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Characterization of bispecific antibodies that drive synthetic agonistic receptor - transduced T cells to mediate specific and conditional therapy in human pancreatic cancer models

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Mohamed-Reda Benmebarek

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Introduction

1.1 Cancer immunotherapy

1.1.1 Hallmarks of cancer

Two decades ago, Hanahan and Weinberg eloquently dubbed the rules that govern the neoplastic transformation of healthy cells and tissues into malignant cancer as the hallmarks of cancer (Hanahan *et al*. 2000) . They broke these hallmarks down into six acquired alterations in cellular physiology: self-sufficiency in growth signals, evasion of apoptosis, insensitivity to anti-growth signals, limitless replicative potential, tissue invasion and metastasis and sustained angiogenesis. These hallmarks are all underpinned and accelerated by genomic instability, creating heterogeneity and inflammation (Hanahan *et al*. 2011).

Over the time period it takes for a tissue pathology to develop into a neoplastic lesion, intermediate evolutionary pre-malignant steps are passed, as cells begin to circumvent natural cell breaks and suppressive mechanisms. An accumulation of multiple driver mutations each conferring a type of growth advantage, is a sequential and stochastic process that slowly turns healthy cells cancerous (Balmain *et al*. 1993, Negrini *et al*. 2010) . Underlying factors behind these alterations can be both inherited and acquired. These mutations can affect two categories of genes, oncogenes and tumor suppressor genes. Oncogenes bestow cancer cells with a dominant gain of function, and often play a role in the stimulation of controlled cellular proliferation. Tumor suppressor genes are behind the changes that result in a recessive loss of function (Weinberg *et al*. 1994) . One example is p53, a tumor suppressor gene that plays a role in the regulation or progression of a cell through the cell cycle, and its arrest in the presence of DNA damage. If mutated, the ability of the cell to control its progression through the cell cycle, as part of the DNA damage response, becomes dysregulated (Negrini *et al*. 2010).

1.1.2 The role of the immune system in tumor progression

The idea that the immune system acts as a sentinel, in recognizing and eliminating newly transformed cells is the basis for the longstanding theory of immunosurveilance, first established by Burnet and Thomas in 1957 (Burnet *et al*. 1957) . Built upon this is the concept of immunoediting, outlining the dual role the immune system plays in protecting the host from tumor formation while simultaneously driving tumor cell evolution (Dunn *et al*. 2002, Dunn *et al*. 2004) . The process by which the immune system copes with tumor growth is understood across three stages of tumor development: elimination, equilibrium and escape. In the elimination phase, transformed cells express differentially expressed antigens that make them more easily targetable by immune cells. This process drives the clonal evolution of tumor cells, eventually allowing them to enter the equilibrium phase. Despite managing to control tumor growth, immune evasion mechanisms prevent complete tumor cell eradication. The progressive development of these mechanisms, together with an ability to shape their environment and the very immune cells they are evading, inevitably enables tumor escape (Schreiber *et al*. 2011, O'Donnell *et al*. 2019).

Tumor cells interact with different types of stromal cells (such as mesenchymal stromal cells), cancer-associated fibroblasts, and immune cells (such as myeloid-derived suppressor cells and T cells), collectively referred to as the tumor microenvironment (TME) (Joyce *et al*. 2009, Rabinovich *et al*. 2007, Sakaguchi *et al*. 2008) The TME plays a crucial role in both the development and survival of cancer cells. Inter-cellular bi-directional cross-talk between stromal and immune cells in the TME, including the release of pro-tumoral cytokines (e.g. IL-6, IL-10 and IL-22), growth factors (e.g. TGF-β, VEGF and PDGF) and matrix remodelling enzymes (e.g. MMPs) supports and shapes this milieu (Voigt *et al*. 2017, Nawaz *et al*. 2018,Gorelik *et al*. 2002,Gerlini *et al*. 2004, Kurte *et al*. 2004) . These play important roles in tumorigenesis, as they shape an environment that is most favourable for tumor growth, enabling malignant cells to escape the host's anti-tumor immune responses (Hanahan *et al*. 2011).

1.2 Adoptive T cell therapy

A disrupted equilibrium within the TME is often a crucial enabling factor for tumor formation. Immunotherapy, which is based on the exploitation of the autologous immune response to

a neoplastic process, is an effective, and in some cases curative cancer therapy that aims to shift the balance back in favour of the anti-tumoral immune response (Mellor *et al*. 2003,Schneider *et al*. 2006,Pardoll *et al*. 2012) . T cells are plastic immune cells that have been implicated to play diverse roles in driving and controlling tumor growth. Nevertheless, T cells have repeatedly been shown to be effective and dynamic mediators of anti-tumoral immunity, and their therapeutic use is termed adoptive T cell therapy (ACT) (June *et al*. 2007, Kalos *et al*. 2013).

ACT requires the harvest, expansion and re-infusion of T_{eff} cells into a patient (Melief *et al*. 1992) . ACT initially depends on the ability to genetically engineer or select for cells with targeted antigen specificity. Beyond this, one must be able to induce these cells to proliferate, persist and survive, and mediate their anti-tumoral effector functions (Rosenberg *et al*. 2015).

Three independent ACT approaches have stemmed from the idea that tumor-specific T cells could eliminate cancer. Tumor infiltrating lymphocyte (TIL) therapy was the first approach to be implemented and relied on the harvest of T cells directly from a tumor, before their *ex vivo* expansion, activation and ultimate reinfusion back into the patient. This approach is advantageous because it does not harbour the risks associated with genetic engineering. However, scarce access to resectable tumors or metastases, labour-intensive T cell preparation and limited numbers of tumor-reactive T cell clones have so far hampered this strategy's success (Rosenberg *et al*. 1988, Wu *et al*. 2012).

The other two approaches rely on genetic engineering to confer T cells, isolated from peripheral blood, with a specificity to recognize tumor antigens via carefully engineered constructs. T cell receptor (TCR)-engineered T cells started being generated to confront some of the drawbacks of TIL therapy. Using viral transduction, isolated T cells are genetically engineered to possess tumor-specificity through recognition of MHC-restricted peptides (June *et al*. 2007) . Binding of these peptides by the TCR activates T cells and induces a cytotoxic response. TCR-mediated specificity is restricted as a result of a reliance on the expression of tumor antigens through their MHC complexes (Harris *et al*. 2016) . The final ACT approach, the use of chimeric antigen receptor (CAR)-modified T cells, will be discussed in greater detail in the next section.

1.3 Synthetic receptors in T cell therapy

CAR T cell therapy is a leading adoptive T cell therapy (ACT) approach. CARs are synthetic constructs use a single-chain variable fragment (scFv) to bind surface-bound target antigens.

In the initial concept, the target-binding domain was linked to a cytoplasmic signalling subunit made up of part of the cluster of differentiation (CD)-3 ζ chain, that would trigger T cell activation following antigen binding. More precisely, these two gene segments are linked via a transmembrane domain and an extracellular hinge module, coming together to form the simplest form of a CAR, known as a first-generation CAR. The TCR CD3ζ chain contains three immuno-tyrosine activation motifs (ITAMs) (Wange *et al*. 1996) . As a result, this module alone can propagate a strong signal 1 without needing the rest of the TCR-CD3 complex (the gamma, delta and epsilon chains) (Geiger *et al*. 1999, Haynes *et al*. 2001).

Signalling is started when ITAMs are phosphorylated by lymphocyte-specific protein tyrosine kinase (Lck) within the endodomain of CD3. Building on this, strides taken to ameliorate the CAR molecule gave rise to second and third generation CAR structures that incorporated signalling cytoplasmic domains, such as CD28, 41BB, and ICOS, in efforts to recapitulate the added signal that is delivered following TCR recognition by antigen presenting cells (APCs) (Porter *et al*. 2011, Guedan *et al*. 2014).

This co-stimulatory signal, where in the case of CD28 for example is driven by phosphoinositide 3-kinase PI3K (Krogsgaard *et al*. 2005, Zhang *et al*. 2017) is needed for full T cell activation (Love *et al*. 2010) . Additional advances into next (fourth or fifth) generation CAR T cells incorporated cytoplasmic signalling components from cytokine receptors or inflammatory cytokines, such as IL-12 or IL-18, with an inducible expression (Kerkar *et al*. 2010, Chmielewski *et al*. 2014).

1.4 Bispecific antibodies

Proteins made up of fragments from two different monoclonal antibodies, brought together to recognize two different epitopes or antigens are broadly referred to as bispecific antibodies (BiAbs). These man-made antibody-based molecules were first described over 50 years ago by Nisonoff and colleagues (Nisonoff *et al*. 1960) who combined two antigen-binding sites in one molecule. Naturally occurring bivalent antibodies contain identical binding sites which are made up of determinants from both the heavy (H) and the light (L) chain. An initial challenge for BiAb development was the chain association issue, which resulted in the unwanted pairings of heavy and light chains. As a result, obtaining a functional BiAb from a mixture of ten potential H_2L_2 recombinations (through the co-expression of two H and two different L chains) was tedious to say the least. Over the years, numerous strategies emerged with the goal of improving the yield as well as homogeneity of the final product (Brinkmann *et al*. 2017).

The generation of fragment-based formats – an approach that simply combines several antibody-binding regions (Ab fragments) – was a simple and effective approach that effectively circumvented the chain-association issue (Birch *et al*. 2006) . The format lacks an Fc region, while facile co-expression of the polypeptide chains in lower expression systems (eukaryotic or prokaryotic) was cost-effective and reaped higher yields. Fc-deficient formats are however susceptible to catabolism by the neonatal Fc receptor and have a shorter half-life as a result (Demarest *et al*. 2008, Lowe *et al*. 2011).

The development of the 'knob-into-hole' (KiH) technology enabled correct heavy chain heterodimerization (Ridgway *et al*. 1996, Merchant *et al*. 1998) . Importantly, correct light chain association in bispecific heterodimeric IgG antibodies was eventually also enabled by the development and description of immunoglobulin domain crossover 'CrossMAb' technology (Schaefer *et al*. 2011) . The technology relies on antibody domain crossover within one Fab-arm of a bispecific IgG antibody in order to allow for correct chain association, whereas correct heavy chain association can be ensured by, amongst other approaches, KiH and electrostatic steering (Ridgway *et al*. 1996, Gunasekaran *et al*. 2010) . Using domain crossover, any antibody pair can be used to derive many of the formats that have

been described since, including bivalent 1+1, trivalent 2+1 and tetravalent 2+2 (Labrijn *et al*. 2019).

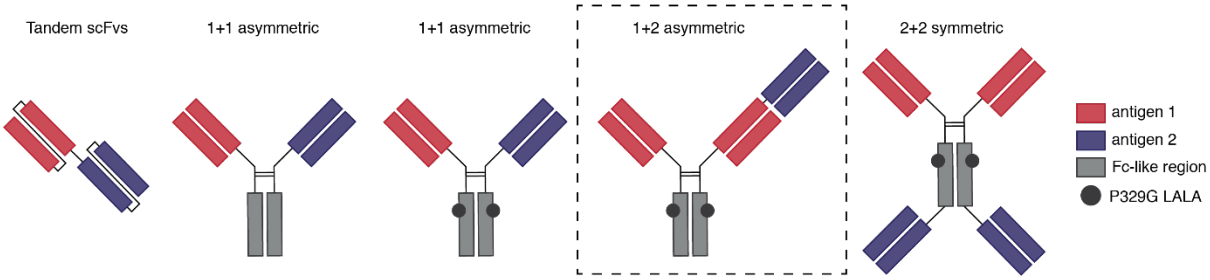

Figure 1: A selection of BiAb formats. Various antibody formats with multiple valencies, binding arms, half-lives and functions. Left to right. Tandem scFvs with two monovalent binding arms and no Fc-region. 1+1 asymmetric with two monovalent binding arms and an Fc region. 1+2 asymmetric with bivalent for one antigen 1 and monovalent for antigen 2, also has an Fc region. 2+2 symmetric bivalent for both antigens and contains an Fc region. BiAbs that are Fc-deficient have a relatively short half-life due to a lack of protection from the neonatal Fc receptor, FcRn. The P329G LALA mutation abolishes FcyR binding, silencing the Fc region.

1.5. The synergy of BiAbs with ACT

Earlier work from our group could demonstrate how BiAbs can be synergised with ACT for improved anti-tumoral immunity. Initial work could show that BiAbs specific for both T cell and tumor cell could bridge T cells with tumor cells and improve the killing efficiency of tumor specific T cells (Kobold *et al*. 2014) . More recently, the group began to develop the approach in efforts to create an MHC-unrestricted platform that could mediate specific and conditional T cell activation and tumor cell lysis. To achieve this, specially designed fusion receptors named synthetic agonistic receptors (SARs) were developed. SARs are constituted of an extracellular domain – itself inert and not present on any naturally occurring T cell – fused to intracellular T cell activating domains.

The unique expression of the SAR ectodomain means an exogenous, controllable, modular and specific molecule is required for its triggering. Bispecific antibodies (BiAbs) perfectly fit that bill, and the importance of BiAb design in the context of SAR activation is described below. The combination of SAR-transduced T cells with BiAbs was initially tested in the murine setting, where SAR-transduced T cells were shown to be functional and specific. Important work that was crucial for this initial characterization was carried out by the former

doctoral students Matthias Kurzay, Moritz Luigi Schmidbauer and Clara Karches (Schmidbauer, 2018, Karches *et al.* 2019).

In the context of the work described herein, the SAR construct is made up of an EGFRvIII extracellular domain, linked to a CD28 transmembrane domain. Intracellularly, signalling is propagated by the intracellular CD28 domain and CD3ζ. EGFRvIII was chosen as the extracellular domain, an antigen not present on normal cells and restricted to particular tumor entities such as glioblastoma. The rationale in choosing the intracellular domains drew from the CAR T cell experience, where several second-generation CARs, including the FDA approved Yescarta successfully employ CD3ζ to initiate signal 1 and CD28 for the costimulatory signal necessary for full blown T cell activation (Boyiadzis *et al*. 2018).

Our collaborators at Roche developed a trivalent and bispecific (two specificities for MSLN and a single specificity for EGFRvIII) CrossMab. This design was developed with knowledge from preliminary studies that demonstrated that a single specificity for EGFRvIII is necessary to avoid unwanted and off-tumor cross-linking of the SAR receptor, and the subsequent T cell activation that would result. The design included two binding arms (Fab) against MSLN to maximize tumor cell binding (Schmidbauer *et al*. 2018, Karches *et al*. 2019).

Following some promising results with the SAR-BiAb platform in preliminary murine studies, the purpose of this work was to determine the validity and efficacy of this approach in the human system. Through the generation of several human pancreatic cancer models, and extensive *in vitro* and *in vivo* testing, we aimed to characterize this platform. Specific T cell activation and target cell lysis, and as well as a demonstration of safety and controllability were critical aims in this regard. Through this characterization, we wanted to determine the translational relevance and significance of the approach as a stand-alone, next-generation adoptive T cell therapy with the potential to plug some pitfalls of current ACT approaches.

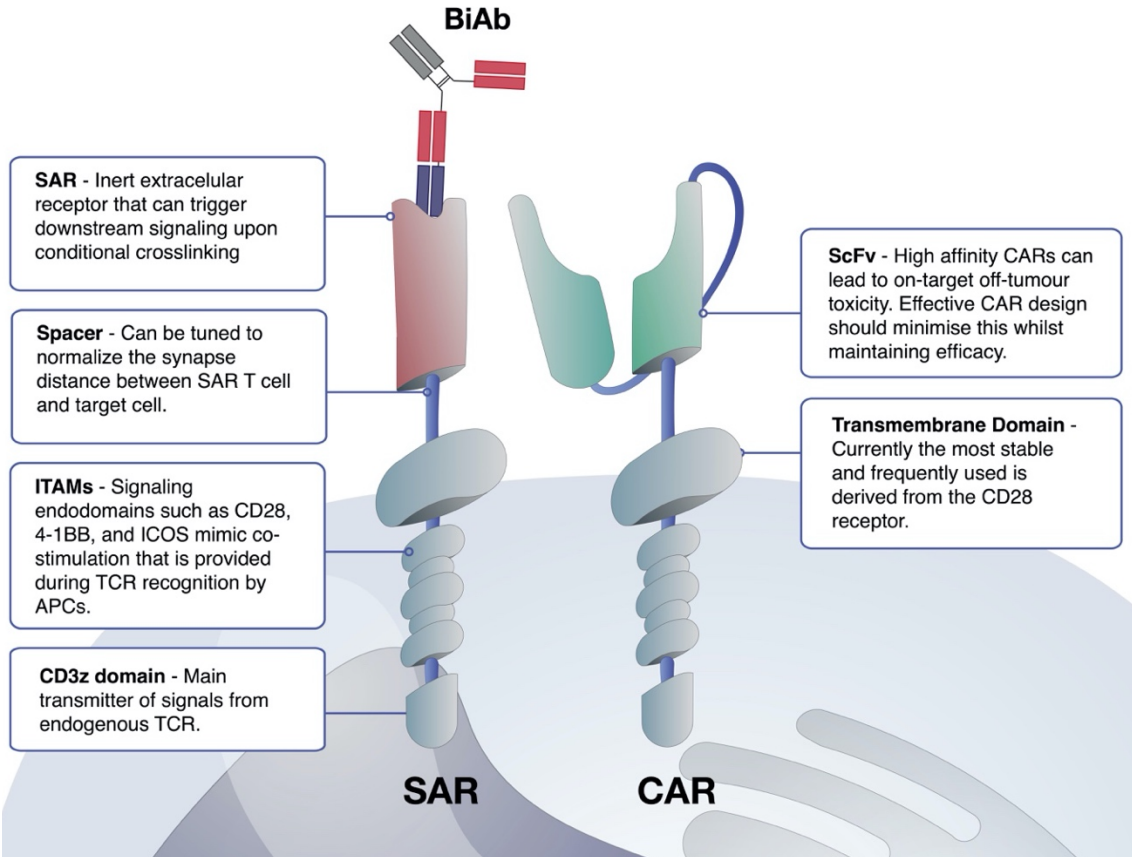

Figure 2: SAR and CAR T cell constructs. Divergence in design lies in the extracellular domain, where the CAR is composed of a scFv, while the SAR has an inert receptor. Additionally, the constructs are made up of several components, each of which leading towards the activation, functionality and persistence of the transduced T cells. Adapted from (Benmebarek *et al.* 2019) .

1.6 Characterizing the SAR-BiAb approach in the pancreatic cancer setting

Over recent years, our understanding of T cell biology, heterogeneity and the functional divergence that arises from their exposure to different stimuli in various environments has drastically improved. T cell stability and plasticity have been shown to be regulated by several components; conditions (costimulation and cytokines), clonality and chromatin (Murphy *et al.* 2010) . Cytokines and costimulation are the prime factors affecting T cell differentiation and stability (Murphy *et al.* 2000) . Circuitry is defined as the network of interactions between transcription factors, and has also been implicated in stability, differentiation, as well as plasticity (Hwang *et al.* 2005, Zheng *et al.* 2010, Nurieva *et al.*

2007) . The effect of chromatin modifications on active or repressed genes can be impactful on the maintenance of T cell phenotype and plasticity (Zhou *et al*. 2009, Wilson *et al*. 2009).

Within the TME, most T cells have been shown to be driven towards a hyporesponsive state of exhaustion, characteristically associated with an upregulation in inhibitory receptors, a decrease in effector cytokines and impaired cytotoxicity (Jiang *et al*. 2015,Wherry *et al*. 2015). In choosing to characterize the approach in human pancreatic tumor models, we sought to learn if SAR T cells can migrate to, infiltrate, and mediate anti-tumoral functions against a dense and immunosuppressive microenvironment. The impact of this milieu on SAR T cell phenotype and function was also of interest, the understanding of which having the potential to inform future SAR designs and treatment combinations.

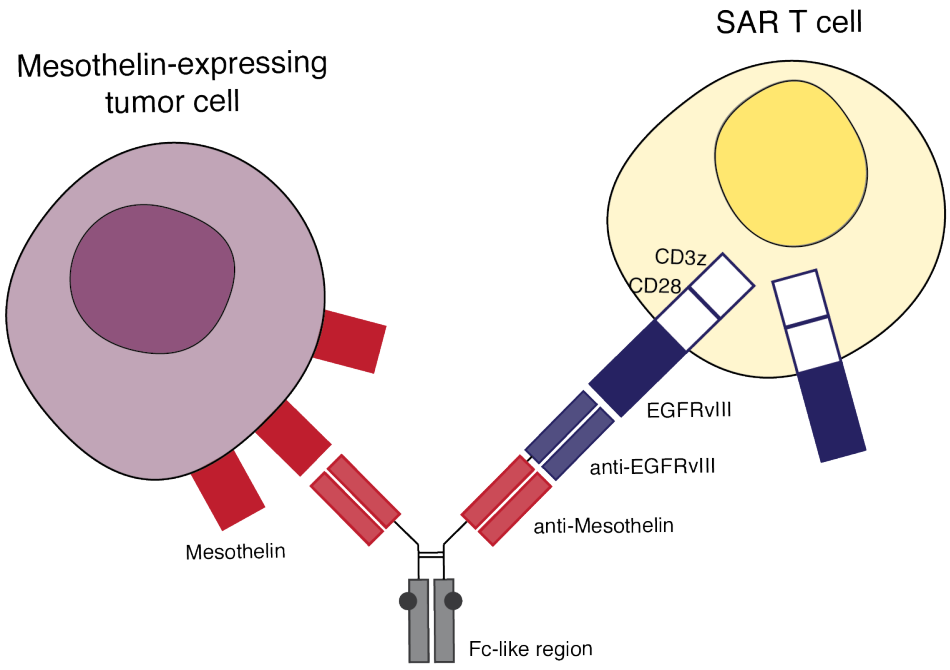

Figure 3: The SAR-BiAb concept illustrated. Specific and conditional SAR T cell activation can only be mediated in a milieu where the tumor target, BiAb and SAR T cell are present.

1.7 Aims

Based on previous findings, this thesis aims to demonstrate the following:

1. To characterize the SAR-BiAb platform in the human system specifically looking at the requirements for T cell activation and target cell lysis. As such, conditional specificity will be evaluated in the presence or absence of the target antigen, as well as determining the range and dose-dependent activity of the BiAb.
2. To visualise the immune synapse at the SAR T cell-target cell interface. Specifically evaluating the function as well as characteristics of the synapse in comparison to those observed at the CAR or TCR-target cell interfaces.
3. To demonstrate the inherent safety, modularity and controllability facets of the platform *in vivo* and *in vitro*
4. To characterize the *in vivo* functionality if the platform in solid tumor xenograft models

2 Material and Methods

2.1 Machines and reagents

Table 1 Machines and devices

Machine	Manufacturer	Origin
Analytical balance	Satorius Laboratory	Göttingen, Germany
Cell culture flow HeraSAFE KS	Heraeus, ThermoFischerScientific	Massachusetts, USA
Centrifuge Rotina 420R	Hettich GmbH	Tuttlingen, Germany
ChemiDoc	BioRad	California, USA
CO_2 – Incubator (BD6220)	Heraeus, ThermoFischerScientific	Massachusetts, USA
FACS Canto II	BD Biosciences	New Jersey, USA
FACS Fortessa	BD Biosciences	New Jersey, USA
iCELLigence	ACEA Biosciences Inc	California, USA
Innova44 Thermoshaker	New Brunswick Scientific, Eppendorf	Hamburg, Germany
Leica TCS SP5 confocal system	Leica Microsystems	Wetzlar, Germany
Light microscope Axiovert 40C	Zeiss	New York, USA
LightCycler480 System	Roche	Mannheim, Deutschland
Multilabel Plate Reader	Mithras LB 940 Berthold	Bad Wildbad, DE
Nanodrop 2000c	ThermoFischerScientific	Massachusetts, USA
pH 720 WTW	inoLab GmbH	Weilheim, DE
PowerPac™ Universal Power Supply	Bio-Rad Laboratories	Munich, Germany
T3 Thermocycler	Biometra	Göttingen, Germany
xCELLigence	ACEA Biosciences Inc.	California, USA

Table 2 Reagents

Reagent	Manufacturer	Origin
Albumin fraction V (BSA)	Sigma-Aldrich	Steinheim, Germany
Annexin A5	Thermofisher	Massachusetts, USA
Batimstat	Sigma-Aldrich	Steinheim, Germany
Calcium chloride	Sigma-Aldrich	Steinheim, Germany
CD178 (Fas Ligand) Monoclonal Antibody (NOK-1), functional grade	ThermoFischerScientific	Massachusetts, USA
CD178 (Fas Ligand) Monoclonal Antibody TNFSF6	RnD systems	Minneapolis, USA
Cetuximab	Apotheke Innenstadt	LMU Munich, Germany
Colagenase D	Sigma-Aldrich	Steinheim, Germany
Count Bright, counting beads	LifeTechnologies	California, USA
Dimethylsulfoxid (DMSO)	Sigma-Aldrich	Steinheim, Germany
DNase I	Roche	Mannheim, Germany
Dithiothreitol	Sigma-Aldrich	Steinheim, Germany
Dulbecco's modified Eagles medium DMEM	PAA	Pasching, Austria
Dulbecco's Phosphate Buffered Saline (PBS)	PAA	Pasching, Austria
Dynabeads human T-activator CD3/CD28	Invitrogen (ThermoFischerScientific)	Massachusetts, USA
EcoRI	NEB	Massachusetts, USA
EcoRV	NEB	Massachusetts, USA
Egtazic acid (EGTA)	Sigma-Aldrich	Steinheim, Germany
Ethanol 100 %	Carl Roth GmbH	Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	Steinheim, Germany
FACSFlow, FACSSafe	BD Biosciences	New Jersey USA
GeneJet plasmid mini prep kit	ThermoFischerScientific	Massachusetts, USA

Heparin-sodium 2.500 IE / 5 ml	Braun AG	Melsungen, Germany
HEPES-buffer 1 M	Sigma-Aldrich	Steinheim, Germany
Human Granzyme B ELISA kit	RnD Systems	Minneapolis, USA
Human Interferon gamma ELISA kit	BD Biosciences	New Jersey USA
Human IL-2 ELISA Kit	BD Biosciences	New Jersey USA
Human serum	Sigma-Aldrich	Steinheim, Germany
Isofluoran	CP PHARMA	Burgdorf, Germany
KasI	NEB	Massachusetts, USA
LB agar	Carl Roth GmbH	Karlsruhe, Germany
LB medium	Carl Roth GmbH	Karlsruhe, Germany
LE agarose	Biozym	Hessisch Oldendorf, Germany
L-glutamin 200 mM	PAA	Pasching, Austria
MEM non-essential amino acids (NEAA, 100x)	Gibco Products	New York, USA
N-Octyl-Beta-Glycopyranoside	Sigma-Aldrich	Steinheim, Germany
NotI	NEB	Massachusetts, USA
OneComp Beads	ThermoFischerScientific	Massachusetts, USA
peqGOLD TriFast	PEQ LABS	Erlangen, Germany
Penicillin/ Streptomycin (100x)	PAA	Pasching, Austria
Puromycin	InvivoGen	California, USA
Propidium Iodide	Thermofisher	Massachusetts, USA
Protease Inhibitor	Sigma-Aldrich	Steinheim, Germany
Q5 enzyme	NEB	Massachusetts, USA
RetroNectin	TaKaRa	Kyoto, Japan
RevertAid first strand cDNA synthesis kit	ThermoFischerScientific	Massachusetts, USA
Recombinant human etc. Amphiregulin	Peprotech	New Jersey USA

Recombinant Human Betacellulin	SinoBiological	Beijing, China
Recombinant Human Betacellulin (Fc Tagged)	SinoBiological	Beijing, China
Recombinant Human EGF	SinoBiological	Beijing, China
Recombinant Human EGF (Fc Tagged)	SinoBiological	Beijing, China
Recombinant human EGFR	SinoBiological	Beijing, China
Recombinant Human Epigen	PeproTech	New Jersey USA
Recombinant Human Epiregulin	PeproTech	New Jersey USA
Recombinant Human Epiregulin (Fc Tagged)	SinoBiological	Beijing, China
Recombinant Human HB-EGF	SinoBiological	Beijing, China
Recombinant Human Mesothelin (Fc tagged)	SinoBiological	Beijing, China
Recombinant Human TGF alpha	SinoBiological	Beijing, China
Rituximab	Apotheke Innenstadt	LMU Munich, Germany
Roswell Park Memorial Institute (RPMI)	PAA	Pasching, Austria
SERVA DNA Stain Clear G	SERVA	Heidlberg, Germany
Sodium chloride	Sigma-Aldrich	Steinheim, Germany
Sodium pyruvate	Gibco Products	Pasching, Austria
Sulphuric acid	Apotheke Innenstadt	LMU Munich, Germany
Tissue freezing medium	Leica biosystems	Nussloch, Germany
TNF alpha monoclonal antibody MP6-XT22	ThermoFischerScientific	Massachusetts, USA
Trypan blue	Sigma-Aldrich	Steinheim, Germany
Trypsin (10x)	PAA	Pasching, Austria
Tween 20	Carl Roth GmbH	Karlsruhe, Germany
Urea	Sigma-Aldrich	Steinheim, Germany

UltraComp eBeads	ThermoFischerScientific	Massachusetts, USA

Table 3 Cell culture media

Medium	Composition	Medium	Composition
Human	VLE RPMI	**RD clone medium**	DMEM
T cell	2 % Human Serum		10 % FBS
	2 mM L-Glutamin		4 mM L-Glutamin
	100 IU/ml Penicillin		100 IU/ml Penicillin
	100 µg/ml Streptomycin		100 µg/ml Streptomycin
	1 mM Natrium-Pyruvat		
	1 % MEM-NEAA		
	50 µM β-Mercaptoethanol		
Tumor	DMEM	**Freezing**	90 % FBS
	10 % FBS		10 % DMSO
	2 mM L-Glutamin		
	100 IU/ml Penicillin		
	100 µg/ml Streptomycin		

2.2 Molecular biology methods

2.2.1 Synthetic agonistic receptor cloning via overlap-extension polymerase chain reaction

The fusion of gene segments by overlap-extension PCR is a fast and effective way to create chimeric genes encoding novel proteins (Heckman *et al*. 2007). The human EGFRvIII-CD28-CD3ζ chimeric receptor construct (E3) is made up of human EGFRvIII (Uniprot Entry P00533 AA 1-29, 298-646), human CD28 (Uniprot Entry P10747 AA 153-220) and human CD3ζ (Uniprot Entry P20963 AA 52-164). The control construct $E3^{del}$ is lacking the intracellular signalling domains CD28 and CD3ζ. SAR constructs were generated by Mathias Kurzay, a

former doctoral student in the laboratory. All SAR constructs were subsequently inserted into the retroviral vector pMP71 (Engels *et al*. 2003).

2.3 Cell lines

2.3.1 Tumor cell lines

The human mesothelin over-expressing cell-lines MIA PaCa-MSLN, SUIT-2-MSLN, and Flp-In-HEK293-MSLN (HEK-MSLN) were generated by transduction with pMXs (Kitamura 2003) containing full-length human mesothelin (MSLN, UNIPROT entry Q13421) cDNA. Likewise, the human glioblastoma cell line U251-MG was generated to express full-length human EGFRvIII (Uniprot Entry P00533 AA 1-29, 298-646). The cell line MSTO-mesothelin-luciferase (MSTO-MSLN-LUC) was generated by transducing the MSTO-211H cells with lentiviruses (VSV-G pseudotyped) encoding full-length human MSLN and full-length firefly luciferase (ffLuc), sequentially. The cells were transduced with lentivirus encoding hMSLN. The gene encoding full-length human MSLN was cloned into the MCS of the lentiviral vector obtained from System Bioscience, pCDH-EF1a-MCS-T2A-Puro cDNA. The cells were then selected with puromycin at 5 μg/mL for 2 to 3 days. Surviving cells were stained and confirmed with surface expression of hMSLN. The generated MSTO-MSLN cells were then transduced with lentivirus encoding ffLuc. The gene encoding full-length ffLuc was cloned into the MCS of the lentiviral vector obtained from System Bioscience, pCDH-CMV-MCS-EF1a-Neo. The cells were then selected with 5 μg/mL of G418 for 2 to 3 days.

2.3.2 Virus production

For virus production retroviral pMP71 (kindly provided by C. Baum, Hannover) vectors carrying the sequence of the relevant receptor were stably expressed in packaging cell lines. Single cell clones were generated and screened for receptor expression levels, then screened for levels of virus production by determining the transduction efficiency of primary T cells. Using this method, we generated the producer cell lines 293Vec-RD114-E3, 293Vec-RD114-E3^{del}, and 293Vec-RD114-CAR-MSLN. 293Vec-Galv and 293Vec-RD114 were grown in DMEM with 10 % fetal bovine serum (FBS, Life Technologies, USA), 1 % penicillin and streptomycin (PS) and 2 % L-glutamine (all from PAA, Germany).

Table 5 Cell lines

HEK-MSLN	**DMEM3+**	Human embryonic kidney cells. Made from Flp-In 293- Cell line	ThermoFischerScientific
A375	**DMEM3+**	Human melanoma cells	Acquired from ATCC
U-251-MG	**DMEM3+**	Human glioblastoma cells	Acquired from ATCC
SUIT-2		Human pancreatic ductal adenocarcinoma cell line derived form a liver metastasis.	
MSTO-211H		Biphasic mesothelioma of the lung	
MIA PaCa-2		Pancreas Carcinoma	
293Vec- Galv	**RD clone medium**	Amphotropic packaging cell line.	Prof. Dr. Manuel Caruso (Québec, Canada)
293Vec-RD114		Amphotropic packaging cell line.	

2.3.3 STR profiling

All human cell lines were short tandem repeat profiled in house to verify their origin. Cells were used for a time period no longer than 2 months.

2.4 Cytotoxicity assays

T cells were coincubated with tumor cells and BiAb at indicated effector to target ratios and concentrations. The coculture conditions between transduced T cells and adherent target cells were set-up as indicated. For impedance-based real-time killing assays the xCELLigence instrument was used. The cell index parameter denoted is a measure of the relative change in electrical impedance, to represent cell status (adherence). All described calculations are based on the RTCA software version 1. The BioGlo cytotoxicity assay (Promega, USA) system was also used where indicated according to the manufacturer's protocol.

2.5 Cytokine release assays

Human T cell stimulation assays were set up at indicated concentrations and effector-to-target ratios. IFN-γ, IL-2 and Granzyme B were quantified by enzyme-linked immunosorbent assay (ELISA).

2.6 Bispecific antibody depletion *in vitro* assay

T cell and tumor cell coculture was set up at indicated antibody concentrations and effector-to-target ratios. SAR T cells were cocultured with BiAb before being added to the tumor cells. Anti-MSLN CAR T cells were used as a control and cocultured with tumor cells following the same procedure (no BiAb was added). After 24 h (time between each depletion) supernatants were collected, and T cells transferred onto a new plate with seeded tumor cells. T cells were either re-suspended with medium only or redosed with BiAb to control for the depletion. Three sequential assay depletions were performed. IFN-γ concentration in the supernatants was quantified by ELISA.

2.7 Animal Experimentation

2.7.1 Xenograft models

NSG mice (NOD.Cg-PrkdcscidIl2rgtm1WjI/SzJ) were purchased from Charles River. Suit-2-MSLN and MiaPaCa-MSLN xenograft models were established by subcutaneously injecting 5 x 10^5 cells into the right flank. MSTO-MSLN-LUC xenograft model was established by subcutaneously injecting 10^6 cells in a 1:1 ratio of PBS and Matrigel (Corning Life Sciences) into the right flank. BiAb and 10^7 T cells were given intravenously as indicated. For the BiAb depletion, redosing schedule and antibody dosage were carried out as indicated. A total of six BiAb treatments were injected per mouse at 3, 6, 9, 13, 17, and 19 days after tumor injection. Mice were bled 6 hours after antibody redosing. All animal experiments were approved by the local regulatory agency (Regierung von Oberbayern) and adhered to the NIH guide for the care and use of laboratory animals. Endpoints were registered by an observer blinded to the treatment groups as previously defined (Karches *et al*. 2019).

2.7.2 *In vivo* imaging

For *in vivo* imaging, mice were injected with 100 µl of luciferin substrate (prepared according to the manufacturer's instructions) 10 minutes prior to imaging (Xenolight D-Luciferin potassium salt, Perkin Elmer, USA). IVIS Lumina X5 (Caliper Life Sciences, Perkin Elmer, USA) was used to acquire *in vivo* images. The Living Image Software 4.7.2 was used for analysis (Perkin Elmer, USA).

2.8 Single cell analysis by flow cytometry

Flow cytometry was carried out according to a previously published protocol (Voigt *et al*. 2017) Spleens were passed through 30 µm cell strainers, following an erythrocyte lysis protocol. Tumors were digested with 1.5 µg/ml collagenase IV and 50 U/ml DNAse I for 30 minutes at 37ºC. Analysis was carried out with a BD FACS Canto II and Fortessa (BD Bioscience, Germany).

2.8.1 Flow cytometry staining

Dead cells were stained using the fixable viability violet dye (Biolegend) for 15 minutes at RT. This was followed by the blocking of Fc receptors with TruStain fcX (Biolegend) for 20 minutes at 4ºC. For phenotype analysis surface staining was performed by anti-human CD8a (clone HIT8a, Biolegend), anti-human CCR7 (clone G04387, Biolegend), anti-human CD45RO (clone UCHL1, Biolegend), anti-human PD1 (clone EH12.2H7, Biolegend), anti-human EGFR (APC, clone AY13, Biolegend), anti-human CD45 (clone 2D1, Biolegend), anti-human CD3 (clone HIT3a, Biolegend), and anti-human CD4 (clone OKT4, Biolegend). Cells were washed and resuspended in PBS. For cell number quantification CountBright® absolute counting beads (Life Technologies) were added.

2.9 Confocal microscopy

Blinded confocal imaging and conjugate quantification were carried out following the selection of 10 representative areas of each slide. Cells in or out of conjugate within each area were quantified and a ratio thereof subsequently determined. For each conjugate, the position of the microtubule organizing center (MTOC) was observed, and its polarization to the immune synapse, or lack thereof, was noted. The ratio of polarized to non-polarized

MTOCs was used to determine the ratio of functional synapses out of all conjugates formed. Image acquisition and staining are outlined in the supplementary methods.

2.9.1 Confocal image acquisition

Samples for confocal laser scanning microscopy were examined using a Leica TCS SP5 confocal system (Leica Microsystems, Wetzlar, Germany), with a HCX PL APO CS 63x/1.4 oil objective. For z-axis image reconstruction (stacking), confocal sections were taken 0.2 μm apart, assembled using the Leica application suite v2.7.3.9723 (Leica Camera AG) and further processed by the open access program FIJI, based on image J (https://fiji.sc). For each marker, at least 20 conjugates were recorded by confocal microscopy per condition.

2.9.2 Confocal microscopy staining

SAR T cells were stained with anti-human Granzyme-B (clone GB11, BD), anti-human LFA-1 (clone CB5.4, BioLegend), anti-alpha tubulin, (clone DM1A, Abcam), anti-human Lck (BD Biosciences). F-actin was stained using Phalloidin-iFluor reagent (Abcam). Following conjugation in a 96-well V-bottom plate, cells were transferred onto a poly-L-lysine-coated (1 μg/ml – Sigma, Germany) microscope slide (ThermoFisherScientific), and were allowed to adhere for 30 minutes, Then, fixation, permeabilization (BD Cytofix, BD Biosciences) and antibody staining were carried out according to the manufacturer's instructions.

2.10 Statistical Analysis

Statistical evaluation was performed using GraphPad Prism software V8.3.1 (San Diego, CA, USA). Differences between experimental conditions were analysed as described in the figures. P values < 0.05 were considered to be significant. Data are shown as mean values with a SEM of a minimum of three biological replicates or independent experiments, as indicated.

3 Results

3.1. SAR expression and BiAb binding

In efforts to equip T cells with a controllable activating receptor, we generated a SAR construct made up of the extracellular domain of human EGFRvIII, linked to the intracellular CD28 and CD3ζ domains - E3. Control construct lacking the intracellular costimulatory domains was also generated, herein referred to as - E3^{del} (Figure 1A). Constructs could be retrovirally transduced into human T cells with high and comparable efficiencies (Figure 1B).

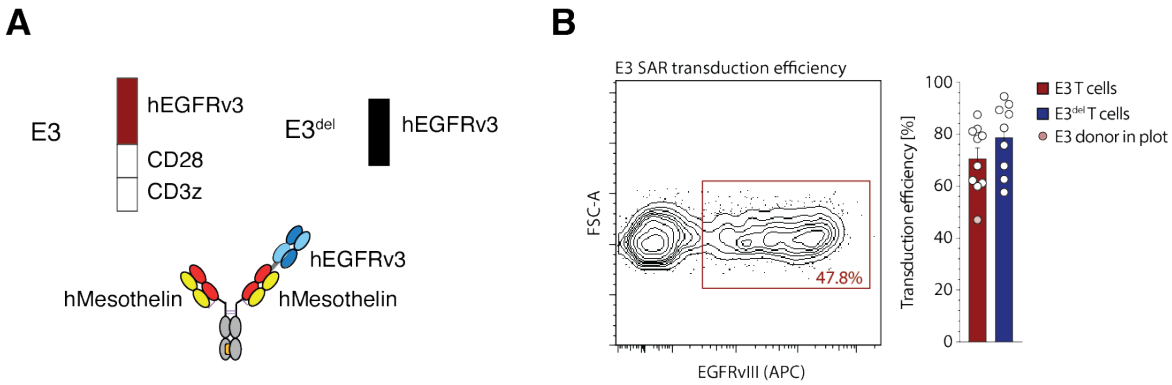

Figure 1: Schematic drawing of constructs and molecule and retroviral transduction of human CD3⁺ T cells. (A) E3 SAR and E3^{del} construct designs as well as schematic depiction of the 2+1 CrossMab. B) Flow cytometry plot depicting exemplary shift in SAR expression of transduced cells, with graph depicting range of transduction efficiencies from ten representative donors. Figure showing absolute transduction efficiencies from 10 healthy donors.

Using the CrossMab technology (Schaefer *et al*. 2011) a trivalent yet bispecific (2+1) antibody (BiAb) with a single specificity for the SAR receptor (EGFRvIII) and two specificities against the tumor antigen (MSLN) was generated by collaborators at Roche Glycart (Schlieren, Switzerland) (Figure 1A). Previous studies (in the murine system) could demonstrate that BiAbs that are bivalent for the SAR receptor resulted in unwanted crosslinking of the receptor upon binding. This receptor crosslinking was shown to be abolished when a trivalent format monovalent for the fusion protein on the T cell was used.

Before BiAb characterization, its binding curve and kinetics were assessed. To establish a binding curve, SAR T cells were coincubated with ascending concentrations of the E3-MSLN BiAb, and

binding activity was shown to begin at a concentration of 0.01 µg/ml, before SAR T cell saturation was reached at 10 µg/ml (Figure 2A). The binding kinetics of the CrossMab to both its targets (MSLN and EGFRvIII) were assessed by recording binding over time using flow cytometry. Using a saturating BiAb concentration (15 µg/ml) we could show that the binding kinetics were comparable for both targets, with E3 T cells used as the EGFRvIII target and Suit-MSLN tumor cells used as the MSLN target (Figure 2B).

We initially assessed whether the trivalent BiAb format (monovalent for the fusion receptor) could specifically and conditionally activate the receptor in the human system. Binding assays were set-up, where SAR T cells were cultured with coated BiAb (immobilized) or soluble BiAb (free), with the anti-EGFR monoclonal antibody (mAb) cetuximab used as a positive control, and the anti-CD20 mAb rituximab used as a negative control. Results demonstrated that only the immobilized BiAb was able to induce T cell stimulation in E3 T cells, while E3^{del} and UT T cells were not. As expected, cetuximab mediated SAR T cell stimulation while rituximab did not. This SAR T cell activation was also shown to occur in a dose dependent manner (Figure 2C and D).

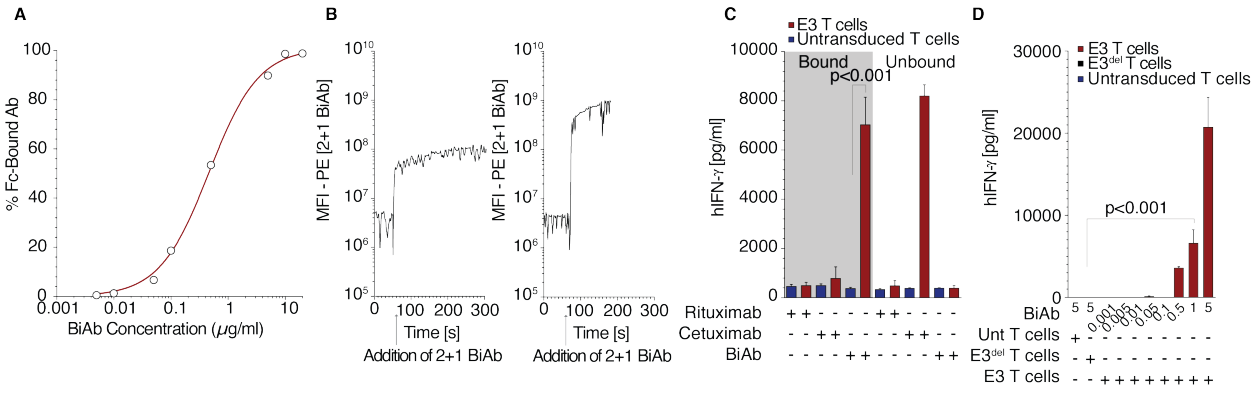

Figure 2: BiAb binding kinetics and its capacity to mediate SAR T cell activation. (A) E3-MSLN BiAb binding curve. 10^7 T cells were co-incubated for 1 hour with ascending concentrations of E3-MSLN BiAb – (0.005 µg/ml to 15.0 µg/ml). (B) 2 + 1 BiAb (EGFRv3 x hMSLN) was pre-labeled with R-Phycoerythrin (PE). A mixture of human E3 SAR T cells (Left) and MSTO-MSLN tumor cells (Right) were acquired at the flow cytometer for approximately 30 sec. The respective 2 + 1 BiAb was added within 5 sec and BiAb binding was recorded for an additional 200-300 sec. BiAb binding was immediate and equal for both target and effector cells. (C) In a 96 well plate, BiAb (5 µg/ml) was either coated overnight (bound) or added in soluble form (unbound) with E3, E3^{del} or UT T cells. Soluble and bound monoclonal antibodies (rituximab – anti-CD20; cetuximab – anti-EGFR) were added as negative and positive controls, respectively. (D) In a 96 well plate, BiAb (0.001 µg/ml to 5.0 µg/ml) was coated overnight (bound) before E3, E3^{del} and UT T cells were added. (C and D) Readouts were carried out at 48 h. For each readout, supernatants were collected

and a hIFN-γ ELISA readout subsequently carried out. For statistical analysis the unpaired two-tailed student's t test was used. Cytokine release experiments show mean values ± SEM and are representative of three independent experiments.

3.2 A closer look at SAR activation requirements

To further analyze the requirements for T cell activation upon SAR T cell engagement, we used natural ligands of EGFR. We found that soluble EGFR ligands did not trigger E3 T cell activation when used under physiological conditions (Figure 3A and B). Furthermore, following the inhibition of EGFR ligand shedding from the tumor cell surface (using the A375 cell line) via the addition of the matrix metalloprotease inhibitor batimastat, SAR T cells were not activated by the upregulated membrane bound ligands compared to the positive control, though basal activation – lower by more than a 100-fold – was observed (Figure 3C). In contrast, Fc-tagged EGF immobilized to the well induced similar activation to EGFR-binding antibodies immobilized in the same way. This indicates that T cell activation is mediated by crosslinking of the SAR.

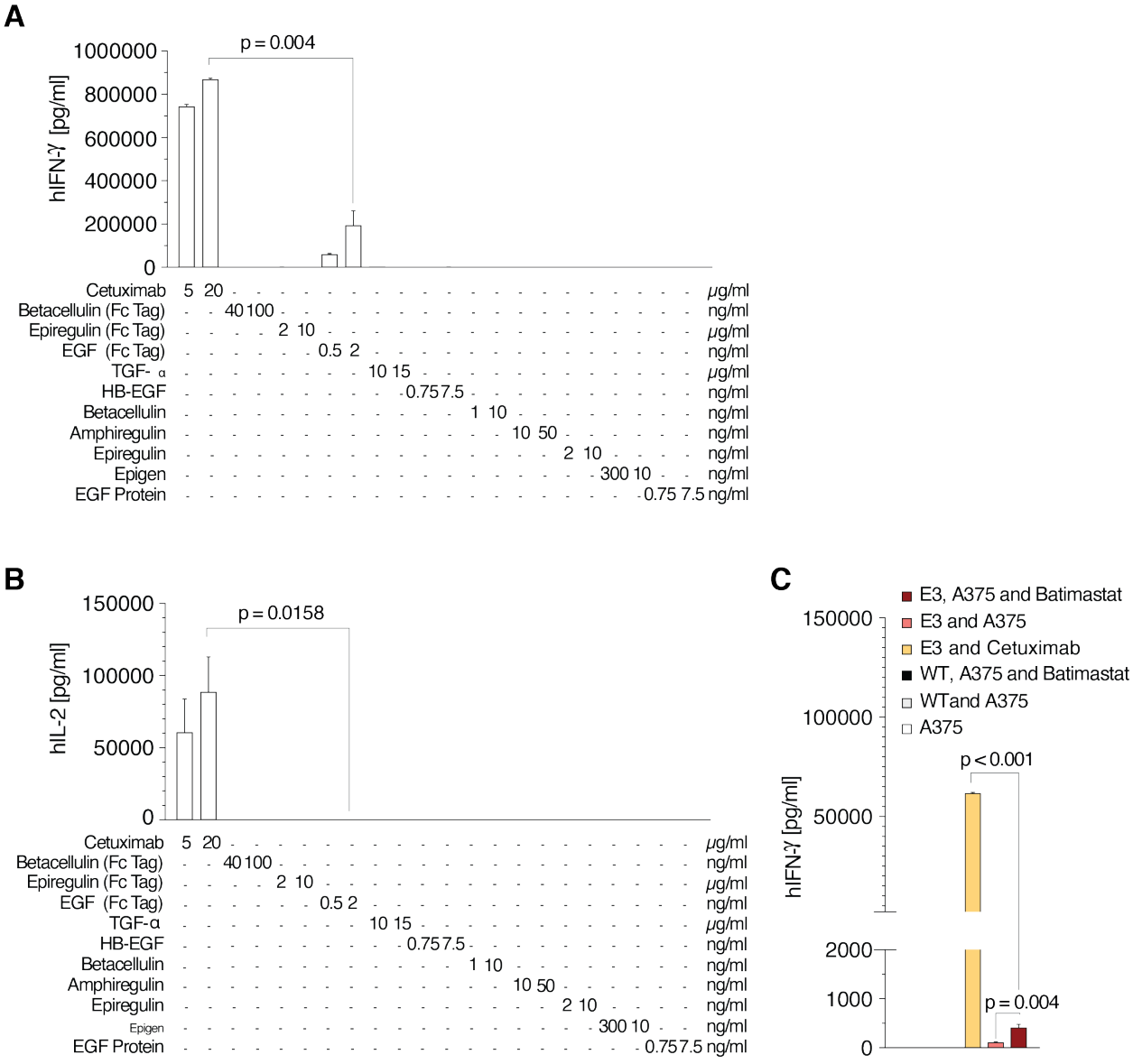

Figure 3: The impact of natural and soluble EGFR ligands on SAR T cell activation. (A) Quantification of T cell stimulation as measured by IFN-γ (A) and IL-2 (B), following coculture of E3 T cells with known EGFR ligands in their soluble as well as Fc-bound forms. Plate-bound cetuximab (5 µg/ml) was used as a positive control. (C) Quantification of T cell stimulation following coculture of E3 T cells with A375 tumor cells (E:T 10:1). The metalloprotease inhibitor Batimastat was added (10 uM) to prevent EGFR ligand shedding. Readouts were carried out at 48 h. For statistical analysis the unpaired two-tailed student's t test was used. Experiments show mean values ± SEM and are representative of two independent experiments.

3.3 BiAb-mediated effects on SAR T cell activation in presence of target cells

Having shown that BiAb-mediated T cell activation is strictly dependent on the crosslink to the target cells, we further assessed this conditional T cell activation. We incubated healthy donor SAR-transduced T cells with the 2+1 CrossMab in the absence or presence of MSLN-expressing pancreatic and mesothelioma cancer cell-lines MiaPaca-MSLN, Suit-MSLN, and MSTO-MSLN, with untransduced and E3^{del}-transduced T cells serving as controls. Importantly, only E3 T cells in the presence of the BiAb as well as the target antigen were shown to produce IFN-γ in a dose-dependent manner, whereas UT and E3^{del}-transduced T cells were not stimulated even in the presence of both BiAb and target molecules (Figure 4A, B and C).

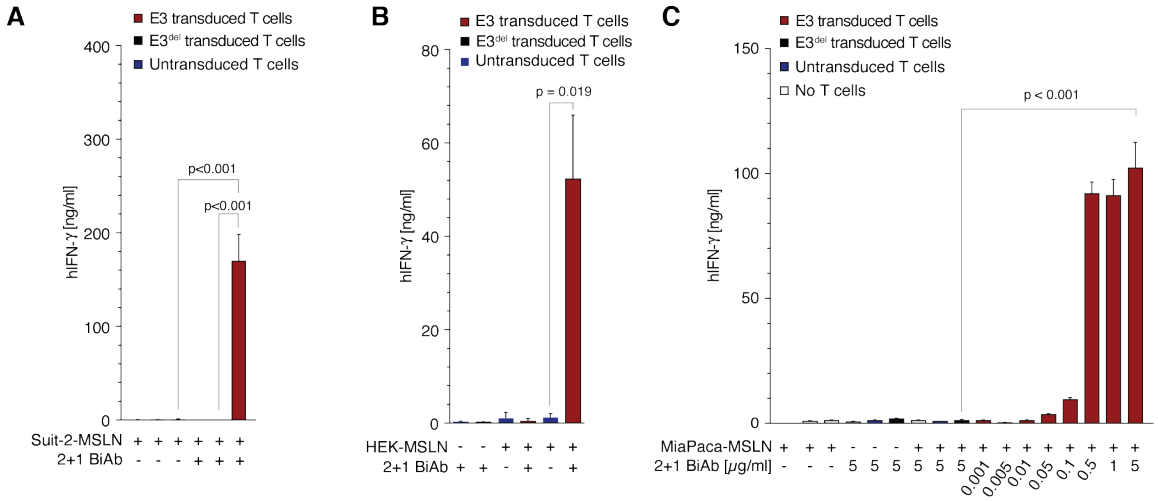

Figure 4: Human SAR T cells are specifically stimulated by anti-human mesothelin x anti-EGFRv3 2 + 1 BiAb in presence of mesothelin^{+} target cells *in vitro*. (A and B) Quantification of E3, E3^{del} or UT T cell activation when co-cultured with Suit-2-MSLN (A) or HEK-MSLN (B) cells and 2 + 1 BiAb (5 µg/ml, E:T - 10:1). (C) Quantification of E3, E3^{del} or UT T cell activation when co-cultured with MiaPaca-MSLN tumor cell and increasing concentrations of 2 + 1 BiAb (0.001, 0.005, 0.01, 0.05, 1 or 5 µg/ml, E:T 10:1). For statistical analysis the unpaired two-tailed Student's t test was used. Experiments show mean values ± SEM and are representative of three independent experiments.

3.4 SAR-transduced T cells form functional immunological synapses, to mediate efficient tumor-cell lysis

MSLN-expressing tumor cells (Suit-02-MSLN and MiaPaca-MSLN) were effectively targeted and lysed by E3 T cells together with E3-MSLN BiAb, but not by $E3^{del}$ and UT T cells. By assessing killing data in real-time, and by employing various effector to target ratios (10:1, 5:1, 1:1), we were able to observe that (Figure 5A and B).

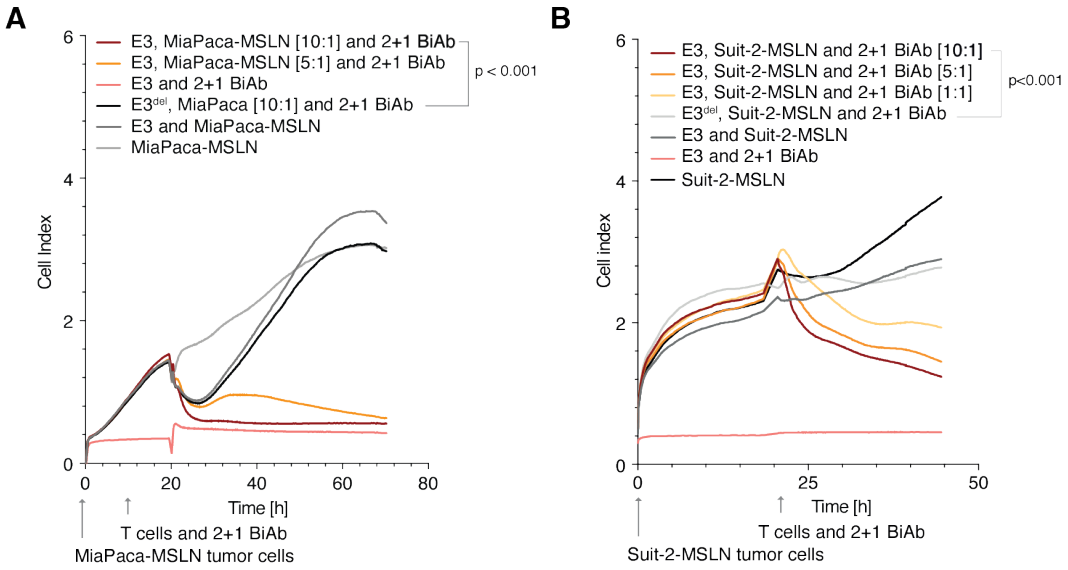

Figure 5: Human SAR T cells mediate specific and conditional tumor cell lysis of mesothelin+ target cells. (A and B) Real-time quantification of MiaPaca-MSLN (A) or Suit-MSLN (B) tumor cell lysis by E3 or $E3^{del}$ T cells and 2 + 1 BiAb at either 10:1, 5:1 or 1:1 effector to target ratios. Experiments shown are representative of 3 independent experiments. Impedance-based cytotoxicity assays were performed in duplicates for technical reasons. For statistical analysis, the total curve over time was compared.

To further control for the specificity of T cell activation and killing, we coincubated E3 SAR T cells with two cell lines, one expressing EGFRvIII (U251) and the other expressing MSLN (MiaPaca-MSLN). We could show that the EGFRvIII expressing cell line could not induce E3 T cell activation, in contrast to the MSLN-expressing one (Figure 6A). This was further supported by comparing the two in a real-time killing assay, where we saw no killing of the EGFRvIII-expressing cell-line compared to the MSLN expressing one (Figure 6B and C).

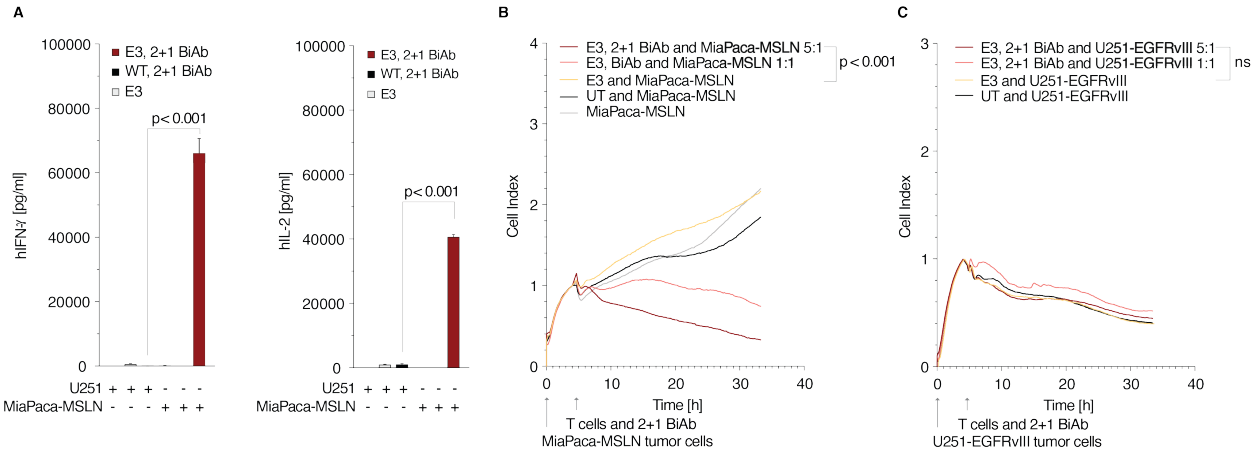

Figure 6: E3-MSLN BiAb activates and redirects SAR T cells in presence of cells expressing mesothelin but not EGFRvIII. (A) Quantification of T cell stimulation as measured by IFN-γ (left) and IL-2 (right), following coculture of E3 or UT (WT) T cells with MSLN-expressing MiaPaca-MSLN or EGFRvIII-expressing U251-EGFRvIII and 2 + 1 BiAb (5 µg/ml). (B) Real-time quantification of MiaPaca-MSLN (B) or U251-EGFRvIII (C) tumor cell lysis by E3 or UT (WT) T cells and 2 + 1 BiAb. Experiments show mean values ± SEM and are representative of 3 independent experiments. Impedance-based cytotoxicity assays were performed in duplicates for technical reasons. For statistical analysis, an unpaired student t test was used (A and B). For impedance-based readouts (C and D), the total curve over time was compared.

To further dissect the mode of action of SAR T cells in these settings, we next thought to analyze the interface between both cell types. Cell conjugates and synapses formed between the T cells and tumor cells were subjected to double color immunofluorescence labeling. The number of T cell-target cell conjugates were quantified. To probe the nature of the immunological synapse, we assessed F-actin and CD11a-LFA-1 accumulation. IS functionality was judged by the polarization of the microtubule organizing center (MTOC).

E3 SAR T cell-target cell conjugates occurred much more frequently than UT T cell-target cell conjugates, while $E3^{del}$ T cells interestingly formed a comparable number of synapses (Figure 7A). Indicative of a functional immune synapse is the strong accumulation of F-actin, which was observed to span the entire area of the synapse. MTOC polarization was significantly higher in SAR-target cell conjugates compared to UT and $E3^{del}$ controls (Figures 7B). A moderate accumulation of LFA-1 signal was seen at the immune synapse, although the LFA-1 signal was also observed across the T cell surface (Figure 7C). Human SAR T cells appeared to utilize the granzyme-perforin axis for target cell lysis under these settings, as granzyme B accumulation and degranulation at the immunological synapse was also observed (Figure 7C).

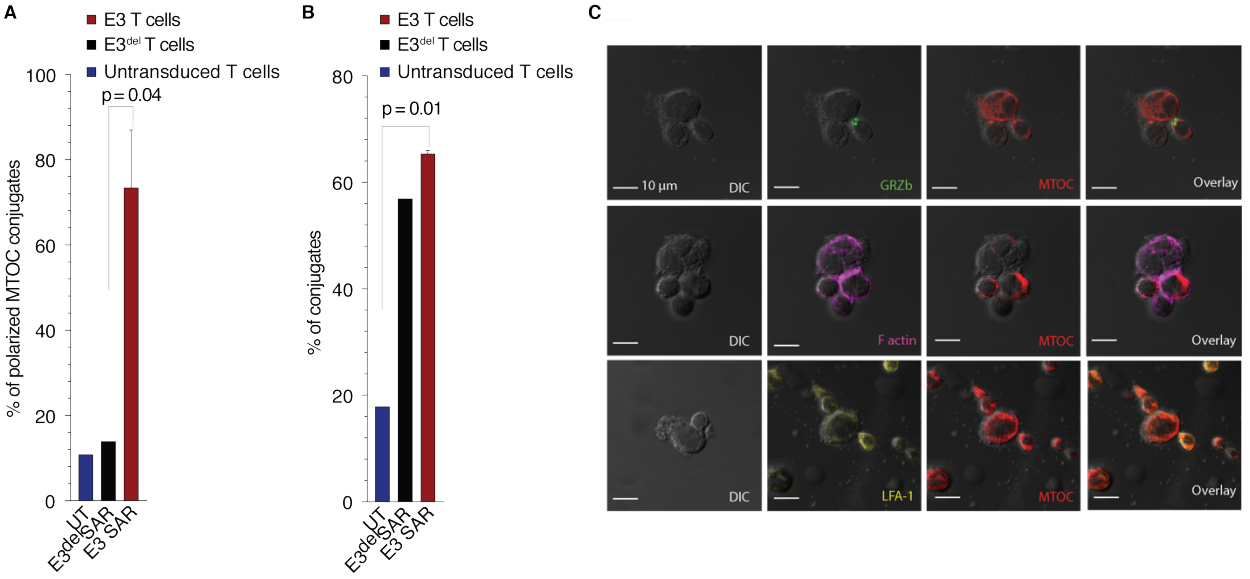

Figure 7: E3 SAR T cells form functional immune synapses. (A and B) E3, E3^{del} and UT T cells were cocultured with Suit-MSLN tumor cells in a V-well plate following the described conjugation assay method. The percentage of T cells in conjugate with tumor cells was quantified (A), as well as the percentage of those conjugates which has a polarized MTOC (E3 formed significantly more conjugates than E3^{del} ($p = 0.04$)) (B). (C) Double Immunofluorescence labelling was carried out to look at the polarization of the MTOC, Granzyme B, LFA-1 and F-actin at the IS. For statistical analysis the unpaired two-tailed student's t test was used. Experiments in subfigures (A and B) show mean values ± SEM and are representative of two independent experiments. Subfigure D is representative of two independent experiments. Leica TCS SP5 confocal system with a HCX PL APO CS 63x/1.4 oil objective was used for image acquisition on Leica application suite v2.7.3.9723. Tumor cells were GFP positive. Fluorochromes used: MTOC (AF594) Granzyme B (AF647); F-actin (AF647); LFA-1 (AF647); Lck (AF647).

3.5 Recombinant protein inhibition of SAR-BiAb activity

We wanted to assess the potential impact of non-tumor or T cell-derived MSLN and EGFRvIII proteins on platform functionality. To achieve this, we used recombinant MSLN and EGFRvIII proteins. Proteins were added in ascending concentrations to a T cell-tumor cell coculture to look at T cell killing efficiency and kinetics.

Saturating concentration of EGFRvIII did not appear to have an impact on killing efficiency nor killing kinetics. However, ascending concentrations of recombinant MSLN appeared to have an inhibitory effect on T cell killing (B). Nevertheless, the concentrations where this effect was

observed were supra-physiological. Thus, it appears that the SAR T cell- BiAb platform is not easily impacted by alternative sources of targeted proteins.

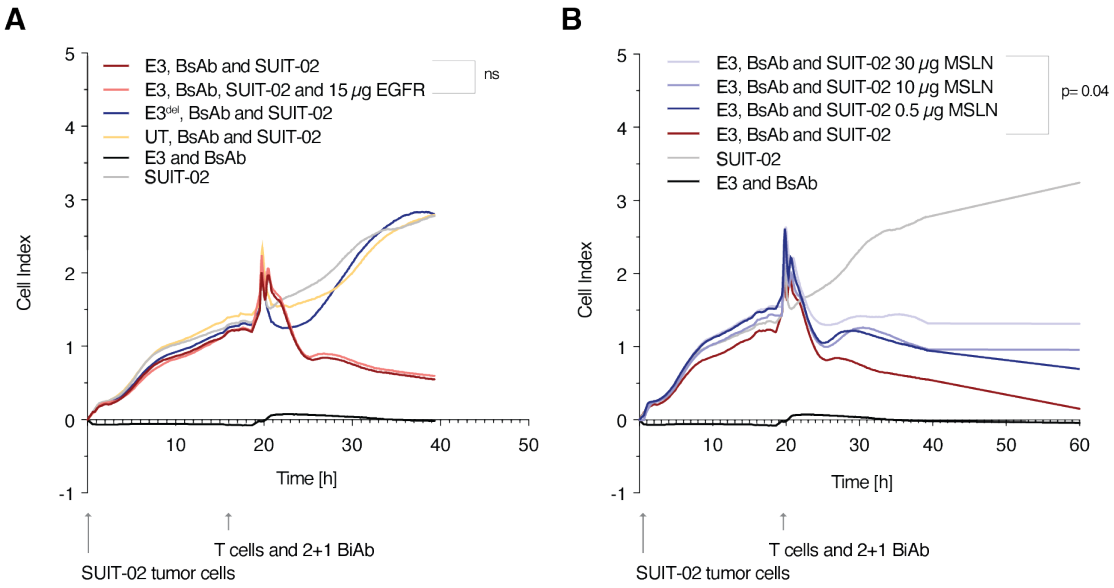

Figure 8: Recombinant protein inhibition of SAR-BiAb axis. (A) Real-time quantification of the impact of recombinant EGFR (15 µg/ml) (A) and MSLN (0.5, 10 and 30 µg/ml) (B) on Suit-MSLN-mediated killing by E3 SAR T cells and BiAb combination. (A and B) 2 x 10^5 T cells were cocultured with 2 x 10^4 Suit-MSLN tumor cells. A 5µg/ml concentration of BiAb was used. Experiments shown are representative of 2 independent experiments. These impedance-based cytotoxicity assays were performed in duplicates for technical reasons. For statistical analysis, the total curve over time was compared.

3.6 Selective and reversible activation of SAR T cells and their applied safety-switches

To demonstrate the selective advantages of the SAR x BiAb platform over BiTE, E3 SAR T cells were serially titrated in a PBMC mix, then cocultured with target cells and either a pan T cell-targeting T cell bispecific antibody (TCB) (anti-CD3 x anti-MSLN) or a SAR-specific BiAb (anti-E3 x anti-MSLN). The selective activation of SAR T cells was evident when the SAR x PBMC mix was coincubated with an anti-EGFRvIII x anti-MSLN BiAb, as IFN-γ levels decreased with lower concentrations of SAR T cells in the mix.

This titrated T cell activation effect was lost when the anti-CD3 x anti-MSLN BiAb was employed at equivalent total cell numbers. Further, the anti-EGFR x anti-MSLN BiAb did not mediate any T cell activation when incubated with a pure PBMC mix devoid of SAR T cells, whereas the anti-

CD3 x anti-MSLN BiAb was non-selective in activating $CD3^+$ T cells in the PBMC mix, as expected (Figure 9A).

An intrinsic safety switch of the SAR x BiAb platform is that the activity of SAR T cells is strictly dependent on the presence of the BiAb. Unlike CAR T cells, the activity of which is irreversible in the presence of the target antigen, SAR T cell activity should quickly dissipate with clearance of the BiAb. This could be demonstrated following coculture with Suit-MSLN tumor cells, as SAR T cell activity was reversible over time in the absence of BiAb redosing, unlike human anti-MSLN CAR T cells. Importantly, repeated dosing of the BiAb molecule could maintain SAR activity at comparable levels to that of the CAR (Figure 9B). This data indicates that the short half-life of the BiAb molecule enables control over SAR activity through half-life engineering of the targeting molecule.

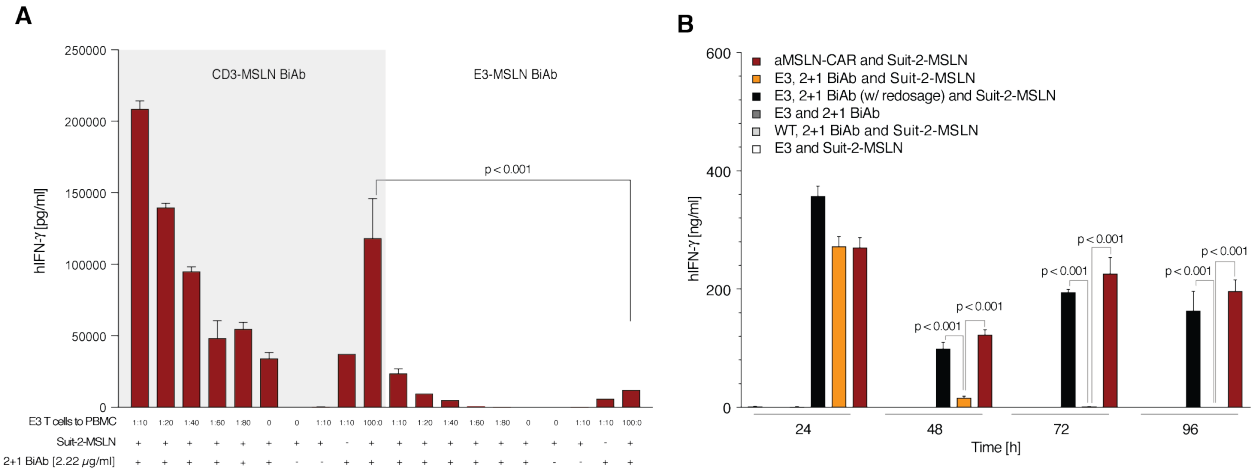

Figure 9: Modular, selective and reversible activation of SAR T cells and their applied safety switches. (A) SAR T cells were serially titrated (1:10, 1:20, 1:40, 1:60, 1:80, 0:100) in a PBMC mix. Cells were then cocultured with Suit-2-MSLN tumor cells (E:T - 10:1), with either a pan-T cell-BiAB (anti-CD3–anti-MSLN, 5 µg/ml) or a SAR-specific BiAb of the same format (anti-E3–anti-MSLN, 5 µg/ml). (B) E3-MSLN BiAb selectively activates E3 T cells while its depletion results in rapid and complete reversibility of SAR T cell stimulation. For BiAb depletion, Suit-2-MSLN tumor cells were repeatedly cocultured with E3 T cells with or without readjustment of the BiAb concentration (5 µg/mL). Control condition utilized anti-MSLN-CAR T cells (E:T 10:1). For statistical analysis the unpaired two-tailed student's t test was used. Experiments show mean values ± SEM and are representative of at least two independent experiments.

3.7 Impact and functionality of SAR-BiAb combination in pancreatic cancer xenograft models

To assess the functionality of the SAR T cells and BiAb combination *in vivo*, pancreatic cancer and mesothelioma xenograft models were developed. The three models developed for testing were MiaPaca-MSLN, Suit-MSLN and MSTO-MSLN. E3 T cells plus BiAb mediated anti-tumor responses in all three tumor models (Figure 10 A-D). In addition, mice where challenged at two tumor sizes (<10 mm^2 and <25 mm^2), with a therapeutic benefit still being observed when larger tumors were treated (Figure 10C and 10D).

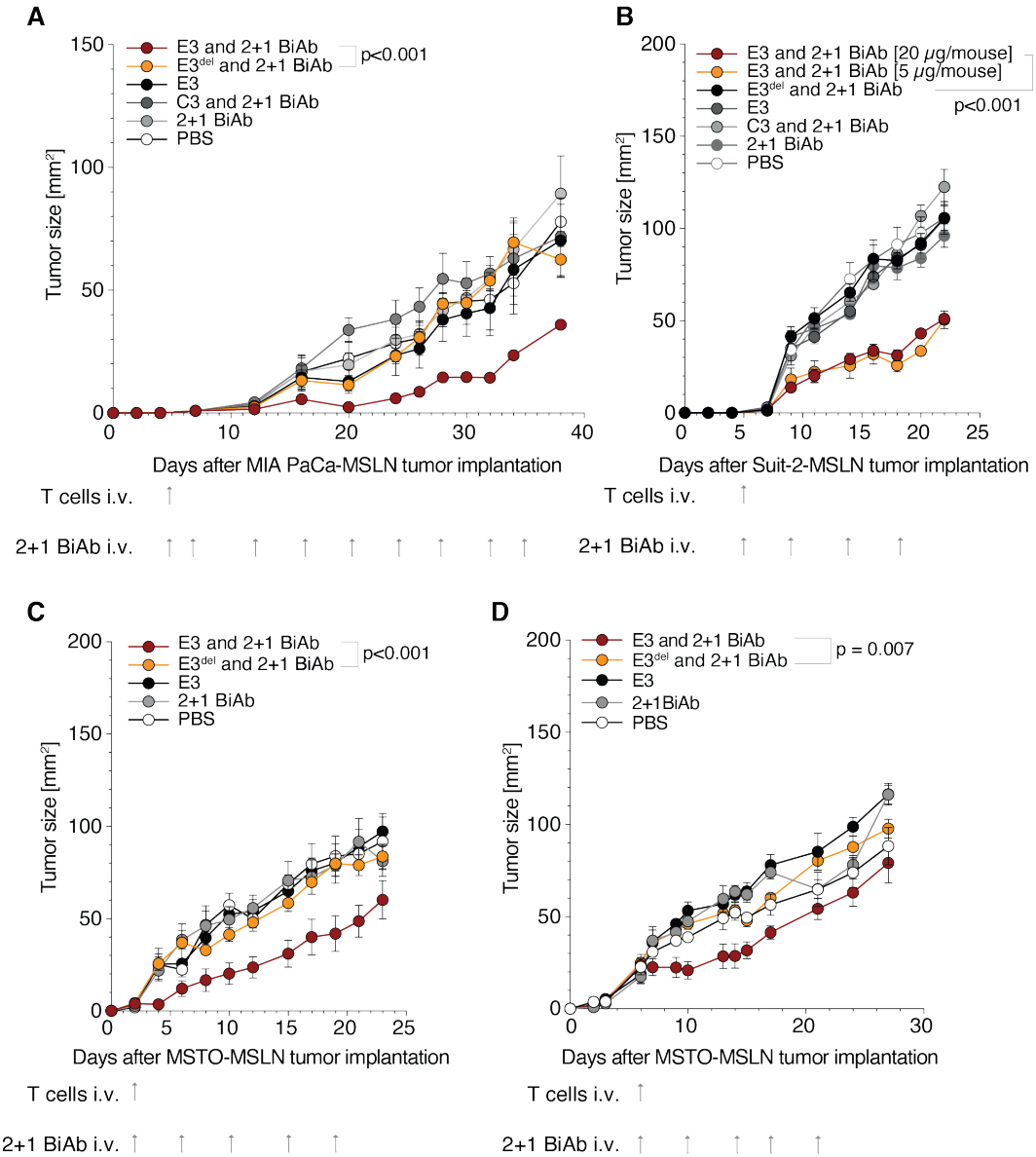

Figure 10. Anti-human mesothelin anti-EGFRvIII 2 + 1 BiAb specifically stimulates SAR T cells and mediates tumor killing in pancreatic cancer xenograft models. (A) NSG mice inoculated s.c. with MIA PaCa-MSLN were treated with a single i.v. injection of E3 T cells only (n = 6), E3 T cells + 10 μg BiAb (n = 10), E3del T cells + 10 μg BiAb (n = 6), C3 T cells + 10 μg BiAb (n = 6), 10 μg BiAb only (n = 6), and PBS (n = 6), followed by a total of 10 BiAb injections as indicated. (B) NSG mice inoculated s.c. with Suit-2-MSLN were treated with a single i.v. injection of E3 T cells only (n = 5), E3 T cells + 20 μg BiAb (n = 10), E3 T cells + 5 g BiAb (n = 5), E3del T cells + 20 μg BiAb (n = 5), C3 T cells + 20 μg BiAb (n = 5), 20 μg BiAb only (n = 5), and PBS (n = 5), followed by a total of four BiAb injections as indicated. NSG mice inoculated s.c. with MSTO-MSLN-LUC were treated with a single i.v. injection of E3 T cells only (n = 5), E3 T cells + 10 μg BiAb (n = 5), 10 μg BiAb only (n = 5), and PBS (n = 5), followed by a total of four BiAb injections as indicated. NSG mice inoculated s.c. with 1 x 10^6 MiaPaca MSLN (A), MSTO-MSLN-LUC cells. When tumors reached the size of < 25 mm^2, mice were treated with a single i.v. injection of E3 T cells only (n=5), E3 T cells + 10 μg BiAb (n=5), 10 μg BiAb only, (n=5), and PBS (n=5), followed by a total of four BiAb injections, as indicated. Analysis of differences between groups for the tumor growth curves was performed using two-way ANOVA with correction for multiple testing by the Bonferroni method.

In the MSTO-MSLN-LUC xenograft model, we could observe significant infiltration and persistence of E3 T cells in the treated mice 23 days following adoptive transfer, which was not seen in control groups (Figure 11A, C and D). This tumor T cell infiltrate consisted predominantly of $CD4^+$ SAR T cells, though $CD8^+$ SAR T cells were also present (Figure 11A) - In the MSTO-MSLN-LUC cell-line, a luciferase signal could be used to quantify and show tumor cell persistence at experimental end-point (Figure 11 B).

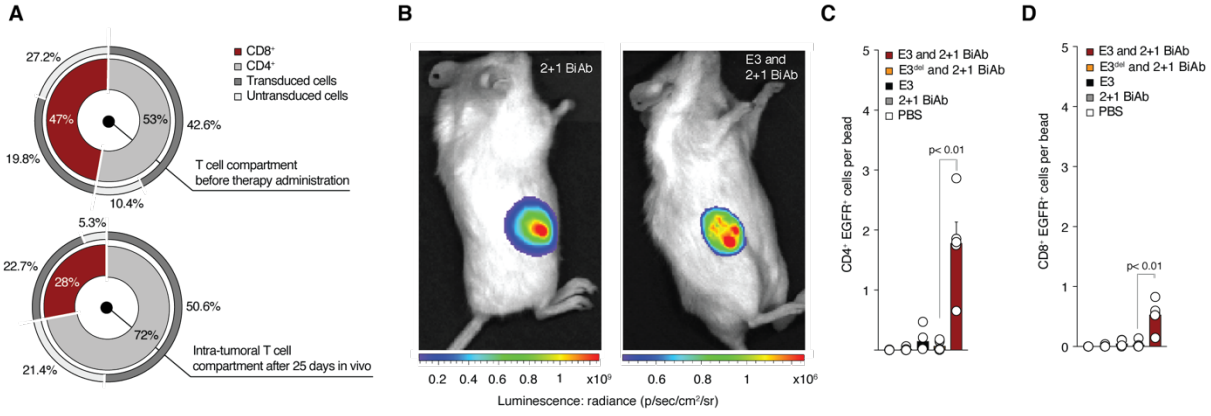

Figure 11. SAR T cell phenotype, infiltration as well as luciferase-based readout from MSTO-MSLN-LUC xenograft experiment. (A) FACS-based quantification of E3 T cell compartments before therapy administration, and the intra-tumoral infiltrate following tumor harvest at day 25 of MSTO-MSLN-LUC xenograft treatment experiment. Inner circle, CD4 and CD8 ratios. Outer circle, ratio of transduced and un-transduced T cells within each compartment. (B) Representative NSG mice from MSTO-MSLN-LUC xenograft treatment experiment are depicted. Tumor persistence in both treated mouse (right hand side) and control treated mouse (left hand side). Differences in luminescence shown in

radiance scales underneath. (C and D) Tumors from the mice in MSTO-MSLN-LUC xenograft experiment (Figure 10 D) were FACS analyzed for T cell infiltration. Following a drill down through the CD45 and CD3 gates, the EGFR^{+} CD4 (C) and CD8 (D) T cell populations were quantified and are depicted as a ratio of cells per bead. In vitro experiments show mean values ± SEM of at least triplicates and are representative of at least three independent experiments. P values are based on two-sided unpaired t test.

LDH, urea, AST and ALT serum levels remained within the normal range for both E3 T cell plus BiAb- and E3^{del} T cell plus BiAb-treated mice, showing no systemic parameters of kidney or liver damage in the treated models (Figure 12 A-D).

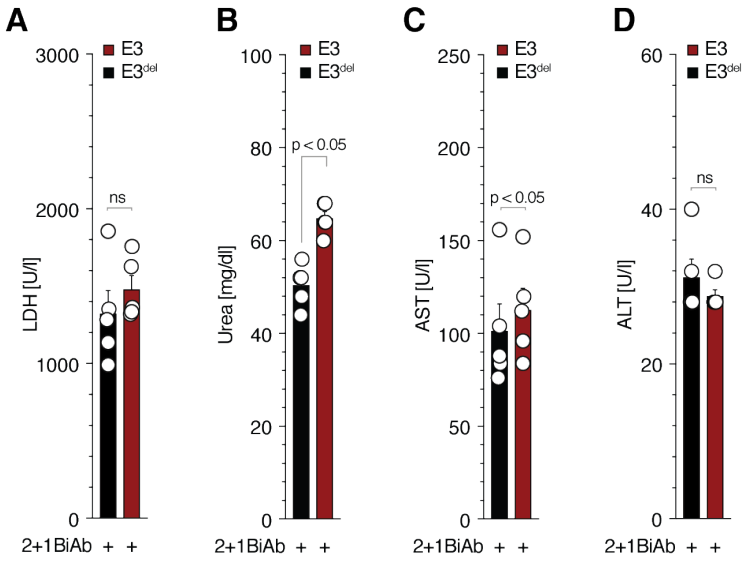

Figure 12: Serum readout of several toxicity markers following SAR-BiAb treatment combination. (A, B, C, D) LDH (A), urea (B), AST (C) and ALT (D) serum levels comparing E3 + 2+1 BiAb treated mice with the control mice receiving E3^{del} + 2+1 BiAb. Serum was collected at indicated experimental endpoint of MSTO-MSLN-LUC xenograft treatment experiment (see Figure 10). Data collected from 5 mice per group. Experiment shows mean values ± SEM. P values are based on two-sided unpaired t test.

To investigate whether the observed *in vitro* reversibility of SAR T cell activity could also be observed *in vivo*, we used the Suit-02-MSLN xenograft model to compare tumor growth in mice that were redosed with BiAb with mice that received a single dose. Despite transient tumor control (P = 0.026 on day 12), non-redosed mice lost tumor control compared with redosed mice (Figure 13A). To link these findings to limited T cell activation, mice were bled repeatedly, and serum IFN-γ concentration levels were quantified. We found T cell stimulation was reversible in non-redosed versus redosed mice as a function of time (Figure 14 B). In summary, we could show

that SAR T cell activity is limited to the continued presence of the BiAb, which unlike for CAR T cells could prevent unwanted prolonged T cell activation.

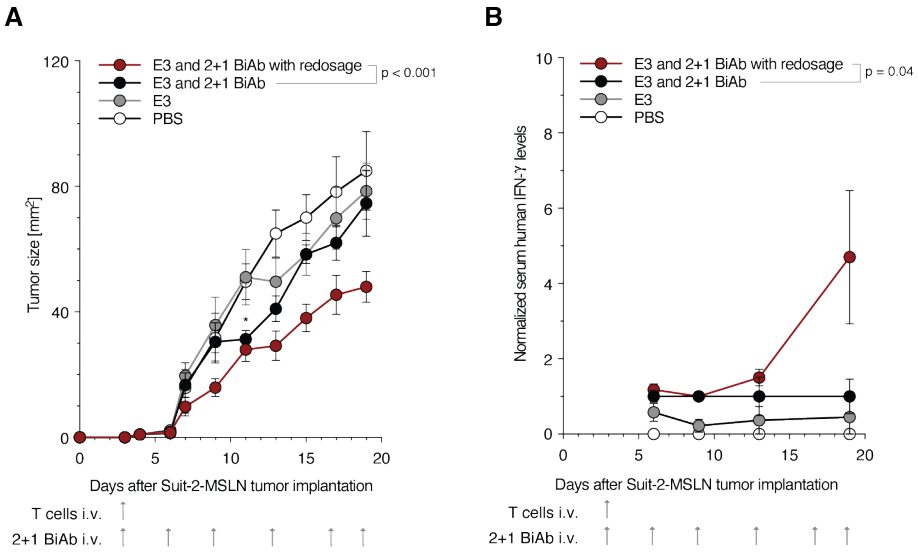

Figure 13: Reversibility of SAR T cell activation upon BiAb depletion, *in vivo.* (A and B) NSG mice inoculated s.c. with Suit-2-MSLN (n = 5 / group) were treated with a single i.v. injection of E3 T cells only, E3 T cells + 10 µg BiAb—redosed, E3 T cells + 10 µg BiAb—single dose and PBS followed by a total of six BiAb injections as indicated. Significance of non-redosed group versus E3 only at day 12 (P = 0.026; E). Mice were bled 3, 6, 10, and 16 days after T cell transfer to quantify T cell activation by IFN-γ serum levels (F). Data are reported normalized to the values obtained from the single-dose group. Experiments show mean values +/- SEM and are representative of one independent experiment. Analysis of differences between groups for the tumor growth curves was performed using two-way ANOVA with correction for multiple testing by the Bonferroni method (A). P value based on two-sided unpaired t test (B). P < 0.05 was considered statistically significant.

4 Discussion

The experiments presented in this thesis set out to characterize and demonstrate the efficacy of the SAR-BiAb platform in the human system. Through this work, we could show that BiAbs can mediate specific and conditional activation of human T cells transduced with the E3 SAR construct. We could further show that SAR T cells are able to form a distinct and functional IS with their target cells to mediate MHC-independent tumor cell lysis. *In vivo*, substantial antitumoral effects could also be shown in two pancreatic cancer and one mesothelioma xenograft model. In the solid tumor models, SAR T cell *in vivo* persistence and tumor infiltration could also be observed. SAR T cell activation was shown to be controllable and reversible with BiAb dosage and depletion, *in vitro* and *in vivo*. Unlike murine SAR T cells, the mechanism of killing employed by human SAR T cells was shown not to be reliant on the Fas-FasL axis, although the exact mechanism is yet to be shown.

4.1 SAR and BiAb design for controlled T cell activation and tumor cell lysis

The rationale upon which the platform was built required the careful design of two entities, the SAR and the BiAb.

4.1.1 SAR Design

A crucial aspect of SAR design was the identification of a rare and inert extracellular receptor domain that could be selectively triggered to crosslink, ultimately propagating an activation signal intracellularly. It was important to the clinical translation of this therapeutic platform that the antigen was restricted to the transduced T cells to avoid unwanted binding of the BiAb to non-tumor tissues. One such receptor is the epidermal growth factor receptor variant III (EGFRvIII). Its reported expression in the human system is restricted to certain tumor entities, notably associated with increased proliferation of glioma cells (Montano *et al*. 2011)). A splice variant of the epidermal growth factor receptor (EGFR), it is an in-frame deletion of exons 2 and 7, followed by the introduction of a glycine residue where exons 1 and 8 meet, that results in its altered transcription. Several reports have cited its inability to bind the natural EGFR ligand EGF, despite its binding site (in the L2 region of domain 3) being conserved in the splice variant (Woltjer *et al*. 1992) . It has been postulated that the altered architecture of EGFRvIII is the cause of this (Gong

et al. 2014,Huang *et al*. 1997) . EGFRvIII is known to mediate a constitutive, ligand-independent signaling due to the formation of transient homodimers which are stabilized by disulfide bonds of free amino-terminal cysteines C16. Although we have not observed ligand-independent signaling in our studies, the dimerization can be abolished by mutating the respective cysteine residue (Ymer *et al*. 2011) . C16 is not involved in BiAb engagement and thus could be mutated if any spontaneous dimerization of the SAR occurs. It is of relevance that the EGFR-specific monoclonal antibodies cetuximab and panitumumab have been shown to possess comparable affinities for EGFRvIII (Gong *et al*. 2014,Patel *et al*. 2007)). The utility of this finding lies in the ability to target genetically engineered SAR T cells, which could be crucial as a safety switch, through which engineered cells expressing the receptor could be depleted (Paszkiewicz *et al*. 2016) .

Although a rare occurrence, the reported expression of EGFRvIII in the human system means the situation where EGFRvIII is no longer an ideal receptor could arise (Montano *et al*. 2011) . In such a case, alternative receptor domains would be required. One such domain is the embryonic gene Cripto-1. It is a member of the EGF-Cripto-1/FRL1/Cryptic family and has been implicated in embryogenesis as well as carcinogenesis. Importantly, its expression is very low in adult tissues and not present on T cells (De Luca *et al*. 2011) . When membrane-bound, it can potentiate ligand-dependent signaling, Although binding to its ligand Activin B has been reported, it has been shown to have no appreciable binding to TGF-B family ligands (Aykul *et al*. 2017) .

Designing the intracellular signaling compartment of the SAR, which is made up of the CD3ζ stimulatory and CD28 costimulatory domains, was informed by the extensive work carried out towards the development of the CAR. Distal to the membrane, the CD3ζ chain was incorporated for its role in initializing T cell activation (signal 1). Its use in CAR T cells was first outlined by Eshhar and colleagues (Eshhar *et al*. 1993) and it remains an integral part of CAR design today. Proximal to the membrane, and linked to its own transmembrane domain, the intracellular domain of the CD28 receptor was added. The signaling domain of the CD28 receptor is important for a full physiological T cell activation to be achieved, as it propagates a necessary costimulatory signal via PI3K (Krogsgaard *et al*. 2005,Zhang *et al*. 2017,Love *et al*. 2010) . Its combination with the CD3ζ domain to form a single chimeric receptor was shown by Maher and colleagues to ameliorate T cell cytotoxicity and proliferation (Maher *et al*. 2002) . The exchange or addition of other signaling endodomains, such as 41BB and ICOS, have been shown to have a marked

impact on T cell activity, metabolic programing and *in vivo* persistence (Guedan *et al*. 2014,Zhong *et al*. 2010).

A SAR containing 41BB as the costimulatory endodomain is an alternative design that warrants testing and characterization. Extensive research has been carried out comparing clinically tested CAR T cells containing a 41BB costimulatory domain with those containing CD28 (Kawalekar *et al*. 2016) . $CD4^+$ and $CD8^+$ CAR T cells with a 41BB domain were shown to have a prolonged proliferation phase and better persistence (through non canonical NF-kB signaling) over those containing CD28 (Philipson *et al*. 2020) . A differentiation status more skewed towards a central memory phenotype was observed with 41BB CAR T cells in comparison to CD28 CAR T cells (Ying *et al*. 2019) . T cell activation is linked to a bioenergetics and biosynthetic flux that is needed to aid T cell function and persistence (Pearce *et al*. 2013,Wang *et al*. 2012) . Naïve and memory T cells primarily depend on mitochondrial oxidation, while effector T cells have been shown to switch to glycolysis or augmented oxidative phosphorylation and aerobic glycolysis (Pearce *et al*. 2009,van der Windt *et al*. 2013) . Consistent with this and the differentiation status observed when comparing 41BB and CD28 CAR T cells, are studies highlighting the metabolic differences between 41BB and CD28 CAR T cells (Kawalekar *et al*. 2016,Menk *et al*. 2018) . They showed that 41BB leads to increased mitochondrial biogenesis and oxidative metabolism, while CD28 results in decreased mitochondrial biogenesis and increased glycolytic metabolism. Whether these findings in CAR T cells could be translated to the SAR-BiAb setting is yet to be shown, although evidence suggests it could improve *in vivo* T cell persistence and antitumoral functions.

Nevertheless, optimal CAR and SAR designs will inevitably depend on the stimulatory or phenotypic advantage that is deemed most necessary to equip the T cell with, depending on the case and setting. Thus, the extensive testing of different costimulatory domains in various settings would still be required, as demonstrated by several preclinical studies in the CAR setting (Guedan *et al*. 2014,Hu *et al*. 2019,Guedan *et al*. 2018).

4.1.2 BiAb design

When designing a BiAb for SAR activation, certain prerequisites must be considered. The binding affinity of the tumor-targeting Fab must be designed with consideration for the relative expression of its target. A balance that would maximize tumor cell binding while minimizing, if not abolishing,

on-target off-tumor binding would be desired. Too low an affinity would compromise tumor targeting, while an affinity that is too high would result in off-tumor toxicity (Liu *et al*. 2015) . If safety considerations allow and depending on the tumor entity that is targeted, future BiAb designs could have a higher affinity for the tumor antigen (Yuraszeck *et al*. 2017) . A higher affinity would allow a BiAb matrix to be formed on the surface of the tumor cells, upon which SAR T cells, with their lower affinity binding to the BiAb, can mediate serial tumor cell killing more efficiently (Hoffmann *et al*. 2005,Herrmann *et al*. 2018) . This design would also minimize antibody trapping in T cell-rich tissues, such as the spleen or lymph nodes, reducing the potential for off-target toxicity (Leong *et al*. 2017,Bortoletto *et al*. 2002).

The number of valencies directed towards the SAR are critical for the facet of targeted specificity. It is imperative that a BiAb must not trigger T cell activation in the absence of the tumor cell. In the presence of the tumor cell, the BiAb can aggregate, thereby enabling the SAR receptor to crosslink and mediate T cell activation. Early studies using CD3-targeting BiTEs showed that it was not possible for the BiTEs to stimulate the T cell lytic capacity simply through binding with their anti-CD3 binder to the receptor complex even when tested at concentrations far exceeding the EC50 values for redirected lysis (Kufer *et al*. 1997) . This distinct feature was attributed to the monomeric nature of BiTEs, because binding of a BiTE with a single monovalent specificity to the CD3 complex was insufficient in mediating a complete T-cell activation. On the other hand, when BiTEs were organized on the target cell surface, thus accessible to the T cells in a polyvalent form, they resulted in strong T-cell signaling and redirected killing, and a cascade of consequent events (Perez *et al*. 1985,Wolf *et al*. 2005) . Although the targeted receptor is not the same in the case of the SAR (EGFRvIII), the same concept applies. As a result, the cross-linking ability of the 2 + 1 BiAb is restored in the presence of an immobilizing tumor- associated binding moiety for the other arm of the BiAb.

Depending on the disease entity and the level of controllability that is required, BiAb design can have a significant impact on both function and half-life. Through binding the FcγR, a functional Fc region can mediate antibody-dependent cellular cytotoxicity (Petricevic *et al*. 2013) . Introduction of the P329G LALA mutation in IgG1 antibodies was previously shown to abolish binding to the FcγR receptor, rendering the Fc region non-functional (Schlothauer *et al*. 2016,Klein *et al*. 2019) . In the context of the SAR-BiAb platform, this Fc function is redundant and therefore the BiAb was designed to include this mutation in the Fc region.

Despite its lack of function, the Fc region of the CrossMab still has an impact on the half-life of the molecule. Fc-deficient formats have been shown to have a relatively short plasma half-life as they are not protected from catabolism by the neonatal Fc-receptor (FcRn). Should it become necessary to modulate this in the future, FcRn binding and recycling can be abolished via the introduction of Triple A (I253A, H310A, H435A) mutations into the Fc region, as previously described (Regula *et al*. 2016).

4.2 The importance of the immunological synapse to SAR functionality

In response to cell-surface stimulation, lymphocytes have the capacity to totally change their cellular architecture. It is a structural plasticity that helps to promote and specify interactions between T cells and tumor cells. The immunological synapse (IS) is a well-defined cell-cell contact formed after the association of adhesion and signaling proteins, as well as cell surface receptors into concentric rings (Dustin *et al*. 2010) . Cytotoxic T cells rapidly and specifically lyse their target cell, following the formation of an IS once the TCR has been engaged (Monks *et al*. 1998) . Upon engagement of the (TCR), several concentric rings, known as the supramolecular activating clusters (SMAC) make up the IS. Clustered molecules conferring specific functions make up each cluster. The central SMAC (cSMAC) is made up of clusters of Lck and TCR, that strengthen the cytotoxic response via the buildup of activation signals and the transfer of cytotoxic particles. Surrounding the cSMAC is the peripheral SMAC (pSMAC), an accumulation of adhesion molecules (made up of LFA-1)) stabilizes the synapse itself as well as target cell binding. An aggregation of actin known as the distal SMAC (dSMAC) (Dustin *et al*. 2014) The microtubule organizing center (MTOC), otherwise known as the centrosome, is an important structure that is rapidly reorganized (within 5 minutes of T cell stimulation) to carry with it key vascular components such as the golgi apparatus. The reorientation of the MTOC to a point below the cell-cell boundary is indicative of functional IS formation. This remodeling of the cytoskeleton in an event which aligns secretory organelles within the T cell with the IS (Martín-Cófreces *et al*. 2008).

It is known that the signaling machinery of CARs shares many aspects with that of the conventional TCR (Maher *et al*. 2002) . It was thus surprising when a study demonstrated considerable differences between the IS structure formed by a CAR to that formed by a TCR. They could show that LFA-1 is expendable for the IS formation at CAR as well as TCR interfaces.

Further, Lck did not cluster within the cSMAC of the CAR IS, with its micropatches forming a more disordered pattern. As a result, the inner diameter of the CAR synapse was a lot smaller by comparison and is congruent with the earlier CAR T cell detachment that was observed. CAR T cells were also shown to deliver lytic granules more rapidly to the synapse, resulting in speedier target cell lysis (Davenport *et al*. 2018) . On the other hand, CD3-targeting BiAbs were shown to form functional immune synapses that were comparable to those formed following a classical TCR-target cell interaction (Offner *et al*. 2006).

Given the differences observed at the T cell-tumor cell interface of several ACT approaches, it was important to establish both the nature and functionality of the IS formed at the interface between SAR T cells and their target cells, and how it compares to the CAR and TCR approaches described above. A polarized MTOC and a large F-actin area demonstrated that the SAR-BiAb combination could induce the formation of a functional synapse. MTOC translocation towards the nascent IS has previously been shown to be an important early step in lymphocyte activation initiated by the TCR (Martín-Cófreces *et al*. 2008) , as well as the CAR (Davenport *et al*. 2018) . Interestingly, MTOC polarization in the CAR T cell IS was more distal from the membrane when compared to TCR T cells, the localization in which was more proximal to the membrane, and close to Lck. In this regard, the MTOC polarization observed in SAR T cells was more comparable to that observed in TCR T cells, given its close proximity to the membrane. It remains to be shown whether MTOC polarization in SAR T cells also localizes in proximity to Lck. Granzyme B (GZMB) granules were seen to be polarized at the SAR IS in comparable fashion to both TCR and CAR T cells (Xiong *et al*. 2018,Jenkins *et al*. 2010) . The LFA-1 organization pattern at the SAR T cell IS was disorganized and comparable to that observed at the IS formed by CARs (Davenport et al. 2018). This is contrary to the LFA-1 organization pattern at the IS of TCR T cells, which is comprised of an organized bull's eye structure (Davenport et al. 2018). A more comprehensive side-by-side comparison of the IS formed by SAR, CAR and TCR T cells will need to be carried out to further validate and better understand the differences observed.

4.3 Target antigen specificity and the conditional activation of human SAR T cells for tumor cell lysis

Specific and conditional SAR stimulation and targeted tumor cell lysis was observed when the SAR, BiAb and targeted antigen were present. Furthermore, the targeted specificity of the

platform was tested to investigate whether the expression of EGFRvIII could stimulate our T cells, and whether SAR T cells would subsequently mediate cytotoxicity of EGFRvIII-positive cells. Cytotoxicity assays as well as IFNγ and IL-2 cytokine release assays revealed that E3 SAR T cells were not able to lyse EGFRvIII-positive tumor cells when compared to MSLN-positive ones, which were effectively lysed. We also questioned whether free MSLN or EGFRvIII proteins could inhibit or interfere with SAR-BiAb activity and found that ascending concentrations of recombinant MSLN could inhibit tumor cell killing. Free targeted protein in the TME must be considered as a potential hindrance to the efficacy of the SAR-BiAb combination. This could become especially problematic in targeting cleavable proteins, such as MSLN, and the targeting of membrane-associated forms of proteins could maximize efficiency in this regard (Asgarov *et al*. 2017) . In any case, testing under physiological conditions must be carried out to further validate this finding.

4.4 The selective and modular nature of the SAR-BiAb platform in cancer therapy

While insufficient tumor infiltration and a dense immunosuppressive microenvironment are notable hindrances to the success of T cell therapies in solid tumor settings, antigen heterogeneity and on-target off-tumor toxicity remain problematic in this regard (Runa *et al*. 2017).

To overcome these challenges, highly modular and controllable approaches are needed to offer flexibility when targeting a diverse set of tumor-associated antigens, and control over the risk of toxicities due to attraction to healthy tissue that expresses the antigen (Lesch *et al*. 2019) . These risks cannot be adequately predicted from preclinical data (Fitzgerald *et al*. 2017,Watanabe *et al*. 2018) . The ‘safety switches’ that have been incorporated into the SAR-BiAb platform have been shown to have the potential to limit possibly lethal side effects.

A major advantage of the platform we describe is its reversibility with the elimination of the BiAb. Elimination of the BiAb, in the event of toxicity or on-target-off-tumor activity, would reverse SAR T cell activity. Moreover, the BiAb concentration could then be adjusted accordingly, to maintain T cell efficacy and manage possible toxicity. Furthermore, as previously mentioned, SAR T cell depletion with anti-EGFR antibodies can act as an additional safety switch.

Inter-patient heterogeneity and the diverse antigen profile of many cancer types highlights the importance of a modular platform with the potential for redirection towards different targets. In terms of modularity, once the T cell arm of the therapy is adoptively transferred, the redirection and subsequent activation of SAR T cells is completely dependent on the BiAb. Further or sequential targeting of the tumor through the redirection of pre-existing SAR T cells could then be achieved through the introduction of a new BiAb with a different antigen specificity.

In the event of target downregulation as an escape mechanism following treatment (most prevalent resistance mechanism observed following CD19 targeted treatment in ALL patients) (Ruella *et al*. 2016,Majzner *et al*. 2018) , platform modularity would again be advantageous. In a clinical setting, this treatment approach would allow for the careful monitoring of patients for the potential emergence of side effects.

4.5 Approaches to improve SAR T cell functionality

The SAR-BiAb platform distinguishes between two T cell populations in the patient (engineered and non-engineered). This was shown when directly comparing a pan-T cell targeting molecule with a SAR-specific one, substantiating the claim that non-engineered T cells are not affected by the platform, and differentiating the approach from those using pan-T cell–activating BiAbs. SAR T cells can therefore be carefully selected and tailored. In this line, identifying the optimal T cell differentiation state that would result in maximal efficacy is an important axis through which the SAR-BiAb can be improved.

The impact of defined $CD4^+$ and $CD8^+$ T cell subsets is one important consideration. Several reports have shown that defined compositions can have a significant impact on anti-tumoral efficacy. One such study in the glioblastoma disease setting showed that $CD4^+$ CAR T cells (anti-IL13Ra2) mediated superior tumor control *in vivo* when compared to a mix of $CD4^+$ and $CD8^+$ CAR T cells (Wang *et al*. 2018) A clinical study by Turtle and colleagues in the B cell ALL setting employed CD19 CAR T cells of a defined $CD4^+$ and $CD8^+$ composition, with 93 % of patients achieving remission (Turtle *et al*. 2016) . Discerning if various differing $CD4^+$ and $CD8^+$ ratios can have an impact on SAR T cell functionality would be an important finding in this regard.

In addition to the introduction of the synthetic construct, additional genetic modifications, including targeted transgene integration is another potential approach to improve SAR T cell function, and has already been shown to improve anti-tumoral efficacy in CAR T cells. Directing transgenes towards specific genomic locations has the potential advantages of optimizing transgene function while mitigating unforeseen genotoxicity. One study could reduce tonic signaling (resulting in more optimal CAR surface expression levels) in CAR T cells by directing the CAR to the T cell receptor alpha constant (TRAC) locus (Eyquem *et al*. 2017) . In a case study, random lentiviral vector integration had serendipitously disrupted the methylcytosin dioxygenase TET2 gene on one allele. As the patient harbored a hypomorphic mutation in their second allele, it led to the loss of function of TET2 in the patient's CAR T cell. Astonishingly, 94 % of the patient's $CD8^+$ T cell compartment was TET2 deficient at the peak of response (Fraietta *et al*. 2018) . The phenotypic and functional changes observed in the patient's CAR T cell subset that were correlated with improved persistence and response were recapitulated in further *in vitro* studies, showing TET2 to be a potential axis for improved CAR T cell function.

Several gene-editing approaches including CRISPR/Cas9, zinc finger nucleases and TALENs have been successfully used to disrupt T cell suppressive genes in T cells. A recent clinical study was conducted in patients with refractory cancer and showed the potential of CRISPR-cas9 edited T cells for cancer therapy (Stadtmauer *et al*. 2020,Hamilton *et al*. 2020,Lu *et al*. 2020) . The application of these approaches for the improvement of SAR T cell functionality has a lot of promise.

4.6 *in vivo* functionality – Understanding the shortcomings and room for improvement

As previously mentioned, the use of both $CD4^+$ and $CD8^+$ T cell subpopulations has been shown to be required for efficient and durable clinical responses to ACT (Turtle *et al*. 2016) . The SAR used in this study could be successfully transduced in $CD4^+$ and $CD8^+$ human T cells with great efficiency. Higher tumor infiltration and longer persistence *in vivo* by $CD8^+$, and especially $CD4^+$ SAR T cells emphasize the necessity of both cell types for the observed anti-tumoral responses. Impaired migration and activation of T cells within tumor tissue are major limiting factors linked to the poor success of advanced T cell–based therapies in solid tumor settings (Slaney *et al*. 2014,Kmiecik *et al*. 2013,Galon *et al*. 2006,Piersma *et al*. 2007) . SAR T cells achieved long-

lasting tumor control *in vivo* but failed to completely eradicate established tumors, and these limiting factors are likely to play a role.

As described above, exchange of the signaling endodomains or the addition of structural components was observed to boost the *in vivo* performance of various CARs. As such, the impact of further modifications to SAR design must be investigated in search of strategies to ameliorate the *in vivo* functionality (Lim *et al*. 2017) . The additional engineering of SAR T cells with selected chemokine receptors might overcome their limited infiltration into tumor tissue (Rapp *et al*. 2016) . Further, the use of dominant- negative TGF-β receptors (Foster *et al*. 2008) or PD-1-CD28 (Kobold *et al*. 2014) switch receptors could help shield the modified T cells from the immunosuppressive tumor microenvironment. It is thus reasonable to postulate that treatment failure occurs due to insufficient persistence, recruitment and infiltration of the transferred T cells into the tumor and is a matter of ongoing investigations to improve SAR T cell efficacy.

The *in vivo* downregulation of the MSLN antigen on the tumor cell surface is another potential contributing factor. We applied ex-vivo flow cytometry analysis of tumor tissue in an attempt to do this. However, MSLN expression could not be accurately quantified, as a result of suboptimal staining and shedding of the protein.

5 Conclusions and outlook

In conclusion, we describe a novel modular platform for MHC- unrestricted SAR T cell–based therapy with properties distinct from current approaches. Taken together, the SAR–BiAb platform aligns the advantages of antibody therapy (controllable dosing and reversibility) with that of adoptive T cell therapy (potent anti-tumoral effectors) (Karches *et al*. 2019,Slaney *et al*. 2018) .

Beyond what has been demonstrated in the solid tumor setting, the application of this platform for the treatment of hematological diseases is very appealing. The most prevalent resistance mechanism observed following CD19-targeted therapy is target downregulation (Ruella *et al*. 2016) . The modularity of the SAR-BiAb platform would offer a flexibility to change the target and redirect the already transferred effector cells if such an event were to occur. In addition, its application in the AML disease setting could also be of interest. Perhaps the greatest challenge hindering the success of adoptive T cell therapy in AML is target specificity, which results from disease heterogeneity and diverse antigen expression on leukemic stem cells (Haubner *et al*. 2019,Perna *et al*. 2017) . A patient-specific multi-targeting approach, where BiAbs are added in a simultaneous or sequential manner is an approach that warrants further investigation.

While further development of the separate components, and more extensive testing within and across tumor models is still required before its application in a clinical setting, the SAR-BiAb platform undoubtedly offers new solutions in tumor settings in need of more modular and controllable approaches.

6 Abstract

Adoptive T cell therapy, namely chimeric antigen receptor (CAR) T cell therapy has been a groundbreaking and effective treatment of relapsed or refractory haematological malignancies. Still, many patients do not respond or relapse with treatment-resistant disease. Additionally, toxicities such as cytokine release syndrome remain problematic. Cancer heterogeneity, beyond cancer types and inter-patient differences, is present within every individual patient. This heterogeneity, especially in the context of solid tumors, has meant that targeted immunotherapies have fared relatively poorly, creating a need for a modular platform with a capacity to target multiple antigens simultaneously and/or sequentially. Likewise, treatment-related toxicities have limited the therapeutic efficacy and breadth of patient selection.

To tackle these caveats through a modular and controllable approach, we equipped T cells with synthetic agonistic receptors (SARs) that are only activated when a tumor-associated antigen and a cross-linking bispecific antibody (BiAb) specific for both SAR T cell and tumor cell are also present. The SAR itself is constituted of an inert extracellular domain in the form of EGFRvIII, that is fused to the T cell activating domains CD28 and CD3ζ. The BiAb employed is a trivalent CrossMab, with two binding arms (2 x Fab) for the tumor-associated antigen (mesothelin), and one binding arm for the SAR receptor (EGFRvIII).

We showed that BiAb triggering of the SAR is conditional upon the binding of the second BiAb specificity. What is particularly advantageous with this approach is that T cell activation may only occur when the BiAb is present and in proximity to the antibody-targeted tumor cell. This conditional T cell activation is an inherent safety feature of the platform, whereby if unwanted levels of T cell activation are observed, depletion of the BiAb from the system could result in the reversal of said activation, thus managing the potential toxicity.

This work was able to determine the validity and efficacy of the approach. Through the generation of several human pancreatic cancer models, and extensive *in vitro* and *in vivo* testing, the platform could be characterized. Its translational relevance and significance as a next-generation adoptive T cell therapy with the potential to plug some gaping pitfalls of current ACT approaches were also shown.

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8 Abbreviations

ACT	Adoptive T cell therapy
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen presenting cell
BiAb	Bispecific antibody
BiTE	Bispecific T cell engager
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CRISPR	Clustered regularly interspaced short palindromic repeats
CrossMab	Immunoglobulin domain crossover
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant three
EGTA	Egtazic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay

FcRn	Neonatal Fc receptor
FcγR	Fc gamma receptor
FasL	Fas ligand
FDA	Food and Drug Administration
ffLuc	Firefly luciferase
GZMB	Granzyme B
ICOS	Inducible T cell costimulator
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IS	Immunological synapse
ITAM	Immunotyrosine activation motif
KiH	Knob into hole
Lck	Lymphocyte-specific protein tyrosine kinase
LFA-1	Lymphocyte function-associated antigen 1
MCS	Multiple cloning site
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase

MSLN	Mesothelin
MTOC	Microtubule organizing centre
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
SAR	Synthetic agonistic receptor
scFv	Single chain variable fragment
SEM	Standard error of the mean
SMAC	Supramolecular activation cluster
TALEN	Transcription activator-like effector nuclease
TCR	T cell receptor
TET2	Tet methylcytosine dioxygenase 2
TME	Tumor microenvironment
TIL	Tumor infiltrating lymphocyte
TRAC	T cell receptor α constant
TGF-β	Tumor growth factor-β
TNF	Tumor necrosis factor

TNFR	Tumor necrosis factor receptor
UT	Untransduced
VEGF	Vascular endothelial growth factor

9 Publications

1. 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.
International Journal of Molecular Science 2019; 20:1283

2. Clara Helke Karches*, **Mohamed-Reda Benmebarek***, Moritz Luigi Schmidbauer, Mathias Kurzay, Richard Klaus, Martina Geiger, Felicitas Rataj, Bruno Loureiro Cadilha, Stefanie Lesch, Constanze Heise, Ramona Murr, Johannes vom Berg, Martin Jastroch, Daniel Lamp, Jian Ding, Peter Duewell, Gerhard Niederfellner, Claudio Sustmann, Stefan Endres, Christian Klein and Sebastian Kobold (* contributed equally).
Bispecific antibodies enable synthetic agonistic receptor-transduced T cells for tumor immunotherapy.
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3. Stefan Stoiber, Bruno Loureiro Cadilha, **Mohamed-Reda Benmebarek**, Stefanie Lesch, Stefan Endres, Sebastian Kobold.
Limitations in the design of chimeric antigen receptors for cancer therapy.
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4. Stefanie Lesch*, **Mohamed-Reda Benmebarek***, Bruno Loureiro Cadilha*, Stefan Stoiber*, Marion Subklewe, Stefan Endres, Sebastian Kobold (* contributed equally).
Determinants of response and resistance to car t cell therapy.
Seminars in Cancer Biology 2019.

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