthermore, additional engineering of the BiAb should be taken into consideration. Here, dual targeting of various TAAs, the addition of an anti-PD-1 scFv or the incorporation of T cell stimulatory cytokines such as IL-12 might bear the potential to ameliorate the therapeutic outcome of SAR T cell therapy.

The suitable dosage and administration of both the SAR T cells and the BiAb should not be overlooked. Here, the most commonly recommended method for T cell application is the intravenous infusion of the cell product. For the BiAb, intravenous or intratumoral injection or the implantation of a subdermal sponge-like biomimetic cryogel releasing the agent *in vivo* (Bachmann et al., 2018) are possible strategies to deliver the antibody. Furthermore, combination therapy with immunomodulatory small molecules as IDO inhibitors or lenalidomide and the blockage of checkpoint molecules such as PD-1 or CTLA-4 can provide additional synergistic effects to the SAR T cell treatment (Beatty et al., 2017, John et al., 2013, Ramsay et al., 2012).

7 Summary

Genetically engineered T cells are powerful anti-cancer treatments but are limited by safety and specificity issues. By utilizing the advantages of currently used approaches based on cellular and antibody constituents, our group created a new therapeutic platform for adoptive T cell therapy of solid malignancies. Combing autologous T cells, expressing a targetable activating receptor (SAR) with bispecific antibodies (BiAb) provides MHC-independent tumor antigen recognition, selective recruitment and simultaneous activation of T cells with integrated safety switches.

This thesis identifies the importance of the BiAb format used for the platform, the mode of action used by SAR T cells to mediate an anti-tumoral response and the functionality of the implemented safety mechanisms to control unwanted T cell activation.

In previous studies, the BiAb used was bivalent for both, the SAR and the tumor-associated antigen, leading to unspecific T cell activation due to crosslinking of two SAR T cells. Here, a selective activation of SAR T cells dependent on BiAb monovalency for the SAR was demonstrated. Unexpectedly, FasL appeared to have a major impact in SAR T cell-mediated tumor cell lysis. In contrast, CAR T cells targeting the same tumor antigen utilized predominantly granzyme and perforin for target cell killing.

T cell activation vanished with the clearance of the BiAb, demonstrating the superior safety profile of the therapeutic strategy. In addition, adoptively transferred SAR T cells could be effectively depleted *in vivo* by administration of the clinical grade antibody cetuximab, allowing fast elimination of the cell product if required. Overall, the functional data presented here display a potent anti-tumor activity, a distinct mode of action and enhanced safety of the SAR T cell therapeutic approach. This provides the basis for further translation into human studies.

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9 List of abbreviations

ACT	Adoptive cell transfer
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ALL	Acute lymphatic leukemia
APC	Allophycocyanin
BiAb	Bispecific antibody
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CDR	Complementarity determining region
CMC	Complement-mediated cytotoxicity
CRS	Cytokine release syndrome
CTL	Cytotoxic T lymphocyte
ddH2O	Double deionized water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EGF	Epithelial growth factor
EGFR	Epithelial growth factor receptor
EGFRv3	Epithelial growth factor receptor, transcript variant III
ELISA	Enzyme-linked immunosorbent assay
EpCAM	Epithelial cell adhesion molecule
Fab	Fragment antigen binding
FasL	Fas ligand
Fc	Fragment crystallizable
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GzmB	Granzyme B
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

mAb	Monoclonal antibody
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NHL	Non-Hodgkin lymphoma
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerithrin
PerCP	Peridinin chlorophyll A protein
PerCPCy5.5	Peridinin chlorophyll A protein-cyanin 5.5
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RTK	Receptor tyrosine kinases
SAR	Synthetic agonistic receptor
scvF	Single chain variable fragment
SEM	Standard error of mean
TAA	Tumor-associated antigen
TCR	T cell receptor
TIL	Tumor-infiltrating T cell
TME	Tumor microenvironment
Tregs	Regulatory T cells
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
WHO	World health organization

10 Publications

Mohamed-Reda Benmebarek*, **Clara Helke Karches***, Bruno Loureiro Cadilha, Stefanie Lesch, Stefan Endres and Sebastian Kobold (* contributed equally).
Killing Mechanisms of Chimeric Antigen Receptor (CAR) T Cells.
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