CAR modified T cells resist TGF-β mediated repression through engineered IL-7 triggered IL-2 signaling

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“Felfedezni valamit annyit tesz, mint látni, amit mindenki lát,
és közben arra gondolni, amire még senki.”

“Discovery consist of seeing what everybody has seen
and thinking what nobody else has thought.”

Szent-Györgyi Albert

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A teljes AG Abken csapatnak szeretném megköszönni, hogy a kezdeti nyelvi nehézségek ellenére is nagy szerettel fogadtak be maguk közé és tették a hétköznapokat családias hangulatúvá sok szép, emlékezetes pillanattal. Egy nagyon szép időszakként fogok mindig visszaemlékezni az itt töltött időkre. Külön nagy köszönet jár Johannesnek, aki mindig minden kérdésemmel, problémámmal komolyan foglakozott és segített a megoldás elérésében. Hálás köszönettel tartozom Dr. Gunter Rappl-nak is a metodikai tanácsaiért, melyek mindig nagy segítségemre szolgáltak. Nagy köszönet illeti Petra-t és Nicole-t is a kiválló technikai segítségért, melyre főleg a munkám utolsó szakaszában volt nagy szükségem.

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És végül, de nem utolsó sorban a szüleimnek és testvéreimnek szól a hála, akiknek nem is tudok eleggé hálásnak lenni a szeretetükért, a támogatásukért, hogy hittek bennem, hogy biztosították szabadságot számomra, hogy „elengedtek" külföldre, de legfőképp azért, hogy mindig velem voltak és vannak.

ABSTRACT

Adoptive cell therapy with chimeric antigen receptor (CAR) redirected T cells induces spectacular regressions of leukemia and lymphoma, but failed so far in the treatment of solid cancer. One of the causes is the repression of T cell activity, especially T cell proliferation through TGF-β present in the tumor microenvironment. Here we show that T cells with a second generation CAR containing a CD28 signaling domain can overcome the suppression in T cell proliferation, in contrast to T cells with a 4-1BB-containing CAR. The resistance to TGF-β activity depends on the secretion of IL-2, which is induced *via* CD28-mediated activation of the kinase LCK. Deletion in the LCK binding motif of the CD28 domain of the CAR (CD28ΔLCK-ζ) was able to abrogate CAR-induced IL-2 secretion and the resistance of T cell proliferation in the presence of TGF-β. However, IL-2 secreted from activated CAR T cells also sustains suppressive Treg cells at the tumor site, thus impairing the anti-tumor response. To generate enhanced CAR T cells we sought to replace CD28-mediated IL-2 secretion by an alternative cytokine, which mediates an IL-2-like signal in trans for providing TGF-β resistance. In this context, expression of IL-7 in CD28ΔLCK-ζ CAR T cells mediated TGF-β resistance equivalent to IL-2. Since the IL-7 receptor is downregulated after T cell activation we further modified CD28ΔLCK-ζ CAR T cells with a hybrid cytokine receptor, which provides IL-2R β-chain signaling upon binding of co-expressed transgenic IL-7. The strategy minimizes the detrimental effects of secreted IL-2 and at the same time improves the CAR T cell activity against TGF-β^+ tumors *in vivo*. Our data provide proof that editing the CD28 signaling capacities and establishing a CAR induced autocrine loop by synthetic biology can make CAR T cells more potent in the hostile environment of solid tumors.

ZUSAMMENFASSUNG

Die adoptive Zelltherapie mit einem chimären Antigen-Rezeptor (CAR)-modifizierten T-Zellen hat spektakuläre Regressionen von Leukämie und Lymphom induziert, ist aber bisher bei der Behandlung von soliden Tumoren gescheitert. Eine der Ursachen ist die Repression der T-Zell-Aktivität, insbesondere T-Zell-Proliferation durch TGF-β vorhanden in der Tumormikroumgebung. Hier zeigen wir, dass T-Zellen mit einem CAR der zweiten Generation, das eine CD28-Signaldomäne enthält, die Repression in dem Proliferation von T-Zellen überwinden können, im Gegensatz zu T-Zellen mit einem 4-1BB enthaltenden CAR. Diese Resistenz gegen TGF-β Aktivität hängt von der Sekretion von IL-2 ab, die durch CD28-vermittelte Aktivierung der Kinase LCK induziert wird. IL-2, das von aktivierten CAR-T-Zellen entstammen wird, unterstützt jedoch auch suppressive Treg-Zellen an der Tumorstelle und beeinträchtigt so die Antitumorantwort. Um verbesserte CAR-T-Zellen zu erschaffen, versuchten wir, die CD28-vermittelte IL-2-Sekretion zu eliminieren und ein zweites intrinsisches Signal in trans zu liefern, die TGF-β Resistenz von T-Zell Proliferation vermittelt. Die Mutation des LCK-Bindungsmotivs in der CD28-Domäne des CAR (CD28ΔLCK-ζ) konnte die CAR-induzierte IL-2-Sekretion und die Resistenz von T-Zell Proliferation in der Anwesenheit von TGF-β aufheben. Darüber hinaus könnte die Expression des Zytokins IL-7 in CD28ΔLCK-ζ CAR T-Zellen die TGF-β Resistenz vermitteln, der IL-2 äquivalent. Da der IL-7-Rezeptor nach der Aktivierung der T-Zellen herunterreguliert wird, haben wir die CD28ΔLCK-ζ CAR-T-Zellen mit einem hybriden Zytokinrezeptor weiter modifiziert, der bei Bindung von co-exprimiertem transgenem IL-7 eine IL-2R β-Kettensignalisierung bereitstellt. Diese Strategie minimiert die schädlichen Auswirkungen von sekretiertem IL-2 und verbessert gleichzeitig die CAR-T-Zellaktivität gegen TGF-β^+-Tumore *in vivo*. Unsere Daten liefern den Beweis, dass die Veränderung der CD28-Signalkapazitäten und die Etablierung einer CAR-induzierten autokrinen Schleife durch die synthetische Biologie die CAR-T-Zellen in der feindlichen Umgebung von soliden Tumoren verbessern können.

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LIST OF ABBREVIATIONS

4-1BB	tumor necrosis factor receptor superfamily member 9 (CD137)
7-AAD	7-Amino-Actinomycin D
Ab	antibody
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACT	Adoptive Cell Therapy
Amp	Amplicilin
APC	allophycocyanin
ATCC	American type culture collection
Bcl-2	B cell lymphoma 2
Bcl-x	B cell lymphoma-extra large
BMPs	bone morphogenetic proteins
bp	base pair
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CAR	Chimeric Antigen Receptor
CD	cluster of differentiation
CD122	IL-2 Receptor β-chain
CD127	IL-7 Receptor α-chain
CD132	common γ-chain
CD215	IL-15 Receptor α-chain
CD25	IL-2 Receptor α-chain
CDK2	cyclin-dependent kinase 2
CEA	carcinoembrionic antigen
CFSE	5-carboxylfluorescein diacetate succinimidyl ester
CH2-CH3	constant region 2/3 of heavy chain
$C_{H/L}$	constant region of heavy/light chain
CKIs	cyclin-dependent kinase inhibitors
c-myc	myelocytomatosis oncogene
CO_2	carbon dioxid
CTLA-4	cytotoxic T lymphocyte-associated protein 4
CTLs	cytotoxic T lymphocytes
DAPI	4′,6-diamidino-2-phenylindole
DC	dentritic cells
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNTGFβ	dominant-negative TGF-β Receptor II
E. coli	*Escherichia coli*

EBV	Epstein–Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FACS	Fluorescence-activated cell sorting
Fas/FasL	CD95/Fas ligand
Fc	fragment crystallizable
FcεRIg	Fc epsilon receptor I
FcγRs	IgG Fc gamma receptor
FCS	fetal calf serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
FoxP3	forkhead box protein 3
g	gravitational acceleration
GALV	gamma-retroviral envelope protein
gBlocks	gene fragments
γ_c	common cytokine-receptor gamma-chain (CD132)
GDFs	growth and differentiation factors
GrB	Granzyme B
H+L	heavy+light chain
$H2O_{dd}$	water
HCl	hydrogen chloride
HEK	human embrionic kidney (cell)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI-FCS	heat-inactivated fetal calf serum
i.v.	lat.: *intra vena*
IDT	Intergrated DNA Technologies
IC	intracellular
IFN-γ	interferon-gamma
Ig	immunoglobulin
IL	Interleukin
JAK	Janus kinase
KO	knock-out (mause)
LAKs	lymphokine-activated killer cells
LAP	Latency Associated Peptide
LB	lysogeny broth
LCK	lyphocyte-specific protein tyrosine kinase
mAb	monoclonal antibody
MAGE-A3	melanoma-associated antigen 3
MDSCs	myeloid-derived suppressor cells
MFI	mean fluorescence intensity
MHC	major histocompatibility complex

min	minute
MUC1	mucin 1
mRNA	messenger ribonucleic acid
NK cell	natural killer cell
NY-ESO-1	cancer-testis antigen
OD	optical density
OX40	Tumor necrosis factor receptor superfamily, member 4 (CD134)
p	phospho
P2A	“self-cleaving” peptide
PBLs	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline tween
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PDL	poly-D-lysine
PE	phycoerythrin
PenStrep	Penicillin Streptomycin mixtures
pH	lat.: *potetntia Hidrogenii*
pHIT	gamma-retroviral gag/pol
PI3K	phosphatidylinositide 3-kinases
PMS	N-Methylphenazonium methyl sulfate
POD	peroxidase
PSCA	prostate stem cell antigen
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute (Medium)
sec	seconds
S.D.	standard deviation
scFv	single chain variable fragment
SCID	severe combined immunodeficiency
STAT	signal transducer and activator of transcription
T2A	“self-cleaving” peptide
TAA	tumor associated antigen
TAE	Tris-acetate-EDTA
TAMs	tumor-associated macrophages
TANs	tumor-associated neutrophils
TCR	T cell receptor
TE	Tris-EDTA
TGF-β	Transforming growth factor beta
TILs	tumor-infiltrating lymphocytes
TM	transmembrane

TNF-α	tumor necrosis factor alfa
Tregs	regulatory T cells
Tris	tris(hydroxymethyl)aminomethane
TRUCKs	T cells re-directed for universal cytokine-mediated killing
U	Unit
UV	ultraviolet
v/v	volume concentration (volume/volume)
$V_{H/L}$	variable region of the immunoglobulin heavy/light chain
w/o	without
w/v	mass concentration (weight/volume)
WHO	World Health Organisation
XSCID	X-linked severe combined immunodeficiency
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide
X-vivo	serum-free, haematopoietic media

1 INTRODUCTION

1.1 Cancer

Cancer is still the one of the leading causes for death globally. In 2012 worldwide, 14.1 million new cases of cancer and 8.2 million cancer caused deaths were registered according to GLOBOCAN (Torre et al., 2015). This compares to 18.1 million new cancer cases and 9.6 million cancer related deaths in 2018 (World Health Organization). The reasons for the rising occurrences are diverse; among the causes there are increasing risk factors such as obesity, physical inactivity, smoking and environmental pollutants. The leading cause of cancer death among males is lung cancer, whilst among females it is breast cancer (Torre et al., 2015). Malignant diseases are a large and very diverse disease group, which can affect any part of the body. The malignances can originate either from the hematopoietic system to induce hematological cancer diseases such as leukemia or lymphoma or originates from other tissues to induce sarcoma or carcinoma. In the second case we talk about solid tumors (cancerresearchuk.org). In most cases genome alteration with a gain or loss of function mutation are the causes of cancer (Hanahan and Weinberg, 2000). Development of the disease is a multistep process towards the malignant phenotype which is characterized by six biological capabilities including to sustain proliferative signaling, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis, and activate invasion and metastasis (Hanahan and Weinberg, 2011). Metastasis is one of the major causes of death in cancer disease. Cancer is a heterogeneous system with a very complex network, which makes the treatment very difficult and requires multi-target therapies (Floor et al., 2012). Nonetheless, the majority of cancer deaths can be prevented by early diagnosis or through prevention, (Vineis and Wild, 2014) but in most cases cancer therapy is needed. The classical treatment options are surgery, chemotherapy and radiotherapy. These treatments remain the primary choices for cancer patients despite their known side effects. However, continued progress in medicine has made other beneficial therapies available nowadays. Immunotherapy is a novel therapy form, which has revolutionized the treatment of cancer patients and was named as Breakthrough of the Year 2013 (Couzin-Frankel, 2013). In a specific cancer immunotherapy the patient's own immune system is modified in such a way that can fight specifically against the targeted tumor cells, but there are more approaches within immunotherapy. Immunotherapy's strength compared to other cancer treatment therapies is it´s higher degree of cancer selectivity and specificity.

1.2 Immunotherapy

Immunotherapy is a new principle in cancer treatment in which the immune system of the patient is used to achieve more effective responses in the cancer cells when attacked by the body's own immune cells. There are many different types of immunotherapy available in the treatment of cancer, such as monoclonal antibodies, cytokine therapy or adoptive cell transfer. One example is in the use of monoclonal antibodies against CTLA-4 (cytotoxic T lymphocyte-associated protein 4) or PD-1 (programmed cell death protein 1). By binding the antibodies to regulatory molecules, the inhibitory signal on T cells is turned off and cytotoxic T lymphocytes (CTLs) can destroy the cancer cells successfully. Monoclonal antibody against CTLA-4 was approved by FDA (Food and Drug Administration, USA) in 2011 as one of the first immune system targeted therapy. For the discovery of both the protein receptor and it is role in cancer therapy, James P. Allison and Tasuku Honjo were awarded the Nobel Prize in Physiology or Medicine in 2018 (nobelprize.org).

A further very promising therapy form is adoptive T cell therapy, where the patient's own T cells are utilized in the fight against the cancer cells (Rosenberg, 1984).

1.2.1 Adoptive T cell immunotherapy

T cells from the tumor lesion are able to recognize and eliminate cancer cells, but not in a very effective manner. To achieve an improved anti-tumor response, the patient's own T cells are collected, expanded and/or modified ex vivo and transferred back into the host patient. The main advantage of this type of therapy is, that the transferred cells are specific to destroy cancer cells with minimal attacking of normal tissue.

A major limitation of the therapy is the resting state of most isolated T cells. In order to activate isolated T cells prior re-infusion, lymphokine-activated killer cells (LAKs) were used (Fagan and Eddleston, 1987). These cells are generated *in vitro* from patient lymphocytes by stimulation with interleukin-2 (IL-2) *in vitro*. Those activated cells are able to recognize and eliminate cancer cells while do not lyse normal cells. The therapy was successful *in vitro* and *in vivo* in mice as well as with human patient (Mazumder, 1984). Adoptive transfer of LAK cells to mice with repeated administration of IL-2 was highly effective in the reduction of the number and size of tumor lesions (Mule et al., 1984; Mulé et al., 1986). LAK cells were efficient in cytolytic activity in human patient (Rosenberg et al., 1985). However, this therapy is not specific enough and almost every patient suffered from some toxic side effect -

especially when used in conjunction with the systemic administration of IL-2 (Glassman, 1989).

Another approach of adoptive transfer of T cells is the use of tumor-infiltrating lymphocytes (TILs) (Fagan and Eddleston, 1987). TIL cells are isolated from human tumors of the patients and expanded ex vivo with high doses of IL-2. TILs are more effective in their therapeutic potency than LAK cells (Rosenberg, 1988), although this effectiveness is only achieved when used in conjunction with IL-2. With the combination of prior lymphodepletion by cyclophosphamide and simultaneous administration of IL-2 with TIL transfer, almost all mice with colon adenocarcinoma and metastasis were cured (Rosenberg et al., 1986). However, the administration of high-doses of IL-2 to cancer patients has contributed to toxic side effects (Rosenberg et al., 1989). Further, TILs have an unknown specificity, which is a potential risk for autoreactivity with normal, non-malignant tissue. Nevertheless, improvements in TILs therapy are still being developed (Dudley et al., 2013; Sim et al., 2014).

In order to equip T cells with known specificity for targeting tumor antigen, a new approach in adoptive immunotherapy was established and so the personalized immunotherapy was created. Human T cells are engineered to express the artificial receptor, either a Chimeric Antigen Receptor (CAR) or αβ T cell receptor (TCR) with defined specificity. These modified T cells are re-infused into the patient. Both approaches have advantages and disadvantages but above all both are potential treatment options for patients suffering from cancer. There are more comparative studies where the two approaches are examined, however it is hard to compare them because of many different parameters such as affinity, ligand structure or ligand density (Barrett et al., 2015; Harris and Kranz, 2016). Recently, more clinical trials related to both therapies have been reported (Holzinger et al., 2016; Kunert et al., 2013). Many promising results have been observed by using CARs especially against hematological cancer but not against solid tumors. The most successful CAR in clinical use is the CD19 specific CAR targeting B cell malignancies (Brentjens et al., 2013; Grupp et al., 2013; Lee et al., 2015; Porter et al., 2011); FDA approval was granted in 2018. Kymriah™ is approved for the treatment of pediatric and young adult patients with acute lymphoblastic leukemia (ALL). Yescerta™ is approved for the treatment of adult patients with large B cell lymphoma (Zheng et al., 2018). In addition, Kymriah™ has recently achieved EMA (European Medicines Agency) approval for use in Europa. Recombinant TCRs used in the clinic have shown some promising results with both hematological and solid tumors, although severe side effects were also experienced. For example, targeting the MAGE-A3 tumor

antigen in myeloma and melanoma induced off-target toxicity, destruction of non-cancerous tissue and even led to the death of the patient (Linette et al., 2013; Morgan et al., 2013). Nonetheless, by targeting cancer testis antigen NY-ESO-1, no off-target toxicity has been reported (Robbins et al., 2015, 2011). A general limitation of this approach is that each artificial TCR is specific for a certain MHC-peptide complex (major histocompatibility complex) and therefore is only suitable for MHC-matched tumor patients (Cartellieri et al., 2010). Moreover, further limitation is the downregulation of different components of MHC class I antigen presenting machinery (Sadelain et al., 2003). The benefit for TCR T cell therapy is the targeting of intracellular proteins as long as they are sufficiently presented on the MHC (Figure 1).

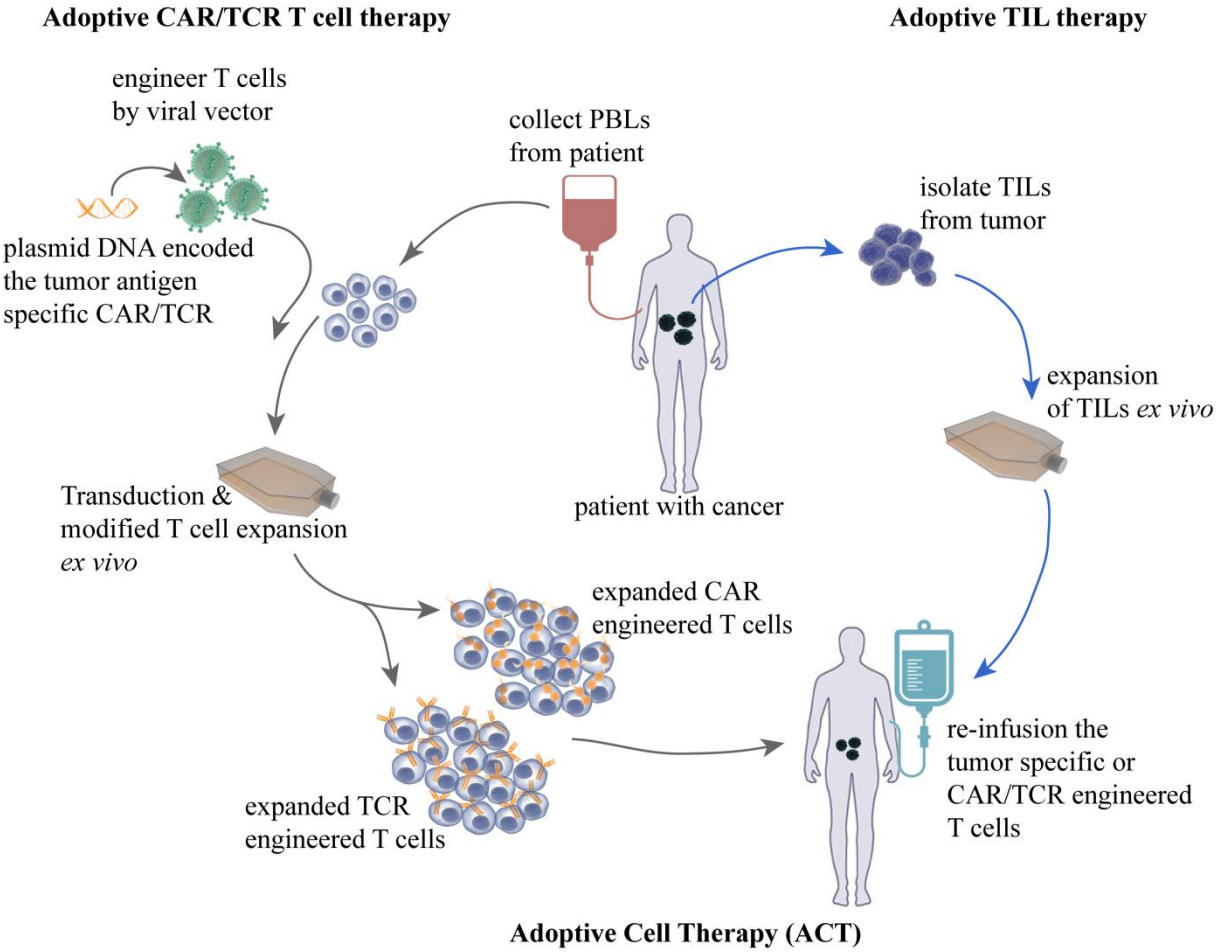


Figure 1 Demonstration of adoptive cell therapy for cancer

1.2.2 Adoptive CAR T cell immunotherapy

Adoptive transfer of CAR engineered T cells is a very promising field of immunotherapy. Chimeric Antigen Receptor is an artificial receptor, which consists of different units (Harris and Kranz, 2016). A classical CAR comprises an antigen-binding domain, a hinge domain, a transmembrane domain and one or more signaling domains (Figure 2).

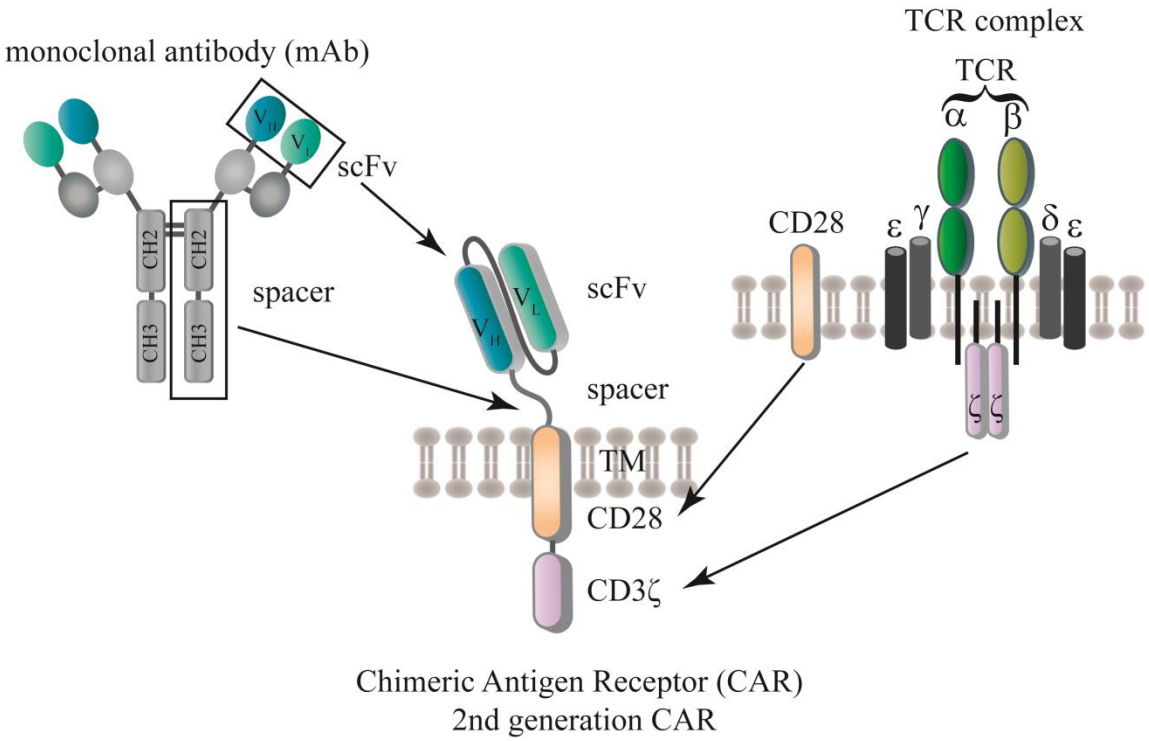


Figure 2 Illustration of a classical CAR

In the first CAR the antigen-binding single chain was directly connected with CD3ζ signaling domain and it was published by Eshhar Z et al. in 1993 (Eshhar et al., 1993). A direct ancestor of the first CAR was the hybrid T cell receptor, which was expressed in T cells and recognized the appropriate antigens as well (Gross et al., 1989; Kuwana et al., 1987).

In the prototype CAR, the antigen-binding domain is a single chain fragment of variable region (scFv) of an antibody and is responsible for the recognition of tumor-associated antigen (TAA) on the target cells. The scFv consists of the variable region of the immunoglobulin heavy chain (V_H) and the variable region of the immunoglobulin light chain (V_L) connected by a short flexible $(Gly_4Ser)_3$ peptide linker and thereby represents the complete antigen-binding site of the antibody. The sensitivity and binding affinity of a CAR is determined by the scFV, which impacts the functionality of the CAR T cells. To target an antigen expressing in low level on target cells, an increase of scFv binding affinity is needed. However, an increase of binding affinity above a specific threshold does not mean increased activation of T cell (Chmielewski et al., 2004). If the target antigen is expressed in a significant level on normal tissue as well, then the use of lower affinity of antigen-binding fragment is recommended (Caruso et al., 2015; Liu et al., 2015). In this way it is possible to discriminate between different target tumor antigen densities on the cell surface (Alvarez-Vallina and Russell, 1999; Westwood et al., 2009).

The hinge or spacer domain of the CAR is located between the antigen-binding domain and the transmembrane domain and originated from the immunoglobulin CH2-CH3 (Fc) domain from the constant region of immunoglobulin G (IgG) or from the spacer domain of the CD4 or CD8 (Harris and Kranz, 2016). Incorporation of a spacer domain is required for a stable CAR expression on T cells, more stability and flexibility as well as higher accessibility to TAA (Guest et al., 2005). The necessity of a spacer domain is determined by the position of the epitope in the relevant antigen. If the epitope located at the membrane-distal position extracellular spacer element is not needed, then for the membrane-proximal epitope a hinge region is recommended (James et al., 2008). The optimal length of the spacer domain can be decisive for the effector function of the CAR depending on the targeted surface antigen (Hudecek et al., 2015, 2013). The presence of different spacer regions can result in significant differences in CAR modified T cell function including cytokine secretion and specific cytotoxicity (Patel et al., 1999). Moreover, CAR modified T cells could be activated through the Fc spacer domain by IgG Fc gamma receptor (FcγRs) binding resulting in unwanted T cell function including cytokine secretion or cytotoxicity. To avoid this off-target activation and to reduce FcγR binding, modification in the spacer domain is needed (Hombach et al., 2010).

The transmembrane domain can be derived from various components of T cell surface receptor from homo- or heterodimeric type I membrane proteins such as CD3ζ, CD4, CD8, CD28, OX40 or FcεRIγ (Shi et al., 2013) and can also impact on T cell function of the CAR modified T cells. For example CD28 derived transmembran region is related with higher CAR expression level on T cell surface (Pulè et al., 2005).

All parts of the CAR receptor have an important role and effect on the modified T cell function, which is also valid for the case of intracellular domains. Moreover, the attention the intracellular signaling domains receive is dependant on the impact on T cell persistence, activity and efficacy (Harris and Kranz, 2016). A first generation CAR (Figure 3) has only one signaling domain in the intracellular part, which is mostly derived from the CD3ζ or FcεRIγ chain, but CD3ζ receptor have shown better efficacy in tumor eradication (Ren-Heidenreich et al., 2002). Nevertheless, first generation CARs provided some promising results *in vitro* as well as *in vivo* (Gong et al., 1999; Parker et al., 2000), although the engineered T cells achieved minimal anti-tumor efficacy and a short term persistence *in vivo* (Kershaw et al., 2006; Till et al., 2008). In addition, it soon became obvious, that one signal is not enough for a prolonged activation of T cells. Co-stimulatory signal is also needed for the complete T cell function with optimal proliferative ability and cytokine secretion. For this

reason the first generation CAR was equipped with an additional co-stimulatory signal domain which is mostly the CD28, 4-1BB or rarely OX40 or other domain (Hombach and Abken, 2007) and thereby the so-called second generation CARs (Figure 3) have been created (Finney et al., 1998; Hombach et al., 2001b; Maher et al., 2002). By adding co-stimulatory domain, enhanced T cell function was observed with stronger signaling, improved proliferative ability and cytokine secretion. Furthermore, better persistance and anti-tumor activity were observed (Imai et al., 2004; Kowolik et al., 2006; Milone et al., 2009; Song et al., 2011). The different co-stimulatory domains modulate differently the T cell effector function, CD28 domain has a role rather in primary activation while 4-1BB or OX40 sustain T cell activation (Finney et al., 2004; Hombach and Abken, 2011). However, CD28 co-stimulatory domain with CD3ζ signaling domain appear to be the best combination (Brentjens et al., 2007). Nonetheless, CD28 is an important component for T cell because of the abilty of IL-2 secretion. IL-2 as well as IFN-γ secretion is significantly better than CD3ζ alone or with other co-stimulatory domain combinations (Hombach et al., 2001a). Moreover, the T cell proliferation is enchanced in the presence of CD28 co-stimulatory domain (Beecham et al., 2000). The position of the CD28 domain is also not negligible since the CAR with CD28 proximal and CD3ζ distal positon to the membrane and not in opposite orientation have shown better expression in Jurkat cells (Finney et al., 1998). For further development two different co-stimulatory domains were combined and so three different signaling moieties were located in the intracellular part. They are the 3rd generation of CARs (Figure 3) (Tammana et al., 2010; Zhong et al., 2010). In this way, the CAR engineered T cell functions with enchanced proliferation activity, increased expression of antiapoptitic protein as well as better survival were completed (Carpenito et al., 2009; Hombach et al., 2013; Redmond et al., 2009).

By taking CD28 in combination with CD3ζ could be effective in tumor eradication and show appropriate persistence similar to cytokine production, with further co-stimulation the T cell function is augmented. However, with three signal domain the T cell could be more sensitive and activation could occur in antigen independent manner (Cartellieri et al., 2010).

Recently, the 4th generation of CARs (Figure 3) have also appeared and named as T cells re-directed for universal cytokine-mediated killing (TRUCKs) (Chmielewski and Abken, 2015). With this improvement the T cells are equipped with the abilty to produce a cytokine in an inducible fashion in order to activate other T cells and innate immune cells to eliminate antigen negative tumor cells (Chmielewski et al., 2011). This new approach is opened the way

for a treatment of solid tumors, since in contrast to the successful treatment of hematological cancer by CARs, targeting solid tumors are more difficult because of the immunosuppressive tumor microenvironment in which the function of immune cells are inhibited.

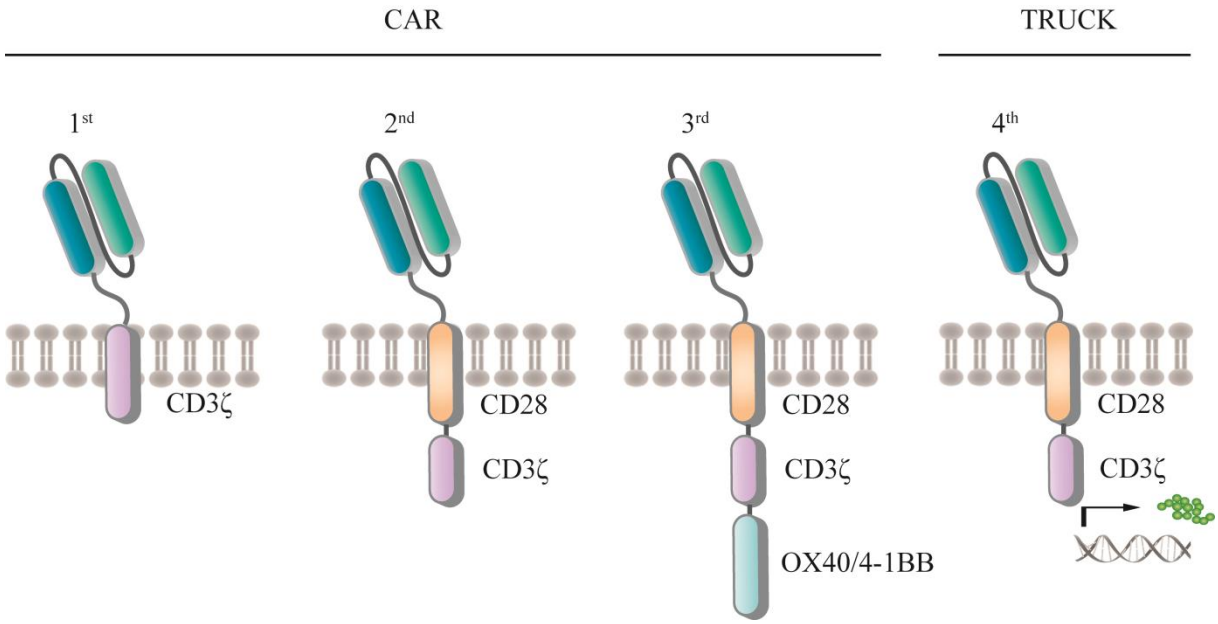


Figure 3 Generation of CARs

CAR takes advantages over artificial TCR by combining the antibody specificity with T cell signaling. In addition, in the CAR concept the antigen recognition works independently from MHC peptide complex in contrast to TCRs. In this way, CAR therapy overtakes TCR therapy since the major immune escape strategy by many tumors is the downregulation of the expression of the MHC class I molecule (Cartellieri et al., 2010).

Once modified T cell become activated by the engagement of tumor antigen with the CAR receptor, T cells start to produce pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-2, which are the results of downstream activation of the signaling pathways. Furthermore, the activation initiates T cell mediated cytotoxicity either by the granzyme/perforin pathway (Hombach et al., 2006) or due to the Fas/Fas-L mediated pathway (Shresta et al., 1998; Yasukawa et al., 2000). However, the answer given for an antigen engagement depends on many factors, such as: the location of the epitope, the antigen binding domain, its affinity, the length and kind of spacer and transmembrane domain and the number and choice of intracellular signaling domains. Moreover, the option between different T cell subset can also influence the outcome of the treatment given to the cancer patient (Riddell et al., 2014).

1.3 Solid tumor and the suppressive tumor microenvironment

CAR T cell therapy has achieved remarkable success in the treatment of hematologic malignances such as lymphoma or leukemia and has already reached clinical application as well. This success is not so apparent in the treatment of solid tumor (Newick et al., 2017). In contrast to hematological cancer, in the case of solid tumor the T cells have to reach and find the tumor cells and infiltrate the stromal elements in order to recognize the targeted antigen, which is mostly downregulated on the tumor cells. To find a tumor antigen, which is exclusively expressed on the tumor cell and not on non-malignant tissue, is difficult anyway. There are approximately 30 solid tumor antigens targeted by CAR T cell therapy. Up to now, none of them has been ideal and as a result the occurrence of "on target-off tumor" toxicity is a possibility (Morgan et al., 2010).

If T cells get through and find the tumor cells successfully they rapidly turn into dysfunctional T cells. There are three significant reasons for this. Firstly, the dominated milieu, which is presented around the tumor cells with hypoxia, acidic pH and oxidative stress (Hatfield et al., 2015) as well as with low level of nutrient (Fischer et al., 2007; Jacobs et al., 2008). Secondly, the presence of the tumor resident suppressive immune cells, such as regulatory T cells (Tregs) (Nishikawa and Sakaguchi, 2010), myeloid-derived suppressor cells (MDSCs) (Gabrilovich and Nagaraj, 2009), tumor-associated macrophages (TAMs) or tumor-associated neutrophils (TANs). These suppressive cells are responsible (beside the tumor cells) for the production of inhibitory soluble factors (Goodwin et al., 1977) and inhibitory cytokines. One of the most important inhibitory cytokines, which is mostly responsible for the unsuccessful tumor targeting, is the Transforming Growth Factor-β (TGF-β) produced by tumor cells, Tregs, MDSCs, M2 TAMs and N2 TANs (Massagué, 2008; Pickup et al., 2013). Thirdly, the induced regulatory mechanisms including upregulation of cytoplasmic and surface inhibitory receptors such as PD-1 or CTLA-4 (John et al., 2013). (Newick et al., 2017)

1.4 Targeting Transforming Growth Factor-β (TGF-β)

Transforming Growth Factor-β is a multifunctional polypeptide that has an important role in proliferation, differentiation, embryonic development, angiogenesis, wound healing, and other functions in many cell types (Nagaraj and Datta, 2010). TGF-β belongs to a large superfamily with 33 known human family participants including bone morphogenetic proteins (BMPs), activins and inhibins, growth and differentiation factors (GDFs) and three isoforms of TGF-β

(TGF-β1, TGF-β2, TGF-β3) (Morikawa et al., 2016). The members of the family are evolutionary conserved proteins and there is 70% - 80% homology among the TGF-β isoforms. Among the three isoforms, TGF-β1 has the largest role in the regulation of immune cells. TGF-β is expressed in epithelial, endothelial, hematopoietic and connective tissue cells (Achyut and Yang, 2011). Many cancer cells are able to produce a vast amount of TGF-β as well and so to form the suppressive tumor microenvironment. TGF-β promotes tumor formation, progression and metastasis in many human tumors, therefore tremendous effort was given to develop strategies in order to target TGF-β, TGF-β receptor or TGF-β signaling (Yingling et al., 2004).

There are many approaches to block TGF-β including monoclonal antibodies, antisense oligonucleotides, small molecule inhibitors, soluble TGF-β receptor or dominant-negative TGF-β receptor. It is important to note, that a systemic block of TGF-β can lead to serious consequences; the lack of TGF-β1 in TGF-β1 KO mice causes multifocal inflammatory disease and embryonic lethality (more than 50%) (Kulkarni and Karlsson, 1993; Shull et al., 1992) whilst the lack of TGF-β2 or TGF-β3 results in 100% embryonic lethality (Kaartinen et al., 1995; Sanford et al., 1997).

The first treatment option was the development of antisense oligonucleotides that inhibit the production of protein at molecular level. Antisense oligonucleotide against TGF-β2 (AP 12009) was applied in clinical trial I/II and the results have shown that this is a promising therapeutic approach for tumor therapy (Hau et al., 2007; Schlingensiepen et al., 2008). Application of monoclonal antibodies was well tolerated in the mouse model; nonetheless the treatment alone was not sufficient to induce tumor reduction (Takaku et al., 2010; Terabe et al., 2009). Other treatment options are the targeting of specific TGF-β signaling components by small molecule inhibitors (Vogt et al., 2011). Soluble TGF-β receptor II could bind with TGF-β1 and therefore inhibited the TGF-β-dependent transcription in target cells. This approach was successfully applied in human breast cancer therapy (Hu et al., 2011; Seth et al., 2006).

One more option to target TGF-β signaling is the expression of the dominant-negative TGF-β receptor II. In the early 2000s, two groups developed transgenic mice with overexpression of a dominant-negative TGF-β receptor, where TGF-β signaling was selectively blocked in T cells. They found a similar impact, the mice developed autoimmune disease, Gorelik et al.

described about spontaneously T cell differentiation into Type1/Type2 cytokine secreting cell, while Lucas et al. wrote about $CD8^+$ T cell lymphoproliferative disorder (Gorelik and Flavell, 2000; Lucas et al., 2000). In addition, in a dominant-negative TGF-β II (DNTGFβ) transgenic mice have shown better anti-tumor effects, due to the enhanced generation of tumor specific $CD8^+$ CTLs in the absence of TGF-β signaling (Gorelik and Flavell, 2001). Dominant-negative TGF-β II receptor modified EBV specific CTLs were used against EBV positive Hodgkin lymphoma, where they reached almost complete resistance to TGF-β without impact on CTL function in the long term *in vitro* (Bollard et al., 2002). Lacuesta et al. demonstrated the safe usage of DNTGFβ II receptor modified E7 specific CTLs *in vivo* (Lacuesta et al., 2006). Targeting solid tumor with the help of DNTGFβ II receptor engineered T cells had benefit in an *in vivo* B16 melanoma model, while soluble TGF-β receptor in the same model had no improvement (Zhang et al., 2013). Recently, the first clinical trial with DNTGFβ II receptor was published. Eight patients with EBV-positive Hodgkin lymphoma received DNTGFβ II receptor modified T cells. Four patients achieved clinical responses, including one with complete response. No toxicity, no uncontrolled T cell proliferation was detected during the 4 years observation time (Bollard et al., 2018). DNTGFβ II receptor combined with CAR modified T cells have shown promising results against prostate cancer (Kloss et al., 2018; Zhang et al., 2018).

All of these approaches targeted either the secreted form of TGF-β or the signaling pathway and therefore block the TGF-β mediated regulation, although the role of TGF-β in immune homeostasis is essential. The regulation by TGF-β is needed to impede the immune response and to prevent unpredictable immune cell growth, cytokine storm or autoimmune responses. Unfortunately, cancer cells have the capacity to avoid the suppressive influence of TGF-β and thus contribute to the tumor growth, to invasion and evasion of immune surveillance as well as to cancer cell dissemination and metastasis (Massagué, 2008).

1.5 Role of cytokines

Cytokines are a very diverse group and mostly responsible for intracellular communication either in autocrine or in paracrine manner. They play an important role in immunity and inflammation as well as in functions related to cellular proliferation, differentiation and survival. The group of type I cytokines involve many interleukins (IL) and some growth and hematopoietic factors; their common feature is the four α-helical bundle structures. One

member of this group is the common cytokine-receptor γ-chain (γ_c) family, which consists of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. All of these cytokines share a γ-chain in their receptor. Among these participants IL-2, IL-7 and IL-15 have particular interest because of the roles in T cell (or other immune cell) development as well as immune regulation (Rochman et al., 2009). Mutation in the γ-chain leads to X-linked severe combined immunodeficiency (XSCID) in the human patient. This disease is characterized by complete or profound T cell defect, which indicates the important role of these cytokines in the regulation of immune homeostasis (Noguchi et al., 1993; Sugamura et al., 1996).

IL-2 is the prototypic member of the family, its functions include as T cells growth factor, regulate the expansion and apoptosis of activated T cells. In addition, IL-2 contributes to generation and function along with the development of Tregs. The main sources of IL-2 are the activated effector T cells. The IL-2 receptor consists of three polypeptide chains, which are the IL-2Rα (CD25), IL-2Rβ (CD122) and γ-chain (γ_c or CD132). These three components form the high affinity IL-2 receptor; for an intermediate affinity receptor the β- and the γ-chain are needed, while α-chain alone is just a low-affinity IL-2 receptor (Kim et al., 2006). IL-15 and IL-2 share not only the γ-chain in their receptor but they use the same β-chain as well. However, the high-affinity IL-15 receptor composes of a third IL-15R α-chain (CD215). In spite of the two common subunits, both cytokines have the individual role in adaptive immune response. For instance, IL-15 is necessary for NK cell development and supports the survival of $CD8^+$ memory T cells. The main IL-15 produced cells are dendritic cells and monocytes (Waldmann, 2006). IL-7 has a central role in immune regulation and it is indispensable for T cell development in humans. IL-7 is a tissue-derived cytokine; primary sources of it are stromal and epithelial cells. IL-7 receptor is heterodimer and consists of the IL-7Rα (CD127) and the common γ-chain. Mutation in the IL-7R α-chain causes severe combined immunodeficiency (SCID) disease (Fry and Mackall, 2002). All γ-cytokines lead the signal through the JAK-STAT pathway and IL-2, IL-7 and IL-15 activate mainly STAT5 protein. Besides, all three cytokines have essential roles in T cell proliferation (Rochman et al., 2009).

Optimal T cell activation requires at least 3 signals: TCR/CAR engagement, co-stimulatory signal and cytokine engagement (Kershaw et al., 2013). The first two signals are gratified by a second generation CAR, while the feasibility of stimulatory cytokine engagement is limited in the suppressive tumor microenvironment. Inhibitory cytokine signal can be turned into stimulatory signal by the use of chimeric cytokine receptor. Wilkie et al. published a hybrid

receptor consists of the IL-4R α-chain as ectodomain and the IL-2/IL-15 receptor β-chain as intracellular domain. This receptor was co-expressed with a MUC1 specific CAR and successfully destroyed MUC1 positive tumor cells *in vitro* (Wilkie et al., 2010). In another publication the IL-4 receptor ectodomain was fused with the IL-7 receptor endodomain and the hybrid receptor was co-expressed with the PSCA (prostate stem cell antigen) specific CAR. This combined approach enhanced the anti-tumor activity in prostate cancer (Mohammed et al., 2017).

1.6 Aims

Beside the successful treatment of hematological cancer by CAR T cell therapy there are no promising results in the CAR T cell treatment of solid tumors. The suppressive tumor microenvironment prevents the CAR T cells from executing their effector functions. For instance, the T cells do not enter the tumor lesion and do not stay active and effective against tumor cells in the presence of repressive signals. TGF-β is one of the dominant suppressive cytokine, which is used by tumor cells in order to escape immune surveillance. Ongoing research focuses on the modulation of the tumor tissue to provide a more supportive microenvironment for effector T cells for a successful anti-tumor attack.

Previous work of our group showed that the suppressive effect of TGF-β is conquerable by CD28 signaling through a second generation CAR. We here wanted to answer how CD28 compensates the suppressive effect of TGF-β and how the suppression can be circumvented to engineer more effective CAR T cells.
We addressed the following questions:

- Is other costimulation, beside CD28, such as 4-1BB also able to overcome the suppressive effect of TGF-β?
- Which part of CD28 signaling domain is responsible for the resistance to TGF-β?

CD28 costimulation induces IL-2 release and the accumulation of IL-2 in the tumor site can lead to the enhanced activation of Tregs. This raises the question whether another γ-cytokines can replace IL-2 to compensate the suppressive effect of TGF-β in T cell proliferation. We identified IL-7 and IL-15 as promising candidates since both of them are able to stimulate T cell proliferation. IL-7 is more suitable, since IL-7 does not activate Tregs because of the lack of IL-7 receptor on Treg cells.

In this part, we addressed the following questions:

- Is IL-7 or IL-15, similar to IL-2 able to overcome the repressive TGF-β in T cell proliferation?
- Is IL-7 or IL-15 TRUCK engineered T cells able to produce an appropriate level of cytokine to compensate the suppressive effect of TGF-β in T cell proliferation?
- Can we overcome the activation-mediated downregulation of the IL-7 receptor by a chimeric cytokine IL7/IL2 receptor?
- Does IL-7 autocrine loop make T cells resistant to TGF-β?

- How do such engineered CAR T cells perform in the attack against TGF-β positive tumors?

In this thesis we provide a novel concept to demonstrate how to make CAR engineered T cells more efficient against tumor cells in the suppressive TGF-β signals enriched tumor environment.

2 MATERIALS

2.1 Chemicals and reagents

chemical, reagents	company
ABTS	Roche Diagnostics, Rotkreuz, Switzerland
ABTS buffer	Roche Diagnostics, Rotkreuz, Switzerland
Aceton	Carl Roth GmbH, Karlsruhe, Germany
Agarose	Invitrogen GmbH, Darmstadt, Germany
Ampicillin	Merck/Calbiochem, Darmstadt, Germany
Background Buster	Innovex biosciences, Richmond, CA, USA
Bacto tryptone	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Bacto Agar	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Blasticidin-Agar	InvivoGen, San Diego, CA, USA
Brij®97	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
BSA (Albumin)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
dGTP	Promega, Mannheim, Germany
DMSO (dimethylesulfoxid)	Carl Roth GmbH, Karlsruhe, Germany
DNA ladder Gene Ruler^{TM} 1kb #SM0313	Fermentas, St. Leon-Rot, Germany
DNA loading dye 6x	Fermentas, St. Leon-Rot, Germany
EDTA (ethylenediaminetetraacetic acid)	Carl Roth GmbH, Karlsruhe, Germany
Ethanol	Carl Roth GmbH, Karlsruhe, Germany
Ethidium bromide	Bio-Rad Laboratories, München, Germany
Fc Receptor Block	Innovex biosciences, Richmond, CA, USA
gBlocks fragments	IDT, Skokie, Illinois, USA
Glycerin	Merck/Calbiochem, Darmstadt, Germany

chemical, reagents	company
HEPES	GIBCO/Invitrogen GmbH, Darmstadt, Germany
Isopropanol	Carl Roth GmbH, Karlsruhe, Germany
IS Mounting medium +/- DAPI	dianova, Hamburg, Germany
Lymphoprep™	Axis-Shield Poc AS, Oslo, Norway
Methanol	Carl Roth GmbH, Karlsruhe, Germany
PEIpro® Transfection Reagent	Polyplus transfection, Illkirch, France
PenStrep	GIBCO/Invitrogen GmbH, Darmstadt, Germany
Peptone	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
PMS (N-Methylphenazonium methyl sulfate)	Biomol GmbH, Hamburg, Germany
Reddot2	Biotium, Fermont, CA, USA
Streptavidine-Peroxidase conjugate	Roche Diagnostics, Rotkreuz, Switzerland
Tissue-Tek® O.C.T.™ Compound	Sakura, Torrance, CA, USA
Tris	Carl Roth GmbH, Karlsruhe, Germany
Trizol	Invitrogen, Carlsbad, CA, USA
Trypan Blue	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Trypsin/EDTA 10x	PAN-Biotech GmbH, Aidenbach, Germany
Tween® 20	Merck, Darmstadt, Germany
Yeast extract	Carl Roth GmbH, Karlsruhe, Germany
7AAD (7-Amino-Actinomycin D)	BD Biosciences, San Jose ,CA, USA

Table 1 List of chemicals and reagents used in this study

2.2 Kits

KIT	company
APC-Annexin V Apoptosis Detection Kit	BD Pharmingen™, Hamburg, Germany
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems GmbH, Darmstadt, Germany
CellTrace CFSE Proliferation Kit	Invitrogen GmbH, Darmstadt, Germany
Cytofix/Cytoperm Kit	BD Bioscience, San Jose, CA, USA
IC Fixation Kit	eBioscience/ThermoFisher Scientific, Waltham, MA, USA
Intrasure Kit	BD Bioscience, San Jose, CA, USA
NucleBond® Xtra Midi Kit	Macherey Nagel GmbH, Düren, Germany
PureLink® Quick Gel Extraction Kit	Invitrogen GmbH, Darmstadt,Germany
PureLink® Quick Plasmid Miniprep Kit	Invitrogen GmbH, Darmstadt,Germany
XTT Cell Proliferation Kit	AppliChem, Darmstadt, Germany
mouse IL-7 DuoSet ELISA, (DY407)	R&D System, Minneapolis, MN, USA
human IL-15 DuoSet ELISA, (DY247)	R&D System, Minneapolis, MN, USA

Table 2 List of Kits used in this study

2.3 Medium for cell culture

Medium	company
D-MEM, „High Glucose“, #61965-026	GIBCO/Invitrogen GmbH, Darmstadt, Germany
RPMI1640-GlutaMAX™, #61870-010	GIBCO/Invitrogen GmbH, Darmstadt, Germany
FCS (Fetal calf serum)	PAN-Biotech GmbH, Aidenbach, Germany
Heat-Inactivated (HI) FCS (56°C;30 min)	PAN-Biotech GmbH, Aidenbach, Germany
X-vivo™ 15	Lonza, Basel, Switzerland

Table 3 List of Medias used in this study

2.4 Buffers and gels

Buffers and gels	composition
Agarose gel	1%: boil up 1g / 100 ml TAE buffer 1x, after cooling down add 100 ng/ml ethidium bromide
Blocking buffer (ELISA)	1% BSA (w/v), PBS 1x
$CaCl_2$ buffer (for chemically competent bacteria)	60mM $CaCl_2$ dihydrate, 10mM PIPES, 15 % (w/v) Glycerin, H_2O_{dd}; pH 7 (3M NaOH)
Coating buffer (ELISA)	0.1M Na_2HPO_4, pH 9
(Intracellular) staining buffer	1% (v/v) HI-FCS in PBS
LB agar	1l LB medium, 15 g Bacto Agar
LB medium	10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl
PBS 10x	1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4, H_2O_{dd}; pH 7.4
PBS/Tween (PBS-T)	1x PBS, 0.1% (v/v) Tween®-20
TAE buffer 50x	2M Tris, 1M pure acetic acid, 50 mM EDTA pH 8.0, H_2O_{dd}; pH 7.6 – 7.8
TE buffer 1x	10 mM Tris-HCl pH 8, 1 mM EDTA, H_2O_{dd}
Tris-HCl	10 mM Tris, H_2O_{dd}; pH 6.8- 9 (HCl)
XTT reagent solution	per well: 50 µl 1 mg/ml XTT in RPMI1640 + 50 µl RPMI1640

Table 4 List of buffers and gels used in this study

2.5 Antibodies

2.5.1 unconjugated antibodies

antibody	species/isotype	company/source
anti-human IL-2, clone 5344-111	mouse IgG1	BD Pharmingen™, Hamburg, Germany
anti-human IFN-γ, clone NIB42	mouse IgG1	BD Pharmingen™, Hamburg, Germany
IgG1 isotype control, clone 15H6	mouse IgG1	Southern Biotechnology, AL, USA
anti-human CD3, clone OKT3	mouse IgG2a	hybridoma cell line; CRL-8001, ATCC
anti-human CD28, clone 15E8	mouse IgG1	hybridoma cell line, (van Lier *et al.*, 1988)

antibody	species/isotype	company/source
anti-human BW431/26scFv, clone BW2064/36	mouse IgG1	hybridoma cell line, (Bosslet *et al.*, 1985)
anti-TGF-β, mouse monoclonal, clone 3C11	IgG1	Santa Cruz Biotecnology, TX, USA

Table 5 List of unconjugated antibodies used in this study

2.5.2 conjugated antibodies

antibody	species/isotype	conjugate	company
anti-human IL-2, clone B33.2	mouse IgG1	Biotin	BD Pharmingen™, Hamburg, Germany
anti-human IFN-γ, clone 4S.B3	mouse IgG1	Biotin	BD Pharmingen™, Hamburg, Germany
anti-human CD3, clone BW264/56	mouse IgG2aκ	FITC	Miltenyi Biotec, Bergisch Gladbach, Germany
anti-human CD3, clone BW264/56	mouse IgG2aκ	APC	Miltenyi Biotec, Bergisch Gladbach, Germany
anti-human CD66a/c/e (anti-CEA), clone ASL-32	mouse IgG2b	AlexaFluor 488	BioLegend®, CA, USA
anti-human IgG, mouse ads, polyclonal	goat $F(ab')_2$ IgG	PE	Southern Biotechnology, AL, USA
anti-human IgG	goat $F(ab')_2$ IgG	AlexaFluor 555	Southern Biotechnology, AL, USA
mouse anti-human Lck pY505 (Phosflow, clone:4/LCK-Y505)	Mouse IgG1	PE	BD Biosciences, San Jose ,CA, USA
mouse anti-human Granzyme B (clone:GB11)	Mouse BALB/c IgG_1, κ	FITC	BD Biosciences, San Jose ,CA, USA
anti-Hu/Mo pSTAT5 (Tyr694), clone:SRBCZX	Mouse / IgG1, kappa	PE	eBioscience/ThermoFisher Scientific, Waltham, MA USA
anti-human CD127 (clone:MB15-18C9)	mouse IgG2aκ	APC	Miltenyi Biotec, Bergisch Gladbach, Germany
anti-human LAP (TGF-β1), clone:TW4-2F8	Mouse IgG1, κ	PE	BioLegend®, CA, USA

Table 6 List of conjugated antibodies used in this study

2.5.3 conjugated secunder antibody

antibody	species/isotype	conjugate	company
goat anti-mouse IgG (H+L), polyclonal	goat/IgG	AlexaFluor 555	Invitrogen, Carlsbad, CA, USA

Table 7 Conjugated seconder antibody used in this study

2.6 Cytokines

Cytokine	company
recombinat IL-7	
recombinat IL-15	Miltenyi Biotec, Bergish Gladbach, Germany
recombinant TGF-β	
Imukin® (IFN-γ standard)	Boehringer Ingelheim, Ingelheim, Germany
Proleukin® (IL-2 standard)	Novartis Pharma GmbH, Basel, Switzerland

Table 8 List of cytokines used in this study

2.7 Synthetic oligonucleotides

2.7.1 Oligonucleotides used for plasmid generation

ID	name	direction	5´- 3´sequence
#1257	OL1 for	sense	GTGGATCCCGCCGAGCCCAAATC
#1269	OL1-P2A rev neu	antisense	CTGCTTGCTTTAACAGAGAGAAGTTCGTGGCTCCAGATCCGCGAGGGGGCAGGGCCTGCATG
#1018	P2A-IL7s	sense	ACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCTATGTTCCATGTTTCTTTTAGATAT
#1261	OL2 rev neu	antisense	CGCTCGAGTTATATACTGCCCTTCAAAATTTTATTCCAACAAG

Table 9 List of primers used for plasmid generation

2.7.2 Oligonucleotides used for sequencing

ID	name	direction	5´- 3´sequence
#933	-	sense	AAAATGAATATCAGTGAAGAGTTCAATGTCCACTTACTAAC
#934	-	antisense	GTTAGTAAGTGGACATTGAACTCTTCACTGATATTCATTTT
#1071	-	antisense	AGCCGCGGGAGGAGCAGTAC
#1098	-	antisense	TTTAATGTGGCACTCAGATGA
#1152	-	sense	TCGGCCAAGGGACCAAGG
#1166	-	sense	AAGCATTACCAGGCCTATGCCGCCGCACGCGACTTCGCAGCCTAT

ID	name	direction	5´- 3´sequence
#1238	pJet for	sense	AACTTGGAGCAGGTTCCATTC
#1239	pJet rev	antisense	CCTGATGAGGTGGTTAGCATAG
#1272	-	sense	GCAGTACAACAGCACG
#1299	-	antisense	GAGCCTGGACCACTGATATCC
#1448	-	sense	TGCACGATGTAGCTTACCGCCAGG
#1449	-	antisense	CCACCGAGGAGACTGGGGGAGAAG

Table 10 List of primers used for sequencing

2.7.3 gBlocks ordered from IDT

gBlock	description
ApaI-P2A-IL2-XhoI	DNA fragment, part of #1746 vector
ApaI-P2A-IL15-XhoI	DNA fragment, part of #1764 vector
SanDI-CD3ζ-T2A-BspEI	DNA fragment, part of #1941 vector
KpnI-IL7Rα/IL2Rβ-BspEI	DNA fragment, part of #1908 and #1941 vector
BclI-IL7-T2A-IL7Rα-KpnI	DNA fragment, part of #1908 vector

Table 11 List of gBlocks ordered from IDT

2.8 Plasmid vectors

ID	plasmid	characteristics and references
#392	pCOLT-GALV	retroviral vector containing the expression cassette of the gibbon ape leukemia virus envelope (GALV env) protein (Weijtens et al., 1998)
#393	pHIT 60	retroviral vector containing the expression cassette of the Moloney murine leukemia virus (M-MulV) proteins gag and pol (Weijtens et al., 1998)
#422	pBullet	pBullet empty vector (Weijtens et al., 1998)
#607	pBullet-Lk-BW431/26scFv-Fc-CD28-CD3ζ	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-CD28-CD3ζ (Hombach et al., 2001a)
#908	pBullet-Lk-BW431/26scFv-Fc-4-1BB-CD3ζ	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-4-1BB-CD3ζ (Hombach and Abken, 2011)

ID	plasmid	characteristics and references
#946	pBullet-Lk-BW431/26scFv-Fc-CD28ΔLCK-CD3ζ	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-CD28ΔLCK-CD3ζ (Kofler et al., 2011)
#947	pBullet-Lk-BW431/26scFv-Fc-CD28ΔPI3K-CD3ζ	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-CD28ΔPI3K-CD3ζ (Golumba-Nagy et al., 2018)
#958	pBullet-Lk-BW431/26scFv-Fc-CD28ΔLCKΔPI3K-CD3ζ	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-CD28ΔLCKΔPI3K-CD3ζ (Golumba-Nagy et al., 2018)
#1024	pJR-mIL7	contains the mouse IL-7 cDNA (Hock et al., 1991)
#1610	pJET	pJET empty vector (CloneJet KIT, Thermo Scientific)
#1645	pBullet-Lk-BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-IL7	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-IL7 with transgenic mouse IL-7 (Golumba-Nagy et al., 2018)
#1746	pBullet-Lk-BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-IL2	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-IL2 with transgenic human IL-2 (Golumba-Nagy et al., 2018)
#1764	pBullet-Lk-BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-IL15	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-IL15 with transgenic human IL-15 (Golumba-Nagy et al., 2018)
#1908	pBullet-Lk-BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-IL7-IL7Rα/IL2Rβ	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-IL7-IL7Rα/IL2Rβ with transgenic mouse IL-7 and the chimeric IL7/IL2 cytokine receptor (Golumba-Nagy et al., 2018)
#1941	pBullet-Lk-BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-IL7Rα/IL2Rβ	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-IL7Rα/IL2Rβ with the chimeric IL7/IL2 cytokine receptor (Golumba-Nagy et al., 2018)

Table 12 List of plasmids used in this study

2.9 Restrictions enzymes

Enzyme	Buffer	company
ApaI	B	ThermoFisher Scientific, Waltham, MA USA
BamHI	BamHI	
BclI	G	
BglII	O	

Enzyme	Buffer	company
BspEI/Kpn2I	Tango	ThermoFisher Scientific, Waltham, MA USA
KpnI	KpnI	
FastDigest SanDI/KflI	FastDigest	
SpeI/BcuI	Tango	
XhoI	R	
FastAP	every type of buffers	
T4 DNA Ligase	T4 DNA Ligase Buffer	Fermentas, St. Leon-Rot, Germany

Table 13 List of restrictions enzyme used for cloning

2.10 Bacterial strains

Bacterial strains	description
E.coli DH5α:	F-, end A1, hsd R17 (rκ-, mκ-) sup E44, thi-1, lambda-, recA1, gyrA96, Φ 80 d lacZ δ M15
XL10-Gold	Tet^rΔ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F´ proAB $lacI^qZ$ΔM15 Tn10(Tet^r) Amy Cam^r]

Table 14 List of bacterial strains

2.11 Cell lines

cell line	characteristics	reference
HEK293T	human embryonic kidney 293 cell line (ATCC CRL-3216™) stably expressing the SV40 large T antigen; neomycin resistant	(DuBridge et al., 1987)
C15A3	MC38 cell line stably expressing CEA; neomycin resistant (kindly provided by Dr. M. Neumaier, Universität Heidelberg-Mannheim)	(Robbins et al., 1991)
LS174T	human CEA-positive colorectal adenocarcinoma cell line	ATCC: CL-188™
OKT3	mouse hybridoma cell line, (ATCC CRL 8001) secretes monoclonal antibody OKT3 directed against human CD3	(Kung et al., 1979)
15E8	mouse hybridoma cell line, secretes monoclonal antibody 15E8 directed against human CD28 (kindly provided by Dr. Van Lier, NCB Amsterdam, Netherlands)	(Lier et al., 1988)
BW2064/36	mouse hybridoma cell line, secretes monoclonal anti BW431/26 idiotypic antibody BW2064/36 (kindly provided by K Bosslet, Behring-Werke, Marburg, Germany)	(Kaulen et al., 1993)

Table 15 List of cell lines used in this study

2.12 Primary cells

Human peripheral blood mononuclear cells (PBMC) were isolated from fresh blood or buffy coats (platelet and leukocyte fraction from blood donations) of healthy donors. Buffy coats were obtained from Transfusionsmedizin der Uniklinik Köln. All studies involving human blood cells were approved by the Institutional Review Board of the University Hospital of Cologne (reference no. 01-090 and 11-319).

2.13 Mouse model

$Rag2^{-/-}\gamma c^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All animal experiments were performed according to the Animal Experiments Committee regulations and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Recklinghausen, Germany (K17/35-05).

2.14 Software

Software	company
Adobe Illustrator	Adobe Systems
ContingExpress	-
FACSDiva	BD Bioscience
FlowJo	FlowJo LLC
GraphPad Prism	GraphPad Software, Inc
Microsoft Excel	Microsoft
Microsoft Word	Microsoft
Olympus Fluoview (FV1000)	Olympus
SkanIt™	ThermoFisher Scientific
Vector NTI	ThermoFisher Scientific

Table 16 List of software used for data analysis

3 METHODS

3.1 Bacteria

Bacterial cells were handled in gene technology labs of security level *S1*.

3.1.1 Bacteria culture

Bacterial cells *Escherichia coli (E. coli)* DH5α and XL10-Gold were cultured overnight at 37°C and shaking at 200 rpm in liquid LB medium or –alternatively- on LB plates. The medium was supplemented with 100 µg/ml ampicillin (Amp) to select for grow of genetically modified bacteria, with Amp-resistance gene. In case of the long-term storage, 700 µl bacteria cultures were mixed with 300 µl of 87% (v/v) glycerol and stored at -80°C.

3.1.2 Generation of chemically competent *E.coli* DH5α or XL10-Gold bacteria

Bacteria clones were cultured in 400 ml of LB medium without antibiotic at 37°C with 200 rpm shaking until the suspension reached an OD_{590nm} of 0.4 (optical density). The suspension was cooled down on ice for 10 minutes, and the cells were centrifuged without brakes for 10 minutes (1600 x *g*, 4°C), the pellet was immediately resuspended in 80 ml of ice-cold $CaCl_2$ buffer and centrifuged again for 7 minutes (1100 x *g*, 4°C). After the resuspension of the pellet in 80 ml of ice-cold $CaCl_2$ buffer the cells were incubated on ice for 30 minutes. Then, the suspension was centrifuged for 7 minutes (1100 x *g*, 4°C) and resuspended in 16 ml ice-cold $CaCl_2$ buffer. Finally, the suspension was split into 100 µl aliquots, which were directly put into liquid nitrogen for freezing and were then stored at -80 °C.

3.1.3 Heat-shock transformation of bacteria

Competent bacteria (stored at -80°C) were defrosted on ice and 1-100 ng plasmid DNA was added. The mixed bacteria were heat-pulsed for 90 seconds at 42°C and subsequently cooled down on ice for 1-2 minutes. To gain antibiotic resistance, the bacteria were incubated in 900 µl bacterial growth medium (without antibiotic) at 37°C and 200 rpm for 60 minutes. Finally, the suspension was centrifuged for 5 minutes (1100 x g), resuspended in 100 µl LB medium and spread out on agar plates containing antibiotics using a Drigalski spatula for overnight culture at 37°C.

3.2 Working with DNA

3.2.1 Preparation of plasmid DNA

For plasmid DNA preparation, the "PureLink® Quick Plasmid Miniprep Kit" (Invitrogen GmbH) (for DNA amounts up to 40 µg) and the "NucleBond® Xtra Midi Kit" (Macherey Nagel GmbH) (for DNA amounts up to 400 µg) were used according to the manufacturer's instructions. In case of Midi Kit 150 ml of bacteria suspension was prepared, while in case of Mini Kit 3-5 ml of bacteria suspension was used. The eluted plasmid DNA was diluted in sterile H_2O_{dd} and stored at -20°C.

3.2.2 Quantification of DNA

Before freezing the plasmid DNA, the DNA concentration was determined spectrophotometrically by using the NanoDrop™1000 spectrophotometer (ThermoFisher Scientific). In the process, 1 µl DNA sample was applied onto the NanoDrop™ pedestal, and the DNA concentration was analyzed at a wavelength of 260 nm, while protein concentration was measured at 280 nm. Purity from protein contaminations of the preparation is defined by the ratio O.D. 260 nm / abs 280 nm. A ratio of 1.8 marks a high purity of DNA, whereas lower values indicate contaminations with proteins and aromatic substances and higher ratios suggest contaminations with RNA.

3.2.3 Restriction endonuclease digestion of DNA

Restriction enzymes were applied for DNA digestion according to the goal of the cloning strategies. Restriction enzymes were used with the appropriate buffers as stated in the manufacturer's instructions. Digestions with two or more different restriction enzymes were accomplished in one step if the reaction conditions and buffers were similar or compatible.

3.2.4 Agarose gel electrophoresis

Digested DNA fragments were separated by electrophoresis in 1% (w/v) agarose gels. Ethidium bromide (100 ng/ml)-supplemented gels were run at 8-12 V/cm in 1x TAE buffer. 6x DNA Loading Dye was added to DNA samples before their application into the gel and the GeneRuler™ 1 kb DNA ladder was applied as DNA sizing standard. For DNA visualization the Biometra BioDocAnalyze Live (BDAlive) workstation with UVstar UV transilluminator with bandpass filter (254 nm) was used.

3.2.5 DNA isolation from agarose gels

Gel electrophoresis separated DNA fragments (DNA band of interest) were excised under UV light (254 nm) by using a scalpel. DNA purification was performed by using the PureLink® Quick Gel Extraction Kit (Invitrogen GmbH) according to the manufacturer's instructions. Purified DNA fragments were diluted in sterile H_2O_{dd} and stored at -20°C.

3.2.6 Ligation of DNA fragments

DNA fragments with appropriate restriction ends were ligated using the T4 DNA ligase and the 1x ligase buffer (Fermentas) according to the manufacturer's instructions. Plasmid and insert DNA were mixed in a molar ratio of 1:3 to yield 50-100 ng plasmid DNA in a total volume of 20 µl. The mixture was incubated overnight at 14°C and transformed into highly competent bacteria on the next day.

3.2.7 Polymerase chain reaction (PCR)

In most cases new DNA sequences were ordered from Integrated DNA Technologies (IDT, Coralville, IO, USA) as "gBlocks", howevere, in other cases the required DNA fragment was amplified out of existing vectors by PCR using thermal cyclers T3000 Thermocycler (Biometra).

The following reaction mix was used for the amplification:

template DNA	50 ng to 100 ng
upstream primer	1 µl (10 pmol)
downstream primer	1 µl (10 pmol)
High Fidelity Master Mix (2x)	25 µl
sterile H_2O_{dd}	ad 50 µl

The following PCR reaction conditions were used for DNA amplification:

PCR step	Time	Temperature	Number of cycles
Denaturation	240 sec	96°C	1
Denaturation	60 sec	96°C	33 - 35
Annealing	30 – 60 sec	45 – 65°C	33 - 35
Elongation	30 – 150 sec	72°C	33 - 35
Final elongation	240 – 360 sec	72°C	1
Final hold	∞	4°C	N/A

Annealing time and temperature were chosen according to the sequence and the melting temperature of the used primer oligonucleotides. Typical annealing temperatures are 5°C below the lowest primer's melting temperature. The length of the amplified DNA fragment defined the elongation time (e.g. for Taq polymerase: 1 minute per 1000 base pairs).

3.2.8 DNA sequencing

The BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems GmbH) and a thermal cycler (Eppendorf) were utilized for DNA sequencing *via* the dideoxy chain termination method.

The following reaction mixture was used:

template DNA	X µl (50-200 ng)
BigDye sequencing buffer (5x)	2.25 µl
sequencing primer oligonucleotides	1 µl (10 pmol)
BigDye v3.1	0.25 µl
sterile H_2O_{dd}	*ad* 10 µl

The following PCR cycler program was applied:

PCR step	Time	Temperature	Number of cycles
Denaturation	60 sec	96°C	1
Denaturation	30 sec	96°C	90
Annealing	15 sec	50°C	90
Elongation	240 sec	60°C	90
Final hold	∞	4°C	N/A

DNA sequencing was performed at the Cologne Center for Genomics (University of Cologne) using a 3730 DNA analyzer (Applied Biosystems). Sequencing data files were analyzed by using the software program ContingExpress®.

3.3 Working with cells

3.3.1 General cell culture conditions

Cell culture was carried out in laboratories of the Biosafety Levels S2 using laminar flow cabinets of *Sicherheitsklasse II* (Heraeus, Hanau, Germany). The culture conditions for the cells were humidified atmosphere (95-100%) with 5% or 10% (v/v) CO_2 and 37°C. The culture media DMEM and RPMI1640 with GlutaMAX™ were supplemented with 10% (v/v) FCS, 25 U/ml penicillin and 25 µg/ml streptomycin and 10 mM HEPES before use, and then stored at 4°C. Moreover, the media, additives, and solutions were autoclaved or sterile-filtered in advanced. Prior to application, the media and other solutions were pre-warmed up to 37°C.

3.3.2 Passaging of adherent cells

For passaging, the medium of adherent cells was carefully removed and the cells were washed once with 10 ml pre-warmed PBS. Trypsin/EDTA (1x; 2-3 ml) was added to cover the entire surface of the culture flask and to detach the cells from it. As followed, cells were incubated for 5-10 minutes at 37°C and after that 10-20 ml fresh culture medium was added to block the trypsin/EDTA. Finally, the cells were collected and centrifuged at 300 x g for 5 minutes, the supernatant was discarded and the cells were resuspended in fresh culture medium in a ratio of 1:5 up to 1:20 depending on the growth progress and the cell line.

3.3.3 Passaging of suspension cells

Suspension cells were collected and centrifuged at 300 x g for 5-10 minutes, the supernatant was discarded and the cells were resuspended in fresh culture medium in a ratio of 1:5 up to 1:20 depending on the growth progress. In case of peripheral blood lymphocytes (PBLs), IL-2 supplementation was applied in an appropriate concentration (100-250 U/ml) depending on the growth progress.

3.3.4 Freezing and thawing of cells

For long-term storage, the cells (1-5 x 10^7) were centrifuged at 300 x g for 5-10 minutes. The supernatant was discarded and the cells were resuspended in 900 µl fresh medium or 900 µl FCS (depending on the cell line and primary cell type) and pipetted into a cryovial. Cells were mixed carefully with 100 µl of DMSO and the cryovials were placed in a "Mr. Frosty™ Freezing Container" (ThermoFisher Scientific) to achieve a rate of cooling close to minus 1°C/minute. The Mr. Frosty™ Freezing Container was then put into a -80°C freezer and the cryovial was replaced and stored in a -150°C freezer on the following day.

For the thawing of cells, cryotubes were taken out of the -150°C freezer. The frozen cells were carefully mixed with pre-warmed culture medium and immediately transferred into a 30 ml tube and washed twice by centrifugation at 300 x g for 10 minutes to remove the cell-toxic DMSO. In the end, the cell pellet was resuspended in fresh culture medium and stored in fully humidified 5% or 10% (v/v) CO_2 incubator at 37°C.

3.3.5 Determination of cell counts

The cell count and cell viability were determined with the help of the Vi-Cell^{TM}XR Cell Viability Analyzer (Beckman Coulter). For the measurement, the cell suspensions were diluted with medium or PBS 1:2 up to 1:10 (total volume of 1 ml). Alternatively, cells were diluted (1:1) with trypan blue to identify and exclude dead cells. Out of said mixture, 10 µl cell suspension was applied to a "Neubauer" counting chamber (celeromics). The average cell count in four squares was calculated. The number of cells (n) in one square equals to n x 10^4/ml divided by the dilution factor.

3.3.6 Isolation of human peripheral blood mononuclear cells (PBMCs)

Human PBMCs were isolated from fresh blood and buffy coats, respectively, which were obtained from healthy donors *via* the *Transfusionsmedizin der Uniklinik Köln.* The isolation was accomplished through density gradient centrifugation with the aid of the Lymphoprep^{TM} solution (Axis-Shield Poc AS). The separation procedure is based on a polysaccharide solution with a density of 1.077 g/ml. Since mononuclear cells have a lower density than erythrocytes and granulocytes, they do not sediment during centrifugation. In the process first, blood samples were diluted with an equal volume of PBS. Then, 25 ml of the diluted blood was carefully layered on the top of 15 ml Lymphoprep^{TM} into a 50 ml reaction tube. The reaction tubes were centrifuged at 800 x g without brakes at room temperature for 25 minutes. After centrifugation, the accumulated lymphocytes were carefully collected into a new reaction tube with a pipet. The collected PBMCs were washed with PBS three times (10 minutes at 250 x g) to remove the Lymphoprep^{TM} solution and potential serum residue. Afterwards, the cells were resuspended in RPMI1640 medium supplemented with 10% FCS (v/v), 10mM HEPES, 100 IU/mL penicillin/streptomycin, 500 IU/ml human IL-2, 50 ng/ml OKT3 (anti-CD3 mAb), and with or without 50 ng/ml 15E8 (anti-CD28 mAb) depending on the planned experiment. In the application to proliferation assay the PBMC were activated without 15E8. In the first 24 hours the culture flask was laid down flat in the incubator (5% v/v CO_2) in order to separate semi-adherent monocytes from the non-adherent lymphocytes. After two days, the lymphocytes were collected and centrifuged at 300 x g for 10 minutes.

Then the pellet was resuspended in fresh RPMI1640 medium supplemented with 10% (v/v) FCS, 10mM HEPES, 100 IU/mL penicillin/streptomycin and 200-250 IU/ml human IL-2 depending on the growth progress of the cells.

3.3.7 Transfection of HEK 293T cells

Transfection of HEK 293T cells was carried out with the aid of the polymer-based transfection reagent PEIpro® (Polyplus transfection) according to the manufacturer's instructions. One day before transfection, the HEK 293T cells were cultured in DME medium supplemented with 10% (v/v) heat inactivated (HI) FCS in a 10 cm petri dish. On the day of transfection, the confluency of the cells was ought to be about 70%. Construct plasmid DNA was mixed with helper plasmid DNA in DME medium without supplementation. PEIpro transfection reagent was diluted separately in DME medium without supplementation. Both solutions were vortexed thoroughly before being mixed together. Then the mixture was vortexed again and incubated for 15 minutes at room temperature. During this time, the supernatant of the HEK 293T cells was changed to X-vivo 15 medium supplemented with 5% (v/v) HI-FCS. In the end, the DNA-transfection reagent mixture was added to the cells dropwise, and the cells were incubated in 5% (v/v) CO_2 incubator at 37°C for 16-20 hours (Golumba-Nagy et al., 2017).

The following transfection scheme was used:

Culture vessel	Number of HEK 293T cells	Total volume of Medium	Amount of DNA added*	Volume of Medium for DNA	Volume of PEIpro® reagent	Volume of Medium for PEIpro	Volume of mixture added to the cells
10 cm petri dish	$3x10^6$	10 ml	20 µg	250 µl	20 µl	250 µl	500 µl

*whole DNA amount consists of: 6 µg DNA of a helper plasmid encoding the γ-retroviral gag/pol (e.g., pHIT), 6 µg DNA of the helper plasmid encoding the GALV envelope protein and 8 µg plasmid DNA encoding the CAR.

3.3.8 Retroviral transduction of human T cells

Virus supernatant was produced by retroviral transfection, which was collected 16-20 hours after transfection. Fresh medium (10 ml X-vivo 15 medium with 5% (v/v) HI-FCS) was added to the transfected HEK 293T cells for a second harvest the day after. Half of the amount of the supernatant (5 ml) was used to coat two wells of a 6 well plate or a T25 cell culture flask, which had been coated with poly-D-lysine (PDL, 10 µg/ml in 2 ml sterile distilled H_2O) 24

hours in advance. The cell culture plate or flask with the virus supernatant was centrifuged at 2600 x g for 30 minutes at 32°C. Two days after PBLs isolation 5 x 10^6 cells were resuspended in the remaining 5 ml virus supernatant, which had been supplemented with 150 U/ml IL-2. After the first centrifugation step, the virus supernatant was removed and exchanged for the T cell-virus supernatant suspension and centrifuged at 1600 x g and 32°C for 90 minutes. At the end, the cells were incubated for 24 hours at 37°C in a 5% (v/v) CO_2 incubator. On the next day, the whole procedure was repeated once more with the newly produced and harvested virus supernatant, however, without the addition of IL-2 (Golumba-Nagy et al., 2017).

3.3.9 Fluorescence-activated cell sorting (FACS)

For the purpose of determining the presence of cell surface receptors such as a CAR receptor, other cell surface molecules, and intracellular components, the cells were stained with a fluorochrome-conjugated antibody for Flow Cytometry analysis. Transfected or transduced cells were prepared as a single-cell suspension to the staining procedure.

3.3.9.1 Direct Immunofluorescence

Transfected or transduced cells an amount of 5 x 10^5 cells were used for FACS staining. The labeling was prepared in a 5 ml BD Falcon™ tube (BD Biosciences). The single cell suspension was washed with 2 ml PBS twice, the supernatant was discarded and the cell pellet was resuspended in 100 µl PBS. For staining the appropriate fluorochrome-conjugated antibody was used according to the manufacturer's instructions. In general, the cells were incubated with the fluorochrome-conjugated antibody for 30 minutes at 4°C or on ice in the dark. In the end, the cells were washed with 2 ml PBS twice. Then the cell pellet was resuspended in 200-400 µl PBS. CAR detection was carried out by the PE-conjugated Goat F(ab´)2 anti-human IgG antibody, while for T cell labeling the FITC-conjugated anti-human CD3 antibody was used. Data were recorded using the BD FACS Canto II cytofluorometer equipped with the FACS Diva software (Becton Dickinson) and the data were analyzed using the FlowJo v10 software (FlowJo LLC).

3.3.9.2 Intracellular staining

Detection of pLCK:

For the detection of pLCK, 5 x 10^5 CAR-modified T cells were stimulated with the cognate immobilized BW2064/36 antibody through the anti-CEA CAR for 5 minutes before the cells were harvested into 5 ml BD Falcon™ tubes. For the fixation and permeabilization of the cells, the "Intrasure Kit" was used. Reagent A was used for fixation (5 minutes in the dark at room temperature), while Reagent B was used together with the PE-conjugated anti-phosphoLCK antibody (5 µl) for the permeabilization and staining of the cells. The cells were incubated in the dark at room temperature for 30 minutes, and then washed with 2 ml PBS at 800 x g for 5 minutes. The cell pellet was resuspended in 200 µl PBS for the FACS analysis.

Detection of Granzyme B:

The CAR modified T cells (5 x 10^4 CAR^+ T cells pro well) were stimulated on BW2064/36 coated plates over 2 days in the presence or absence of TGF-β. After 2 days, the cells were harvested into 5 ml BD Falcon™ tubes and then fixated and permeabilized using the "Cytofix/Cytoperm" Kit. Firstly, the cells were washed twice with 1 ml PBS supplemented with 1% (v/v) HI-FCS (staining buffer), then resuspended in 250 µl Fix/Perm solution, vortexed and incubated at 4°C for 20 minutes. Afterwards, cells were washed twice with 1 ml 1X Perm/Wash buffer, resuspended in 50 µl Perm/Wash buffer with 20 µl FITC-conjugated Granzyme B antibodies, and incubated at 4°C in the dark for 30 minutes. Before FACS analysis, the cells were washed twice with 1 ml 1X Perm/Wash buffer and resuspended 100-200 µl staining buffer.

Detection of pSTAT5:

For the detection of pSTAT5, 5 x 10^5 CAR-modified T cells were stimulated with the cognate immobilized BW2064/36 antibody through the CAR for 30 minutes or 16 hours. After the activation the cells were harvested into 5 ml BD Falcon™ tubes, fixated at room temperature in the dark for 30 minutes using "IC Fixation Buffer". The permeabilization was performed with 1 ml ice-cold methanol (99,9%) and on ice for 30 minutes. Afterwards, the cells were washed twice with 1 ml staining buffer and stained with the PE-conjugated anti-phosphoSTAT5 antibody (5 µl) in 100 µl staining buffer. Before FACS analysis, the cells were washed twice with 1 ml staining buffer and resuspended in 100-200 µl staining buffer.

All data were recorded by using the BD FACS Canto II cytofluorometer equipped with the FACS Diva software (Becton Dickinson) and the data were analyzed using the FlowJo v10 software (FlowJo LLC).

3.3.10 5-carboxylfluorescein diacetate succinimidyl ester (CFSE)-labeling of cells

In order to track CAR-engineered T cell proliferation, the cells were stained with CFSE. First, 5-10 x 10^6 cells were washed twice with 10 ml PBS to remove any serum or medium and were resuspended in 1 ml PBS. The staining was performed with 0.5 µM CFSE at room temperature in the dark for 10 minutes. The labeling was stopped by the addition of 5 ml cold RPMI1640 medium supplemented with 10% (v/v) FCS and the cells were then incubated on ice for 5 minutes. The cells were washed twice with 10 ml PBS at 300 x g for 5 minutes and the cell count was determined in order to prepare to the proliferation assay.

3.3.11 Staining of apoptotic and living cells

In order to detect the apoptotic and non-living cells in the CAR-modified T cell population, the cells were stained with AnnexinV to label the apoptotic cells and with 7-AAD to be able to exclude dead cells. The modified T cells (2.5 x 10^4 CAR^+ T cells) were activated for 2 days on plates coated with BW2064/36 antibody for CAR stimulation. After the incubation time the cells were harvested into 5 ml BD Falcon™ tubes and washed with AnnexinV buffer (1X). The staining was performed with an APC-conjugated AnnexinV antibody together with a PE-conjugated anti-IgG antibody for CAR labeling in 100 µl 1X AnnexinV buffer and the cells were incubated on ice in the dark for 30 minutes. Finally, the cells were washed twice with 1 ml 1X AnnexinV buffer and resuspended in 200 µl AnnexinV buffer. Before flow cytometry (~10 minutes earlier) 7-AAD (5 µl) was added to the cells.

3.4 Assays

3.4.1 Cultivation of CAR-engineered T cells on solid-phase-bound antibodies

Sterile cell culture 96 well round-bottom microtiter plates (TPP® Techno Plastic Products AG) were coated with antibodies (50 µl/well in PBS) t at 4°C overnigh or at 37°C for at least 2 hours before assay start. CAR-modified T cells were cultivated without any stimulation for 24-48 hours after transduction. CAR expression on the T cell surface was verified by flow cytometry and the same number of CAR-expressing T cells was adjusted for the assay. The cells were washed with cell culture medium and 2.5 x 10^4 cells/well were cultivated/activated with solid-phase bound antibodies at 37°C in 5% (v/v) CO_2 in a total volume of 200 µl for 48-96 hours in the presence or absence of TGF-β or other cytokines. After stimulation, 100-150 µl of culture supernatant were carefully harvested and the cells and the supernatant were used for further experiments.

3.4.2 Co-cultivation of CAR-engineered T cells with target cells

Before the co-cultivation experiments, the CAR-engineered T cells were cultivated without stimulation for 24-48 hours. The number of CAR-expressing T cells was verified by flow cytometry and the appropriate number of CAR T cells was co-cultivated with antigen positive target cells in different effector-to-target cell ratios in 96 well round-bottom microtiter plates (TPP® Techno Plastic Products AG) with or without the presence of TGF-β or other cytokines at 37°C and 5% (v/v) CO_2 in a total volume of 200 µl for 48 hours. An aliquot of 100 µl of culture supernatant was carefully harvested for further experiments and the viability of target cells was determined by an XTT-based cell viability assay.

3.4.3 Proliferation assay

Amplification of CAR modified T cells was followed through the CFSE labeled cell dividing. CFSE is a fluorescent dye, which can be incorporated within cells for a long term. During cell division only half of the dye is transferred to each daughter cell. Thereby, a CFSE dilution is developed over the time in order to track proliferation. For this assay, CAR-engineered T cells were cultivated without stimulation for 24-48 hours at 37°C after the transduction. Then, they were stained with CFSE as described in section 3.3.10. The CAR-modified and CFSE labeled cells (2.5 x 10^4 cells/well) were transferred to a pre-coted 96 well round-bottom plates and were incubated with or without TGF-β or other cytokines in a total volume of 200 µl. The cells were stimulated with an appropriate antibody for CAR stimulation at 37°C for 48-96 hours. Tests were prepared in triplicates. After incubation, 100-150 µl of cell culture

supernatant were collected for further experiments and the CFSE-labeled cells were stained on the plate for the CAR receptor with the PE-conjugated Goat F(ab´)2 anti-human IgG antibody then analyzed using the BD FACS Canto II cytofluorometer equipped with the FACS Diva software (Becton Dickinson) and the data were analyzed using the FlowJo v10 software (FlowJo LLC).

3.4.4 Enzyme-linked Immunosorbent Assay (ELISA)

MaxiSorp™ microtitre plates (Nunc GmbH) were coated with capture antibodies (50 µl/well in coating buffer) at room temperature for 2 hours. The plates were blocked with 1% (w/v) BSA dissolved in PBS (200 µl/well) for 2 hours at room temperature to prevent unspecific binding, before being washed three times with PBS-T (200 µl/well). Afterwards, the plates were incubated with supernatants (50 µl/well) and with standard samples (100 µl/well) overnight at 4°C on a shaker and then washed four times with PBS-T on the following day. Incubation with biotinylated detection antibodies for 1 hour followed in 1% (w/v) BSA in PBS-T (50 µl/well) at room temperature and then the plates were washed four times with PBS-T. Bound detection antibodies were conjugated to Streptavidin-POD for 30 minutes (1:10,000 in 1% (w/v) BSA in PBS-T, 50 µl/well). As next that followed the last washing step with PBS-T (five times) thereafter came the visualization of bound proteins by incubation with ABTS (1 mg/ml in ABTS buffer, 100 µl/well) and detection in an ELISA plate reader (Multiskan Go Microplate Spectrophotometer, SkanIt Software, ThermoFisher Scientific) at 405 nm and with 490 nm set as reference. In the end, the concentration of bound proteins was calculated according to the absorption curve of a standard sequence with known concentration. The tests were prepared in triplicates.

The following ELISA setups were used:

	IFN-γ ELISA	**IL-2 ELISA**	**IL-7 ELISA**	**IL-15 ELISA**
Capture Ab	1 µg/ml mouse anti-human IFN-	1 µg/ml mouse anti-human IL-2	1.6 µg/ml goat anti-mouse IL-7	2 µg/ml mouse anti-human IL-
Detection Ab	0.5 µg/ml biotin mouse anti-human IFN-γ	1 µg/ml biotin mouse anti-human IL-2	0.4 µg/ml biotin goat anti-mouse IL-7	0.5 µg/ml biotin mouse anti-human IL-15
Standard	1:10,000 of stock solution Imukin® equals 20 ng/ml IFN-γ in 1% (w/v) BSA in PBS-T	1:900 of stock solution Proleukin® equals 2000 U/ml IL-2 in 1% (w/v) BSA in PBS-T	1:60 of stock solution DuoSet® ELSIA equals 2 µg/ml IL-7 in 1% (w/v) BSA in PBS-T	1:95 of stock solution DuoSet® ELSIA equals 1 µg/ml IL-15 in 1% (w/v) BSA in PBS-T

3.4.5 XTT-based cell viability assay

CAR-engineered T cell-mediated specific cytotoxicity was monitored *via* an XTT-based cell viability assay. The assay was described earlier (Jost et al., 1992). The tetrazolium salt XTT is reduced to formazan by metabolically active cells. The dye intensity of formazan is measurable with a spectrophotometer and proportional to the number of metabolically active cells. For this assay, CAR-modified T cells were co-cultivated in increasing numbers with antigen positive target cells in a total volume of 200 µl in 96-well round-bottom plates (TPP® Techno Plastic Products AG) in the presence or absence of TGF-β or other cytokines. After 48 hours of co-cultivation at 37°C, 100 µl of culture supernatant were carefully collected from each well for further experiments. The visualization of the target cell viability was performed by adding 100 µl XTT reagent solution and 1 µl electron coupling reagent solution (PMS) to each well. Then, the plates were incubated again at 37°C and the absorption was detected using an ELISA plate reader (Multiskan Go Microplate Spectrophotometer, SkanIt Software, ThermoFisher Scientific) at 450 nm and with 650 nm set as reference every half an hour until no more change was visible. Maximal reduction of XTT was determined as the mean of twelve wells containing target cells only, and the background as the mean of twelve wells containing only medium. Non-specific formation of formazan due to the presence of T cells was determined from three wells containing only T cells in the same number as in the corresponding co-culture wells.

Viability of target cells was calculated as follows:

$$\text{viability [\%]} = [(OD_{E+T} - OD_{E}) / (OD_{T} - OD_{Med})] \times 100$$

The cytotoxicity was calculated as follows:

$$\text{cytotoxicity [\%]} = 100 - \text{viability [\%]}$$

(E = effector cell; T = target cell; Med = medium; OD = optical density)

3.4.6 Serial killing

CAR-modified T cell-mediated specific cytotoxicity in a serial killing setup was performed in the same manner as described in section 3.4.5. After 48 hours co-cultivation, cells from the parallel assay were centrifuged while still on the plate. Then, the supernatant was removed and fresh target cells were added to each well in the same number as at the beginning of the experiments for a second 48 hour incubation in a total volume of 200 µl. The same procedure was repeated until day 6. The cytotoxicity of each test was calculated as described in section 3.4.5.

3.5 Mouse experiments

$Rag2^{-/-}\gamma c^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All animal experiments were performed according to the Animal Experiments Committee regulations and approved by the *Landesamt für Natur, Umwelt und Verbraucherschutz*, Recklinghausen, Germany (K17/35-05).

3.5.1 Assay for tumor growth

CEA^+ TGF-β secreting C15A3 tumor cells were subcutaneously injected into $Rag2^{-/-}\gamma c^{-/-}$ mice (1×10^6 tumor cells per mouse; 4 mice per group). At day 16, when the tumor size had reached about 200 mm^3, CAR-engineered or non-modified human T cells were injected into the mice intravenously (1.5×10^6 CAR T cells per mouse). The mice were divided for 4 groups, the first group did not get any T cells (w/o T cell), the second group were infused with T cells without CAR (w/o CAR), while the third and fourth groups were treated with CAR-modified T cells (3th group: CD28ΔLCK-ζ, 4th group: CD28ΔLCK-ζ-IL7-IL7/IL2R). Tumor growth was measured on weakly basis after CAR T cell injection. After 2 weeks, the mice were sacrificed and the tumors were removed for immune-histological analysis.

3.5.2 Immune-histological analysis

The whole procedure was performed in a humid environment to protect the tumors from drying out and at room temperature and on shaker if it not stated otherwise.

In order to prepare tumor section from the tumor tissue samples, the samples were first embedded in TissueTek to freeze them. Afterwards, the tumor sections were prepared with the help of Kryotom (Leica) and transferred to microscope slide (engelbrecht) to perform immune-histological staining. After the tumor sections had dried out on the slide, were fixated with ice-cold acetone for 5 minutes at room temperature and were then washed twice with PBS for 5 minutes. As next step, „Fc Block" and „Background Buster" (Innovex biosciences) treatments followed to avoid unspecific binding, which took 30 minutes each. For CAR^+ T cell detection AlexaFluor555-conjugated Goat F(ab´)2 anti-human IgG antibodies (dilution 1:250) were used overnight at 4°C. The next day, the slides were washed with PBS three times for 5 minutes and then treated with Background Buster for 30 minutes. In the following 2 hours, the tumor cells were stained with AlexaFluor 488-conjugated anti-human CD66/a/c/e (anti-CEA antibody; clone: ASL-32; dilution 1:50). The slides were washed with PBS three times for 5 minutes and the cell nuclei were labeled with "Reddot2 nuclear dye" (dilution 1:200) for 30 minutes. The prepared slides were covered with a mounting medium and a

cover glass. For the TGF-β staining the anti-TGF-β antibody (mouse monoclonal IgG_1, clone 3C11; dilution 1:50) was applied overnight at 4°C. Then on the next day, the AlexaFluor555-conjugated goat anti-mouse IgG (H+L) antibody (dilution 1:200) was used. In this case, the cell nuclei were labeled with DAPI in the mounting medium. For imaging the Olympus IX81 microscope and the Olympus Fluoview (FV1000) software (Olympus, Center Valley, PA, USA) were applied.

The results are in part demonstrated in the publication of Golumba-Nagy et al., 2018

Golumba-Nagy V, Kuehle J, Hombach AA, Abken H (2018) CD28-ζ CAR T Cells Resist TGF-β Repression through IL-2 Signaling, Which Can Be Mimicked by an Engineered IL-7 Autocrine Loop. *Mol Ther*. 5;26(9):2218-2230

4 RESULTS

4.1 Identifying the mechanism of TGF-β-mediated resistance of CD28-ζ CAR T cells

4.1.1 CD3ζ CAR-modified T cells are sensitive to the suppressive effect of TGF-β

In this study we aimed to investigate the impact of CAR signaling on TGF-β-mediated repression of T cell function with respect to T cell amplification, cytokine release, and specific cytotoxicity.

4.1.1.1 T cell amplification by CD3ζ CAR T cells is repressed by TGF-β

Human T cells were modified with a CEA-specific CAR, including a CD3ζ (ζ) endodomain, and analysed with respect to the TGF-β-mediated suppression of CAR T cell amplification. In the cause of this, we labeled the CAR T cells with CFSE, stimulated the cells through immobilized antigen in the presence of TGF-β, and thereby determined the T cell proliferation. The successful CFSE labeling of the remaining population was verified after one day using flow cytometry (Figure 4).

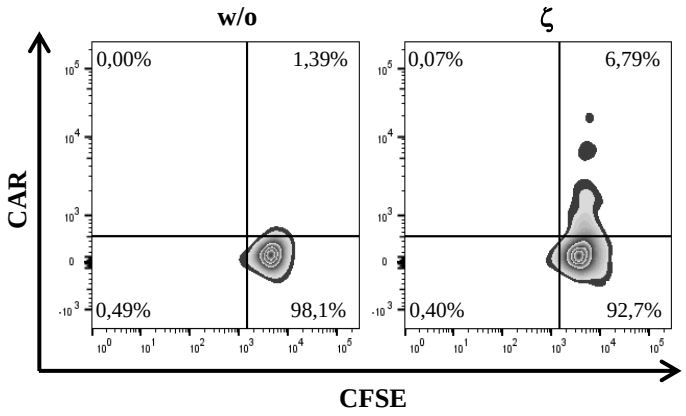


Figure 4 CFSE labeling of CAR modified T cells

Human T cells were modified by the CEA specific CD3ζ CAR (ζ) and labeled with CFSE to track the proliferation of the cells. One day after the CFSE staining, the remaining cells without stimulation were analyzed by flow cytometry to verify the successful labeling. CAR receptors were stained with the PE-conjugated anti-IgG antibody, which binds to the CAR in the extracellular IgG1 spacer region. The percentage represents the cell number in each quadrat, w/o means T cells without CAR.

T cells were activated for 4 days by CAR engagement through the immobilized BW2064/36 antibody, which is an anti-idiotypic antibody for the anti-CEA scFv of the CAR. The amplification of the CAR T cells was determined by analyzing the CFSE dilution over the time *via* flow cytometry. We saw, that ζ CAR T cell proliferation was inhibited in the presence of TGF-β (Figure 5).

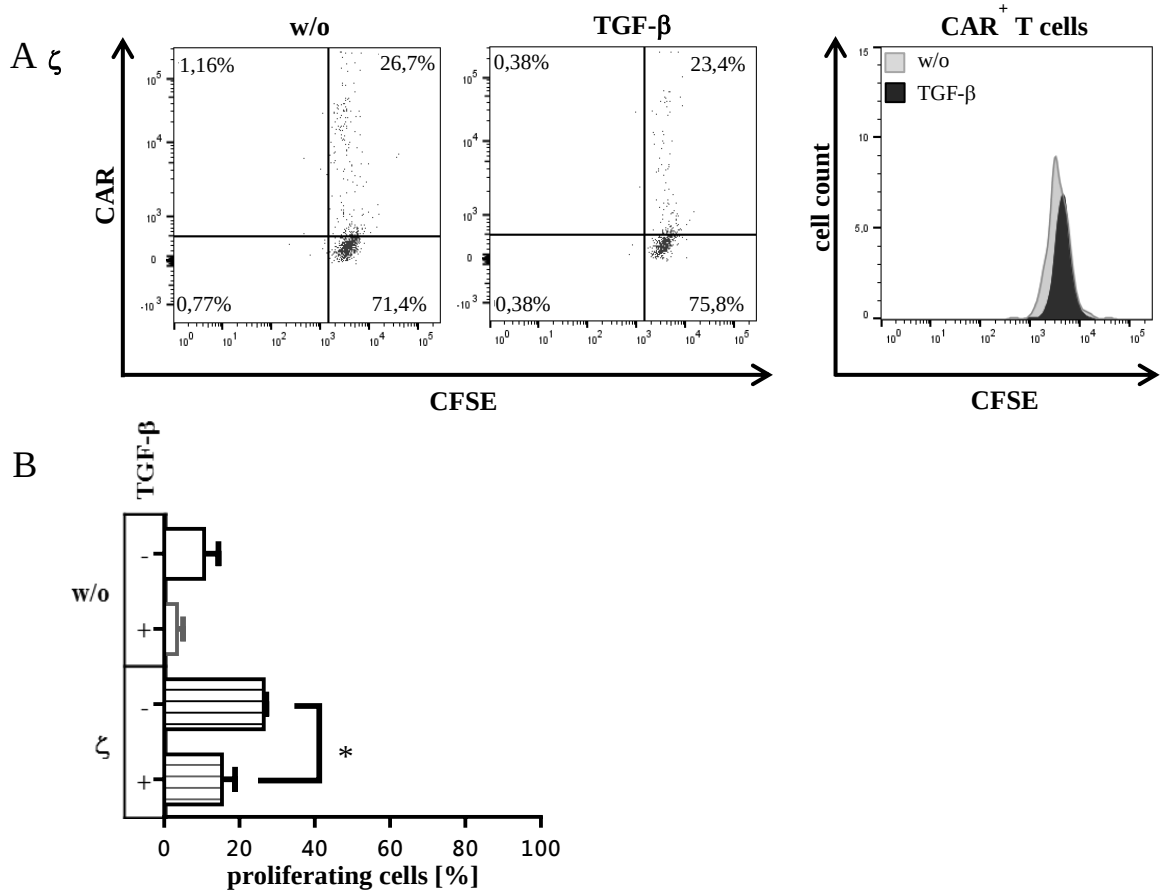


Figure 5 T cell amplification by CD3ζ CAR T cells is repressed by TGF-β

(A) Human T cells were modified by the ζ CAR and labeled with CFSE. CAR engineered T cells (2.5 x 10^4 T cells per well) were incubated on 96-well plates coated with the anti-idiotypic mAb BW2064/36 (1.5 µg/ml) in the presence or absence of TGF-β (10 ng/ml) for 4 days. CFSE dilution was recorded by flow cytometry to identify CAR T cell amplification, CAR receptor were stained with the PE-conjugated anti-IgG antibody which binds to the CAR in the extracellular IgG1 spacer region. The percentage represents the cell number in each quadrat. The histogram shows the CAR^+, CFSE labeled cells, w/o means without cytokine. **(B)** The data represented on the diagram are the proliferated CAR^+ T cells on the 4^{th} day, comparing the incubation with or without TGF-β. The assay were repeated three times, data are demonstrated by a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means T cells without CAR. Statistical analyses were executed using a two-tailed Student´s t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The diagram B was adapted from the publication of Golumba-Nagy et al., 2018.

4.1.1.2 TGF-β decreased CAR T cell IFN-γ and IL-2 secretion

To analyze cytokine production the modified T cells with ζ CAR were activated through the immobilized BW2064/36 antibody for 2 days and the accumulated IFN-γ and IL-2

concentrations were measured in the cell culture supernatant by ELISA. The release of both cytokines was repressed in the presence of TGF-β, however, the ζ CAR T cells did not produce a significant amount of IFN-γ and IL-2 (Figure 6).

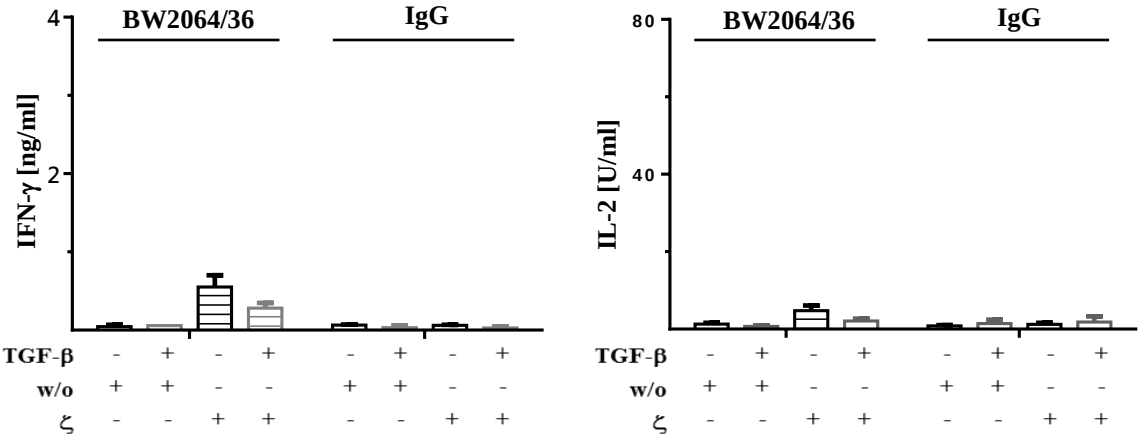


Figure 6 TGF-β decreased CAR T cell IFN-γ and IL-2 secretion

CAR T cells with a ζ signaling domain were incubated for 2 days on plates (2.5 x 10^4 CAR T cells pro well) coated with the anti-idiotypic mAb BW2064/36 or mouse IgG1 as isotype control (1.5 µg/ml each). The modified T cells were incubated in the presence or absence of TGF-β (10 ng/ml). Secreted IFN-γ and IL-2 concentration were measured in the cell culture supernatants with ELISA. The assays were repeated three times; the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means T cells without CAR, served as control. The diagrams were adapted from the publication of Golumba-Nagy et al., 2018.

4.1.1.3 TGF-β altered the specific cytotoxicity of ζ CAR T cells

To record the specific cytotoxicity of CAR T cells in the presence of TGF-β, T cells were engineered with the CAR and co-incubated with CEA^+ LS174T tumor cells in the presence or absence of TGF-β for 2 days. The cytotoxic activity was determined by the XTT-based viability assay. The CAR-modified T cell-mediated tumor cell killing was altered in the presence of TGF-β (Figure 7).

Taken together, the most profound effect of TGF-β on CAR T cell effector functions was on the CAR T cell amplification. We therefore used T cell proliferation as a marker to record the activity of various costimuli against TGF-β repression.

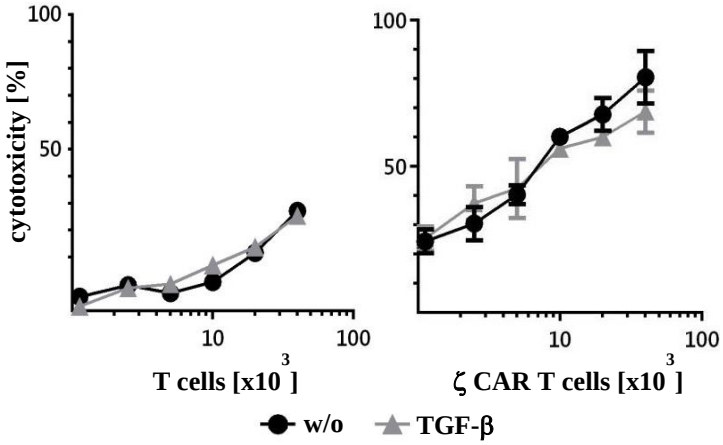


Figure 7 TGF-β altered the specific cytotoxicity of ζ CAR T cells

T cells with or without CAR (0.125 x 10^4 – 4 x 10^4 CAR T cells per well) were incubated in the presence or absence of TGF-β (10 ng/ml) with CEA^+ LS174T cells (2 x 10^4 tumor cells per well). The specific cytotoxicity was determined by the XTT-based viability assay after 2 days. The assays were repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means without cytokine. These diagrams were taken from the publication of Golumba-Nagy et al., 2018.

4.1.2 Adding a costimulatory domain can compensate the suppressive effect of TGF-β

Costimulation by CD28 or 4-1BB makes T cell functions more effective; therefore, we investigated T cell functions initiated by the second generation CAR in the presence of TGF-β.

4.1.2.1 CD28 overcomes the inhibitory effect of TGF-β on T cell proliferation, while 4-1BB does not

Human T cells were engineered with either a CAR with a 4-1BB-ζ or a CAR with a CD28-ζ endodomain, and in order to record T cell proliferation, the cells were stained with CFSE and stimulated through the CAR using immobilized antigen. One day after the CFSE staining, the successful labeling was verified by flow cytometry (Figure 8).

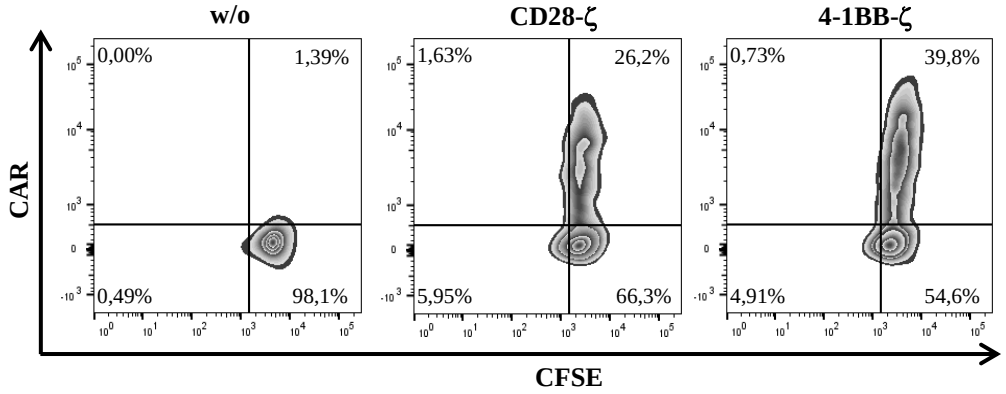


Figure 8 CFSE labeling of CAR modified T cells

Human T cells were modified by CAR with different costimulatory domains (CD28 or 4-1BB), and labeled with CFSE to track the proliferation of the cells. One day after the CFSE staining, the remaining cells without stimulation were analyzed by flow cytometry to verify the successful labeling. CAR receptors were stained with the PE-conjugated anti-IgG antibody which binds to the CAR in the extracellular IgG1 spacer region. The percentage represents the cell number in each quadrat, w/o means T cells without CAR.

After 4 days of activation the proliferative activity was analyzed by determination of the CFSE dilution. While the proliferation of CD28-ζ CAR T cells was not inhibited in the presence of TGF-β, it was suppressed in the absence of CD28 (Figure 5) as well as in the presence of the 4-1BB costimulatory domain (Figure 9). Consequently, the CD28 domain, but not 4-1BB, has a protective role in T cell proliferation in the presence of suppressive TGF-β.

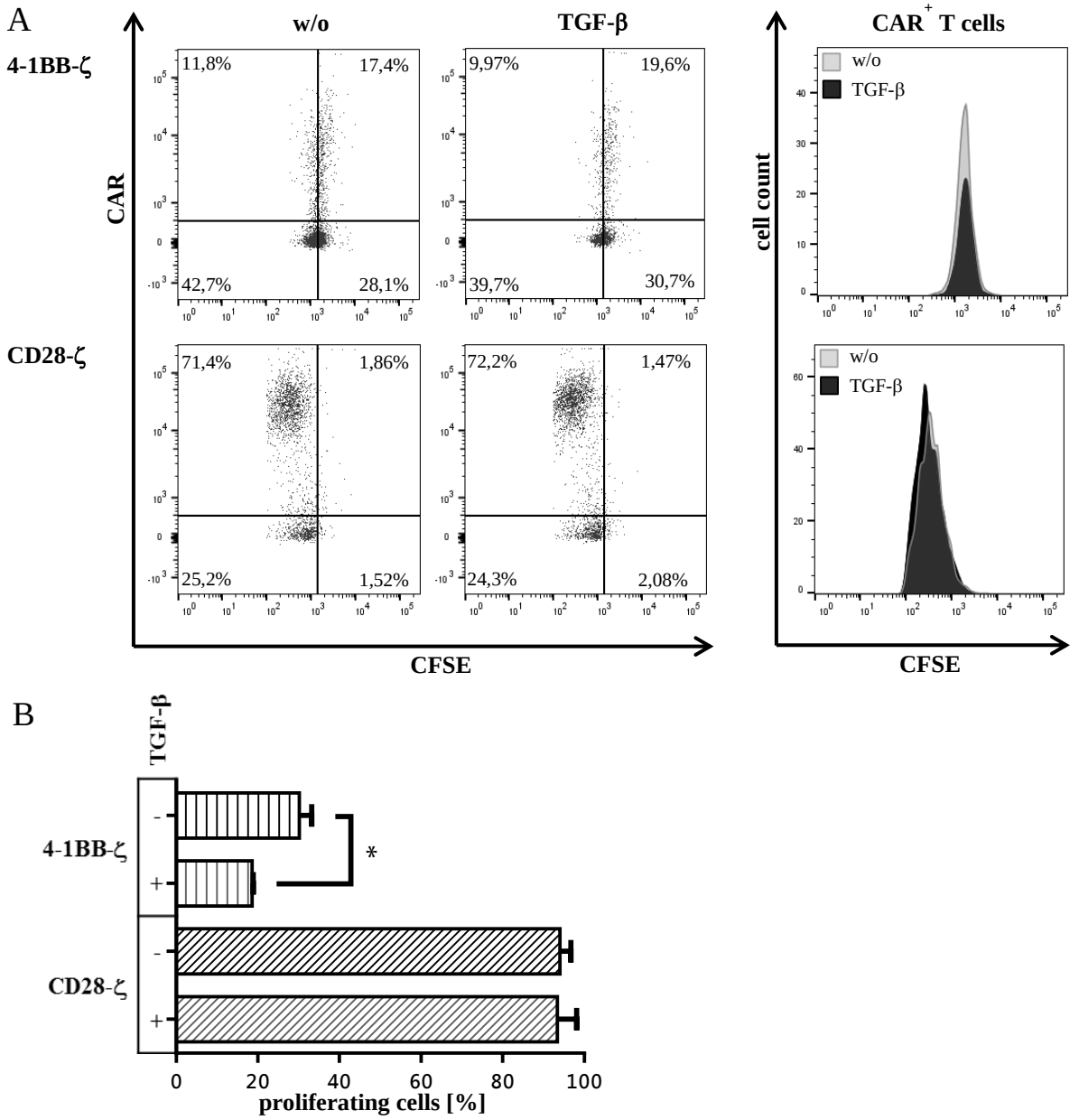


Figure 9 CD28 overcomes the inhibitory effect of TGF-β on T cell proliferation, while 4-1BB does not

(A) Human T cells were modified by the CD28-ζ or 4-1BB-ζ CAR and labeled with CFSE. CAR engineered T cells (2.5 x 10^4 T cells per well) were incubated on 96-well plates coated with the anti-idiotypic mAb BW2064/36 (1.5 µg/ml) in the presence or absence of TGF-β (10 ng/ml) for 4 days. CFSE dilution was recorded by flow cytometry to identify CAR T cell amplification, CAR receptor were stained with the PE-conjugated anti-IgG antibody which binds to the CAR in the extracellular IgG1 spacer region. The percentage represents the cell number in each quadrat. The histogram shows the CAR^+, CFSE labeled cells, w/o means without cytokine. **(B)** The data represented on the diagram are the proliferated CAR^+ T cells on the 4^{th} day, comparing the incubation with or without TGF-β. The assay were repeated three times, data are demonstrated by a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown. Statistical analyses were executed using a two-tailed Student´s t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The diagram B was adapted from the publication of Golumba-Nagy et al., 2018.

4.1.2.2 TGF-β decreased CAR T cell IFN-γ and IL-2 secretion

CAR T cells were activated through antigen engagement for 2 days. In the cause of this, the immobilised antibody BW2064/36 served as the antigen. The concentration of IFN-γ and IL-2 released by CAR-modified T cells was measured by ELISA. Release of both cytokines was inhibited by TGF-β in the case of CD28-ζ and 4-1BB-ζ CAR-modified T cells (Figure 10).

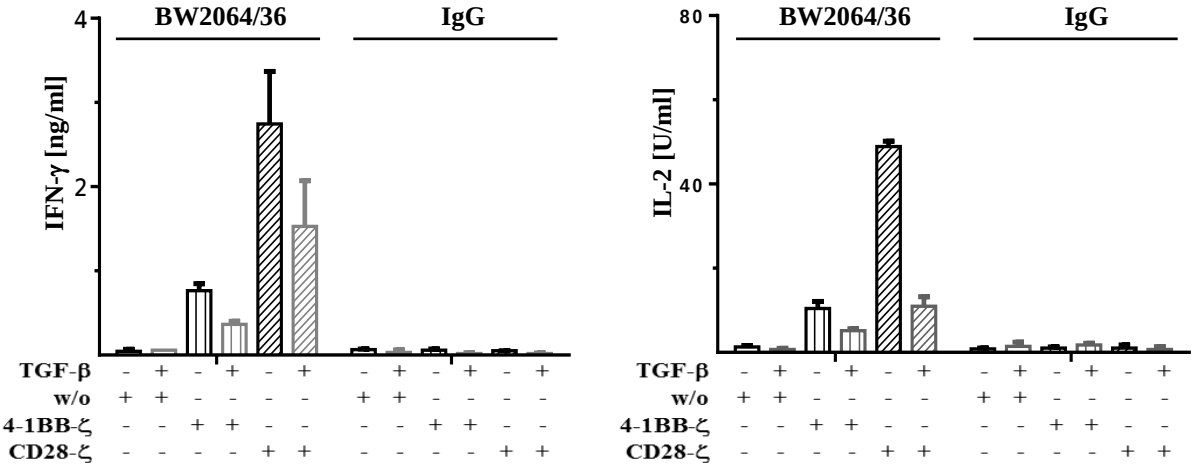


Figure 10 TGF-β decreased CAR T cell IFN-γ and IL-2 secretion

CAR T cells with different costimulatory domain were incubated on plates (2.5 x 10^4 CAR T cells pro well) coated with the anti-idiotypic mAb BW2064/36 or mouse IgG1 as isotype control (1.5 µg/ml each) for 2 days. The modified T cells were incubated in the presence or absence of TGF-β (10 ng/ml). Secreted IFN-γ and IL-2 concentration were measured in the cell culture supernatants with ELISA. The assays were repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means T cells without CAR, served as control. The diagrams were adapted from the publication of Golumba-Nagy et al., 2018.

4.1.2.3 CAR T cell-mediated specific cytotoxicity is not inhibited in the presence of TGF-β

To investigate the antigen specific killing of tumor cells, CAR-modified T cells were co-incubated with the CEA^+ LS174T tumor cells for 2 days in the presence or absence of TGF-β. Cytolytic activity of 4-1BB-ζ CAR engineered T cells was altered by TGF-β, while it had not significant effect on CD28-ζ CAR T cell-mediated tumor cell killing (Figure 11).

In conclusion, TGF-β significantly suppresses T cell proliferation and 4-1BB costimulation is not capable of overcoming TGF-β suppression. However, in the presence of CD28 costimulation, T cells become resistant against TGF-β.

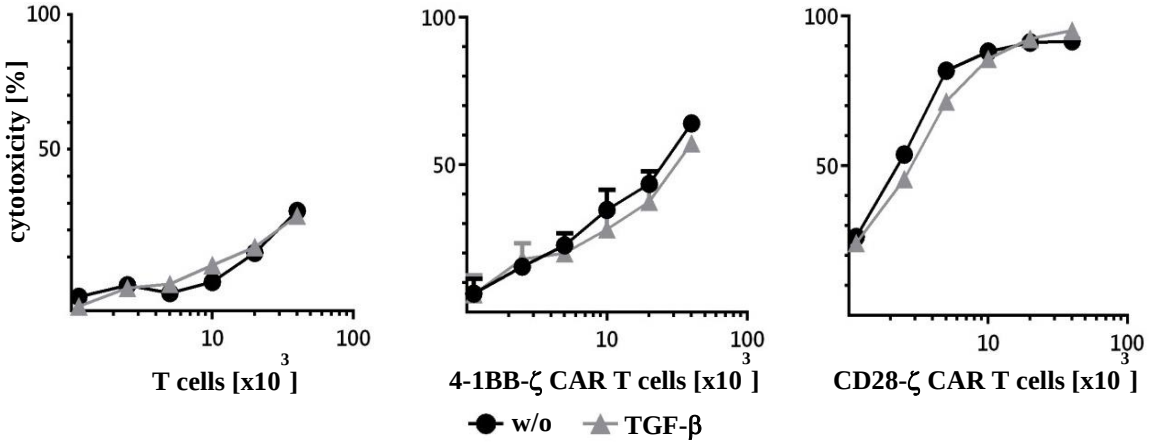


Figure 11 CAR T cell-mediated specific cytotoxicity is not inhibited in the presence of TGF-β

T cells with or without CAR (0.125 x 10^4 – 4 x 10^4 CAR T cells per well) were incubated in the presence or absence of TGF-β (10 ng/ml) with CEA^+ LS174T cells (2 x 10^4 tumor cells per well). The specific cytotoxicity was determined by the XTT-based viability assay after 2 days. The assays were repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means without cytokine. These figures were taken from the publication of Golumba-Nagy et al., 2018.

4.1.3 Mutation in the CD28 domain abolishes some T cells function

To identify the responsible CD28 function responsible for the mediation of the TGF-β resistance in T cell proliferation, we dissected the major signaling moieties of CD28 by deletion of the binding motifs for LCK, PI3K, or both.

4.1.3.1 Amino acid sequence of the wild type CD28 costimulatory domain and mutated variants

To abolish LCK binding we introduced point mutations in the respective CD28 domain by the substitution of the prolins P187 and P190 by alanin (A). The PI3K binding site was eliminated by replacing tyrosin Y170 by phenylalanin (F) (Tai et al., 2005) (Figure 12).

CD28 wt	…NSRRNRLLQSD**Y**MNMTPRRPGLTRKPYQ**P**YA**P**ARDFAAYRP…
CD28ΔLCK	…NSRRNRLLQSD**Y**MNMTPRRPGLTRKPYQ**A**YA**A**ARDFAAYRP…
CD28ΔPI3K	…NSRRNRLLQSD**F**MNMTPRRPGLTRKPYQ**P**YA**P**ARDFAAYRP…
CD28ΔLCKΔPI3K	…NSRRNRLLQSD**F**MNMTPRRPGLTRKPYQ**A**YA**A**ARDFAAYRP…

Figure 12 Amino acid sequence of the wild type CD28 costimulatory domain and mutated variants

Mutation in CD28 domain was performed by substitution of Prolin (P187 and P190) by alanin (A) to destroy the LCK binding as well as tyrosin (Y170) was replaced by phenylalanin (F) to eliminate the PI3K binding side. The figure was taken from the publication of Golumba-Nagy et al., 2018.

4.1.3.2 Deletion of the LCK binding site results in a decreased phosphorylation of LCK

To verify that the mutation at the LCK binding motif of CD28 impaired LCK signaling, we recorded LCK phosphorylation upon activation. T cells were transduced with the wild type CD28 CAR or with the mutated CD28ΔLCK CAR and activated through the CAR by incubation with immobilized antigen for 5 minutes; the phospho-LCK was stained intracellularly and recorded *via* flow cytometry. T cells with the CD28ΔLCK-ζ CAR showed abolished LCK phosphorylation compared to T cells with the CD28-ζ CAR. T cells without CAR were used as control and displayed background phosphorylation compared to CAR bearing T cells (Figure 13).

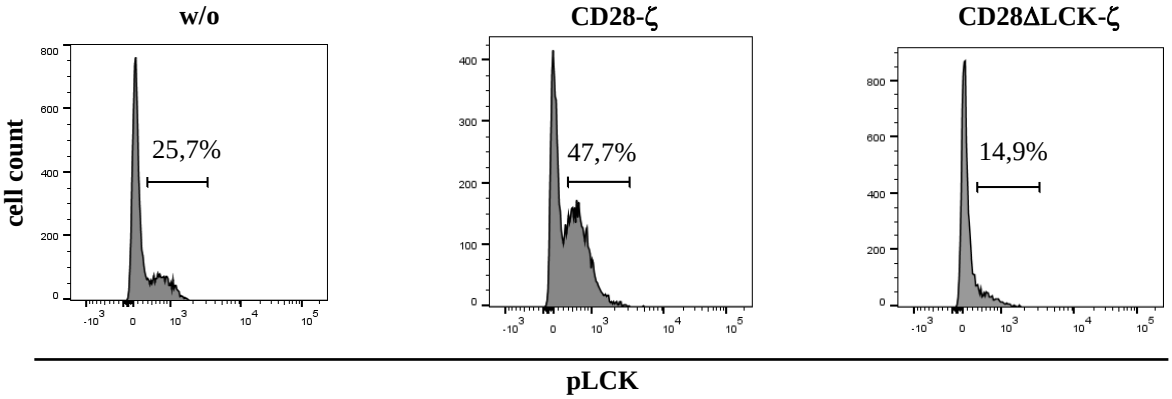


Figure 13 Deletion in LCK binding site results in a decreased phosphorylation of LCK

Upon CAR signaling LCK is less phosphorylated with mutation of the LCK binding side. T cells with the CD28-ζ or the CD28ΔLCK-ζ CAR (1 x 10^6 T cells per well) were incubated on BW2064/36 coated 96-well plates (1.5 µg/ml) for 5 minutes, then collected, fixated, permeabilized and stained with the PE-conjugated anti-phospho-LCK antibody. The cells were analyzed by flow cytometric measurement. On the histogram, the number of pLCK stained positive cells is shown, w/o means T cells without CAR, served as control. The figures were taken from the publication of Golumba-Nagy et al., 2018.

4.1.3.3 Deletion in the LCK binding site selectively abolishes the release of IL-2, but not of IFN-γ

We investigated the IL-2 and IFN-γ secretion of engineered T cells after CAR stimulation. The cells were activated through the CAR on plates coated with the cognate BW2064/36 antigen. After 2 days of incubation, IL-2 and IFN-γ were measured in the culture supernatant. The CD28ΔLCK-ζ CAR-modified T cells were deficient in IL-2 release, while IL-2 secretion was not impaired in the case of CAR T cells harboring the mutation in the PI3K binding domain. On the other hand, CAR induced IFN-γ production was not altered in either case, indicating that the mutations in the CD28 domain did not impact all T cell functions (Figure 14).

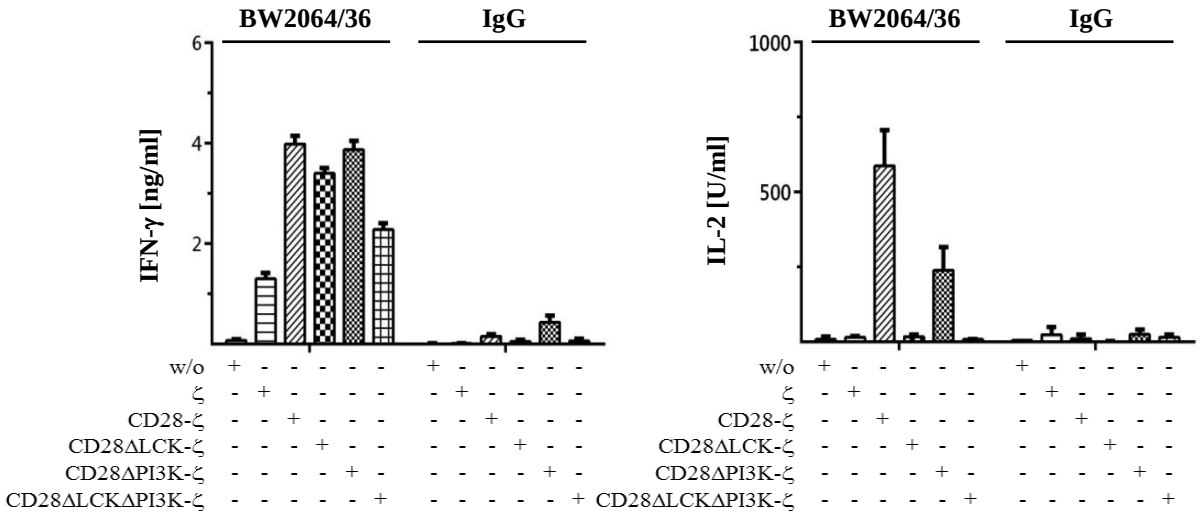


Figure 14 Deletion in the LCK binding site selectively abolishes the release of IL-2, but not of IFN-γ

T cells with or without CAR were incubated on 96 well plates (2.5 x 10^4 CAR T cells pro well) coated with anti-idiotypic mAb BW2064/36 or mouse IgG1 (1.5 µg/ml each) as an isotype control for 2 days. Secreted IFN-γ and IL-2 concentration were recorded in culture supernatants with ELISA. The assays were repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means T cells without CAR, served as control. These diagrams were adapted from the publication of Golumba-Nagy et al., 2018.

4.1.3.4 T cell proliferation is inhibited by TGF-β in the presence of the mutation in the LCK binding moiety of CD28

In order to address which CD28 domain is required for TGF-β resistance we engineered T cells with the wild-type CAR, the CAR with a LCK mutation, or a PI3K mutation, or both, respectively, and the T cell amplification was recorded by CSFE dilution. Successful CFSE labeling of the remaining population was verified one day after staining by flow cytometry (Figure 15).

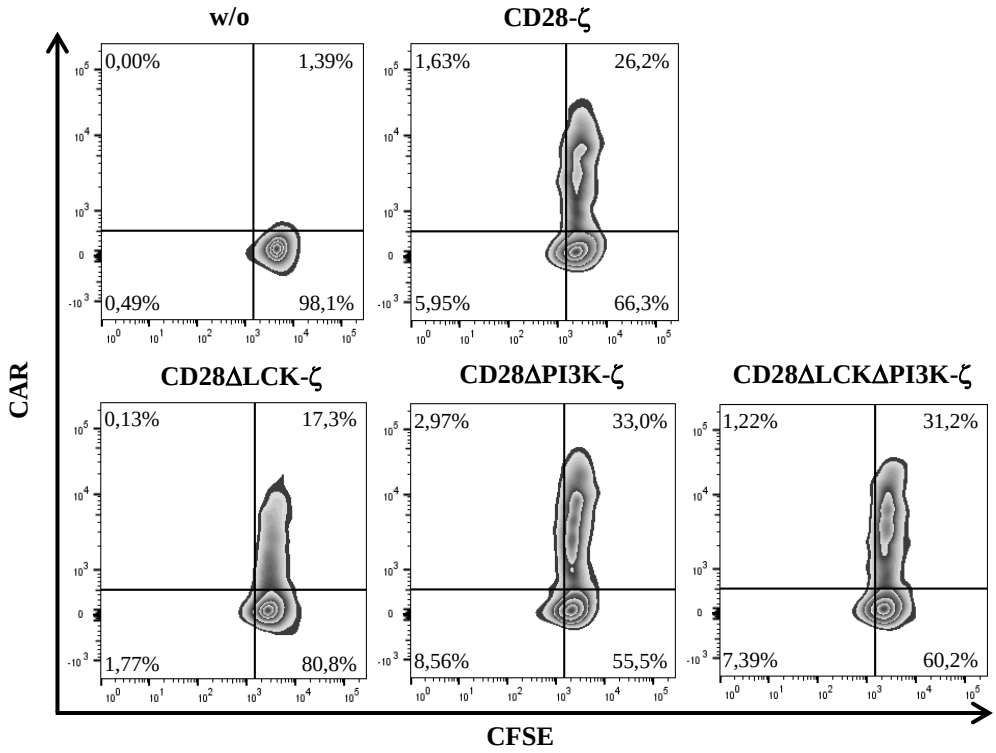


Figure 15 CFSE labeling of CAR modified T cells

Human T cells were modified by CAR with different signaling domain, and labeled with CFSE to track the proliferation of the cells. One day after the CFSE staining, the remaining cells without stimulation were analyzed by flow cytometry to verify the successful labeling. CAR receptors were stained with the PE-conjugated anti-IgG antibody which binds to the CAR in the extracellular IgG1 spacer region. The percentage represents the cell number in each quadrat, w/o means T cells without CAR.

The modified and CFSE-labeled T cells were incubated for 4 days on plates coated with the cognate BW2064/36 antigen with or without TGF-β. LCK mutation altered the proliferative ability of CD28ΔLCK-ζ CAR T cells, whereas PI3K mutation had no effect on T cell amplification in the presence of TGF-β. Furthermore, TGF-β significantly decreased the proliferation of LCK mutated CAR T cells (Figure 16). Mutations in the LCK binding site within the CD28 domain convert CAR T cells sensitive to TGF-β repression.

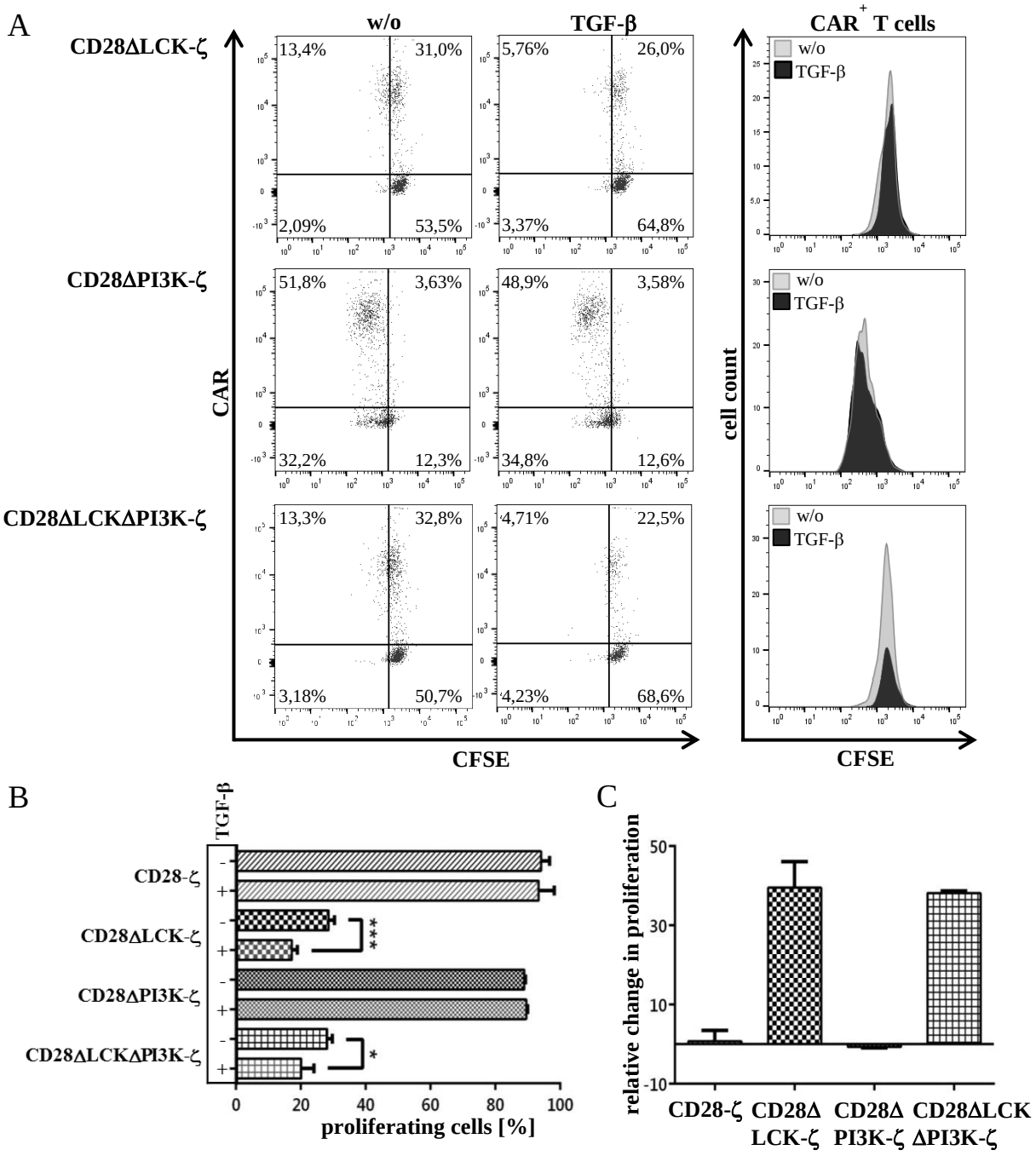


Figure 16 T cell proliferation is inhibited by TGF-β in the presence of the mutation in the LCK binding moiety of CD28

(A) T cells were modified with the respective CAR, labeled with CFSE and incubated on 96-well plates (2.5 x 10^4 T cells per well) coated with anti-idiotypic mAb BW2064/36 (1.5 µg/ml) in the presence or absence of TGF-β (10 ng/ml) for 4 days. T cell amplification was measured by flow cytometric recording of CSFE dilution, CAR receptors were stained with the PE-conjugated anti-IgG antibody.The percentage represents the cell number in each quadrat. The histogram shows the CAR^+, CFSE labeled cells, w/o means without cytokine. **(B)** The data represented on the diagram are the proliferated CAR^+ T cells, comparing the incubation with or without TGF-β. **(C)** Data were transformed to present the loss or gain in CAR T cell proliferation in the presence of TGF-β; [number of proliferating cells without TGF-β – number of proliferating cells in the presence of TGF-β / proliferating cells without TGF-β] x 100. The assays were repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown. Statistical analyses were executed using a two-tailed Student´s t-test (*p < 0.05; **p < 0.01; ***p < 0.001). Diagrams B and C were adapted from the publication of Golumba-Nagy et al., 2018.

Moreover, not only T cell proliferation, but also their specific cytotoxicity was inhibited by TGF-β in the presence of the CD28 LCK mutations. The modified T cells were co-incubated with the CEA^+ LS174T tumor cells for 2 days. On the second day the specific cytotoxicity was measured by XXT-based viability assay. The cytolytic activity by CD28ΔLCK-ζ CAR transduced T cells was suppressed in the presence of TGF-β, compared to the wild type receptor (Figure 17).

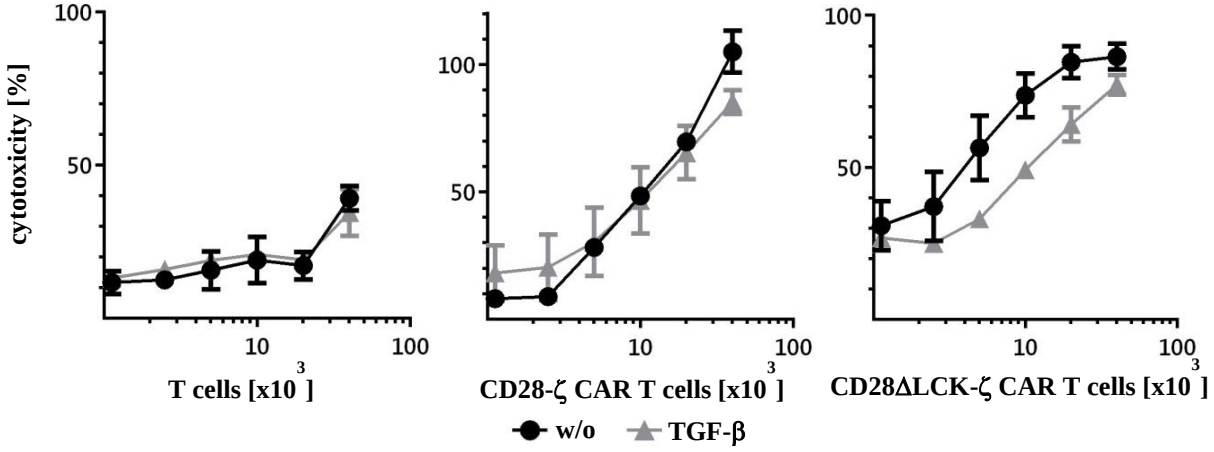


Figure 17 TGF-β inhibits the specific cytotoxicity in the presence of the mutation in the LCK binding moiety

T cells with or without CAR (0.125 x 10^4 – 4 x 10^4 CAR T cells per well) were incubated in the presence or absence of TGF-β (10 ng/ml) with CEA^+ LS174T cells (2 x 10^4 tumor cells per well). The specific cytotoxicity was determined by the XTT-based viability assay after 2 days. The assays were repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means without cytokines, served as control. These figures were taken from the publication of Golumba-Nagy et al., 2018.

Taken together, CD28 costimulation is crucial to provide resistance against TGF-β. CD28-mediated LCK activation, and not PI3K activation, is required for this compensatory effect.

4.1.4 Administration of IL-2 restores the original state of T cell proliferation in the presence of mutations in the LCK binding site and TGF-β

The binding site mutations in the LCK motif abolished IL-2 secretion and consequently IL-2-driven autocrine T cell proliferation was inhibited. To exclude that other LCK dependent functions are responsible for resistance to TGF-β, we added back IL-2 to the modified CD28ΔLCK-ζ CAR T cells through administration into the culture supernatant or restored IL-2 production independently of LCK through transgenic expression and investigated the T cell functions in the presence of TGF-β.

4.1.4.1 Restitution of IL-2 overcomes the suppressive effect of TGF-β

In the first experiment, the amplification of modified T cells was measured in the presence and absence of TGF-β with or without added IL-2. T cells were stained with CFSE after transduction with the CD28ΔLCK-ζ CAR and stimulated by the immobilized BW2064/36 antigen for 4 days. The proliferation with TGF-β plus IL-2 was as effective as without TGF-β. Administration of IL-2 remediated the TGF-β-caused decreased proliferation capacity (Figure 18). We assume that IL-2 is a relevant factor in facilitating TGF-β resistance of CAR T cells.

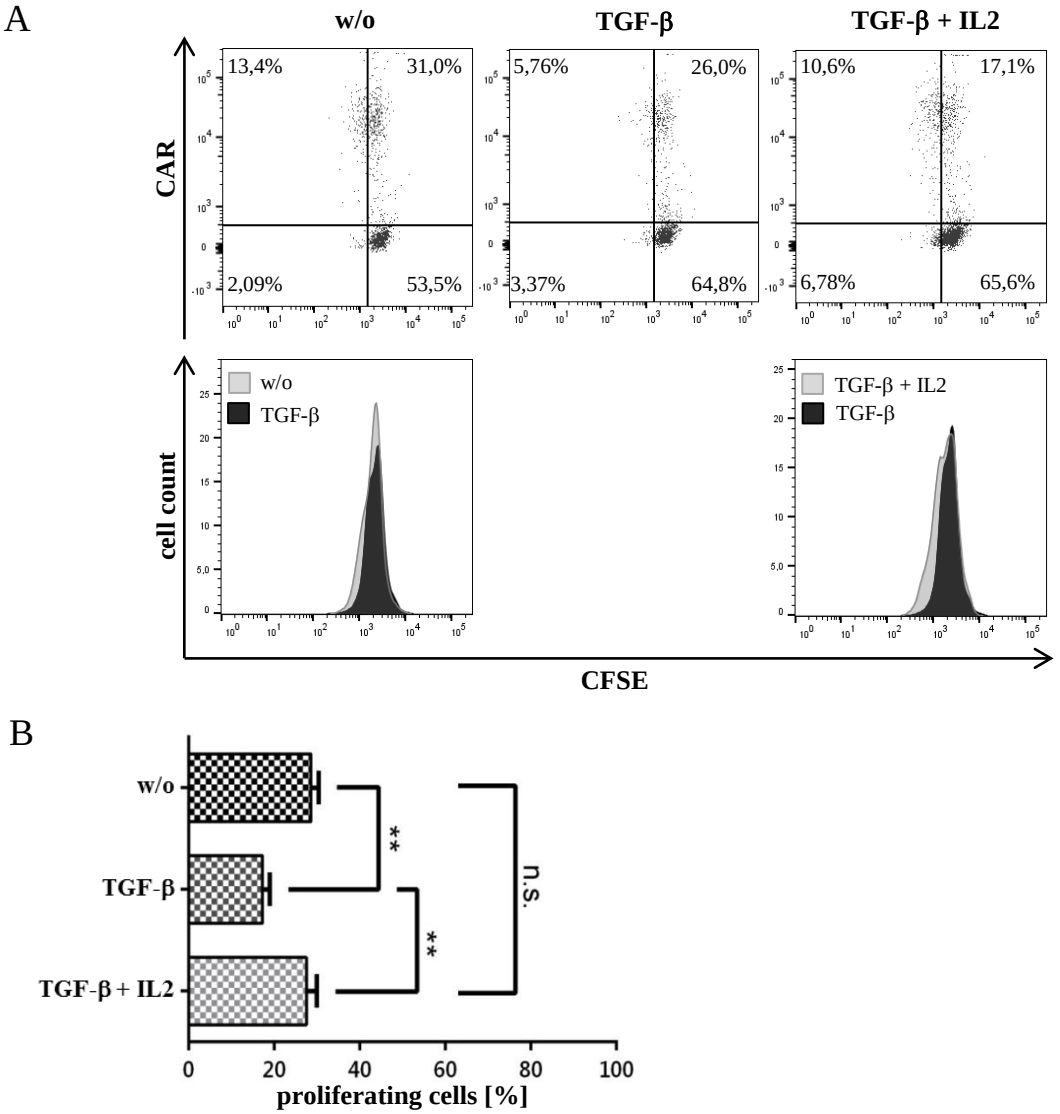


Figure 18 Restitution of IL-2 overcomes the suppressive effect of TGF-β

(A) CD28ΔLCK-ζ CAR engineered T cells (2.5 x 10^4 T cells per well) were stained with CFSE and incubated with TGF-β (10 ng/ml) or TGF-β plus IL-2 (500 U/ml) on plates coated with the mAb BW2064/36 (1.5 µg/ml) for 4 days. T cell proliferation was recorded by flow cytometry through monitoring the CSFE dilution, CAR^+ T cells were identified by staining with the PE-conjugated anti-IgG antibody. The percentage represents the cell number in each quadrat. The histogram shows the CAR^+, CFSE labeled cells.

(B) The data represented on the diagram are the proliferated CAR$^+$ T cells, comparing the incubation with or without TGF-β or with TGF-β + IL-2. The assay was repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means without cytokines, served as control. Statistical analyses were executed using a two-tailed Student´s t-test (*p < 0.05; **p < 0.01; ***p < 0.001). The diagram B was adapted from the publication of Golumba-Nagy et al., 2018.

4.1.4.2 Generation of a pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL2 CAR (#1746)

To confirm our conclusion that released IL-2 can promote TGF-β resistance in an autocrine fashion, we generated a TRUCK (originated from ΔLCK CAR) with IL-2 release and tested the functions of these modified T cells (Figure 19).

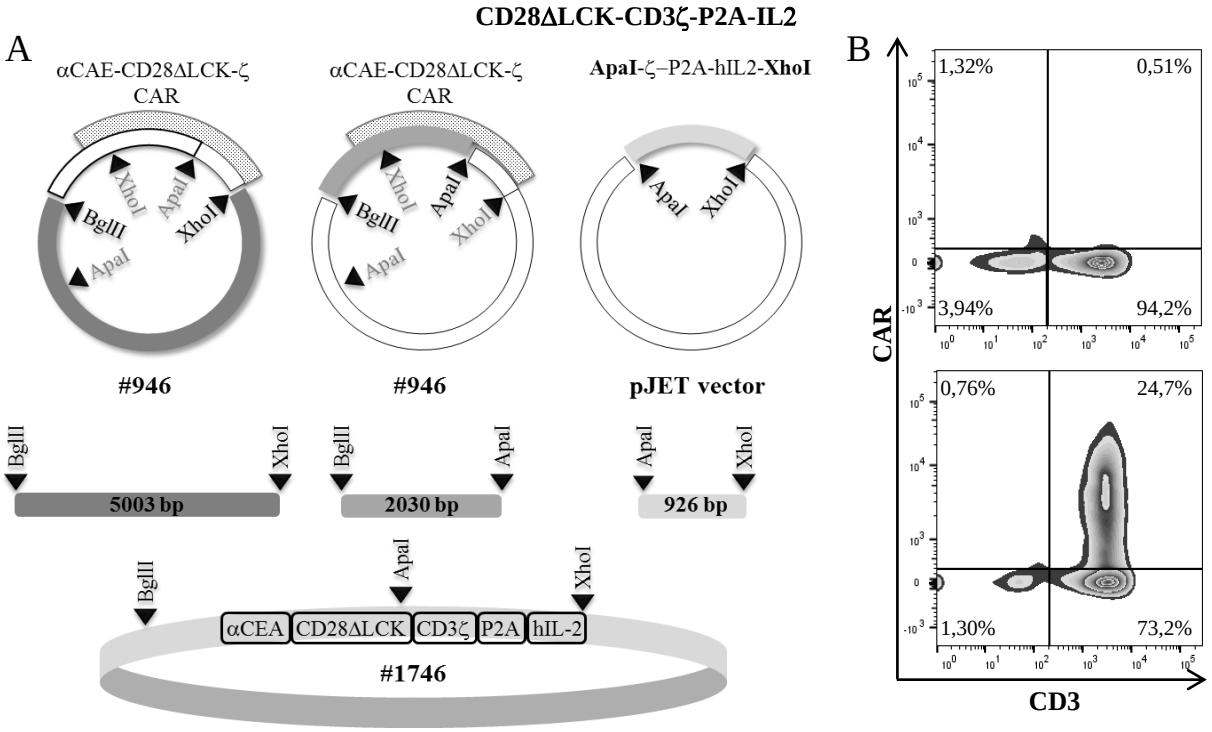


Figure 19 Schematic demonstration of a pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL2 CAR (#1746)

(A) Cloning strategy of CD28ΔLCK-CD3ζ-P2A-IL2 CAR. **(B)** CD28ΔLCK-ζ-IL2 CAR was expressed on T cell surface; human T cells were retrovirally transduced with CD28ΔLCK-ζ-IL2 CAR by spinfection. CAR expression was detected by flow cytometry using the PE-conjugated anti-IgG antibody and for the identification of the T cells, the FITC-conjugated anti CD3 antibody was applied. The percentage represents the cell number in each quadrat.

BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL2 (#1746) was generated from the vector #946 by molecular cloning. The DNA sequence encoding transgenic IL-2 was linked by the self-cleaving P2A element to the CAR encoding sequence. The DNA sequence for P2A-IL2 was ordered as a Gene Fragment (gBlock) from Intergrated DNA Technologies (IDT) and cloned into the pJet vector restricted by EcoRV (creates blunt ends) first for sequencing. Then the

fragment was cloned between the ApaI and XhoI sites of the pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ CAR (#946). For this purpose, #946 was restricted by BglII/XhoI and BglII/ApaI (Figure 19A). After retroviral transduction the CAR was detected on the T cell surface by flow cytometry (Figure 19B).

4.1.4.3 CD28ΔLCK CAR with IL-2 release can produce IL-2 despite of the mutation in the LCK binding site

First, the cytokine production of the CD28ΔLCK-ζ modified T cells with transgenic IL-2 secretion was tested. IFN-γ and IL-2 were measured in the culture supernatant of transduced T cells after 2 days of stimulation through the CAR. As control, the cells were stimulated with an isotype mouse IgG as irrelevant antigen. As further controls, the cytokine production of non-transduced cells, as well as of the wild type, and ΔLCK CAR-transduced T cells without transgenic IL-2 was analyzed. IFN-γ secretion was not altered in the presence of any modification. CD28ΔLCK-ζ CAR with IL-2 release produces IL-2 despite of the mutation in the LCK binding site. As mentioned above, this was not the case for CD28ΔLCK-ζ CAR T cells without IL-2 production (Figure 20). Interestingly, despite of the constitutive structure of the construct we could not record any IL-2 secretion without CAR stimulation.

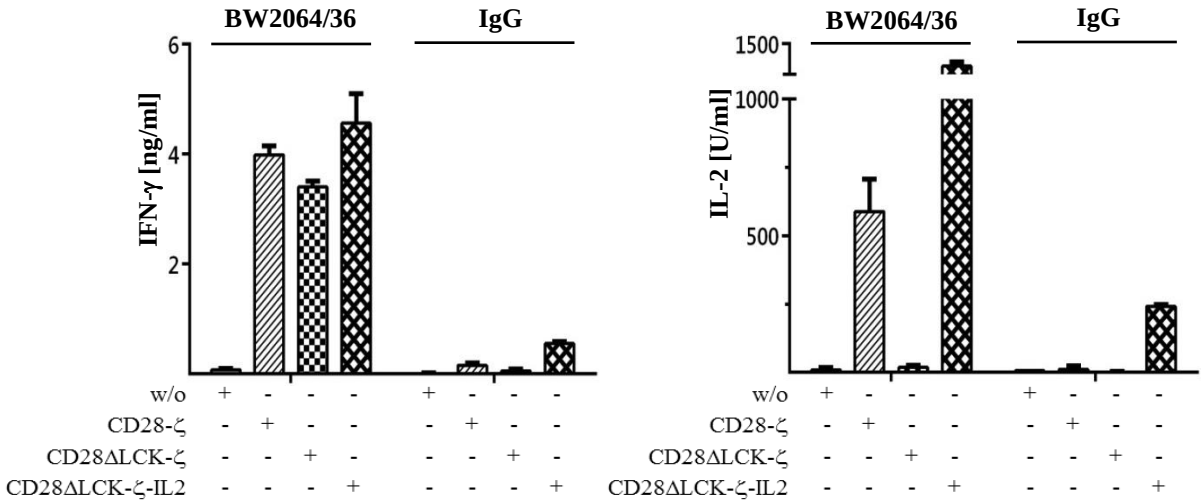


Figure 20 CD28ΔLCK CAR with IL-2 release can produce IL-2 despite of the mutation in the LCK binding site

CAR modified T cells with or without constitutive IL-2 release were incubated on 96 well plates (2.5 x 10^4 CAR T cells pro well) coated with anti-idiotypic mAb BW2064/36 or mouse IgG1 (1.5 µg/ml each) as an isotype control for 2 days. Secreted IFN-γ and IL-2 concentration were recorded in culture supernatants with ELISA. The assays were repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means T cells without CAR, served as control. The diagrams were adapted from the publication of Golumba-Nagy et al., 2018.

4.1.4.4 TGF-β has no effect on the proliferation of T cells modified with CD28ΔLCK-ζ CAR plus transgenic IL-2

The proliferation of the CD28ΔLCK-ζ-IL2-modified T cells was examined to explore whether transgenic IL-2 restores TGF-β resistance. After transduction the engineered T cells were stained by CFSE and stimulated on plates coated with the BW2064/36 antigen in the presence or absence of TGF-β for 4 days. The successful CFSE labeling of the remaining population was confirmed one day after CFSE staining (Figure 21).

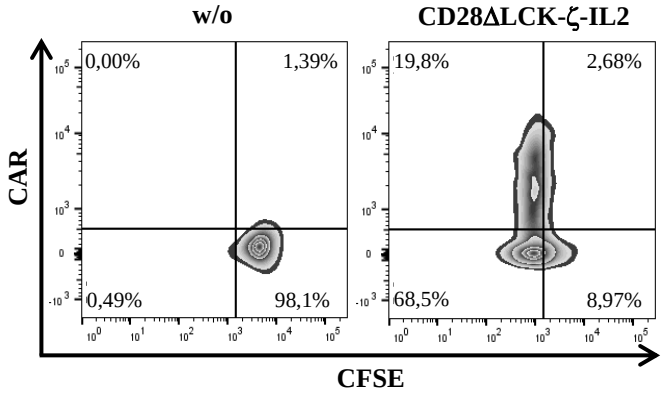


Figure 21 CFSE labeling of CAR modified T cells

Human T cells were modified by CD28ΔLCK-ζ-IL2 CAR and labeled with CFSE to track the proliferation of the cells. One day after the CFSE staining, the remaining cells without stimulation were analyzed by flow cytometry to verify the successful labeling. CAR receptors were stained with the PE-conjugated anti-IgG antibody which binds to the CAR in the extracellular IgG1 spacer region. The percentage represents the cell number in each quadrat, w/o means T cells without CAR.

As shown earlier, TGF-β inhibited the amplification of CD28ΔLCK-ζ CAR-modified T cells, which can be overcome by a CD28ΔLCK-ζ CAR with IL-2 release (Figure 22).

These results sustained our hypothesis, that IL-2 has a central role in the TGF-β resistance of CAR T cell amplification.

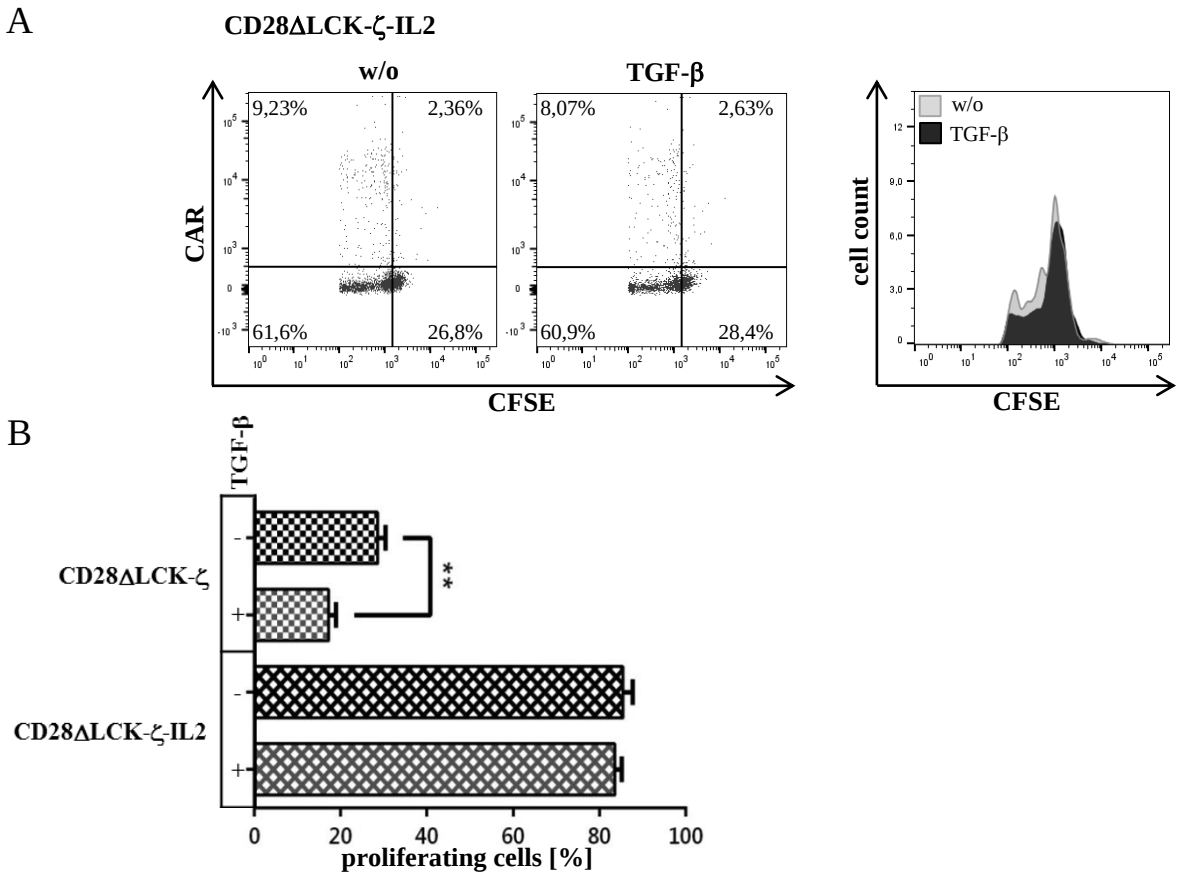


Figure 22 TGF-β has no effect on proliferation of T cells modified with CD28ΔLCK-ζ CAR plus transgenic IL-2

(A) CD28ΔLCK-CD3ζ CAR engineered T cells without or with constitutive transgenic IL-2 release (2.5 x 10^4 T cells per well) were stained with CFSE and incubated with or without TGF-β (10 ng/ml) on BW2064/36 coated plates (1.5 µg/ml) for 4 days. T cell proliferation was recorded by flow cytometry through monitoring the CSFE dilution, CAR^+ T cells were identified by staining with the PE-conjugated anti-IgG antibody. The percentage represents the cell number in each quadrat. The histogram shows the CAR^+, CFSE labeled cells, w/o means without cytokines, served as control. **(B)** The data represented on the diagram are the proliferated CAR^+ T cells, comparing the incubation with or without TGF-β. The assays were repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown. Statistical analyses were executed using a two-tailed Student´s t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The diagram B was adapted from the publication of Golumba-Nagy et al., 2018.

4.2 Identification of other cytokines capable to overcome the suppressive effect of TGF-β

We asked whether other cytokines with a similar capability as IL-2 can overcome the suppressive effect of TGF-β . The rational for the screen is that IL-2 will sustain regulatory T cells in the tumor environment and thereby support TGF-β-mediated T cell repression (Kofler et al., 2011). Ideally, a cytokine with similar effect on CAR T cells but without sustainment of Treg activation would replace the transgenic IL-2 in this context.

We tested two other group members of the γ-cytokine family for the described potential on CAR T cells. We selected IL-7 and IL-15, because they are also activator cytokines for T cells and are able to induce T cell proliferation. IL-7 is a more potential candidate because of the lack of IL-7 receptor expression on Tregs and therefore does not sustain Treg cells. (Perna et al., 2014).

4.2.1 Administration of IL-7 and IL-15 can overcome the suppressive effect of TGF-β

T cells were transduced with the CD28ΔLCK-ζ CAR and labeled with CSFE to track proliferation. T cells were stimulated on plates coated by immobilized BW2064/36 antigen in the presence or absence of TGF-β, or TGF-β plus IL-7 for 4 days. While TGF-β alone inhibited T cell amplification, together with IL-7 the proliferation of the CAR-modified T cells was as efficient as without TGF-β and IL-7. IL-7, similar to IL-2, compensated the suppressive effect of TGF-β (Figure 23).

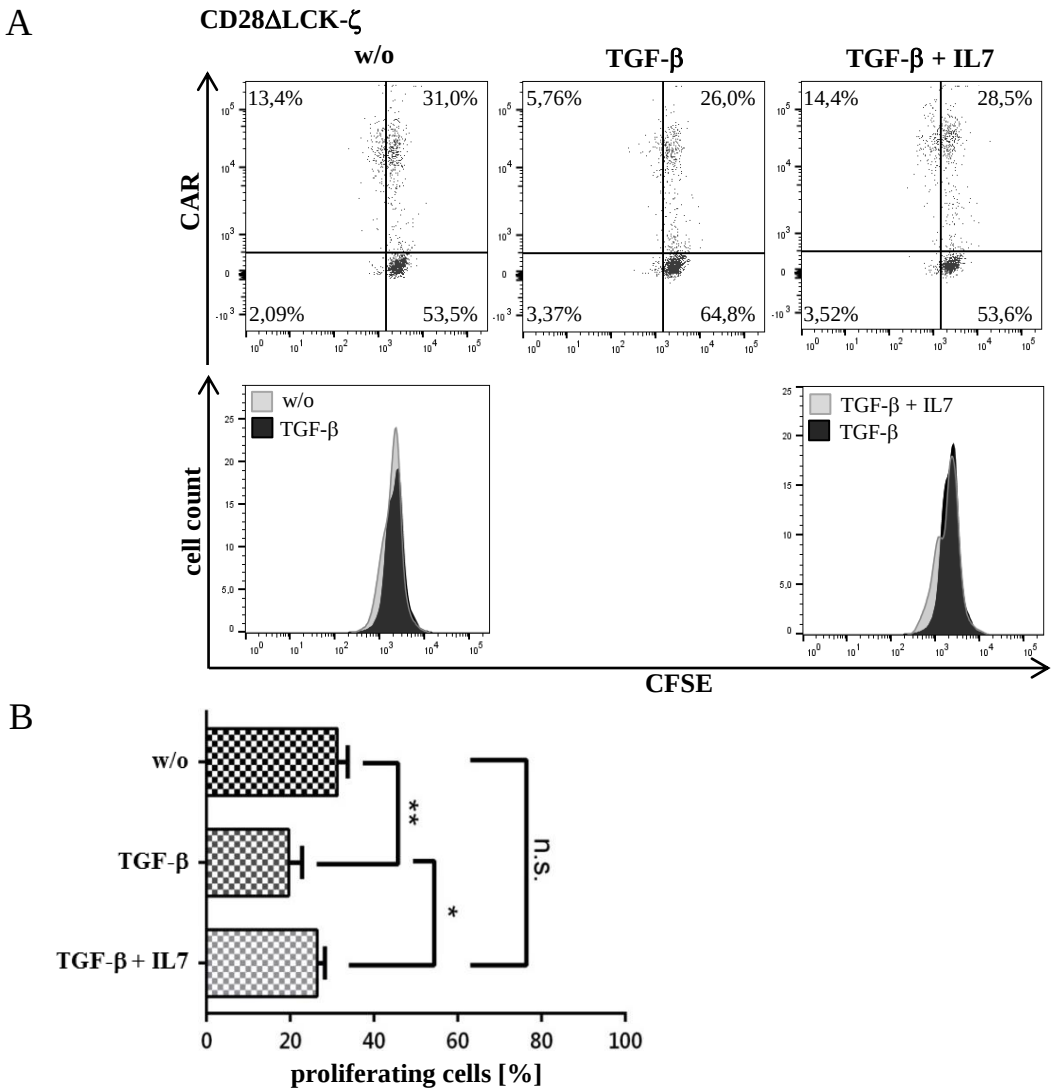


Figure 23 Administration of IL-7 can overcome the suppressive effect of TGF-β

(A) CD28ΔLCK-ζ CAR engineered T cells (2.5 x 10^4 T cells per well) were stained with CFSE and incubated with TGF-β or TGF-β plus IL-7 (10 ng/ml each) on plates coated with the mAb BW2064/36 (1.5 µg/ml) for 4 days. T cell proliferation was recorded by flow cytometry through monitoring the CSFE dilution and the staining with the PE-conjugated anti-IgG antibody identified the CAR^+ T cells. The percentage represents the cell number in each quadrat. The histogram shows the CAR^+, CFSE labeled cells. **(B)** The data represented on the diagram are the proliferated CAR^+ T cells, comparing the incubation with or without TGF-β or with TGF-β + IL-7. The assay was repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means without cytokines, served as control. Statistical analyses were executed using a two-tailed Student´s t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The diagram B was adapted from the publication of Golumba-Nagy et al., 2018.

In a very similar experiment the CD28ΔLCK-ζ CAR-modified T cells were incubated with IL-15 in the presence or absence of TGF-β. IL-15, similar to IL-2 and IL-7, also overcame the repressive TGF-β effect and the proliferation of the modified T cells was the same compared to the situation without TGF-β and IL-15 (Figure 24).

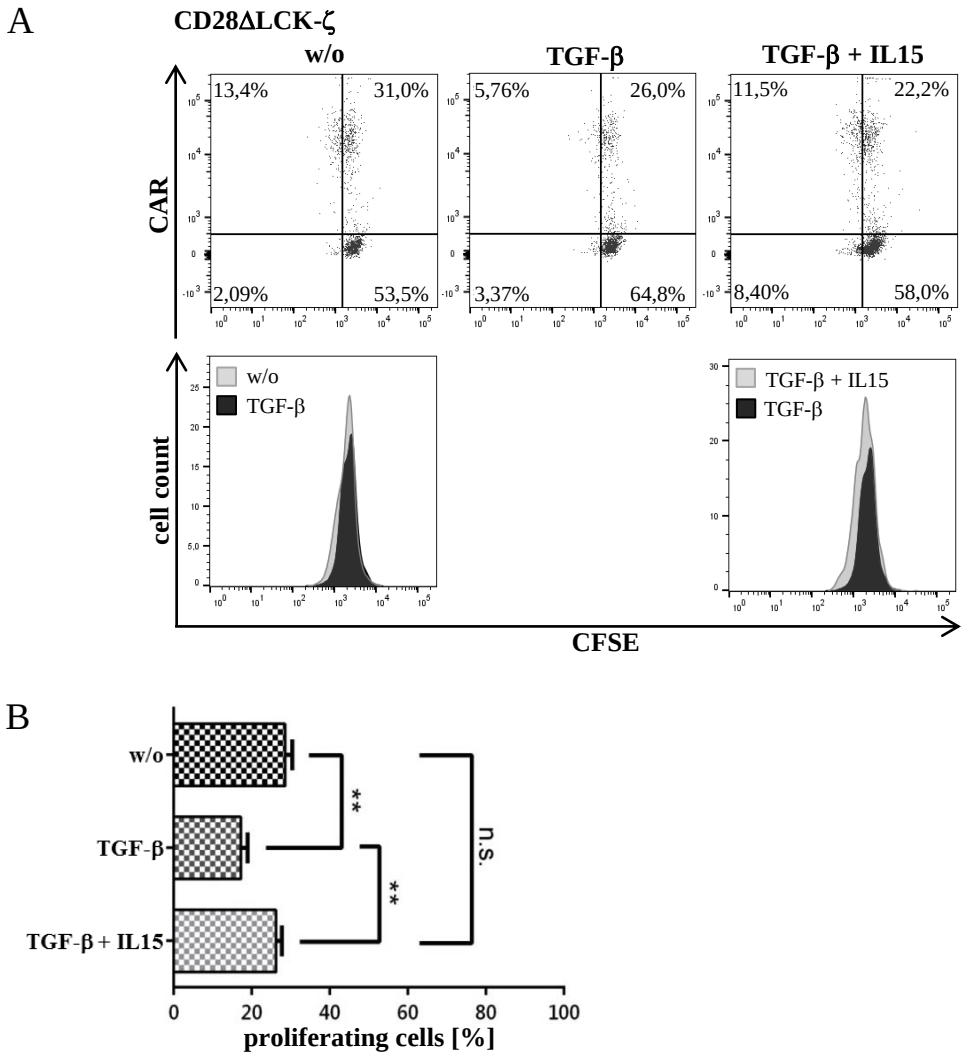


Figure 24 Administration of IL-15 can overcome the suppressive effect of TGF-β

(A) CD28ΔLCK-ζ CAR engineered T cells (2.5 x 10^4 T cells per well) were stained with CFSE and incubated with TGF-β or TGF-β plus IL-15 (10 ng/ml each) on plates coated with the mAb BW2064/36 (1.5 µg/ml) for 4 days. T cell proliferation was recorded by flow cytometry through monitoring the CSFE dilution and the staining with the PE-conjugated anti-IgG antibody identified the CAR^+ T cells. The percentage represents the cell number in each quadrat. The histogram shows the CAR^+, CFSE labeled cells. **(B)** The data represented on the diagram are the proliferated CAR^+ T cells in compare the incubation with or without TGF-β or with TGF-β + IL-15. The assay was repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means without cytokines, served as control Statistical analyses were executed using a two-tailed Student´s t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The diagram B was adapted from the publication of Golumba-Nagy et al., 2018.

4.2.2 Generation of a pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL7 CAR (#1645)

IL-7 was of benefit when we added it to the cells in vitro in the presence of TGF-β, but in clinical applications it is difficult to obtain sufficient IL-7 concentrations especially within solid tumors. For this reason we generated a TRUCK with a coding region that links the transgenic IL-7 to the CD28ΔLCK-ζ CAR with a P2A element to allow constitutive IL-7 production (Figure 25).

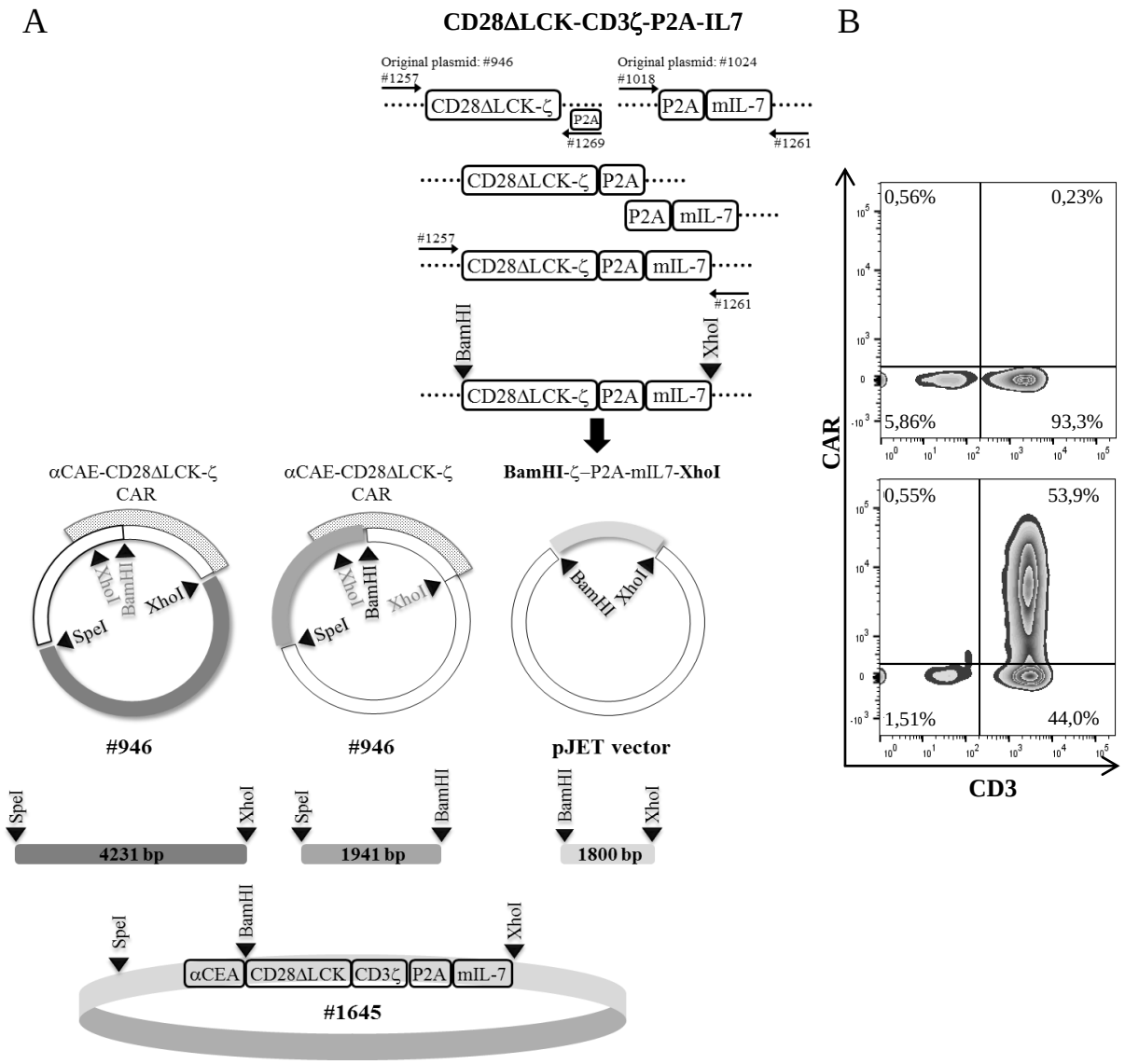


Figure 25 Schematic demonstration of a pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL7 CAR (#1645)

(A) Cloning strategy of CD28ΔLCK-CD3ζ-P2A-IL7 CAR. **(B)** CD28ΔLCK-ζ-IL7 CAR was expressed on T cell surface; human T cells were retrovirally transduced with CD28ΔLCK-ζ-IL7 CAR by spinfection. CAR expression was detected by flow cytometry using the PE-conjugated anti-IgG antibody and for the identification of the T cells, the FITC-conjugated anti CD3 antibody was used. The percentage represents the cell number in each quadrat.

BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL7 (#1645) was generated by assembly PCR. P2A-IL7-XhoI sequence was amplified from the vector #1024 (#1024 vector contained the sequence for IL-7 linked with the P2A element) by using the oligonucleotides #1018 and #1261. BamHI-ΔLCK-P2A sequence was amplified from the vector #946 by using the following set of oligonucleotides: #1257 and #1269. The overlap PCR utilized the oligonucleotides #1257 and #1261. The amplified PCR product was excised with BamHI/XhoI and first cloned into the pJet vector restricted by EcoRV (creates blunt ends) for sequencing and then cloned between BamHI and XhoI sites of the pBullet vector containing

the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ CAR (#946). For this purpose, #946 was restricted by BamHI/SpeI and XhoI/SpeI (Figure 25A). The receptor efficiently expressed on the T cell surface after retroviral transduction; the CAR was detected by flow cytometry (Figure 25B below).

4.2.3 Generation of a pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL15 CAR (#1764)

For the same reason as stated above, we generated another TRUCK, where transgenic IL-15 is linked to the CD28ΔLCK-ζ CAR with a P2A element to allow constitutive IL-15 production (Figure 26).

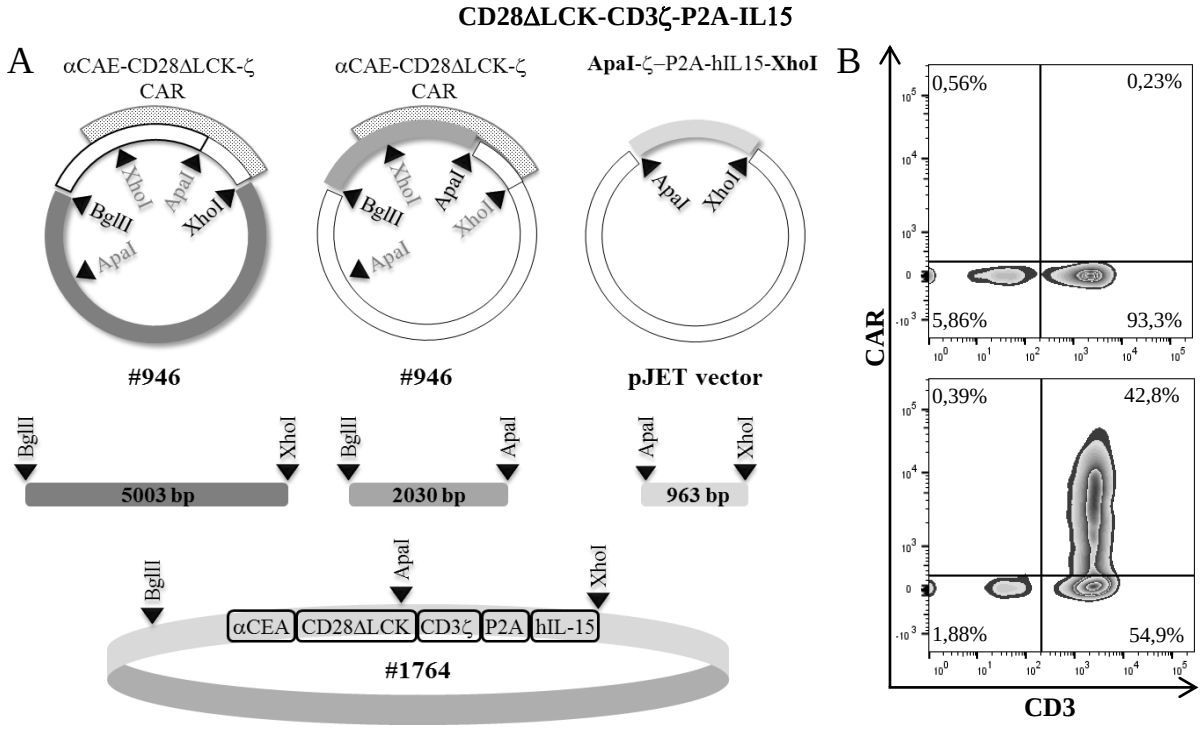


Figure 26 Schematic demonstration of a pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL15 CAR (#1764)

(A) Cloning strategy of CD28ΔLCK-CD3ζ-P2A-IL15 CAR. **(B)** CD28ΔLCK-ζ-IL15 CAR was expressed on T cell surface, human T cells were retrovirally transduced with CD28ΔLCK-ζ-IL15 CAR by spinfection. CAR expression was detected by flow cytometry using the PE-conjugated anti-IgG antibody and for the identification of the T cells, the FITC-conjugated anti CD-3 antibody was used. The percentage represents the cell number in each quadrat.

BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL15 (#1764) was generated from the vector #946 by molecular cloning. DNA sequence of transgenic IL-15 was linked by the self-cleaving P2A element. Sequence for P2A-IL15 was ordered as Gene Fragment (gBlock) from Intergrated DNA Technologies (IDT) and first cloned into the pJet vector restricted by EcoRV (creates blunt ends) for sequencing and then cloned further into the site between ApaI and

XhoI of the pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ CAR (#946). For this purpose, #946 was restricted by BglII/XhoI and BglII/ApaI (Figure 26A). The CAR was detected on the T cell surface by flow cytometry after retroviral transduction (Figure 26B below).

4.2.4 Cytokine production of the T cells modified with CD28ΔLCK CAR plus transgenic IL-7 or IL-15

We investigated the cytokine production of T cells engineered with the TRUCK with transgenic IL-7 and IL-15, respectively. The modified T cells were stimulated on plates coated by the immobilized BW2064/36 antigen for 2 days and IFN-γ, IL-2, IL-7, and IL-15 were measured in the culture supernatant *via* ELISA. Stimulation with an irrelevant mouse IgG served as control. Furthermore, we used not transduced T cells and transduced T cells with the wild type or ΔLCK CAR as control. As we expected IFN-γ secretion was unaltered compared to T cells with the wild type CAR (Figure 27). IL-2 release was detected in the supernatant of T cells modified by the wild type receptor, while the other CAR T cells could not express IL-2 (Figure 27). T cells equipped with a IL-7 or IL-15 expression cassette produce IL-7 or IL-15, respectively, into the supernatant upon CAR stimulation, while incubation of the T cells with irrelevant antigen did not trigger the expression of these cytokines (Figure 27). Interestingly, despite of the constitutive promoter for cytokine transcription, the engineered T cells do not release IL-7 or IL-15 into the culture supernatant without stimulation as observed in the case of the IL-2 TRUCK receptor (Figure 20).

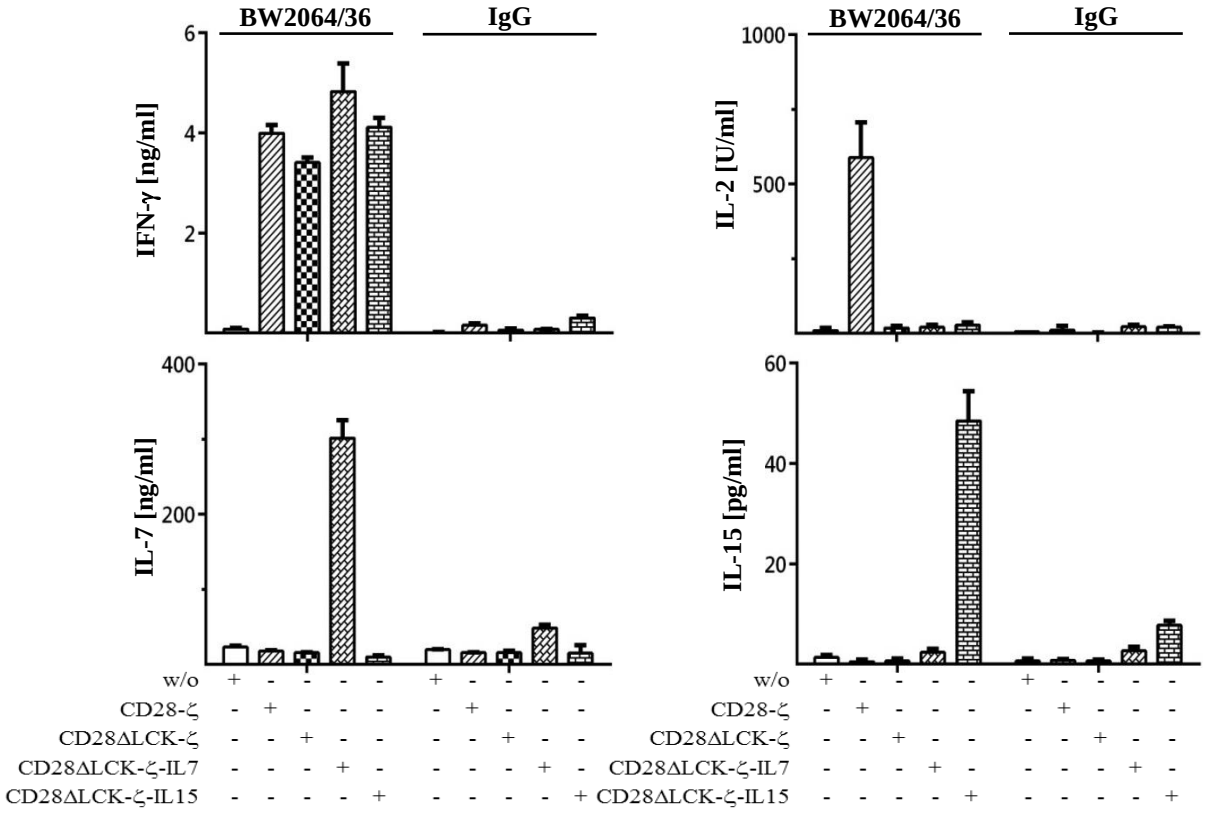


Figure 27 Cytokine production of the T cells modified with CD28DLCK CAR plus transgenic IL-7 or IL-15

CAR modified T cells with or without IL-7 or IL-15 release was incubated on 96 well plates (2.5 x 10^4 CAR T cells pro well) coated with anti-idiotypic mAb BW2064/36 or mouse IgG1 (1.5 µg/ml each) as an isotype control for 2 days. Secreted IFN-γ; IL-2; IL-7 and IL-15 concentration were recorded in culture supernatants with ELISA. The assays were repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means T cells without CAR, served as control. These diagrams were adapted from the publication of Golumba-Nagy et al., 2018.

4.2.5 TGF-β did not impair the proliferation of T cells modified with a CD28ΔLCK-ζ CAR plus transgenic IL-7 or IL-15

The proliferation of T cells modified with the CD28ΔLCK-ζ-IL7 or CD28ΔLCK-ζ-IL15 CAR was determined with the help of CFSE labeling. The CFSE staining of the remaining population was verified one day after the CFSE-labeling *via* flow cytometry (Figure 28).

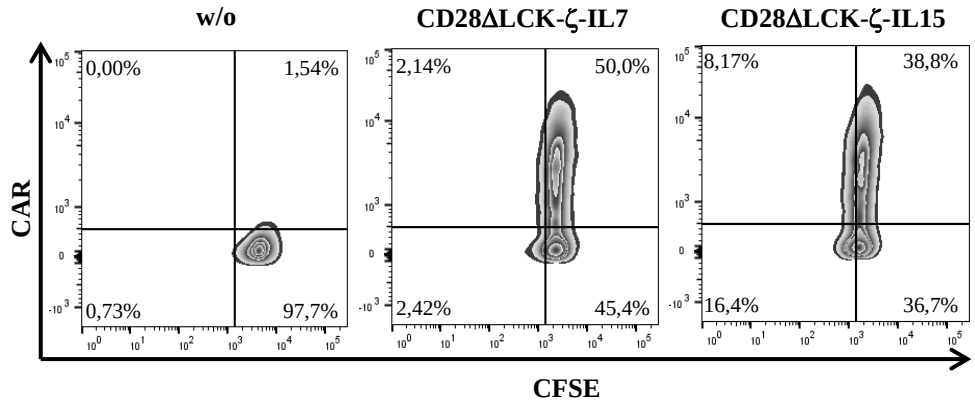


Figure 28 CFSE labeling of CAR modified T cells

Human T cells were modified by CD28ΔLCK-ζ-IL7 or CD28ΔLCK-ζ-IL15 CAR and labeled with CFSE to track the proliferation of the cells. One day after the CFSE staining, the remaining cells without stimulation were analyzed by flow cytometry to verify the successful labeling. CAR receptor was stained with the PE-conjugated anti-IgG antibody which binds to the CAR in the extracellular IgG1 spacer region. The percentage represents the cell number in each quadrat, w/o means T cells without CAR.

The cells were stimulated on plates coated with the immobilized BW2064/36 antigen in the presence or absence of TGF-β for 4 days. As control the CD28ΔLCK-ζ CAR without IL-7 is also shown. TGF-β had no effect on the proliferation of the IL-7-equipped ΔLCK CAR engineered T cells; the CAR T cells proliferated in the presence of TGF-β as efficient as without TGF-β (Figure 29).

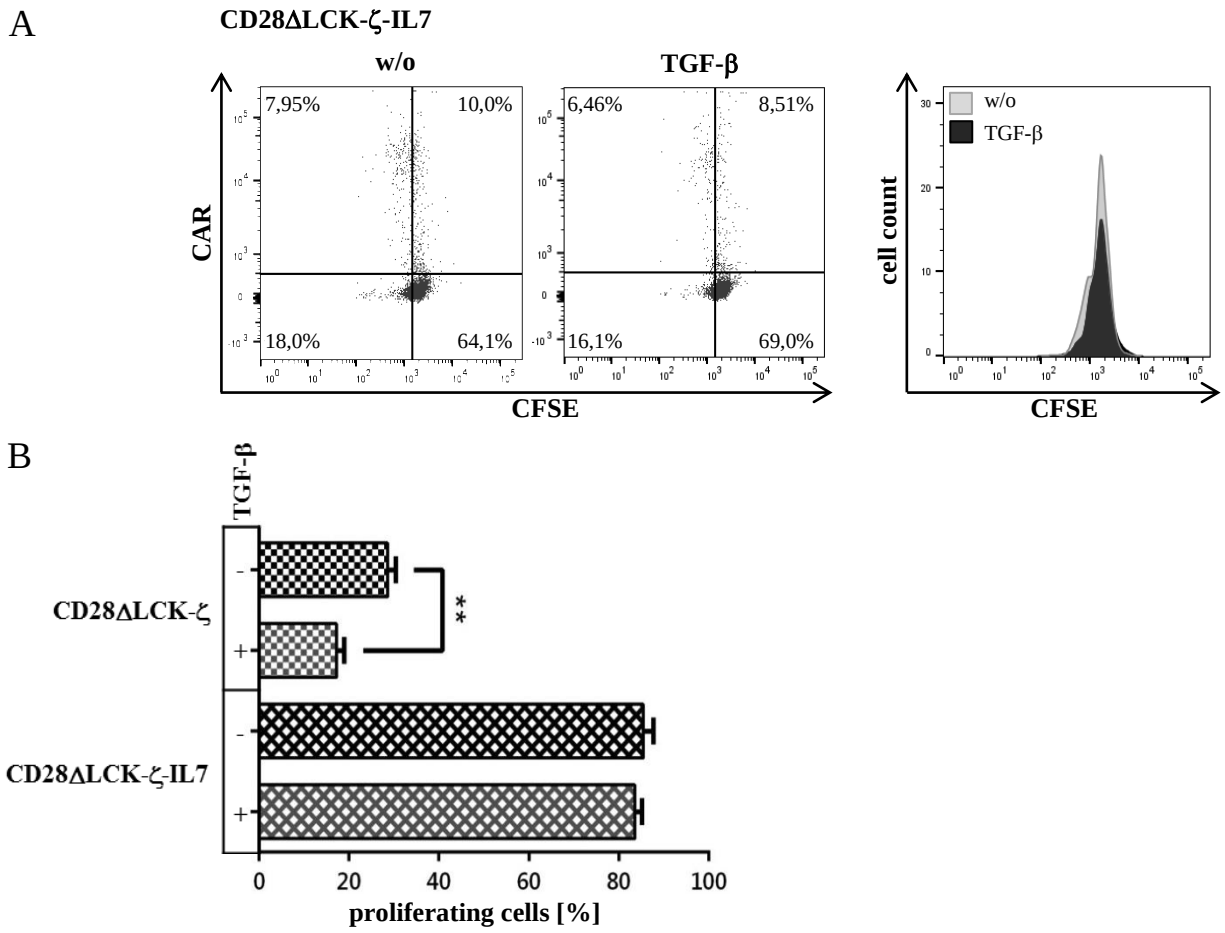


Figure 29 TGF-β did not impair the proliferation of T cells modified with CD28ΔLCK-ζ CAR plus transgenic IL-7

(A) CD28ΔLCK-ζ CAR engineered T cells without or with transgenic IL-7 release (2.5 x 10^4 T cells per well) were stained with CFSE and incubated with or without TGF-β (10 ng/ml) on BW2064/36-coated plates (1.5 µg/ml) for 4 days. T cell proliferation was recorded by flow cytometry through monitoring the CSFE dilution and the CAR^+ T cells were identified by staining with the PE-conjugated anti-IgG antibody. The histogram shows the CAR^+, CFSE labeled cells. The percentage represents the cell number in each quadrat, w/o means without cytokines, served as control. **(B)** The data represented on the diagram are the proliferated CAR^+ T cells, comparing the incubation with or without TGF-β. The assay was repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown. Statistical analyses were executed using a two-tailed Student´s t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The diagram B was adapted from the publication of Golumba-Nagy et al., 2018.

IL-15-equipped ΔLCK CAR engineered T cells have shown the same results as IL-7 equipped T cells. TGF-β had no effect on the proliferation of the IL-15 equipped ΔLCK CAR engineered T cells as well. As control the CD28ΔLCK-ζ CAR without IL-15 is also shown (Figure 30). The same result was obtained with the added IL-7 or IL-15. In a clinical application this is a benefit for CAR engineered T cells producing IL-7 or IL-15 in the tumor microenvironment. The suppressive effect of TGF-β was overcome by both receptors. The modified T cells produce the cytokines required to resist TGF-β repression.

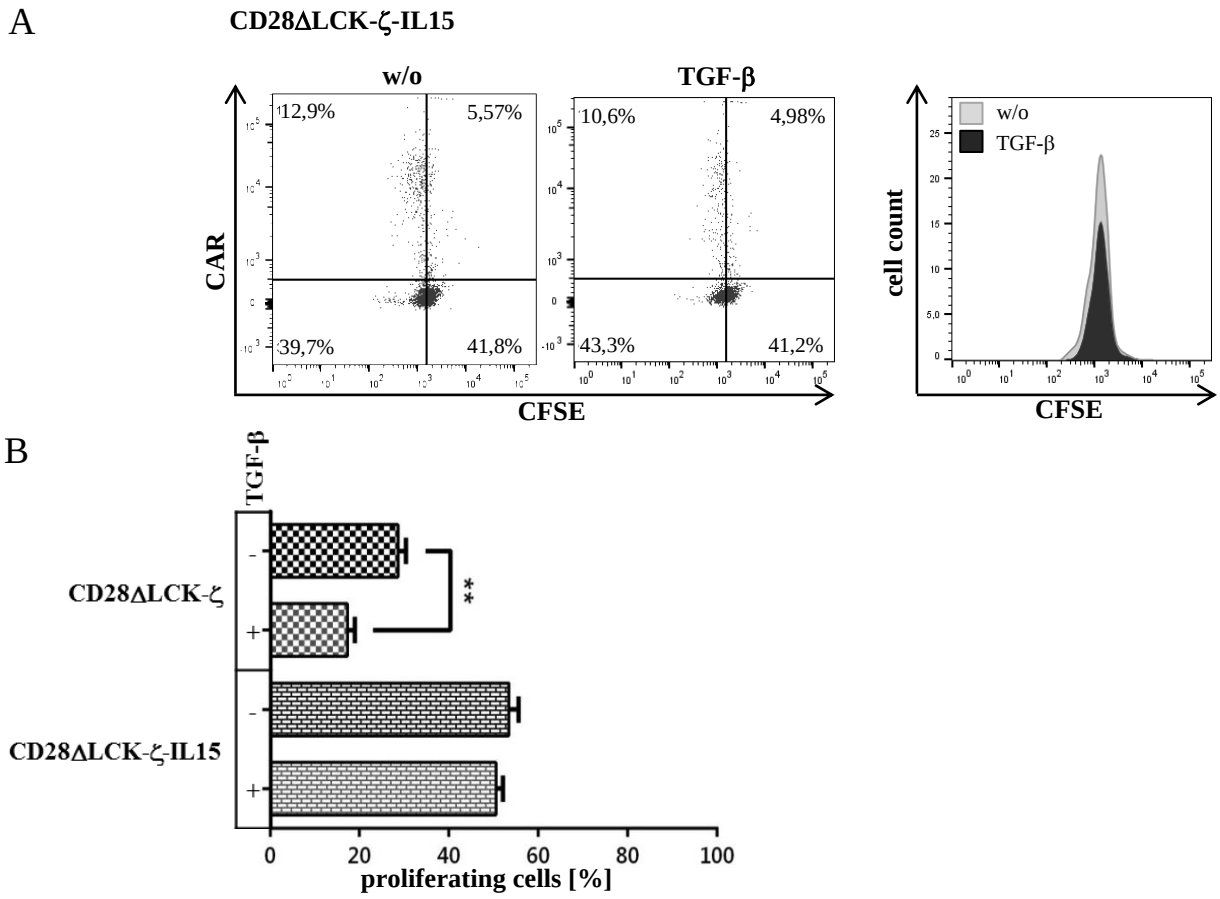


Figure 30 TGF-β did not impair proliferation of the T cells modified with CD28ΔLCK-ζ CAR plus transgenic IL-15 release

(A) CD28ΔLCK-ζ CAR engineered T cells without or with constitutive transgenic IL-15 release (2.5 x 10^4 T cells per well) were stained with CFSE and incubated with or without TGF-β (10 ng/ml) on BW2064/36 coated plates (1.5 µg/ml) for 4 days. T cell proliferation was recorded by flow cytometry through monitoring the CSFE dilution and the CAR^+ T cells were identified by staining with the PE-conjugated anti-IgG antibody. The percentage represents the cell number in each quadrat. The histogram shows the CAR^+, CFSE labeled cells, w/o means without cytokines, served as control. **(B)** The data represented on the diagram are the proliferated CAR^+ T cells, comparing the incubation with or without TGF-β. The assays were repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown. Statistical analyses were executed using a two-tailed Student´s t-test (*p < 0.05; **p < 0.01; ***p < 0.001). The diagram B was adapted from the publication of Golumba-Nagy et al., 2018.

4.2.6 TGF-β did not inhibit the specific cytotoxicity of T cells stimulated via the CD28ΔLCK-ζ-IL7 or CD28ΔLCK-ζ-IL15 CAR

The specific cytotoxicity by CD28ΔLCK-ζ-IL7 or CD28ΔLCK-ζ-IL15 CAR-modified T cells was tested in the presence and absence of TGF-β. The engineered T cells were incubated with the CEA^+ LS174T tumor cells for 2 days; the specific cytotoxicity was measured by the XTT-based viability assay. The results do not show any differences between plus or minus TGF-β (Figure 31), while TGF-β suppressed the specific killing of tumor cells in the case of the CD28ΔLCK-ζ CAR without IL-7 or IL-15 (Figure 17). IL-7 and IL-15 not only support T cell proliferation but also specific killing in the presence of TGF-β.

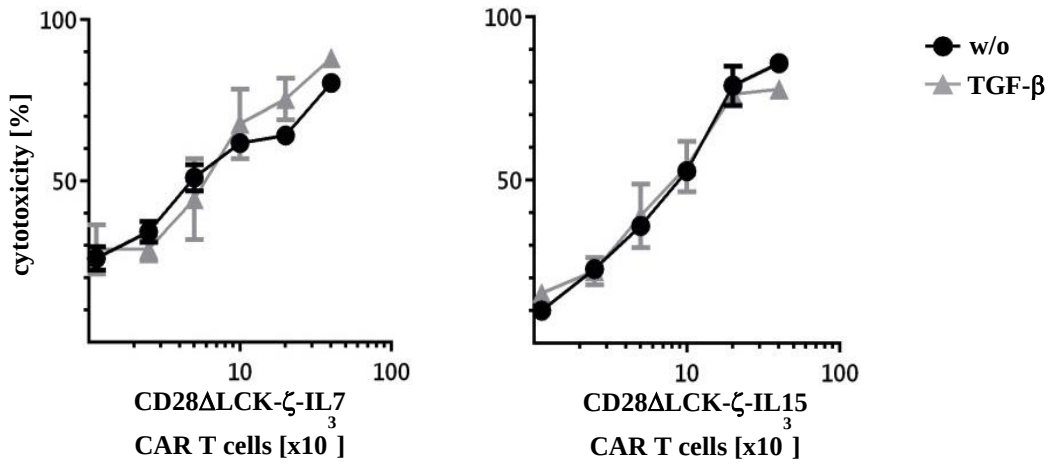


Figure 31 TGF-β did not inhibit the specific cytotoxicity of T cells stimulated via the CD28ΔLCK-ζ-IL7 or CD28ΔLCK-ζ-IL15 CAR

T cells with CD28ΔLCK-ζ-IL7 or CD28ΔLCK-ζ-IL15 CAR (0.125 x 10^4 – 4 x 10^4 CAR T cells per well) were incubated in the presence or absence of TGF-β (10 ng/ml) with CEA^+ LS174T cells (2 x 10^4 tumor cells per well). The specific cytotoxicity was determined by the XTT-based viability assay after 2 days. The assay was repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means without cytokines, served as control. These figures were taken from the publication of Golumba-Nagy et al., 2018.

4.3 Overexpression of the IL-7 receptor helps to maintain a long term activation of modified T cells

One of our goals was to make CAR engineered T cell less sensitive against TGF-β; this can be achieved by the administration of IL-2, IL-7, or IL-15, respectively. For potential clinical application we excluded IL-2 and IL-15, because they sustain Treg cell activation. In further experiments we concentrated on IL-7, because it does not maintain Tregs and is able to overcome the TGF-β-mediated suppression. An added constitutively expressed chimeric cytokine receptor would moreover compensate for the downregulation of IL-7 receptor after T cell activation (Alves et al., 2008).

4.3.1 IL-7 receptor α-chain (CD127) is downregulated after T cell activation

We wanted to monitor the IL-7R downregulation after T cell stimulation. Therefore we engineered human T cells with different CAR receptors, namely the CD28-ζ, CD28ΔLCK-ζ or CD28ΔLCK-ζ-IL7 CAR, respectively. The modified T cells were stimulated on plates coated by OKT3/15E8 antibodies for CAR independent T cell activation, by the BW2064/36 antibody mediating CAR-specific stimulation, and by an irrelevant mouse IgG antibody, or by PBS as negative control. Non-stimulated cells as well as non-transduced T cells are also shown as control (Figure 32). After 2 days incubation time the cells were labeled for the CAR and the IL-7 receptor and the data were evaluated by FACS cytometry. The diagrams show the cell count of each quadrat of a FACS dotplots. A decreased IL-7R expression among CAR^+ T cells can be clearly seen after stimulation either through the TCR, or through the CAR receptor. IL-7R expression stays stable without stimulation. The decreased expression of IL-7R is more obvious in the case of the CD28ΔLCK-ζ-IL7 CAR-modified T cells. Without stimulation these cells produce a fewer amount of IL-7, which –in turn- is able to downregulate the IL-7 receptor (Figure 32).

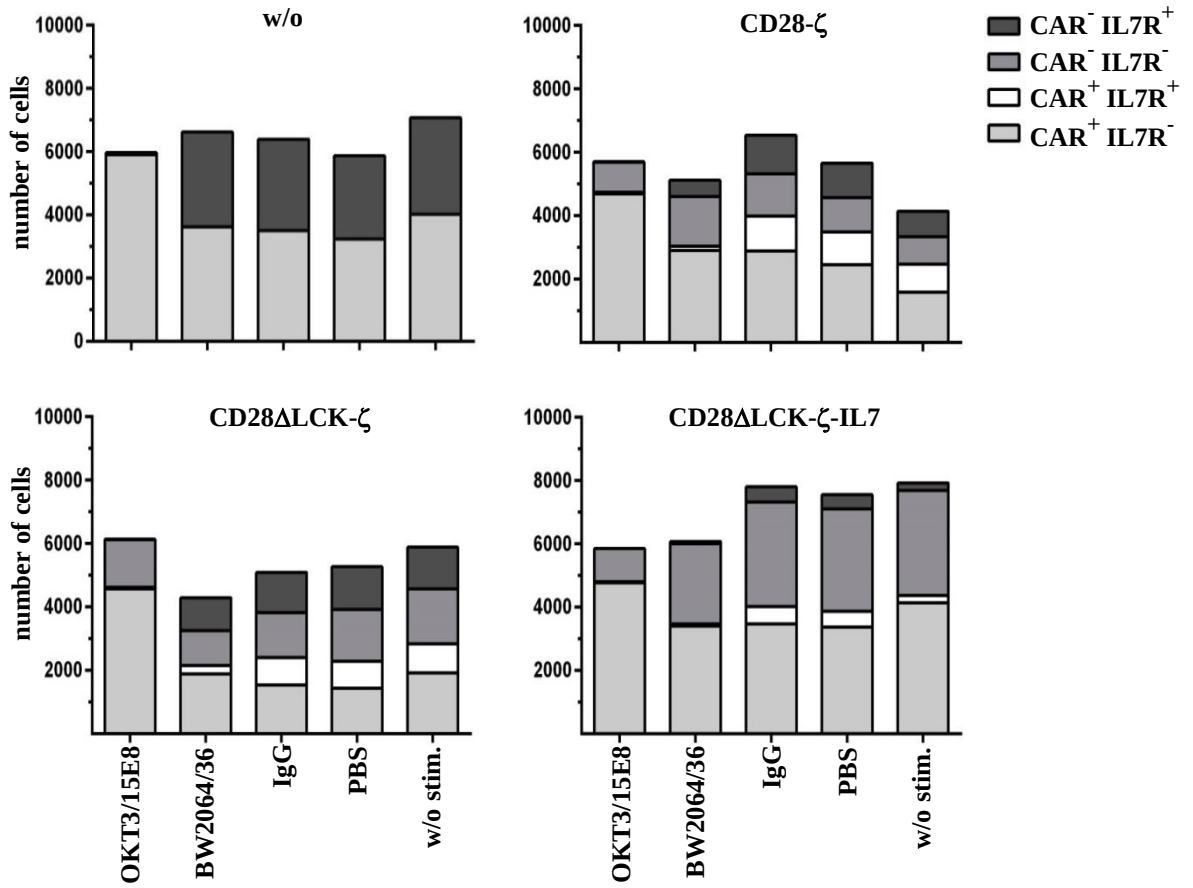


Figure 32 IL-7 receptor α-chain (CD127) is downregulated after T cell activation

CAR modified T cells were incubated on 96 well plates coated with OKT3 (1 µg/ml) plus 15E8 (5 µg/ml) for T cell activation, with BW2064/36 (1.5 µg/ml) for CAR stimulation, with mouse IgG1 (1.5 µg/ml) as isotype control or with PBS or without stimulation (w/o stim.) as control. After 2 days the cells were harvested and stained by PE-conjugated anti-IgG antibody for the identification of the CAR, while the FITC-conjugated CD127 was used for the labeling of the IL-7 receptor α-chain. Data were recorded by flow cytometry. On the diagram the cell number of each quadrat of FACS dot plot are shown. Dark grey bar is the CAR^- $IL\text{-}7R^+$, grey bar labels the CAR^- $IL\text{-}7R^-$, the white bar shows the CAR^+ and $IL\text{-}7R^+$, while the light grey bar indicates the CAR^+ $IL\text{-}7R^-$ T cells. Furthermore, w/o means T cells without CAR.

4.3.2 Generation of a pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-T2A-IL7Rα/IL2Rβ (#1941)

We generated a vector which, besides of the ΔLCK CAR, contains a hybrid IL-7 receptor. The chimeric cytokine receptor contains an IL-7 receptor α-chain in the extracellular part and the IL-2 receptor β-chain in the transmembrane and intracellular part of the receptor (Figure 33). BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-T2A-IL7Rα/IL2Rβ (#1941) was generated from the vector #1908 by molecular cloning. The gene fragment (gBlock) encoding the CD3ζ-T2A fragment with a SanDI and a BspEI restriction sites at the fragment´s ends, respectively was inserted first into pJet vector restricted by EcoRV (creates blunt ends) for sequencing and followed by an insertion between the SanDI and BspEI sites of the pBullet vector containing

the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL7-T2A-IL7Rα/IL2Rβ (#1908). For this purpose, #1908 was restricted by SanDI and SanDI/BspEI (Figure 33).

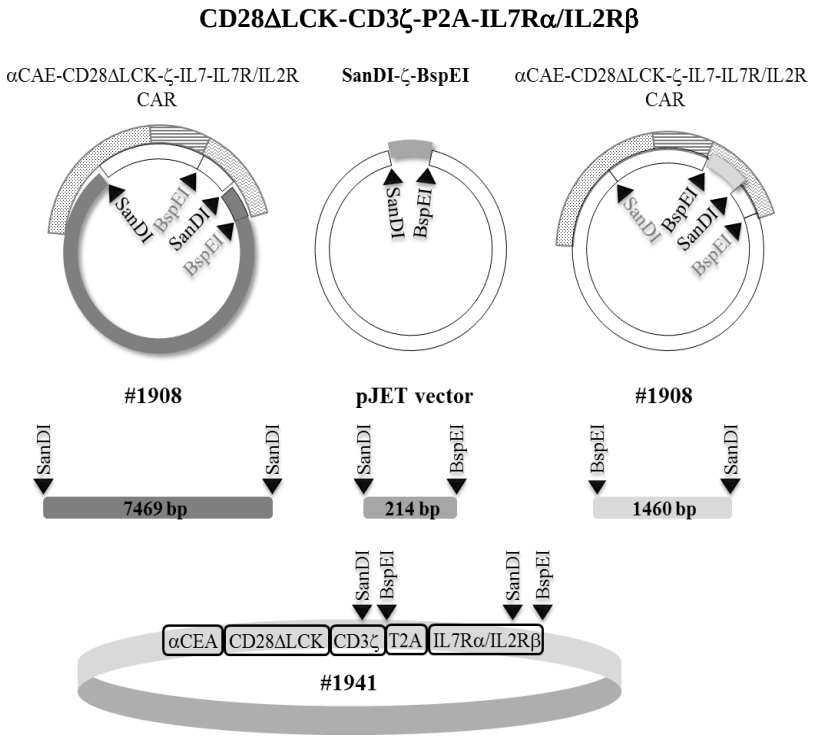


Figure 33 Schematic demonstration of a pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL7Rα/IL2Rβ CAR (#1941)

Cloning strategy of CD28ΔLCK-CD3ζ-P2A-IL7Rα/IL2Rβ CAR.

The CAR receptor was efficiently expressed on the T cell surface after retroviral transduction as well as the CAR and transgenic IL-7R α-chain (CD127) were expressed on the transfected HEK 293T cell surface. CAR and IL-7R were detected by flow cytometry (Figure 34AB).

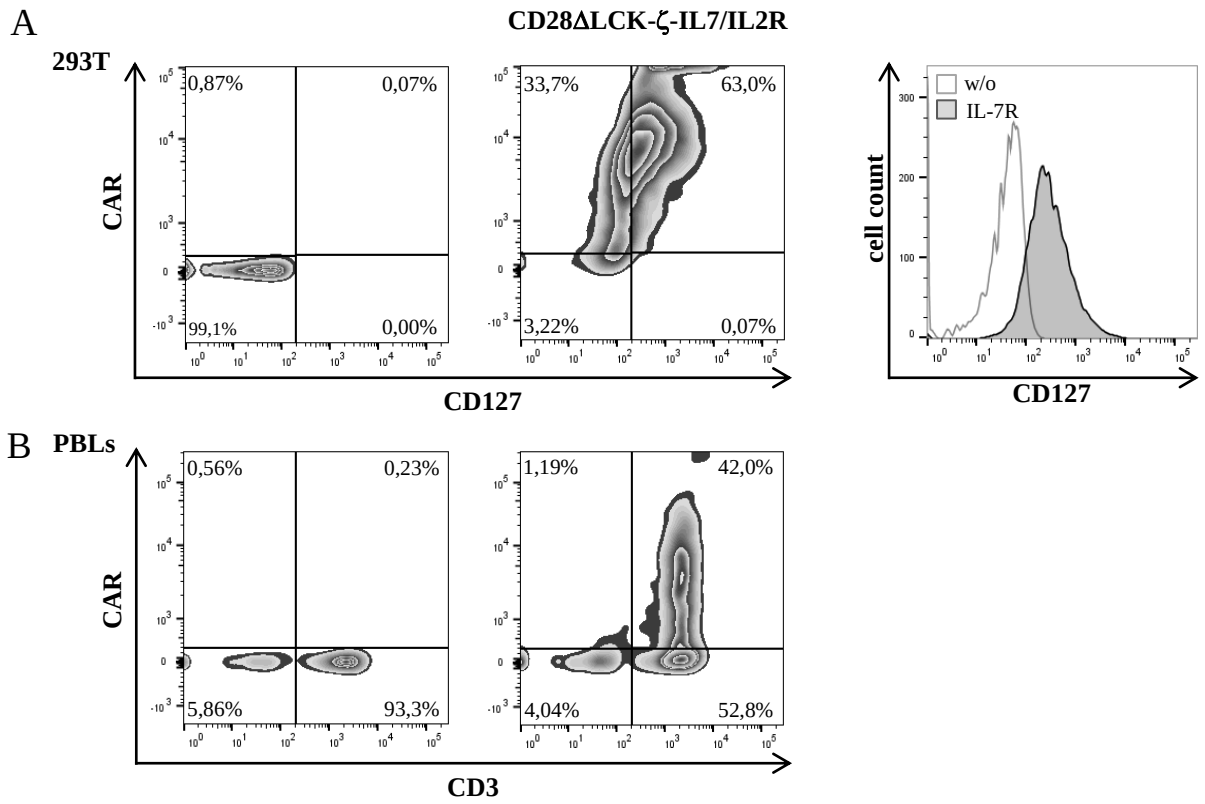


Figure 34 CD28ΔLCK-ζ-IL7/IL2R CAR expression on the cell surface

(A) HEK 293T cells were transfected by the CD28ΔLCK-ζ-IL7/IL2R CAR, then the CAR and the IL-7R expression were detected by the APC-conjugated anti CD127 antibody and the PE-conjugated anti-IgG antibody. **(B)** In human T cells, the CAR expression was detected using the PE-conjugated anti-IgG antibody and for the identification of the T cells the FITC-conjugated anti CD3 antibody was used. Data were recorded by flow cytometry. The percentage represents the cell number in each quadrat.

4.3.3 Cytokine production by CD28ΔLCK-ζ-IL7/IL2R CAR modified T cells

First, we wanted to test the cytokine production of the CAR plus the hybrid receptor engineered T cells. Human T cells were modified with the CD28ΔLCK-ζ-IL7/IL2R and stimulated on plates coated by the immobilized BW2064/36 antigen or the irrelevant mouse IgG antigen in the absence or presence of TGF-β, or IL-7, or both. After 2 days, IFN-γ was measured in the supernatant via ELISA. We observed that TGF-β decreases the IFN-γ secretion, however, the IFN-γ release increased in the presence of IL-7. Without CAR stimulation no IFN-γ production was measured (Figure 35A). The CD28ΔLCK-ζ-IL7/IL2R CAR T cells upon CAR stimulation did not release IL-2. Not transduced cells and T cells with the wild type CAR are also shown as control. IL-2 secretion was not detected in the culture supernatant of T cells engineered with ΔLCK CAR plus the hybrid cytokine receptor, which is what we expected because of the presence of the mutations in the LCK binding site (Figure 35B).

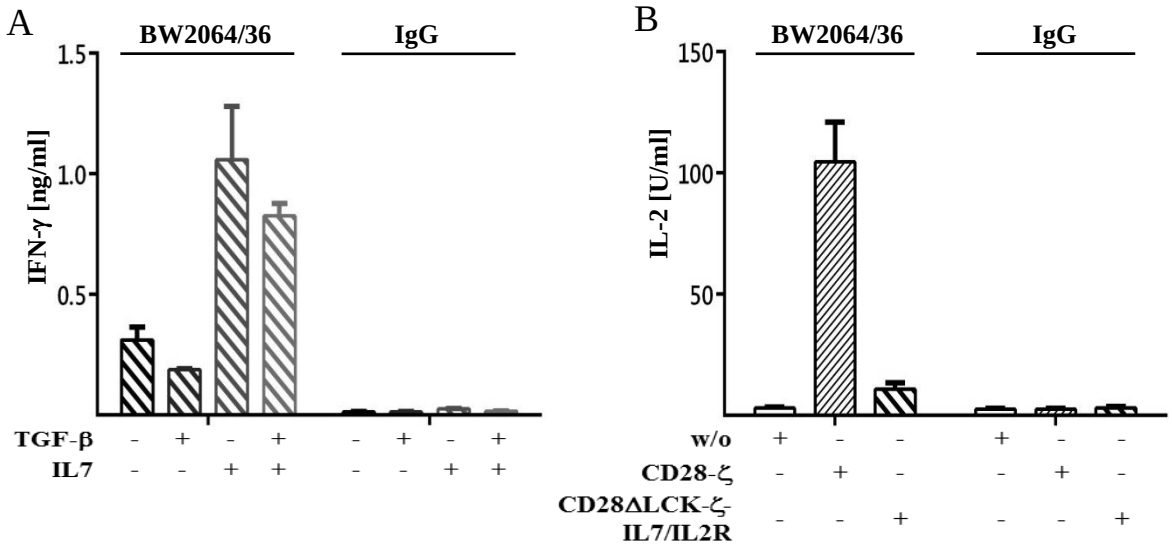


Figure 35 Cytokine production by CD28ΔLCK-ζ-IL7/IL2R CAR modified T cells

(A) IL-7 enhances IFN-γ secretion by CAR modified T cells in the presence of IL7/IL2R
T cells were engineered by CD28ΔLCK-ζ CAR with the hybrid IL-7/IL2 receptor and incubated on 96 well plates (2.5 x 10^4 CAR T cells pro well) to stimulate through the CAR by the engagement of immobilized BW2064/36 antigen or with mouse IgG as irrelevant antigen (1.5 µg/ml each). The cells were co-incubated with or without TGF-β in the presence or absence of IL-7 (10 ng/ml each). Secreted IFN-γ concentration was measured in the cell culture supernatant with ELISA after 2 days activation.
(B) CD28ΔLCK-ζ-IL7/IL2R CAR T cells do not secrete IL-2 because of the mutation in LCK binding domain
T cells were engineered with the mentioned CAR and stimulated on plates coated with the BW2064/36 antigen or with mouse IgG1 as isotype control. Concentration of IL-2 secreted into the culture supernatants was recorded with ELISA after 2 days. The assays were repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means T cells without CAR, served as control. These diagrams were adapted from the publication of Golumba-Nagy et al., 2018.

4.3.4 TGF-β-mediated inhibition on CD28ΔLCK-ζ-IL7/IL2R-modified T cell proliferation and specific killing can be overcome by administration of IL-7

We investigated the proliferative ability of T cells modified with the ΔLCK CAR plus the IL7/IL2 chimeric receptor. After the retroviral transduction the cells were stained with CFSE to track the proliferation. The successful CFSE staining of the remaining population was verified one day later by flow cytometry (Figure 36).

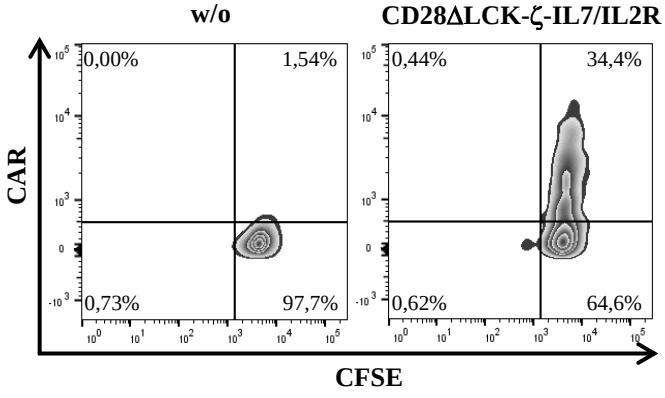


Figure 36 CFSE labeling of CAR modified T cells

Human T cells were modified by CD28ΔLCK-ζ-IL7/IL2R CAR and labeled with CFSE to track the proliferation of the cells. One day after the CFSE staining, the remaining cells without stimulation were analyzed by flow cytometry to verify the successful labeling. CAR receptor was stained with the PE-conjugated anti-IgG antibody which binds to the CAR in the extracellular IgG1 spacer region. The percentage represents the cell number in each quadrat, w/o means T cells without CAR.

The cells were stimulated on plates coated with the immobilized BW2064/36 antibody for 4 days in the presence or absence of TGF-β, IL-7, or both. TGF-β inhibited the proliferation, which was overcome by the presence of IL-7 (Figure 37).

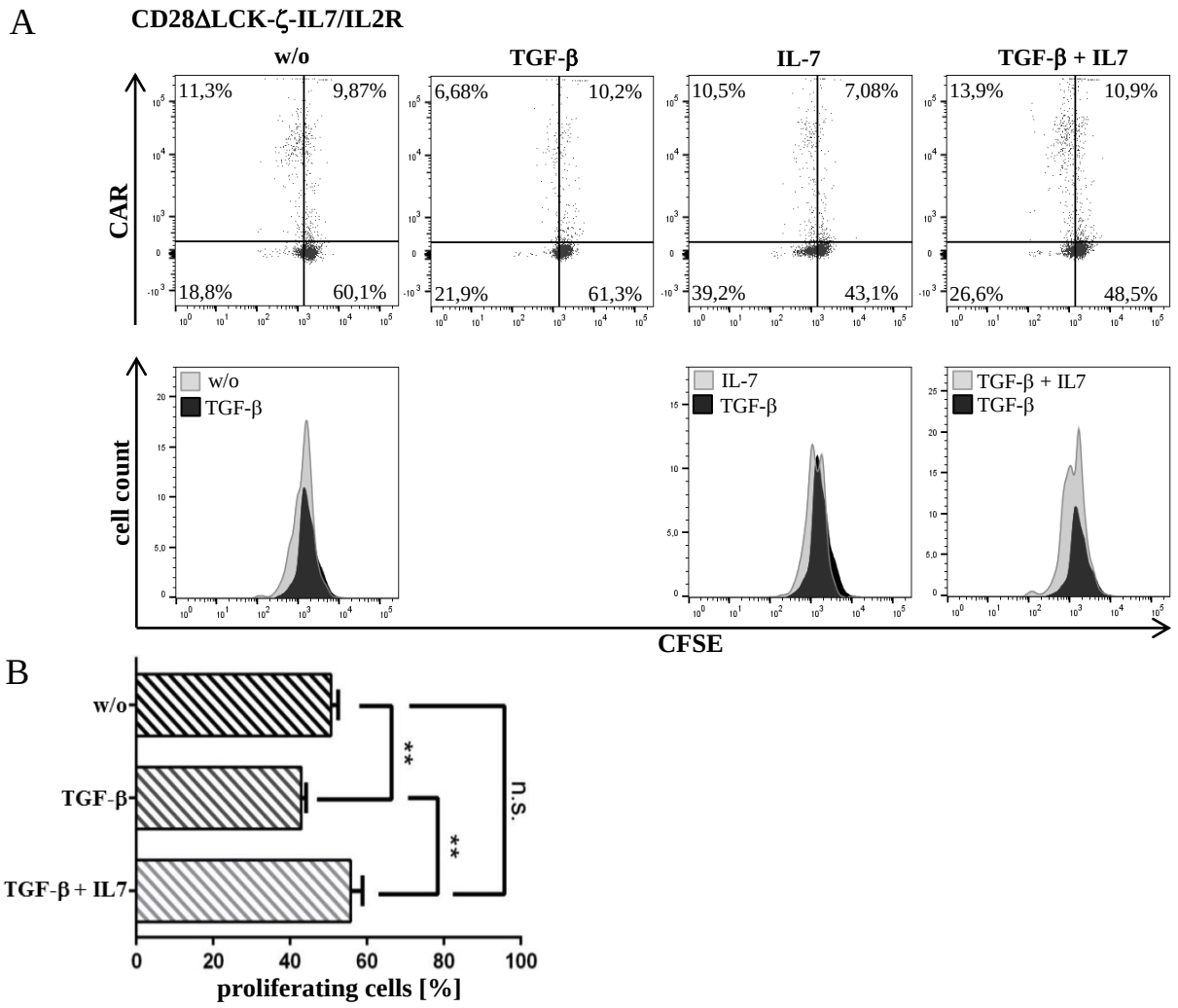


Figure 37 TGF-β-mediated inhibition on CD28ΔLCK-ζ-IL7/IL2R-modified T cell proliferation can be overcome by administration of IL-7

(A) CD28ΔLCK-ζ CAR engineered T cells with the hybrid IL-7/IL2R receptor (2.5 x 10^4 T cells per well) were stained with CFSE and incubated with or without TGF-β or TGF-β plus IL-7 (10 ng/ml each) on BW2064/36 coated plates (1.5 µg/ml) for 4 days. T cell proliferation was recorded by flow cytometry through monitoring the CSFE dilution, while the CAR^+ T cells were identified by the staining with the PE-conjugated anti-IgG antibody. The percentage represents the cell number in each quadrat. The histogram shows the CAR^+, CFSE labeled cells. **(B)** The data represented on the diagram are the proliferated CAR^+ T cells, comparing the incubation with or without TGF-β or TGF-β + IL-7. The assay was repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means without cytokines, served as control. Statistical analyses were executed using a two-tailed Student´s t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The diagram B was adapted from the publication of Golumba-Nagy et al., 2018.

The specific cytotoxicity of the modified T cells was measured in the presence or absence of TGF-β, IL-7, or both. The cells were incubated with the CEA^+ LS174T tumor cells for 2 days and the specific killing was determined by the XTT-based viability assay. TGF-β inhibited the modified T cell-mediated killing compared to the situation where IL-7 is also present. In the presence of IL-7, the modified T cells achieved the same efficiency in killing, as without TGF-β (Figure 38).

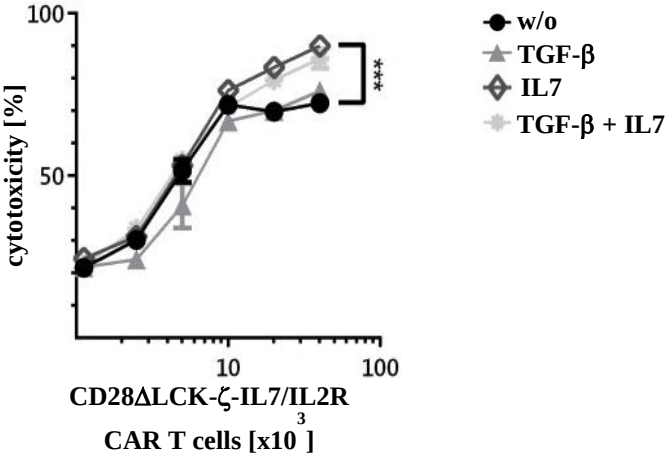


Figure 38 TGF-β inhibits the specific cytotoxicity of CD28ΔLCK-ζ-IL7/IL2R modified T cells, which can be overcome by administration of IL-7

T cells with CD28ΔLCK-ζ-IL7/IL2R CAR (0.125 x 10^4 – 4 x 10^4 CAR T cells per well) were incubated in the presence or absence of TGF-β or IL-7 with or without added TGF-β (10 ng/ml each) with the CEA^+ LS174T cells (2 x 10^4 tumor cells per well). The specific cytotoxicity was determined by the XTT-based viability assay after 2 days. The assay was repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means without cytokines, served as control. The figure was taken from the publication of Golumba-Nagy et al., 2018.

Taken together, the chimeric IL-7 receptor, along with added IL-7, maintains CAR T cell proliferation in the presence of TGF-β.

4.3.5 Generation of the pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL7-T2A-IL7Rα/IL2Rβ (#1908)

In order to complete the artificial autocrine loop, we added a transgenic IL-7 expression cassette to the construct bearing the ΔLCK CAR plus the earlier described hybrid IL7/IL2 receptor. Due to this setup we expect CAR T cells to produce IL-7 upon stimulation *via* the CAR, which in turn binds to the hybrid IL-7 receptor and transmits an IL-2 signal into the CAR T cells through the IL-2 receptor β-chain (Figure 39).

For this purpose, BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL7-T2A-IL7Rα/IL2Rβ (#1908) was generated from the vector #1645 by molecular cloning. The DNA sequence for the hybrid receptor consisting of the IL-7 receptor α-chain for the extracellular region and the

IL-2 receptor β-chain as the transmembrane and intracellular regions was ordered as gene fragment (gBlock) with a KpnI and a BspEI restriction sites at fragments´s ends, respectively. Moreover, the DNA sequence for IL7-T2A-IL7Rα with the restriction sites BclI and KpnI at each end, respectively, were ordered as Gene Fragment (gBlock) from IDT. They were first cloned into the pJet vector restricted by EcoRV (creates blunt ends) for sequencing and then further cloned between the BclI and BspEI sites of the pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-IL7 CAR (#1645) (Figure 39).

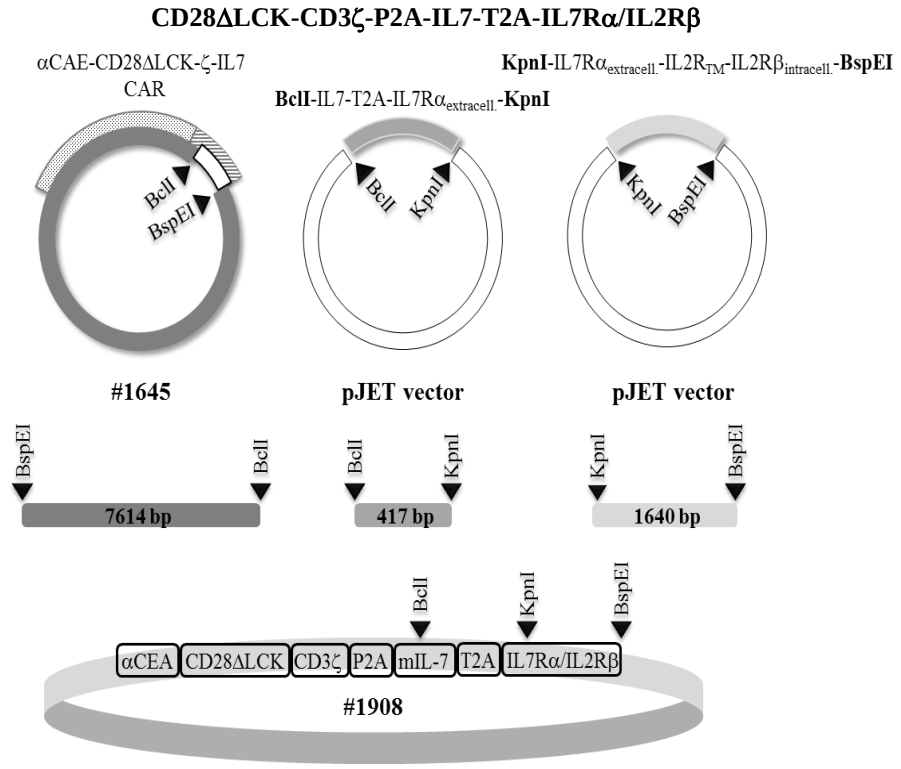


Figure 39 Schematic demonstration of a pBullet vector containing the BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL7-T2A-IL7Rα/IL2Rβ CAR (#1908)

Cloning strategy of CD28ΔLCK-CD3ζ-P2A-IL7-T2A-IL7Rα/IL2Rβ CAR.

The CAR receptor was efficiently expressed on the T cell surface after retroviral transduction. Moreover, the CAR and the transgenic IL-7R α-chain (CD127) expressed on the cell surface of transfected 293T cells. The CAR and the IL-7R were detected by flow cytometry (Figure 40AB).

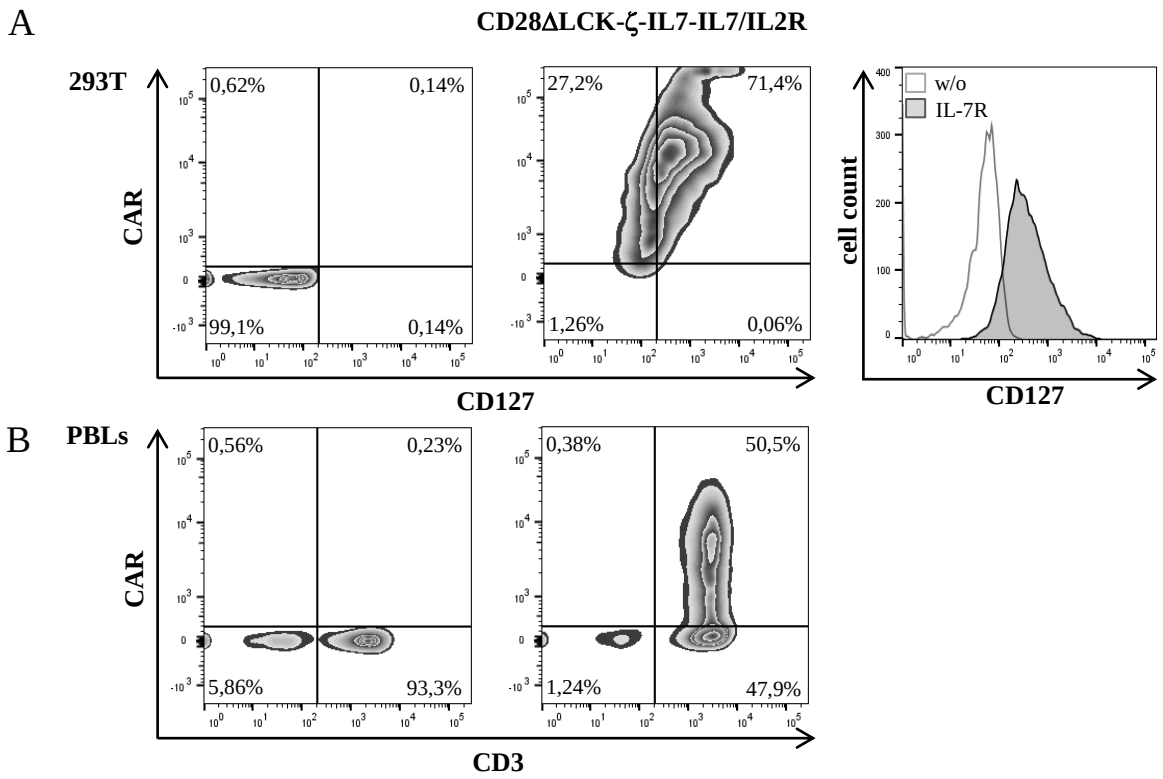


Figure 40 CD28ΔLCK-ζ-IL7-IL7/IL2R CAR expression on the cell surface

(A) HEK 293T cells were transfected by CD28ΔLCK-ζ-IL7-IL7Rα/IL2Rβ CAR, then the CAR and IL-7R expression were detected by the APC-conjugated anti CD127 antibody and the PE-conjugated anti-IgG antibody. **(B)** In human T cells, the CAR expression was detected using the PE-conjugated anti-IgG antibody, while for the identification of the T cells the FITC-conjugated anti CD3 antibody was used. Data were recorded by flow cytometry. The percentage represents the cell number in each quadrat.

4.3.6 IFN-γ and IL-7 secretion by T cells are modified by the CD28ΔLCK-ζ-IL7-IL7/IL2R CAR

First, we tested the cytokine production of the CAR modified T cells. Human T cells were transduced with CD28ΔLCK-ζ-IL7-IL7/IL2R CAR and were stimulated on plates coated with the immobilized BW2064/36 antigen or with irrelevant mouse IgG antigen as control. For further control non-transduced T cells and T cells with the wild type CAR are also shown. IFN-γ and IL-7 were detected in the supernatants by ELISA after two days of incubation. IFN-γ release by the new receptor modified T cell was very similar to that by CD28-ζ CAR modified T cells. As expected, IL-7 was released by the CD28ΔLCK-ζ-IL7-IL7/IL2R CAR modified T cells, while the T cells with the wild type CAR did not produce IL-7 (Figure 41).

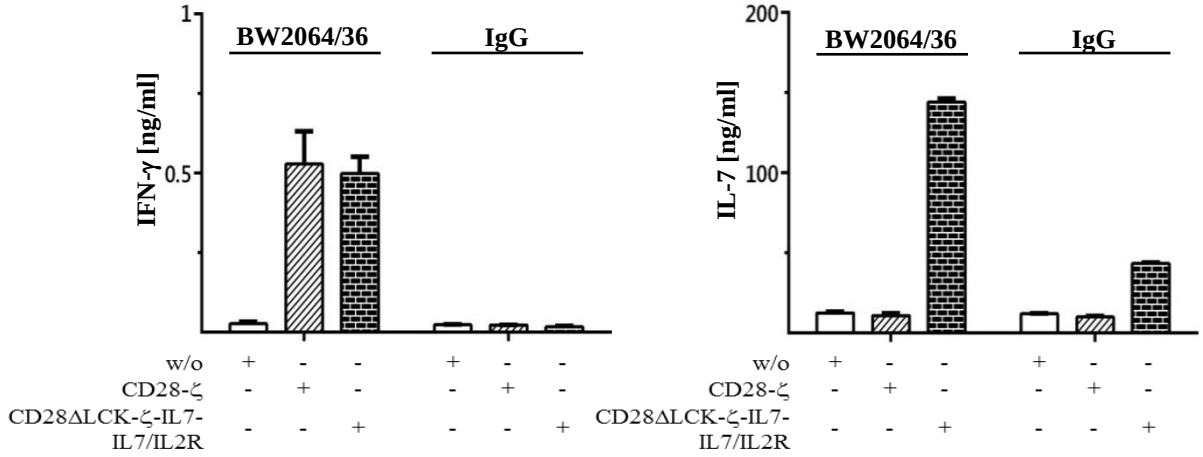


Figure 41 IFN-γ and IL-7 secretion by T cells are modified by the CD28ΔLCK-ζ-IL7-IL7/IL2R CAR

T cells were engineered by CD28ΔLCK-ζ CAR with the hybrid IL-7/IL2R receptor plus transgenic IL-7 and incubated on 96 well plates (2.5 x 10^4 CAR T cells pro well) to stimulate through the CAR by engagement of immobilized antigen BW2064/36 or with mouse IgG as irrelevant antigen (1.5 µg/ml each). Secreted IFN-γ and IL-7 concentration were measured in the culture supernatants of cells with ELISA after 2 days activation The assays were repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown,w/o means T cells without CAR, served as control. These diagrams were adapted from the publication of Golumba-Nagy et al., 2018.

4.3.7 TGF-β had no effect on T cell proliferation or specific cytotoxicity when T cells were modified with the CD28ΔLCK-ζ-IL7-IL7/IL2R CAR

Proliferation of CD28ΔLCK-ζ-IL7-IL7/IL2R CAR modified T cells was investigated after 2 days of stimulation by the immobilized BW2064/36 antibody in the presence or absence of TGF-β. The successful CFSE staining was verified one day after the labeling via flow cytometry (Figure 42).

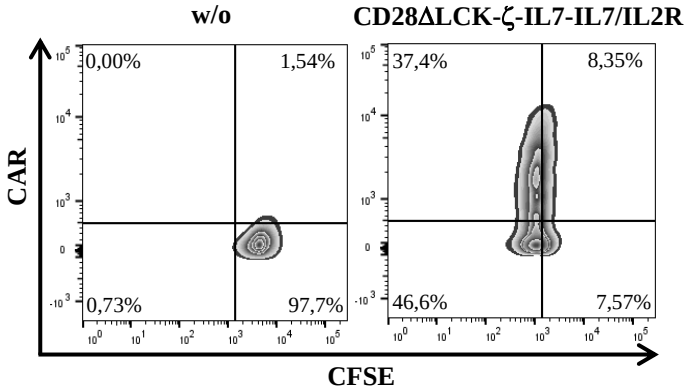


Figure 42 CFSE labeling of CAR modified T cells

Human T cells were modified with the CD28ΔLCK-ζ-IL7-IL7/IL2R CAR and labeled with CFSE to track the proliferation of the cells. One day after the CFSE staining, the remaining cells without stimulation were analyzed by flow cytometry to verify the successful labeling. CAR receptor was stained with the PE-conjugated anti-IgG antibody which binds to the CAR in the extracellular IgG1 spacer region. The percentage represents the cell number in each quadrat, w/o means T cells without CAR.

TGF-β had no effect on T cell proliferation; CAR engineered T cells proliferated with TGF-β as efficient as without TGF-β (Figure 43). The inhibitory effect of TGF-β was overcome by the secreted IL-7 through the artificial autocrine loop.

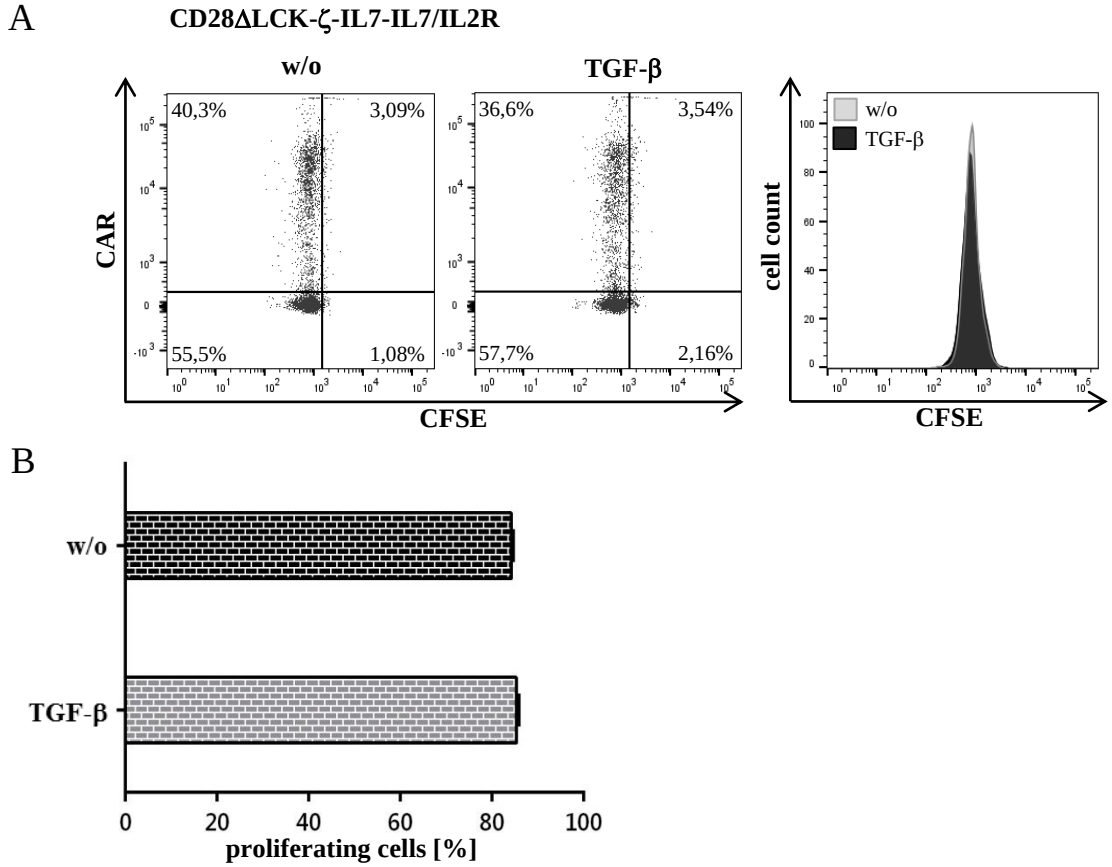


Figure 43 TGF-β had no effect on T cell proliferation when T cells were modified with the CD28ΔLCK-ζ-IL7-IL7/IL2R CAR

(A) CD28ΔLCK-ζ-IL7-IL7/IL2R CAR engineered T cells (2.5 x 10^4 T cells per well) were stained with CFSE and incubated with or without TGF-β (10 ng/ml) on BW2064/36 coated plates (1.5 µg/ml) for 2 days. T cell proliferation was recorded by flow cytometry through monitoring the CSFE dilution, while the CAR^+ T cells were identified by the staining with the PE-conjugated anti-IgG antibody. The percentage represents the cell number in each quadrat. The histogram shows the CAR^+, CFSE labeled cells. **(B)** The data represented on the diagram are the proliferated CAR^+ T cells, comparing the incubation with or without TGF-β. The assay was repeated three times, the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means without cytokines, served as control. The diagram B was adapted from the publication of Golumba-Nagy et al., 2018.

The specific cytotoxicity of the new tricistronic vector was tested with the CEA^+ LS174T tumor cells in the presence or absence of TGF-β. The modified T cells were incubated with the tumor cells for 2 days then the XTT-based viability assay was performed. TGF-β has shown no effect on the specific killing of antigen positive tumor cells by the CD28ΔLCK-ζ-IL7-IL7/IL2R CAR engineered T cell (Figure 44).

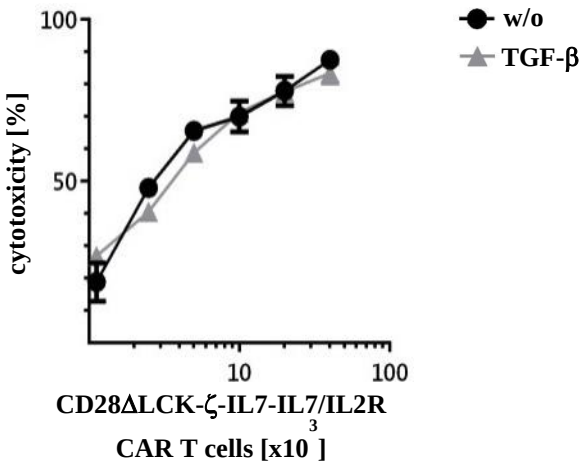


Figure 44 TGF-β has no effect on the specific cytotoxicity of T cells in the presence of self-produced IL-7 and the hybrid IL7/IL2R

T cells with CD28ΔLCK-ζ-IL7-IL7/IL2R CAR (0.125 x 10^4 – 4 x 10^4 CAR T cells per well) were incubated in the presence or absence of TGF-β (10 ng/ml) with the CEA^+ LS174T cells (2 x 10^4 tumor cells per well). The specific cytotoxicity was determined by the XTT-based viability assay after 2 days. The assay was repeated three times; the data demonstrate a representative assay and the mean of triplicates ± standard deviation (S.D.) is shown, w/o means without cytokines, served as control. The figure was taken from the publication of Golumba-Nagy et al., 2018.

4.3.8 CD28ΔLCK-ζ-IL7-IL7/IL2R showed superior activity as wild type CAR in the presence of TGF-β in the long term

To test the ability of the CAR modified T cells to overcome suppression by TGF-β in the long term, the cells were stimulated by repetitive antigen engagement. For this purpose, T cells were engineered either with the wild type CAR or, with the ΔLCK CAR, or with the ΔLCK CAR plus transgenic IL-7 and the hybrid receptor. The cells were cultivated with the CEA^+ LS174T tumor cells for 2 days. Fresh tumor cells were added every two days to the CAR modified T cells until day 6 to stimulate them repetitively (Figure 45A). In this manner, specific serial killing was recorded. CD28ΔLCK-ζ-IL7-IL7/IL2R CAR T cells showed superior cytolytic activity compared to T cells with wild type or the ΔLCK CAR without the autocrine IL-7 loop (Figure 45B).

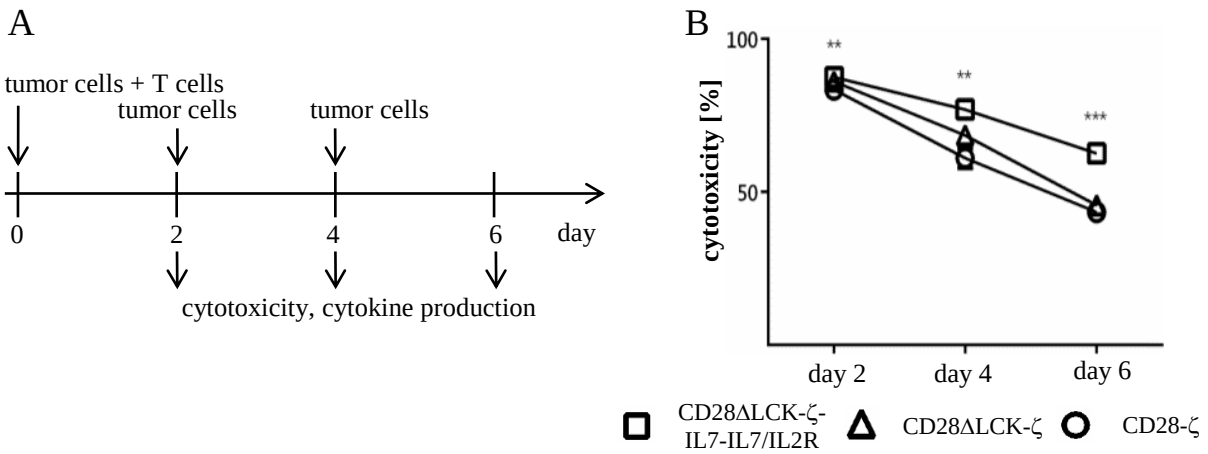


Figure 45 CD28ΔLCK-ζ-IL7-IL7/IL2R showed superior activity as wild type CAR in the presence of TGF-β in the long term

(A) Schematic timeline demonstrates the serial killing assay, addition of target cells, the cytotoxicity and cytokine readings are labeled. **(B)** T cells modified with appropriate CAR (2 x10^4 CAR^+ T cells per well) were incubated with CEA^+ LS174T cells (2 x 10^4 cells per well) and specific cytotoxicity was determined by the XTT-based viability assay after 2 days. The cells from a parallel assay were harvested and incubated again with fresh CEA^+ LS174T cells (2 x 10^4 cells per well) for 2 more days; the same procedure was repeated again until day 6. The data represent the mean of triplicates ± standard deviation (S.D). Statistical analyses were executed using a two-tailed Student´s t-test (*p < 0.05; **p < 0.01; ***p < 0.001), where CAR T cells with IL-7 loop compared with CD28-ζ CAR T cells. The figures were taken from the publication of Golumba-Nagy et al., 2018.

4.3.9 Cytokine production after repetitive stimulation by target cells

After repetitive killing of tumor cells, cytokine concentration in the supernatant was measured every two days. IFN-γ was decreased during the time. However, CD28-ζ CAR T cells produced the same amount of IFN-γ until day 4. Decreasing IL-2 production was measured compared with the wild type CAR, while IL-7 production by the CD28ΔLCK-ζ-IL7-IL7/IL2R CAR T cells remained constant (Figure 46).

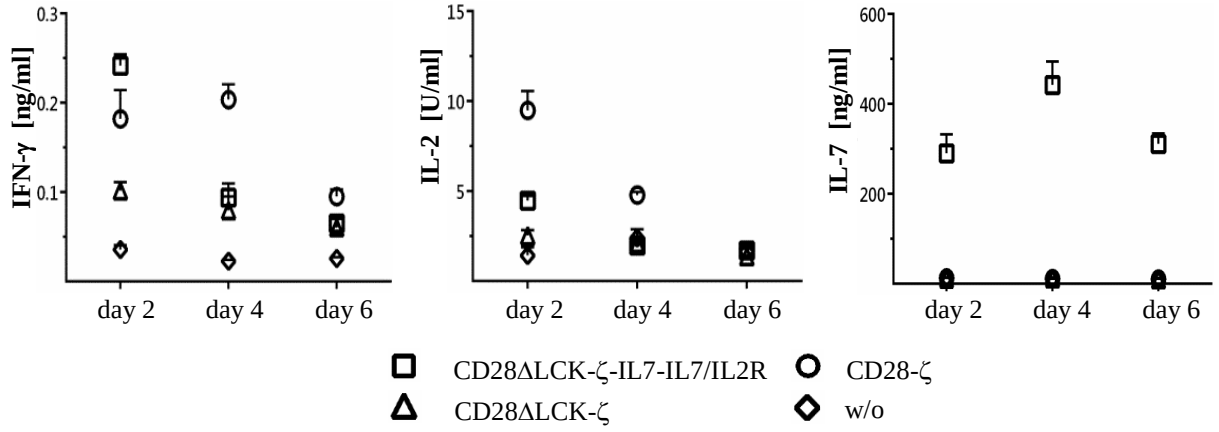


Figure 46 Cytokine production after repetitive stimulation by target cells

Secreted IFN-γ, IL-2 and IL-7 concentration were measured in the cell culture supernatant every two days until day 6 with ELISA after repetitive stimulation with target cells. The data represent the mean of triplicates ± standard deviation (S.D). The diagrams were taken from the publication of Golumba-Nagy et al., 2018.

In conclusion, the chimeric cytokine receptor plus IL-7 release besides the CD28ΔLCK CAR expression made the engineered T cells resistant against TGF-β, not only for a short period, but also for a long term.

4.3.10 Comparative analysis of CAR modified T cells with different functionality assays

4.3.10.1 Intracellular staining of Granzyme B

Human T cells were modified with the wild type, or the CD28ΔLCK-ζ CAR with or without the IL-7 autocrine loop, and incubated on BW2064/36-coated plate for CAR stimulation in the presence or absence of TGF-β. Stimulation with isotype mouse IgG antibody served as control. After two days the produced Granzyme B (Gr B) was measured in the CAR^+ T cell population *via* flow cytometry. There was no difference in the produced amount of Gr B between the different CAR receptor bearing T cells in the absence of TGF-β, while with TGF-β the Gr B level was decreased in the presence of the mutation in the LCK binding site within the CD28 domain. However, the MFI (mean fluorescence intensity) remained the same in all cases independent of the presence of TGF-β (Figure 47).

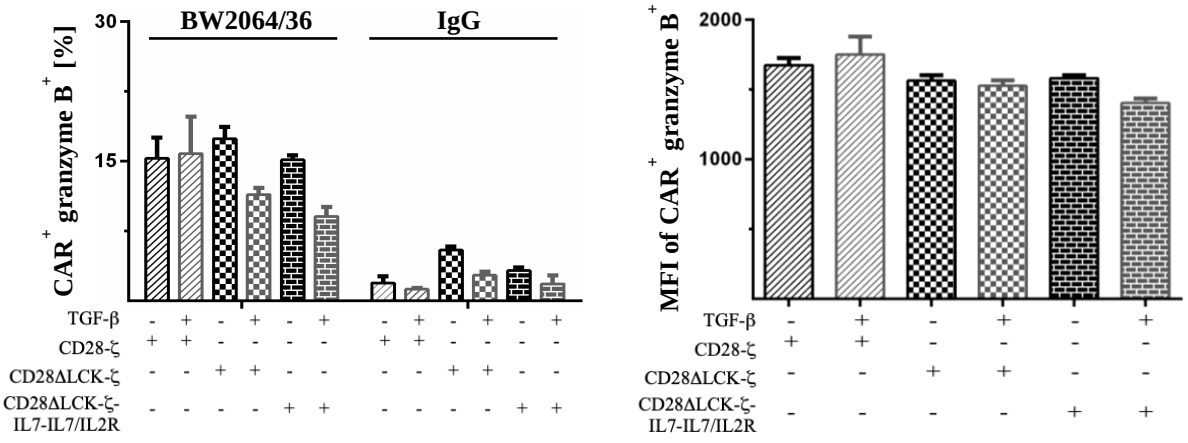


Figure 47 Intracellular staining of granzyme B

T cells with different CAR were incubated on 96 well plates (2.5 x 10^4 CAR^+ T cells pro well) coated with the anti-idiotypic mAb BW2064/36 or mouse IgG1 (1.5 µg/ml each) as an isotype control in the presence or absence of TGF-β (10 ng/ml) for 2 days. CAR^+ T cells were stained with a PE-conjugated anti-human IgG antibody, while granzyme B was labeled with the FITC-conjugated anti-granzyme B antibody. The assay was repeated three times. The data represent the mean of triplicates ± standard deviation (S.D). The figure demonstrates a representative assay. The diagrams were adapted from the publication of Golumba-Nagy et al., 2018.

4.3.10.2 Quantification of apoptotic and living cells after antigen-driven CAR activation

CAR-modified T cells were stimulated on plates coated with BW2064/36 antibody. After 2 days of activation, the cells were harvested. In order to identify the apoptotic and living cells in the CAR^+ T cell population, the cells were stained with Annexin V to label the apoptotic

cells, and with 7-AAD to exclude dead cells. The measurement was performed using flow cytometry. The number of living CD28ΔLCK-ζ-IL7-IL7/IL2R CAR T cells was substantially higher than the number of the wild type CAR modified T cells, while the number of apoptotic cells was higher in the case of CD28ΔLCK-ζ CAR modified T cells (Figure 48). The improved survival of the CD28ΔLCK-ζ CAR with the IL-7 autocrine loop could lead to an improved elimination of cancer cells in the long term (Figure 45).

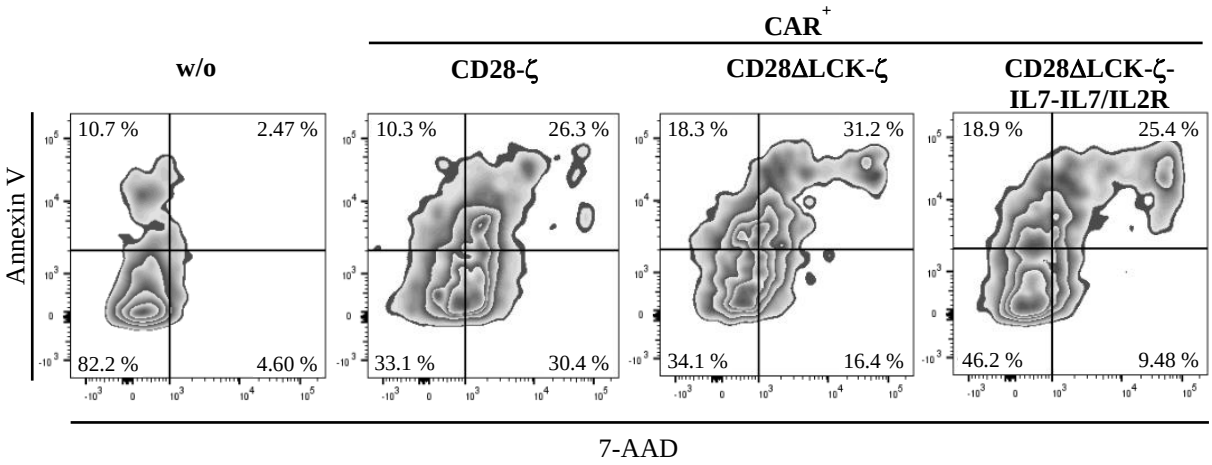


Figure 48 Quantification of apoptotic and living cells after antigen-driven CAR activation

CAR modified T cells were activated on plates coated with the anti-idiotypic mAb BW2064/36 for 2 days. APC-conjugated AnnexinV and 7-AAD staining was used to identify apoptotic and living cells. Hereby the demonstrated cells are gated for CAR^+ cells; the percentages in each quadrat represent the cell number. The assay was repeated three times, the data demonstrate a representative assay, w/o means T cells without CAR. These figures were taken from the publication of Golumba-Nagy et al., 2018.

4.3.10.3 Intracellular staining of pSTAT5

After 30 minutes of antigen engagement with the immobilized BW2064/36 mAb to stimulate the T cells through their CAR, early antigen-mediated T cell activation was detected by STAT5 phosphorylation. Increase in phosphoSTAT5 levels was recorded in T cells with the CD28ΔLCK-ζ -IL7-IL7/IL2R CAR, which was not detectable in the case of the wild type CAR, nor the CD28ΔLCK-ζ CAR modified T cells without the IL-7 autocrine loop. After 16 hours activation through the CAR receptor, increased level of pSTAT5 was detected in all cases. We speculate, that this finding hints at the existence of other STAT5 activation pathways in the complex intracellular signaling matrix (Figure 49).

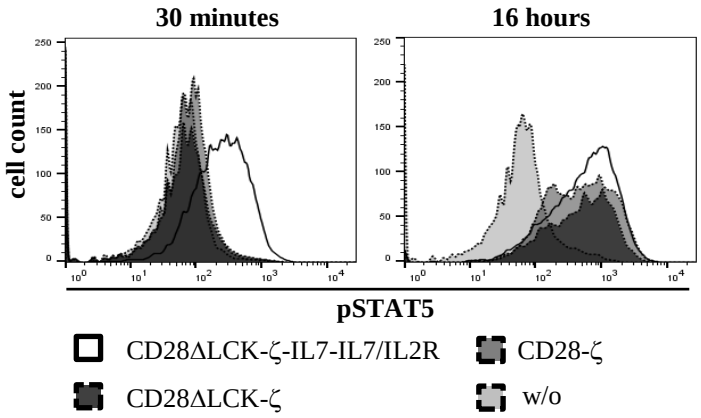


Figure 49 Intracellular staining of pSTAT5

CAR modified T cells were incubated on plates (3.5 x 10^5 CAR T cells pro well) coated with the anti-idiotypic mAb BW2064/36 (1.5 µg/ml each) for 30 minutes or for 16 hours, then the cells were harvested and stained with a PE-conjugated anti-pSTAT5 antibody. T cells without CAR (w/o) served as control. On the histogram the PE-conjugated pSTAT5 staining of the respective cells are demonstrated. The assay was repeated three times, the data show a representative assay. The figures were taken from the publication of Golumba-Nagy et al., 2018.

Taken together and in comparison to the wild type CAR-modified T cells, the CD28ΔLCK-ζ-IL7-IL7/IL2R CAR-modified T cells show advanced T cell function in survival and in early antigen-mediated T cell activation, as well as improved killing of antigen positive tumor cells in the long term.

4.4 In vivo anti-tumor activity of CD28ΔLCK-ζ-IL7-IL-7/IL-2R CAR T cells

4.4.1 Improved anti-tumor activity of CAR T cells with IL-7 autocrine loop in immunodeficient mice

We investigated the novel ΔLCK CAR with transgenic IL-7 and hybrid IL7/IL2 receptor in comparison with the CD28ΔLCK-ζ CAR without IL-7 loop *in vivo*. In the cause of this, $Rag2^{-/-}$ $\gamma c^{-/-}$ immunodeficient mice with established tumor were treated with CAR T cells. Firstly, TGF-β^+ CEA^+ C15A3 tumor cells (1 x 10^6 cells per mouse; Figure 50) were subcutaneously inoculated into the mice and the modified T cells were intravenously injected (1.5 x 10^6 CAR T cells per mouse) at day 16, when the tumor size reached about 200 mm^3.

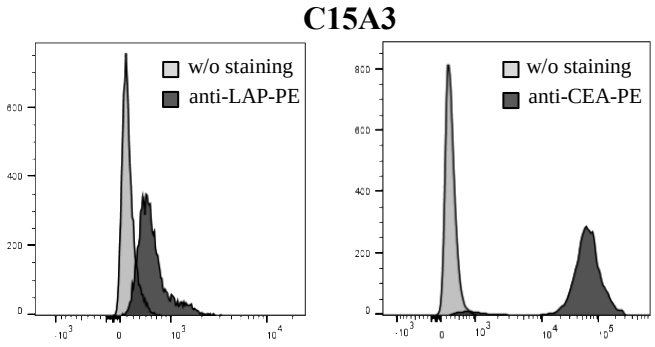


Figure 50 Staining of TGF-β and CEA in C15A3 tumor cells

C15A3 tumor cells were labeled for TGF-β with a PE-conjugated anti-LAP antibody, while for CEA with a PE-conjugated anti-CEA antibody. Tumor cells without staining are shown as control.

The mice were divided into four groups. The control groups were treated either without T cells (w/o T cells), or with T cells without CAR (w/o CAR). The mice treated with CD28ΔLCK-ζ CAR T cells with IL-7 autocrine loop showed slowed tumor progression compare to those treated with CD28ΔLCK-ζ CAR T cells without IL-7 loop. T cells with ΔLCK CAR had no major effect on TGF-β^+ tumor progression (Figure 51A). Two weeks after T cell injection, a significant difference was detected between the two groups of mice treated with CAR T cells with or without IL-7 autocrine loop (Figure 51B).

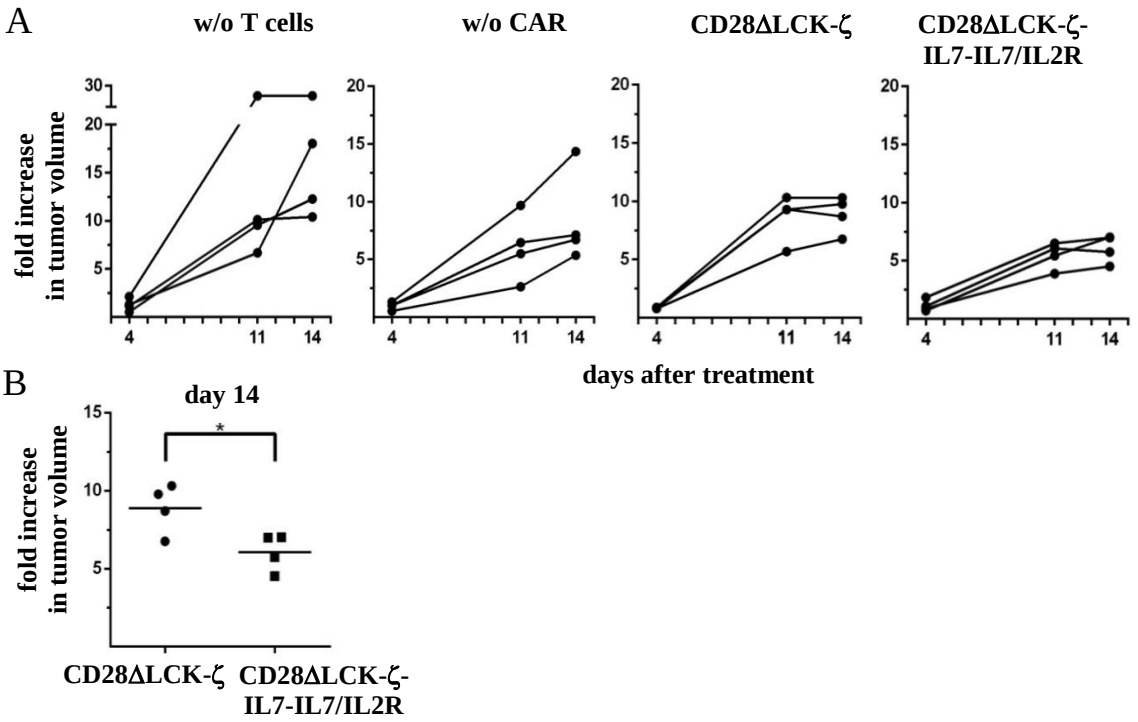


Figure 51 Improved anti-tumor activity of CAR T cells with IL-7 autocrine loop in immunodeficient mice

(A) Rag2$^{-/-}$ γc$^{-/-}$ mice (4 mice per group) were subcutaneously inoculated with TGF-β^{+} CEA^{+} C15A3 tumor cells (10^6 cells per mouse). T cells were modified with the CD28ΔLCK-ζ CAR or additionally with CD28ΔLCK-ζ CAR with IL-7 loop. On day 16, when the tumor size reached about 200 mm^3, the engineered T cells were applied by intravenous injection (1.5 x 10^6 CAR T cells per mouse). Mice without T cells (w/o T cells) or with T cells without CAR (w/o CAR) served as controls. Tumor growth after T cell injection was weekly observed. **(B)** After T cell injection on day 14, the relative tumor growth of mice is shown regarding to the mice recevied CAR modified T cells. Tumor growth at day 14 was referred to the tumor growth at day the treatment with CAR modified T cells was started. Statistical analyses were performed using the two-tailed Student´s t-test (*p < 0.05). The figures were taken from the publication of Golumba-Nagy et al., 2018.

4.4.2 Improved persistence of CAR T cells with IL-7 autocrine loop at the tumor site

The mice were sacrificed at the end of all experiments, and the tumors were used for microscopy imaging to detect CAR^{+} T cell persistence in the tumor tissue. We found more remaining CAR+ T cells in the tumor tissue samples derived from mice treated with CD28ΔLCK-ζ-IL7-IL7/IL2R CAR^{+} T cells, than in the samples derived from mice treated with CD28ΔLCK-ζ CAR+ T cells. This finding is consistent with the improved anti-tumor effect of the CD28ΔLCK-ζ-IL7-IL7/IL2R CAR^{+} T cells observed in the *in vivo* experiment (Figure 52).

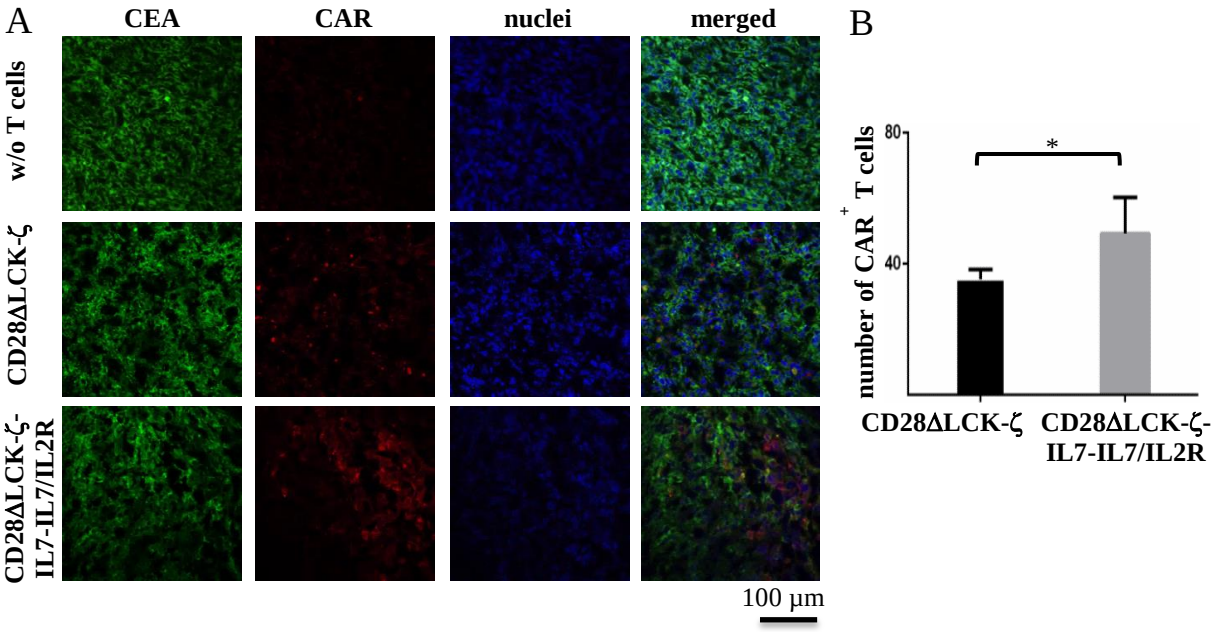


Figure 52 Improved persistence of CAR T cells with IL-7 autocrine loop at the tumor site

(A) CAR^+ T cells were detected in tumor tissue by labeling of CAR receptor on the T cells with AlexaFluor 555-conjugated anti-human IgG antibody (dilution 1:250); the tumor cells were stained with AlexaFluor 488-conjugated anti-CEA (aCD66a/c/e) antibody (dilution 1:50), while cell nuclei was labeled by "Reddot2" (dilution 1:200). Scale bar represents 100 µm. **(B)** CAR^+ T cells was recorded in tumor tissue slides in microscopy imaging. The figures were taken from the publication of Golumba-Nagy et al., 2018.

In order to verify the existed suppressive tumor microenvironment mediated by TGF-β, we stained tumor cells derived TGF-β in the tumor tissue samples. There was no difference in the level of TGF-β at the tumor site in the four groups therefore the same suppressive effect was expected in all cases (Figure 53).

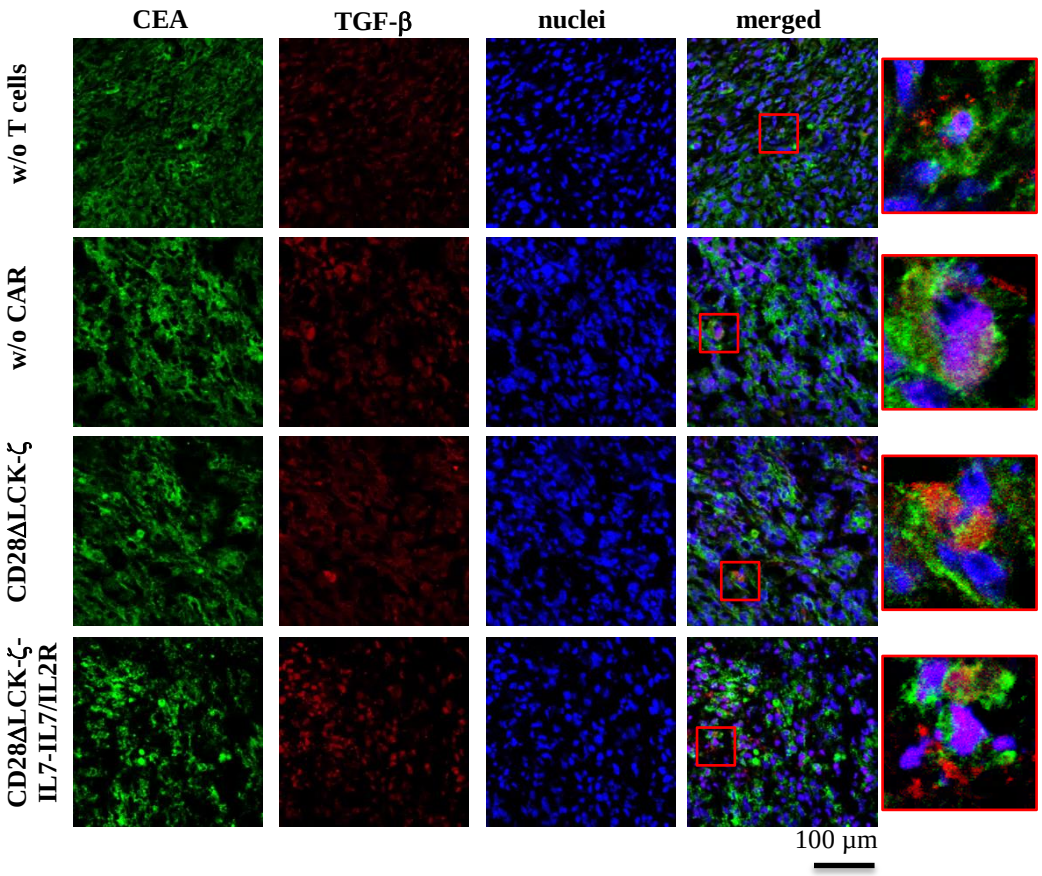


Figure 53 Detection of TGF-β at the tumor site

TGF-β was detected in tumor tissue using the anti-TGF-β antibody (dilution 1:50) and AlexaFluor 555-conjugated anti-mouse IgG (H+L) antibody (dilution 1:200); tumor cells were distinguished with the AlexaFluor 488-conjugated anti-CEA (aCD66a/c/e) antibody (dilution 1:50), cell nuclei were identified with DAPI. Scale bar represents 100 µm. The figures were taken from the publication of Golumba-Nagy et al., 2018.

5 DISCUSSION

The innovation of the second generation CAR contributed to the success of adaptive CAR T cell therapy. Adding one or more costimulatory domains to the CAR structure promoted CAR engineered T cell function such as longer persistence, better ability for cytokine secretion and enhanced specific anti-tumor effects (Kowolik et al., 2006). This is especially true in case of solid tumors, where the tumor-antigen specific T cells have a hard time destroying the tumor cells because of the immunesuppressive tumor microenvironment. TGF-β is a frequent tumor resident suppressive cytokine, which is in most cases responsible for the decreased T cell activity in the tumor milieu. The advanced proliferative capacity of second generation CAR T cells in the presence of TGF-β has been shown earlier for CAR T cells with CD28 as costimulatory domain (Koehler et al., 2007). However, the background mechanisms behind the compensatory effect of the CD28 domain in the TGF-β-mediated suppression were unsolved. The primary object of this study was to disclose the mechanism.

In the absence of costimulatory domains, CAR T cell proliferation is strongly inhibited in the presence of TGF-β (Figure 5). Among other CAR T cell functions such as CAR activation-mediated cytokine secretion or specific killing against antigen positive target cells, the CAR T cell amplification was mostly concerned by the inhibition through TGF-β. Therefore, we used T cell proliferation as primary marker for T cell activity in the presence of TGF-β.

CD28 signaling promoted to the overcoming of the TGF-β-mediated inhibitory effect. We tested two 2nd generation CARs either with the CD28 or the 4-1BB costimulatory domains. The use of different costimulatory domains can have various effect on T cell function: while CD28 has a role in early T cell activation, 4-1BB promotes a long-term activation of T cells (Hombach and Abken, 2011). The mostly used costimulatory domains in CAR applied therapy are the CD28, 4-1BB and OX40 domains. According to Brentjens et al., the CD28 costimulatory domain in combination with CD3ζ domain are testified to be the best in comparison to other combination (Brentjens et al., 2007).

The proliferation ability of CAR-modified T cells with costimulatory domain was analyzed in the presence and absence of TGF-β. While the CD28 domain was protective for CAR T cells in the presence of TGF-β, the 4-1BB domain had no advantages in this process (Figure 9). We speculated that the different cytokine production profiles could be responsible for these divergent attitudes. CD28 is induces IL-2 secretion (Powell et al., 1998; Thomas et al., 2005),

which promotes T cell proliferation. In contrast, CAR T cells with 4-1BB domain release lower amounts of IL-2 (Figure 10). In the presence of TGF-β, IL-2 and IFN-γ production were inhibited in the case of CAR T cells with either of both costimulatory domains. Such observation regarding to the suppressive effect of TGF-β on cytokine production was described earlier (Brabletz et al., 1993; Gorelik and Flavell, 2002; Yoshimura et al., 2010).

In order to explore which CD28 function is responsible for the resistance towards TGF-β-mediated inhibition, we mutated either the LCK or the PI3K binding motif of CD28, or in both. LCK activation is involved a signaling cascade which promotes IL-2 release (Lovatt et al., 2006). For this reason, we supposed that the mutation in the LCK binding site leads to decreased level in LCK phosphorylation in the ΔLCK CAR-modified T cells (Figure 13). Moreover, this mutation within the CD28 domain caused not only impaired LCK signaling but also a decrease in IL-2 release, while this mutation did not affect the IFN-γ release (Figure 14). In contrast, the mutation in the PI3K binding site did not cause any alteration in IFN-γ or in IL-2 production by CAR engineered T cells. Furthermore, CAR T cell amplification was concerned by TGF-β inhibition in case of the mutation in the LCK binding motif (Figure 16) but not in case of the mutation in the PI3K binding site, which indicates that the PI3K binding site within the CD28 domain is not responsible for the resistance against TGF-β in T cell proliferation. However, it would be interesting to investigate weather other binding part of the CD28 domain has a role in this compensatory effect. We supposed that the lack of IL-2 release could be responsible for the suppressed proliferation of the ΔLCK CAR-equipped T cells. Our assumption was confirmed by the observation that the administration of IL-2 restored the ΔLCK CAR T cell proliferation in the presence of TGF-β (Figure 18). This outcome squarely indicates the central role of IL-2 in TGF-β resistance. Furthermore, the transgenically expressed IL-2 along with the CAR results IL-2 production by CAR-modified T cells and T cell proliferation in the presence of TGF-β. The concept confirmed that autocrine IL-2 release independently from the CAR is also able to overcome the suppressive effect of TGF-β in T cell proliferation (Figure 22). Of note, IL-2 release without stimulation is not occurred (Figure 20).

The presence of IL-2 in the tumor environment leads to the activation of regulatory T cells (Yoshimura et al., 2010), which inhibits effector T cell functions through the production of TGF-β (Somasundaram et al., 2002). In addition, in the presence of both suppressive factors, Treg cells and TGF-β, the use of the second generation CARs with CD28 domain is not

sufficient to overcome the inhibition in T cell proliferation (Kofler et al., 2011). Nonetheless, IL-2 and TGF-β together are needed to generate Tregs with stable suppressive function (Chen et al., 2011). For this purpose, we wanted to replace IL-2 with other cytokines, which have the same properties as IL-2, in order to overcome the inhibitory effect of TGF-β without sustaining Tregs.

We identified IL-7 and IL-15 from the γ-cytokine family, and replaced IL-2 by administering IL-7 or IL-15. IL-7 and IL-15 as well, significantly advanced T cell proliferation in the presence of TGF-β (Figure 23 and Figure 24). The rationale for using these cytokines is their role in T cell homeostasis such as the essential role of IL-7 in T cell development (Rich and Leder, 1995). We took advantage of the TRUCK strategy in order to make the cytokines available for effector T cells at the tumor site by using CAR T cells with transgenic IL-7 or IL-15 release. Otherwise it showed not sufficient to obtain adequate IL-7 or IL-15 concentrations during clinical applications. Our approach is supported by the report that the increased IL-7 production within the tumor microenvironment was provided by dendritic cells (DC), which were modified to produce IL-7 and transferred to tumor site. Such modified DCs augmented T cell proliferation and contributed to enhanced anti-tumor responses (Miller et al., 2000; Westermann et al., 1998). Our IL-7- or IL-15-equipped ΔLCK CAR-modified T cells produced IL-7 or IL-15, respectively, after CAR-mediated stimulation, while IL-2 release was not occurred (Figure 27). IL-7 and IL-15 cytokine releases were activation-dependent, a phenomenon which we also observed with the IL-2 TRUCK. We assumed that the produced cytokines are stored in the intracellular milieu when there is no CAR stimulation. The cytokine production is inducible through CAR engagement, which is an important aspect for clinical applications in order to avoid uncontrolled cytokine release. Furthermore, simultaneous administration of cytokines could lead to toxic side effects (Rosenberg et al., 1989). With the TRUCK approach we achieved the same results in T cell proliferation as with the administration of IL-7 or IL-15. In the presence of TGF-β, the proliferation ability of CAR-modified T cells was not changed in comparison to the situation without TGF-β (Figure 29 and Figure 30).

Our aim was to find another cytokine, which is able to compensate the inhibitory effect of TGF-β but without sustaining Treg activation at the same time. The IL-7 receptor is not expressed on the Treg cell surface; therefore IL-7 is not adequate to activate them. The use of IL-7 is moreover underlined by previous reports, because the Rosenberg group reported that the administration of IL-7 to a human patient with lymphopenia resulted in selective increase

in the number of $CD4^+$ and $CD8^+$ lymphocytes and decrease in Treg numbers (Rosenberg et al., 2006). Furthermore, a local accumulation of IL-7 advances to enhanced anti-tumor response of resident tumor infiltrating T cells (Ditonno et al., 1992). In addition, FoxP3 protein expression in Tregs is a marker for Treg identification. The regulation of FoxP3 is antagonistic to the IL-7 receptor expression. Upregulation of FoxP3 leads to the downregulation of IL-7 receptor α-chain (CD127), which results the lack of IL-7 receptor in Treg cells (Seddiki et al., 2006). In contrast, IL-15 plays a rather activatory role in Treg biology. IL-15 increases the expression of CD25 and FoxP3 in $CD4^+$ T cells and furthermore induces the proliferation of Tregs (Imamichi et al., 2008; Xu et al., 2011). These properties make IL-15 less suitable in this context.

Thus, IL-7 is able to stimulate exclusively effector T cells as long as IL-7 receptor is available on the cell surface. However, the IL-7 receptor expression is downregulated after T cell activation: CD127 transcripts as well as the surface protein are downregulated by IL-7 within a few hours. IL-7 treatment decreases the IL-7 receptor half-life from 24 hours to 3 hours (Henriques et al., 2010). In addition, low levels of IL-7 cause smaller and transient decreases in CD127 protein level, while higher concentrations lead to more profound and sustained IL-7R suppression (Ghazawi et al., 2013). Noteworthy, there is a different regulation of the IL-7 receptor by IL-7 and TCR/CD28 stimulation: After IL-7 treatment and withdrawal, CD127 mRNA and surface protein reappeared within 7 hours, while after TCR/CD28 stimulation the re-expression is impeded for a longer time (Alves et al., 2008). We observed a similar outcome when we stimulated T cells either through the TCR or through the CAR in the absence and presence of self-released transgenic IL-7 (Figure 32). The IL-7 receptor is evidently downregulated after T cell stimulation and stays unaltered without stimulation. This observation was seen even stronger in the presence of CAR/TCR stimulation plus IL-7. In the case of wild type CAR, the IL-7 receptor downregulation was also higher than in the case of ΔLCK CAR-modified T cells. This is not surprising, since IL-2 plays a role in CD127 downregulation as well (Xue et al., 2002).

In order to avoid IL-7 receptor downregulation after T cell activation, we expressed a chimeric cytokine receptor along with the CAR in T cells. This cytokine receptor consists of the extracellular part of the IL-7 receptor α-chain and the intracellular part of IL-2 receptor β-chain. This way, IL-7-mediated engagement is converted to IL-2 signaling. We chose the chimeric approach, because IL-2 still has a stronger effect on T cell proliferation than IL-7 has. The chimeric cytokine receptor strategy was supported by previous publications, however

the purpose for the useage was different, in particular, to convert an inhibitory cytokine signal into a stimulatory signal either by linked IL-4 receptor α-chain to IL2/IL15 receptor β-chain (Wilkie et al., 2010) or IL-4 receptor α-chain to IL-7 receptor α-chain (Mohammed et al., 2017).

After IL-7 administration, the engineered T cells showed enhanced IFN-γ release (Figure 35), improved proliferation (Figure 37), and specific cytotoxicity (Figure 38) even in the presence of TGF-β, which indicates the presence of the chimeric IL7/IL2 receptor. In order to take the advantage of the self-produced IL-7 by CAR-modified T cells, we created an autocrine acting circle and linked transgenic IL-7 release with a chimeric cytokine receptor. In this way, the CAR engineered T cells are able to release IL-7 after CAR engagement, which can bind to the chimeric IL7/IL2 receptor and transmit IL-2 signaling. In addition, enhanced T cell proliferation and specific killing by ΔLCK CAR-modified T cells with IL-7 autocrine loop in the presence of TGF-β were registered (Figure 43 and Figure 44). In order to show the benefit of such modified T cells towards wild type CAR or ΔLCK CAR in a long term, we repeatedly stimulated CAR engrafted T cells for 6 days. The IL-7 autocrine loop was compared to IL-2 autocrine loop where IL-7 exceeded IL-2 in repeatedly specific killing mediated by CAR-modified T cells (Figure 45), although a comparison is difficult because of the different circumstances. According to this, Lynch *et al*. published similar results earlier; they found that IL-7 contributed more efficiently to the proliferation of CTLs as well as its antitumor effect compared to the administration of IL-2 (Lynch and Miller, 1994). Cytokine release after repeated stimulation through CAR engagement revealed differences between the different CAR-modified T cells. IFN-γ and IL-2 secretion by wild type CAR was decreased with the time, while IL-7 release by IL-7 autocrine loop-equipped T cells stayed constant during the measurement (Figure 46). The stronger cytotoxicity is attributed to the constitutive presence of IL-7 mediated by T cells with ΔLCK CAR plus IL-7 autocrine loop in the long term. This is underlined with the observation that the granzyme B (Gr B) level after stimulation through the CAR stayed unaltered and there was no difference between the different receptor-modified T cells. In contrast, although TGF-β decreased the level of Gr B in case of the CAR T cells with the mutation of the LCK binding site within the CD28 domain (Figure 47). The reason could be that IL-2 can contribute to the expression of Gr B possibly even in the presence of TGF-β (Janas et al., 2005). IL-7 TRUCK with IL-7 autocrine loop has shown improved survival within the CAR^+ T cell population according to the measurement of living and/or apoptotic cells after CAR stimulation (Figure 48). This finding is well related to the

improved elimination of cancer cells by the CD28ΔLCK-ζ CAR T cells with IL7/IL2R and transgenic IL-7 in the long term (Figure 45). Shortly, after 30 min CAR stimulation results STAT5 phosphorylation in modified T cells with DLCK CAR with IL-7 autocrine loop and indicates the IL-2 receptor signaling pathway. In contrast, CAR T cells without IL-7 loop did not show an increase in pSTAT5. The STAT5 protein is a common signal transduction factor in both, the IL-2 and the IL-7 signaling pathway (Rani and Murphy, 2016). However, after 30 min stimulation, the phosphorylation of STAT5 was not detected in the wild type CAR-modified T cells. Whereas, after 16 hours stimulation with the cognate antigen an increase in pSTAT5 was measured in all CAR-modified T cells (Figure 49). This observation is supported by a previous report, that showed that IL-2 release and STAT5 phosphorylation appeared between 8 and 16 hours (Long and Adler, 2006). Interestingly, in case of ΔLCK CAR-engineered T cells without IL-7 loop, the pSTAT5 signal was detected after 16 hours stimulation, which indicated other activation pathways for the STAT5 protein. The Janus-activated kinase (JAK) is also involved in the activation of the STAT5 protein. Furthermore, many other hematopoietic and non-hematopoietic cytokines use the JAK-STAT signaling pathway, which could explain the late phosphorylation of the STAT5 protein independent of the presence of IL-2 (Rani and Murphy, 2016).

In order to compare ΔLCK CAR plus IL-7 loop with ΔLCK CAR without IL-7 loop *in vivo*, we investigated a tumor challenge in tumor bearing mice. As first step, the mice received the CEA and TGF-β positive C15A3 tumor cells. After the tumor growth reached about 200 mm^3, T cells with or without CAR were injected intravenously into the mice. By using these tumor cells, the presence of TGF-β and therefore the created suppressive tumor microenvironment was provided, a fact we recorded at the tumor site with the help of immune-histological staining of TGF-β (Figure 53). In spite of the suppressive milieu, the ΔLCK CAR with IL-7 loop successfully reduced the tumor growth in comparison to the ΔLCK CAR without IL-7 loop (Figure 51). The increased persistence of CAR-modified T cells with IL-7 loop at the tumor site (Figure 52) verified our expectation that the IL-7 loop enhanced T cell proliferation in the presence of TGF-β even *in vivo*.

Based on our observation and results that IL-2 and other γ-cytokines such as IL-7 and IL-15 are able to compensate the suppressive effect of TGF-β on T cell proliferation, we assume that there is a crossover between the TGF-β and γ-cytokine signaling pathway. TGF-β inhibits several mechanisms e.g. through the upregulation of cell cycle regulators such as cyclin-

dependent kinase inhibitors (CKIs), or through the downregulation of cell cycle promoters such as c-myc, cyclin D2, CDK2, and cyclin E. In addition, TGF-β is able to suppress activated T cells by inhibiting S-phase progression through reduction of the early increases in c-myc mRNA levels (Ruegemer et al., 1990). On the other hand, IL-2 has a role in the accumulation of c-MYC (myelocytomatosis oncogene) protein in activated T cells. After T cell activation, the cells have to upregulate amino acid and glucose uptake in order to sustain all T cell functions required to be effector T cells (Chou and Egawa, 2015). In this metabolic reprogramming the transcriptional regulator c-MYC has one of the most important roles (Wang et al., 2011). The regulation of c-Myc expression is driven by TCR and pro-inflammatory cytokines, such as IL-2, or possibly IL-7 or IL-15 through the JAK-STAT(5) signaling pathway. Stimulation through the TCR is essential to turn on c-myc synthesis, while the IL-2 signal is essential to promote long-term accumulation of c-MYC protein (Preston et al., 2015).

According to the different up- or downregulation of c-myc by TGF-β and IL-2, respectively, c-myc acts as a central modulator in the regulation of T cell proliferation. In our strategy, we used IL-7 stimulated IL-2 signaling through the chimeric cytokine receptor for overcoming the suppressive effect of TGF-β in T cell proliferation. In this way, we could compensate the speculative TGF-β caused downregulation in c-myc synthesis by moving the balance in order to support T cell proliferation. By using IL-7, we expected that IL-7 has a similar role in the regulation of c-myc, since IL-7 is acting in the same final pathway as IL-2 (Rani and Murphy, 2016). This argument is supported by the result of Preston *et al*., since stimulation with IL-7 or IL-2 resulted in equivalent levels of myc mRNA, although different levels of MYC protein (Preston et al., 2015). Besides c-myc, other genes are also upregulated by IL-2 signaling, which have a role in anti-apoptotic mechanisms, such as bcl-2 or bcl-x (Lord et al., 2000). IL-7 also plays a role in the upregulation of bcl-2 family molecules (Fry and Mackall, 2002). Moreover, in IL-7-deficient mice, the BCL-2 protein was markedly decreased (von Freeden-Jeffry et al., 1997). In addition, IL-7 and TGF-β have a reciprocal relationship since each is capable of downregulating the expression of the other (Fry and Mackall, 2002). TGF-β is able to downregulate IL-7 production by stromal cells (Tang et al., 1997), while IL-7 is able to downregulate TGF-β production (Dubinett et al., 1995). Thus, IL-7 is an appropriate candidate to replace IL-2 as antagonist against TGF-β mediated suppression of T cell proliferation. In our strategy we retained the advantages of IL-2 signaling through the chimeric receptor approach, however, with IL-7 triggering.

As a result of the suppressive attitude of TGF-β and its tumor supporting behavior, tremendous effort was made to overcome the suppressive effect of TGF-β around the tumor stroma. There are many approaches to repress the activity of TGF-β on T cells. Monoclonal antibodies and antisense oligonucleotides are concentrated to block TGF-β or TGF-β signaling and thus they impede the whole regulation possibility of TGF-β. Despite of this, they have shown some promising results in the clinics (Schlingensiepen et al., 2008).

The transgenic IL-7/IL-2R approach has an advantage over the transgenic expression of the dominant-negative TGF-β receptor because our strategy tries to compensate the suppressive effect of TGF-β by overcoming it, while the dominant-negative TGF-β receptor tries to reduce or eliminate TGF-β mediated signaling. The DNTGF-β receptor offers a binding site for TGF-β, however without signaling through the receptor, which could lead to a "turned off" signal in TGF-β mediated regulation. Another limitation could be the accessibility of the DNTGF-β receptor in the presence of high TGF-β concentrations. In this situation, TGF-β would bind to the physiological TGF-β receptors as well and thereby would convey some repressive signals even in the presence of the DNTGF-β receptor. However, the first clinical trial with a dominant-negative TGF-β receptor was recently published with promising results (Bollard et al., 2018). It should be noted with respect to that that this strategy shows favorable results against hematological malignancies. In addition, by using transgenic mice with overexpression of the DNTGF-β receptor in T cells, a developed autoimmune disease and lymphoproliferative disorder were demonstrated (Gorelik and Flavell, 2000; Lucas et al., 2000). Such disorders could be avoided by using our strategy in order to overcome the suppressive effect of TGF-β and not to inhibite the regulation through TGF-β.

All of the existing approaches are targeted the TGF-β or any parts of the TGF-β signaling and thereby attempt to repress all possible regulation by TGF-β. Nevertheless, the role of TGF-β in immune homeostasis is essential (Gorelik and Flavell, 2002). This fact is supported by the results of TGF-β1 KO mice, which show several inflammatory diseases and embryonic lethality (Kulkarni and Karlsson, 1993; Shull et al., 1992). In contrast, our strategy is dependent on CAR signaling, which leads to T cell activation and IL-7 production. In this case the process stays inducible and local, and therefore off-target auto-stimulatory activation is not expected. We think with our solution the targeting of solid tumors is feasible even in the presence of Treg cells or presumably high concentrations of TGF-β, which makes our strategy suitable for local activities upon systemic application.

In this thesis, we provide a novel solution and demonstrate a new approach for CAR T cell therapy against solid tumors. Due to our strategy, we make CAR-engineered T cells more efficient against tumor cells in the suppressive TGF-β signal-enriched tumor environment (Figure 54).

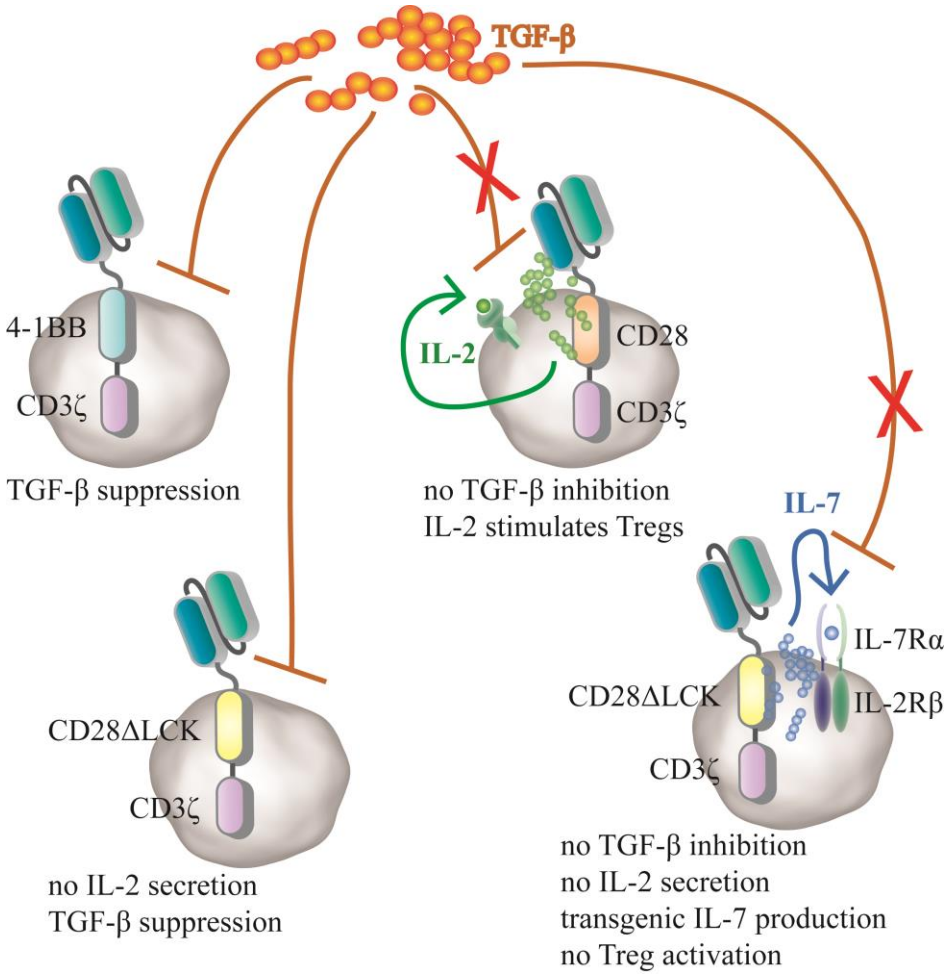


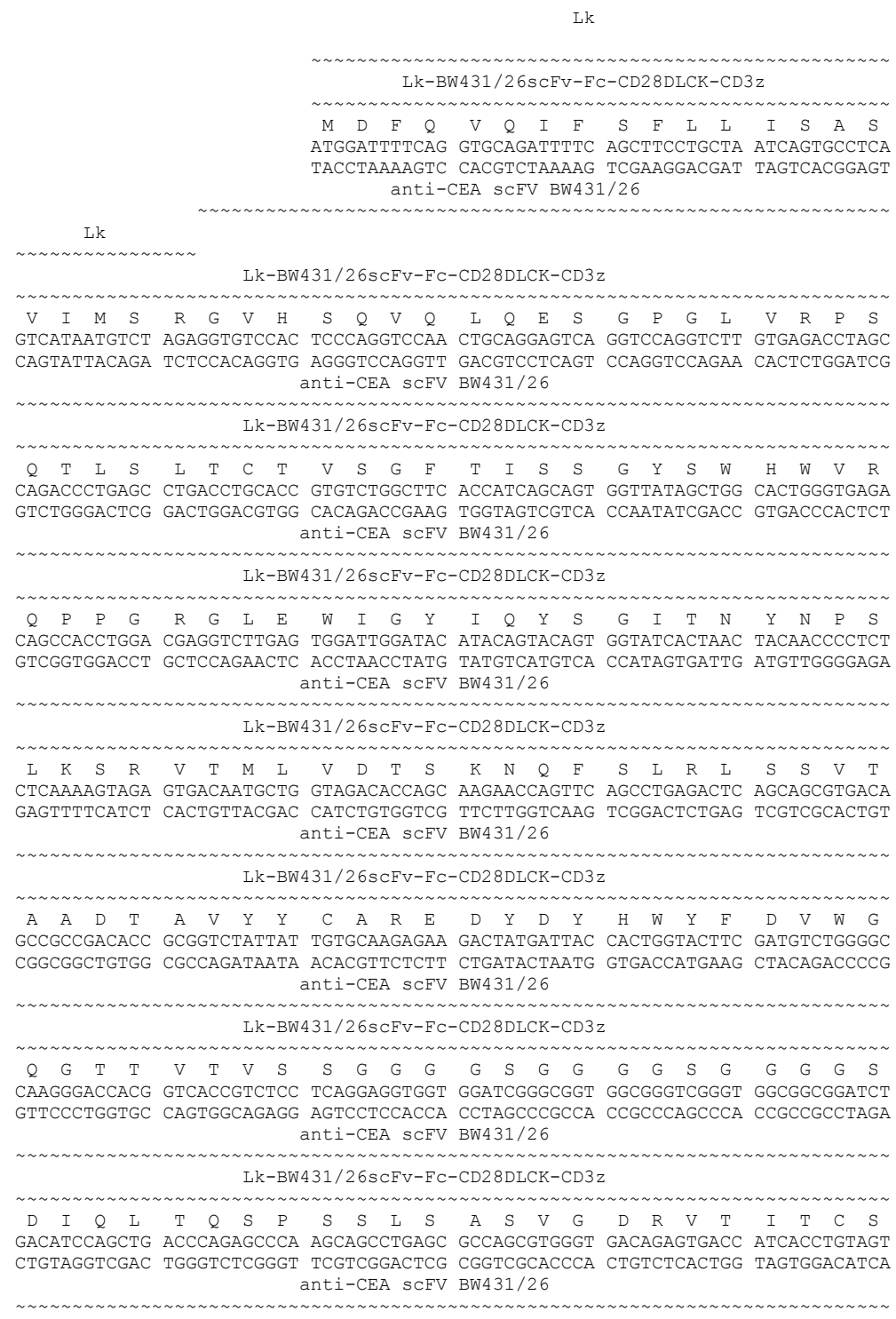
Figure 54 Model of the strategy

The summary of the concept shows the different behavior of various CAR format in T cell proliferation in the presence of TGF-β. The figure is adapted and modified from the publication of Golumba-Nagy et al., 2018.

6 APPENDIX

DNA sequence of each plasmid are demonstrated, which were generated for this work. The amino acid sequence is shown as single-letter amino acid code above the nucleotide sequence. An asterisk (*) is indicated the stop codon.

6.1 BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL7 CAR (#1645)



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2521  ACCAGCTCGAGT GTAAGTTACATG CACTGGTACCAG CAGAAGCCAGGT AAGGCTCCAAAG CTGCTGATCTAC
      TGGTCGAGCTCA CATTCAATGTAC GTGACCATGGTC GTCTTCGGTCCA TTCCGAGGTTTC GACGACTAGATG
                               anti-CEA scFV BW431/26
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       S  T  S  N   L  A  S  G   V  P  S  R   F  S  G  S   G  S  G  T   D  F  T  F
2593  AGCACATCCAAC CTGGCTTCTGGT GTGCCAAGCAGA TTCAGCGGTAGC GGTAGCGGTACC GACTTCACCTTC
      TCGTGTAGGTTG GACCGAAGACCA CACGGTTCGTCT AAGTCGCCATCG CCATCGCCATGG CTGAAGTGGAAG
                               anti-CEA scFV BW431/26
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       T  I  S  S   L  Q  P  E   D  I  A  T   Y  Y  C  H   Q  W  S  S   Y  P  T  F
2665  ACCATCAGCAGC CTCCAGCCAGAG GACATCGCCACC TACTACTGCCAT CAGTGGAGTAGT TATCCCACGTTC
      TGGTAGTCGTCG GAGGTCGGTCTC CTGTAGCGGTGG ATGATGACGGTA GTCACCTCATCA ATAGGGTGCAAG
          anti-CEA scFV BW431/26                                hinge
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  Q  G  T   K  V  E  I   K  V  D  P   A  E  P  K   S  P  D  K   T  H  T  C
2737  GGCCAAGGGACC AAGGTGGAGATC AAAGTGGATCCC GCCGAGCCCAAA TCTCCTGACAAA ACTCACACATGC
      CCGGTTCCCTGG TTCCACCTCTAG TTTCACCTAGGG CGGCTCGGGTTT AGAGGACTGTTT TGAGTGTGTACG
      hinge
      ~~~
                                        humanIgG
         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
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2809  CCACCGTGCCCA GCACCTGAACTC CTGGGGGGACCG TCAGTCTTCCTC TTCCCCCCAAAA CCCAAGGACACC
      GGTGGCACGGGT CGTGGACTTGAG GACCCCCCTGGC AGTCAGAAGGAG AAGGGGGGTTTT GGGTTCCTGTGG
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       L  M  I  S   R  T  P  E   V  T  C  V   V  V  D  V   S  H  E  D   P  E  V  K
2881  CTCATGATCTCC CGGACCCCTGAG GTCACATGCGTG GTGGTGGACGTG AGCCACGAAGAC CCTGAGGTCAAG
      GAGTACTAGAGG GCCTGGGGACTC CAGTGTACGCAC CACCACCTGCAC TCGGTGCTTCTG GGACTCCAGTTC
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       F  N  W  Y   V  D  G  V   E  V  H  N   A  K  T  K   P  R  E  E   Q  Y  N  S
2953  TTCAACTGGTAC GTGGACGGCGTG GAGGTGCATAAT GCCAAGACAAAG CCGCGGGAGGAG CAGTACAACAGC
      AAGTTGACCATG CACCTGCCGCAC CTCCACGTATTA CGGTTCTGTTTC GGCGCCCTCCTC GTCATGTTGTCG
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
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3025  ACGTACCGTGTG GTCAGCGTCCTC ACCGTCCTGCAC CAGGACTGGCTG AATGGCAAGGAG TACAAGTGCAAG
      TGCATGGCACAC CAGTCGCAGGAG TGGCAGGACGTG GTCCTGACCGAC TTACCGTTCCTC ATGTTCACGTTC
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  S  N  K   A  L  P  A   P  I  E  K   T  I  S  K   A  K  G  Q   P  R  E  P
3097  GTCTCCAACAAA GCCCTCCCAGCC CCCATCGAGAAA ACCATCTCCAAA GCCAAAGGGCAG CCCCGAGAACCA
      CAGAGGTTGTTT CGGGAGGGTCGG GGGTAGCTCTTT TGGTAGAGGTTT CGGTTTCCCGTC GGGGCTCTTGGT
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Q  V  Y  T   L  P  P  S   R  D  E  L   T  K  N  Q   V  S  L  T   C  L  V  K
3169  CAGGTGTACACC CTGCCCCCATCC CGGGATGAGCTG ACCAAGAACCAG GTCAGCCTGACC TGCCTGGTCAAA
      GTCCACATGTGG GACGGGGGTAGG GCCCTACTCGAC TGGTTCTTGGTC CAGTCGGACTGG ACGGACCAGTTT
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  F  Y  P   S  D  I  A   V  E  W  E   S  N  G  Q   P  E  N  N   Y  K  T  T
3241  GGCTTCTATCCC AGCGACATCGCC GTGGAGTGGGAG AGCAATGGGCAG CCGGAGAACAAC TACAAGACCACG
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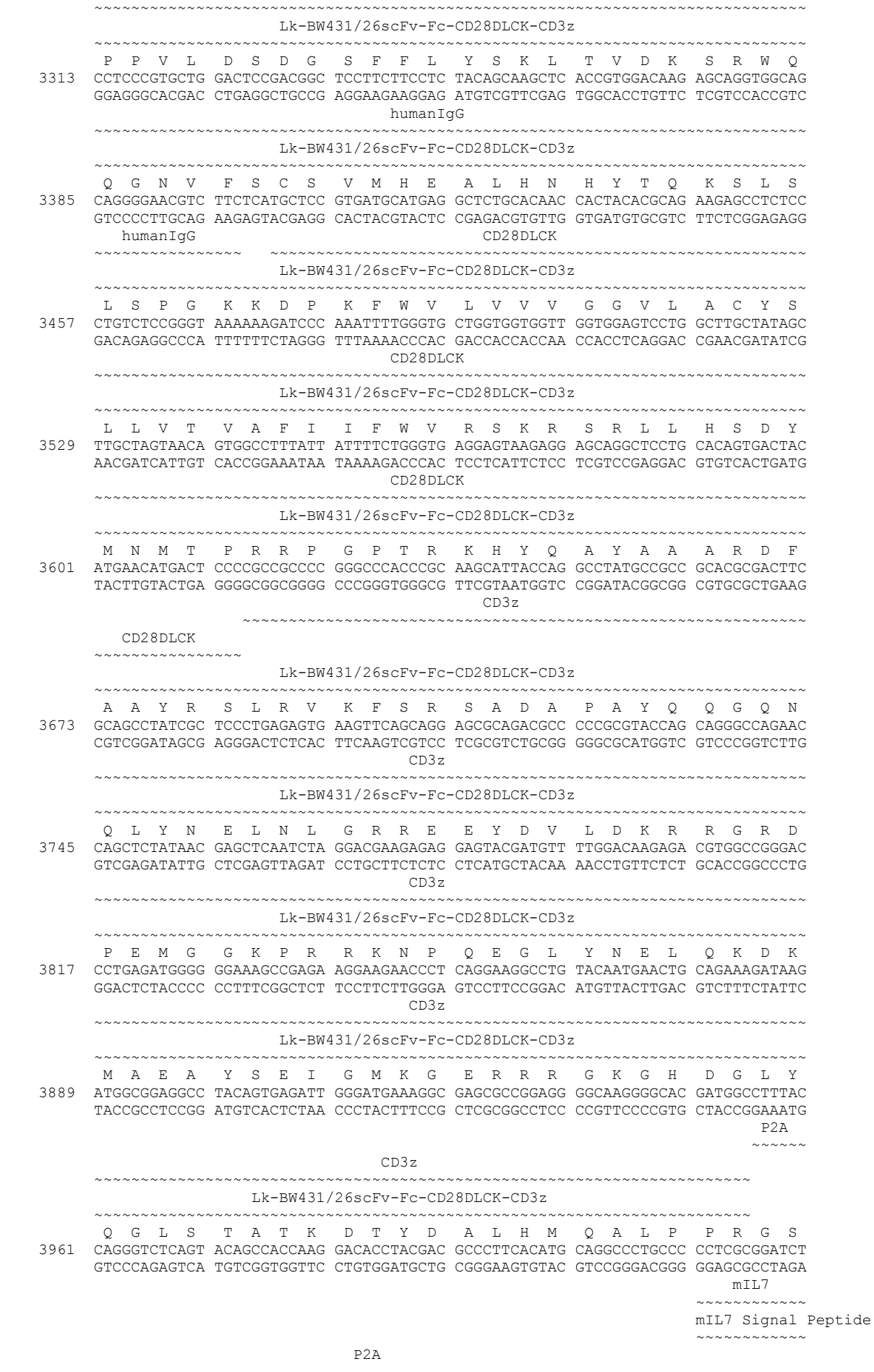
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humanIgG
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Q G N V F S C S V M H E A L H N H Y T Q K S L S
3385 CAGGGGAACGTC TTCTCATGCTCC GTGATGCATGAG GCTCTGCACAAC CACTACACGCAG AAGAGCCTCTCC
GTCCCCTTGCAG AAGAGTACGAGG CACTACGTACTC CGAGACGTGTTG GTGATGTGCGTC TTCTCGGAGAGG
humanIgG CD28DLCK
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
L S P G K K D P K F W V L V V V G G V L A C Y S
3457 CTGTCTCCGGGT AAAAAAGATCCC AAATTTTGGGTG CTGGTGGTGGTT GGTGGAGTCCTG GCTTGCTATAGC
GACAGAGGCCCA TTTTTTCTAGGG TTTAAAACCCAC GACCACCACCAA CCACCTCAGGAC CGAACGATATCG
CD28DLCK
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
L L V T V A F I I F W V R S K R S R L L H S D Y
3529 TTGCTAGTAACA GTGGCCTTTATT ATTTTCTGGGTG AGGAGTAAGAGG AGCAGGCTCCTG CACAGTGACTAC
AACGATCATTGT CACCGGAAATAA TAAAAGACCCAC TCCTCATTCTCC TCGTCCGAGGAC GTGTCACTGATG
CD28DLCK
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
M N M T P R R P G P T R K H Y Q A Y A A A R D F
3601 ATGAACATGACT CCCCGCCGCCCC GGGCCCACCCGC AAGCATTACCAG GCCTATGCCGCC GCACGCGACTTC
TACTTGTACTGA GGGGCGGCGGGG CCCGGGTGGGCG TTCGTAATGGTC CGGATACGGCGG CGTGCGCTGAAG
CD3z
CD28DLCK
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
A A Y R S L R V K F S R S A D A P A Y Q Q G Q N
3673 GCAGCCTATCGC TCCCTGAGAGTG AAGTTCAGCAGG AGCGCAGACGCC CCCGCGTACCAG CAGGGCCAGAAC
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CD3z
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
Q L Y N E L N L G R R E E Y D V L D K R R G R D
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CD3z
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
P E M G G K P R R K N P Q E G L Y N E L Q K D K
3817 CCTGAGATGGGG GGAAAGCCGAGA AGGAAGAACCCT CAGGAAGGCCTG TACAATGAACTG CAGAAAGATAAG
GGACTCTACCCC CCTTTCGGCTCT TCCTTCTTGGGA GTCCTTCCGGAC ATGTTACTTGAC GTCTTTCTATTC
CD3z
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
M A E A Y S E I G M K G E R R R G K G H D G L Y
3889 ATGGCGGAGGCC TACAGTGAGATT GGGATGAAAGGC GAGCGCCGGAGG GGCAAGGGGCAC GATGGCCTTTAC
TACCGCCTCCGG ATGTCACTCTAA CCCTACTTTCCG CTCGCGGCCTCC CCGTTCCCCGTG CTACCGGAAATG
P2A
CD3z
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
Q G L S T A T K D T Y D A L H M Q A L P P R G S
3961 CAGGGTCTCAGT ACAGCCACCAAG GACACCTACGAC GCCCTTCACATG CAGGCCCTGCCC CCTCGCGGATCT
GTCCCAGAGTCA TGTCGGTGGTTC CTGTGGATGCTG CGGGAAGTGTAC GTCCGGGACGGG GGAGCGCCTAGA
mIL7
mIL7 Signal Peptide
P2A

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      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  A  T  N   F  S  L  L   K  Q  A  G   D  V  E  E   N  P  G  P   M  F  H  V
4033  GGAGCCACGAAC TTCTCTCTGTTA AAGCAAGCAGGA GACGTGGAAGAA AACCCCGGTCCT ATGTTCCATGTT
      CCTCGGTGCTTG AAGAGAGACAAT TTCGTTCGTCCT CTGCACCTTCTT TTGGGGCCAGGA TACAAGGTACAA
                           mIL7 Signal Peptide
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                        mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       S  F  R  Y   I  F  G  I   P  P  L  I   L  V  L  L   P  V  T  S   S  E  C  H
4105  TCTTTTAGATAT ATCTTTGGAATT CCTCCACTGATC CTTGTTCTGCTG CCTGTCACATCA TCTGAGTGCCAC
      AGAAAATCTATA TAGAAACCTTAA GGAGGTGACTAG GAACAAGACGAC GGACAGTGTAGT AGACTCACGGTG
                                        mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                           BclI
                                           ~~~~~~
       I  K  D  K   E  G  K  A   Y  E  S  V   L  M  I  S   I  D  E  L   D  K  M  T
4177  ATTAAAGACAAA GAAGGTAAAGCA TATGAGAGTGTA CTGATGATCAGC ATCGATGAATTG GACAAAATGACA
      TAATTTCTGTTT CTTCCATTTCGT ATACTCTCACAT GACTACTAGTCG TAGCTACTTAAC CTGTTTTACTGT
                                        mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  T  D  S   N  C  P  N   N  E  P  N   F  F  R  K   H  V  C  D   D  T  K  E
4249  GGAACTGATAGT AATTGCCCGAAT AATGAACCAAAC TTTTTTAGAAAA CATGTATGTGAT GATACAAAGGAA
      CCTTGACTATCA TTAACGGGCTTA TTACTTGGTTTG AAAAAATCTTTT GTACATACACTA CTATGTTTCCTT
                                        mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  A  F  L   N  R  A  A   R  K  L  K   Q  F  L  K   M  N  I  S   E  E  F  N
4321  GCTGCTTTTCTA AATCGTGCTGCT CGCAAGTTGAAG CAATTTCTTAAA ATGAATATCAGT GAAGAATTCAAT
      CGACGAAAAGAT TTAGCACGACGA GCGTTCAACTTC GTTAAAGAATTT TACTTATAGTCA CTTCTTAAGTTA
                                        mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  H  L  L   T  V  S  Q   G  T  Q  T   L  V  N  C   T  S  K  E   E  K  N  V
4393  GTCCACTTACTA ACAGTATCACAA GGCACACAAACA CTGGTGAACTGC ACGAGTAAGGAA GAAAAAAACGTA
      CAGGTGAATGAT TGTCATAGTGTT CCGTGTGTTTGT GACCACTTGACG TGCTCATTCCTT CTTTTTTTGCAT
                                        mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       K  E  Q  K   K  N  D  A   C  F  L  K   R  L  L  R   E  I  K  T   C  W  N  K
4465  AAGGAACAGAAA AAGAATGATGCA TGTTTCCTAAAG AGACTACTGAGA GAAATAAAAACT TGTTGGAATAAA
      TTCCTTGTCTTT TTCTTACTACGT ACAAAGGATTTC TCTGATGACTCT CTTTATTTTTGA ACAACCTTATTT
              mIL7
      ~~~~~~~~~~~~~~~~~~~~~~
                                     BspEI
                                     ~~~~~~~
       I  L  K  G   S  I  *  L   E  R  S  G
4537  ATTTTGAAGGGC AGTATATAACTC GAGAGATCCGGA
      TAAAACTTCCCG TCATATATTGAG CTCTCTAGGCCT
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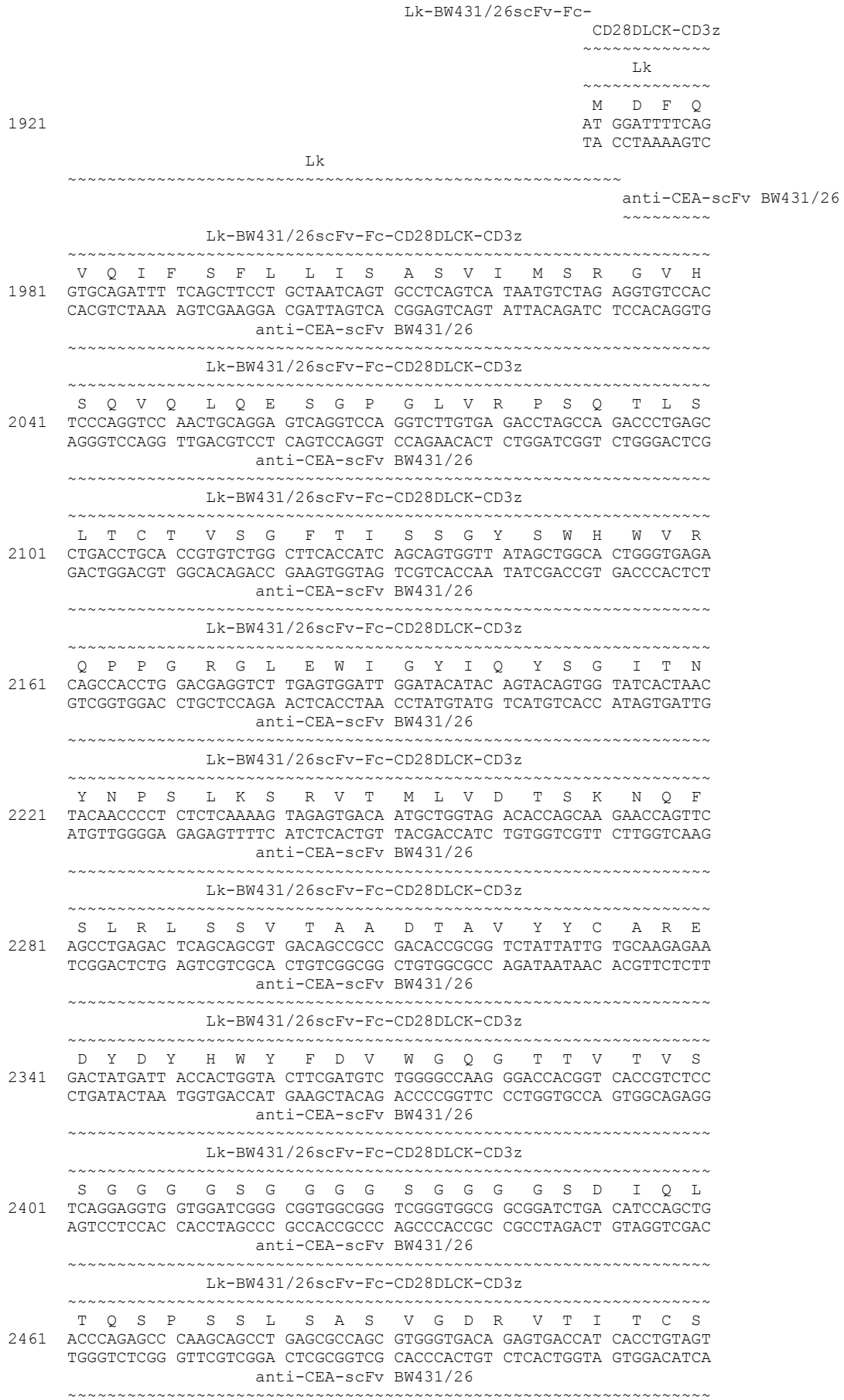
6.2 BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL2 CAR (#1746)



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                Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
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            XhoI
           ~~~~~~~
       T  S  S  S   V  S  Y   M  H  W   Y  Q  Q  K   P  G  K   A  P  K
2521   ACCAGCTCGA GTGTAAGTTA CATGCACTGG TACCAGCAGA AGCCAGGTAA GGCTCCAAAG
       TGGTCGAGCT CACATTCAAT GTACGTGACC ATGGTCGTCT TCGGTCCATT CCGAGGTTTC
                        anti-CEA-scFv BW431/26
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       L  L  I  Y   S  T  S   N  L  A   S  G  V  P   S  R  F   S  G  S
2581   CTGCTGATCT ACAGCACATC CAACCTGGCT TCTGGTGTGC CAAGCAGATT CAGCGGTAGC
       GACGACTAGA TGTCGTGTAG GTTGGACCGA AGACCACACG GTTCGTCTAA GTCGCCATCG
                        anti-CEA-scFv BW431/26
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  S  G  T   D  F  T   F  T  I   S  S  L  Q   P  E  D   I  A  T
2641   GGTAGCGGTA CCGACTTCAC CTTCACCATC AGCAGCCTCC AGCCAGAGGA CATCGCCACC
       CCATCGCCAT GGCTGAAGTG GAAGTGGTAG TCGTCGGAGG TCGGTCTCCT GTAGCGGTGG
                        anti-CEA-scFv BW431/26
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Y  Y  C  H   Q  W  S   S  Y  P   T  F  G  Q   G  T  K   V  E  I
2701   TACTACTGCC ATCAGTGGAG TAGTTATCCC ACGTTCGGCC AAGGGACCAA GGTGGAGATC
       ATGATGACGG TAGTCACCTC ATCAATAGGG TGCAAGCCGG TTCCCTGGTT CCACCTCTAG
       anti-CEA-scFv BW431/26                                   humanIgG
       ~~~~~~                                                   ~~~~~~~~~
                                  hinge
                        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       K  V  D  P   A  E  P   K  S  P   D  K  T  H   T  C  P   P  C  P
2761   AAAGTGGATC CCGCCGAGCC CAAATCTCCT GACAAAACTC ACACATGCCC ACCGTGCCCA
       TTTCACCTAG GGCGGCTCGG GTTTAGAGGA CTGTTTTGAG TGTGTACGGG TGGCACGGGT
                                 humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  P  E  L   L  G  G   P  S  V   F  L  F  P   P  K  P   K  D  T
2821   GCACCTGAAC TCCTGGGGGG ACCGTCAGTC TTCCTCTTCC CCCCAAAACC CAAGGACACC
       CGTGGACTTG AGGACCCCCC TGGCAGTCAG AAGGAGAAGG GGGGTTTTGG GTTCCTGTGG
                                 humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       L  M  I  S   R  T  P   E  V  T   C  V  V  V   D  V  S   H  E  D
2881   CTCATGATCT CCCGGACCCC TGAGGTCACA TGCGTGGTGG TGGACGTGAG CCACGAAGAC
       GAGTACTAGA GGGCCTGGGG ACTCCAGTGT ACGCACCACC ACCTGCACTC GGTGCTTCTG
                                 humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  E  V  K   F  N  W   Y  V  D   G  V  E  V   H  N  A   K  T  K
2941   CCTGAGGTCA AGTTCAACTG GTACGTGGAC GGCGTGGAGG TGCATAATGC CAAGACAAAG
       GGACTCCAGT TCAAGTTGAC CATGCACCTG CCGCACCTCC ACGTATTACG GTTCTGTTTC
                                 humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  R  E  E   Q  Y  N   S  T  Y   R  V  V  S   V  L  T   V  L  H
3001   CCGCGGGAGG AGCAGTACAA CAGCACGTAC CGTGTGGTCA GCGTCCTCAC CGTCCTGCAC
       GGCGCCCTCC TCGTCATGTT GTCGTGCATG GCACACCAGT CGCAGGAGTG GCAGGACGTG
                                 humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Q  D  W  L   N  G  K   E  Y  K   C  K  V  S   N  K  A   L  P  A
3061   CAGGACTGGC TGAATGGCAA GGAGTACAAG TGCAAGGTCT CCAACAAAGC CCTCCCAGCC
       GTCCTGACCG ACTTACCGTT CCTCATGTTC ACGTTCCAGA GGTTGTTTCG GGAGGGTCGG
                                 humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  I  E  K   T  I  S   K  A  K   G  Q  P  R   E  P  Q   V  Y  T
3121   CCCATCGAGA AAACCATCTC CAAAGCCAAA GGGCAGCCCC GAGAACCACA GGTGTACACC
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      GGGTAGCTCT TTTGGTAGAG GTTTCGGTTT CCCGTCGGGG CTCTTGGTGT CCACATGTGG
                                humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       L  P  P  S   R  D  E   L  T  K   N  Q  V  S   L  T  C   L  V  K
3181  CTGCCCCCAT CCCGGGATGA GCTGACCAAG AACCAGGTCA GCCTGACCTG CCTGGTCAAA
      GACGGGGGTA GGGCCCTACT CGACTGGTTC TTGGTCCAGT CGGACTGGAC GGACCAGTTT
                                humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  F  Y  P   S  D  I   A  V  E   W  E  S  N   G  Q  P   E  N  N
3241  GGCTTCTATC CCAGCGACAT CGCCGTGGAG TGGGAGAGCA ATGGGCAGCC GGAGAACAAC
      CCGAAGATAG GGTCGCTGTA GCGGCACCTC ACCCTCTCGT TACCCGTCGG CCTCTTGTTG
                                humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Y  K  T  T   P  P  V   L  D  S   D  G  S  F   F  L  Y   S  K  L
3301  TACAAGACCA CGCCTCCCGT GCTGGACTCC GACGGCTCCT TCTTCCTCTA CAGCAAGCTC
      ATGTTCTGGT GCGGAGGGCA CGACCTGAGG CTGCCGAGGA AGAAGGAGAT GTCGTTCGAG
                                humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       T  V  D  K   S  R  W   Q  Q  G   N  V  F  S   C  S  V   M  H  E
3361  ACCGTGGACA AGAGCAGGTG GCAGCAGGGG AACGTCTTCT CATGCTCCGT GATGCATGAG
      TGGCACCTGT TCTCGTCCAC CGTCGTCCCC TTGCAGAAGA GTACGAGGCA CTACGTACTC
                           humanIgG                                CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~   ~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  L  H  N   H  Y  T   Q  K  S   L  S  L  S   P  G  K   K  D  P
3421  GCTCTGCACA ACCACTACAC GCAGAAGAGC CTCTCCCTGT CTCCGGGTAA AAAAGATCCC
      CGAGACGTGT TGGTGATGTG CGTCTTCTCG GAGAGGGACA GAGGCCCATT TTTTCTAGGG
                                CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       K  F  W  V   L  V  V   V  G  G   V  L  A  C   Y  S  L   L  V  T
3481  AAATTTTGGG TGCTGGTGGT GGTTGGTGGA GTCCTGGCTT GCTATAGCTT GCTAGTAACA
      TTTAAAACCC ACGACCACCA CCAACCACCT CAGGACCGAA CGATATCGAA CGATCATTGT
                                CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  A  F  I   I  F  W   V  R  S   K  R  S  R   L  L  H   S  D  Y
3541  GTGGCCTTTA TTATTTTCTG GGTGAGGAGT AAGAGGAGCA GGCTCCTGCA CAGTGACTAC
      CACCGGAAAT AATAAAAGAC CCACTCCTCA TTCTCCTCGT CCGAGGACGT GTCACTGATG
                                CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                               ApaI
                               ~~~~~~~
       M  N  M  T   P  R  R   P  G  P   T  R  K  H   Y  Q  A   Y  A  A
3601  ATGAACATGA CTCCCCGCCG CCCCGGGCCC ACCCGCAAGC ATTACCAGGC CTATGCCGCC
      TACTTGTACT GAGGGGCGGC GGGGCCCGGG TGGGCGTTCG TAATGGTCCG GATACGGCGG
            CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                CD3z
                                      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  R  D  F   A  A  Y   R  S  L   R  V  K  F   S  R  S   A  D  A
3661  GCACGCGACT TCGCAGCCTA TCGCTCCCTG AGAGTGAAGT TCAGCAGGAG CGCAGACGCC
      CGTGCGCTGA AGCGTCGGAT AGCGAGGGAC TCTCACTTCA AGTCGTCCTC GCGTCTGCGG
                               CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  A  Y  Q   Q  G  Q   N  Q  L   Y  N  E  L   N  L  G   R  R  E
3721  CCCGCGTACC AGCAGGGCCA GAACCAGCTC TATAACGAGC TCAATCTAGG ACGAAGAGAG
      GGGCGCATGG TCGTCCCGGT CTTGGTCGAG ATATTGCTCG AGTTAGATCC TGCTTCTCTC
                               CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
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      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       E  Y  D  V   L  D  K   R  R  G   R  D  P  E   M  G  G   K  P  R
3781  GAGTACGATG TTTTGGACAA GAGACGTGGC CGGGACCCTG AGATGGGGGG AAAGCCGAGA
      CTCATGCTAC AAAACCTGTT CTCTGCACCG GCCCTGGGAC TCTACCCCCC TTTCGGCTCT
                                  CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       R  K  N  P   Q  E  G   L  Y  N   E  L  Q  K   D  K  M   A  E  A
3841  AGGAAGAACC CTCAGGAAGG CCTGTACAAT GAACTGCAGA AAGATAAGAT GGCGGAGGCC
      TCCTTCTTGG GAGTCCTTCC GGACATGTTA CTTGACGTCT TTCTATTCTA CCGCCTCCGG
                                  CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Y  S  E  I   G  M  K   G  E  R   R  R  G  K   G  H  D   G  L  Y
3901  TACAGTGAGA TTGGGATGAA AGGCGAGCGC CGGAGGGGCA AGGGGCACGA TGGCCTTTAC
      ATGTCACTCT AACCCTACTT TCCGCTCGCG GCCTCCCCGT TCCCCGTGCT ACCGGAAATG
                                  CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Q  G  L  S   T  A  T   K  D  T   Y  D  A  L   H  M  Q   A  L  P
3961  CAGGGTCTCA GTACAGCCAC CAAGGACACC TACGACGCCC TTCACATGCA GGCCCTGCCC
      GTCCCAGAGT CATGTCGGTG GTTCCTGTGG ATGCTGCGGG AAGTGTACGT CCGGGACGGG
                                    P2A
           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       CD3z
      ~~~~~~
      Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~
       P  R  G  S   G  A  T   N  F  S   L  L  K  Q   A  G  D   V  E  E
4021  CCTCGCGGAT CTGGAGCCAC GAACTTCTCT CTGTTAAAGC AAGCAGGAGA CGTGGAAGAA
      GGAGCGCCTA GACCTCGGTG CTTGAAGAGA GACAATTTCG TTCGTCCTCT GCACCTTCTT
                                     hIL2
                  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
          P2A
      ~~~~~~~~~~~~~~
       N  P  G  P   M  Y  R   M  Q  L   L  S  C  I   A  L  S   L  A  L
4081  AACCCCGGTC CTATGTACAG GATGCAACTC CTGTCTTGCA TTGCACTAAG TCTTGCACTT
      TTGGGGCCAG GATACATGTC CTACGTTGAG GACAGAACGT AACGTGATTC AGAACGTGAA
                                  hIL2
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  T  N  S   A  P  T   S  S  S   T  K  K  T   Q  L  Q   L  E  H
4141  GTCACAAACA GTGCACCTAC TTCAAGTTCT ACAAAGAAAA CACAGCTACA ACTGGAGCAT
      CAGTGTTTGT CACGTGGATG AAGTTCAAGA TGTTTCTTTT GTGTCGATGT TGACCTCGTA
                                  hIL2
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       L  L  L  D   L  Q  M   I  L  N   G  I  N  N   Y  K  N   P  K  L
4201  TTACTGCTGG ATTTACAGAT GATTTTGAAT GGAATTAATA ATTACAAGAA TCCCAAACTC
      AATGACGACC TAAATGTCTA CTAAAACTTA CCTTAATTAT TAATGTTCTT AGGGTTTGAG
                                  hIL2
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       T  R  M  L   T  F  K   F  Y  M   P  K  K  A   T  E  L   K  H  L
4261  ACCAGGATGC TCACATTTAA GTTTTACATG CCCAAGAAGG CCACAGAACT GAAACATCTT
      TGGTCCTACG AGTGTAAATT CAAAATGTAC GGGTTCTTCC GGTGTCTTGA CTTTGTAGAA
                                  hIL2
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Q  C  L  E   E  E  L   K  P  L   E  E  V  L   N  L  A   Q  S  K
4321  CAGTGTCTAG AAGAAGAACT CAAACCTCTG GAGGAAGTGC TAAATTTAGC TCAAAGCAAA
      GTCACAGATC TTCTTCTTGA GTTTGGAGAC CTCCTTCACG ATTTAAATCG AGTTTCGTTT
                                  hIL2
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       N  F  H  L   R  P  R   D  L  I   S  N  I  N   V  I  V   L  E  L
4381  AACTTTCACT TAAGACCCAG GGACTTAATC AGCAATATCA ACGTAATAGT TCTGGAACTA
      TTGAAAGTGA ATTCTGGGTC CCTGAATTAG TCGTTATAGT TGCATTATCA AGACCTTGAT
                                  hIL2
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       K  G  S  E   T  T  F   M  C  E   Y  A  D  E   T  A  T   I  V  E
4441  AAGGGATCTG AAACAACATT CATGTGTGAA TATGCTGATG AGACAGCAAC CATTGTAGAA
      TTCCCTAGAC TTTGTTGTAA GTACACACTT ATACGACTAC TCTGTCGTTG GTAACATCTT
                                hIL2
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                                  XhoI
                                                                 ~~~~~~
       F  L  N  R   W  I  T   F  C  Q   S  I  I  S   T  L  T   *  L  E
4501  TTTCTGAACA GATGGATTAC CTTTTGTCAA AGCATCATCT CAACACTAAC TTGACTCGAG
      AAAGACTTGT CTACCTAATG GAAAACAGTT TCGTAGTAGA GTTGTGATTG AACTGAGCTC
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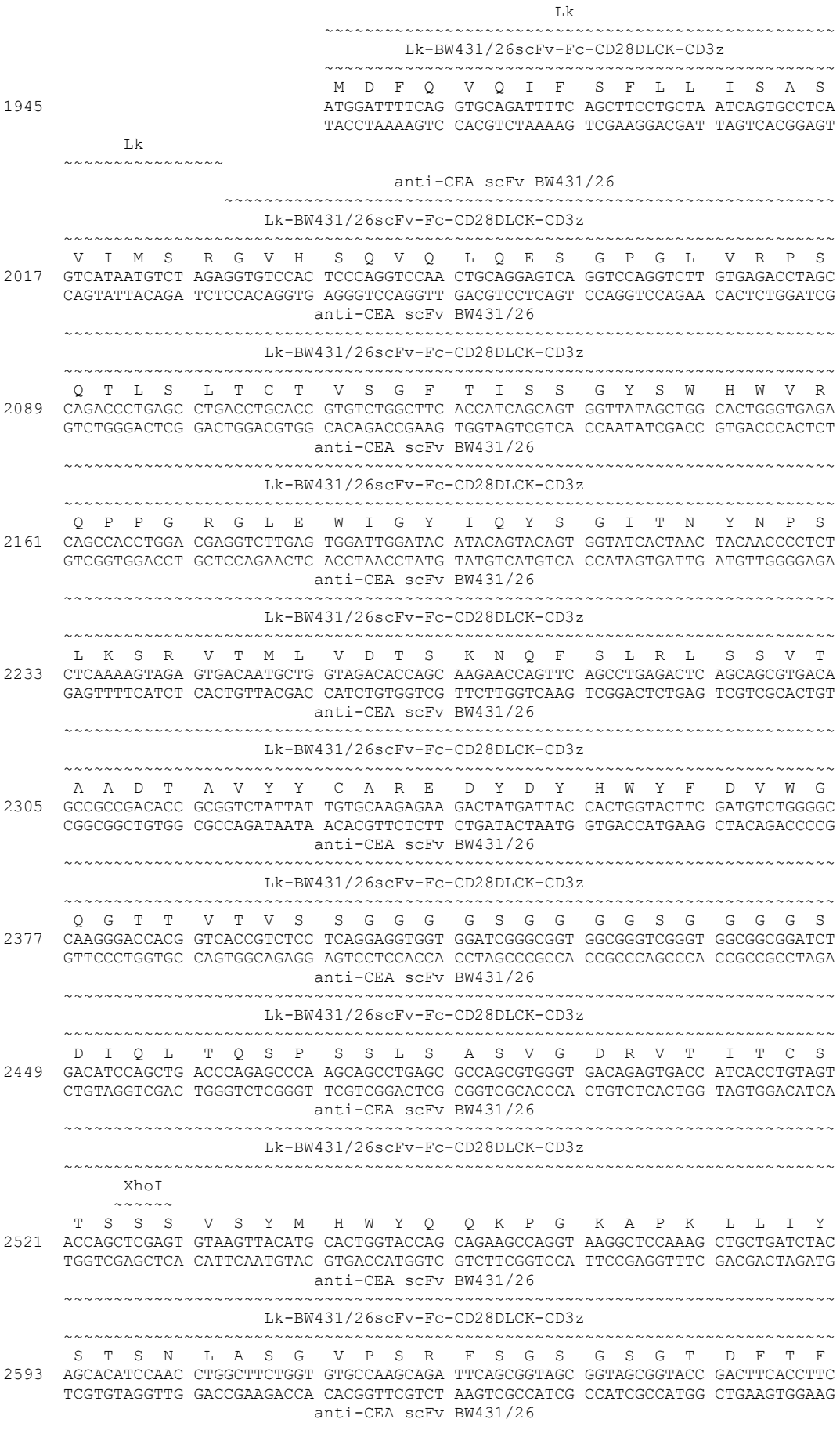
6.3 BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL15 CAR (#1764)



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                            Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       T  I  S  S   L  Q  P  E   D  I  A  T   Y  Y  C  H   Q  W  S  S   Y  P  T  F
2665  ACCATCAGCAGC CTCCAGCCAGAG GACATCGCCACC TACTACTGCCAT CAGTGGAGTAGT TATCCCACGTTC
      TGGTAGTCGTCG GAGGTCGGTCTC CTGTAGCGGTGG ATGATGACGGTA GTCACCTCATCA ATAGGGTGCAAG
          anti-CEA scFv BW431/26                                hinge
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  Q  G  T   K  V  E  I   K  V  D  P   A  E  P  K   S  P  D  K   T  H  T  C
2737  GGCCAAGGGACC AAGGTGGAGATC AAAGTGGATCCC GCCGAGCCCAAA TCTCCTGACAAA ACTCACACATGC
      CCGGTTCCCTGG TTCCACCTCTAG TTTCACCTAGGG CGGCTCGGGTTT AGAGGACTGTTT TGAGTGTGTACG
      hinge
      ~~~
                                        humanIgG
         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  P  C  P   A  P  E  L   L  G  G  P   S  V  F  L   F  P  P  K   P  K  D  T
2809  CCACCGTGCCCA GCACCTGAACTC CTGGGGGGACCG TCAGTCTTCCTC TTCCCCCCAAAA CCCAAGGACACC
      GGTGGCACGGGT CGTGGACTTGAG GACCCCCCTGGC AGTCAGAAGGAG AAGGGGGGTTTT GGGTTCCTGTGG
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       L  M  I  S   R  T  P  E   V  T  C  V   V  V  D  V   S  H  E  D   P  E  V  K
2881  CTCATGATCTCC CGGACCCCTGAG GTCACATGCGTG GTGGTGGACGTG AGCCACGAAGAC CCTGAGGTCAAG
      GAGTACTAGAGG GCCTGGGGACTC CAGTGTACGCAC CACCACCTGCAC TCGGTGCTTCTG GGACTCCAGTTC
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       F  N  W  Y   V  D  G  V   E  V  H  N   A  K  T  K   P  R  E  E   Q  Y  N  S
2953  TTCAACTGGTAC GTGGACGGCGTG GAGGTGCATAAT GCCAAGACAAAG CCGCGGGAGGAG CAGTACAACAGC
      AAGTTGACCATG CACCTGCCGCAC CTCCACGTATTA CGGTTCTGTTTC GGCGCCCTCCTC GTCATGTTGTCG
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       T  Y  R  V   V  S  V  L   T  V  L  H   Q  D  W  L   N  G  K  E   Y  K  C  K
3025  ACGTACCGTGTG GTCAGCGTCCTC ACCGTCCTGCAC CAGGACTGGCTG AATGGCAAGGAG TACAAGTGCAAG
      TGCATGGCACAC CAGTCGCAGGAG TGGCAGGACGTG GTCCTGACCGAC TTACCGTTCCTC ATGTTCACGTTC
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  S  N  K   A  L  P  A   P  I  E  K   T  I  S  K   A  K  G  Q   P  R  E  P
3097  GTCTCCAACAAA GCCCTCCCAGCC CCCATCGAGAAA ACCATCTCCAAA GCCAAAGGGCAG CCCCGAGAACCA
      CAGAGGTTGTTT CGGGAGGGTCGG GGGTAGCTCTTT TGGTAGAGGTTT CGGTTTCCCGTC GGGGCTCTTGGT
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Q  V  Y  T   L  P  P  S   R  D  E  L   T  K  N  Q   V  S  L  T   C  L  V  K
3169  CAGGTGTACACC CTGCCCCCATCC CGGGATGAGCTG ACCAAGAACCAG GTCAGCCTGACC TGCCTGGTCAAA
      GTCCACATGTGG GACGGGGGTAGG GCCCTACTCGAC TGGTTCTTGGTC CAGTCGGACTGG ACGGACCAGTTT
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  F  Y  P   S  D  I  A   V  E  W  E   S  N  G  Q   P  E  N  N   Y  K  T  T
3241  GGCTTCTATCCC AGCGACATCGCC GTGGAGTGGGAG AGCAATGGGCAG CCGGAGAACAAC TACAAGACCACG
      CCGAAGATAGGG TCGCTGTAGCGG CACCTCACCCTC TCGTTACCCGTC GGCCTCTTGTTG ATGTTCTGGTGC
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  P  V  L   D  S  D  G   S  F  F  L   Y  S  K  L   T  V  D  K   S  R  W  Q
3313  CCTCCCGTGCTG GACTCCGACGGC TCCTTCTTCCTC TACAGCAAGCTC ACCGTGGACAAG AGCAGGTGGCAG
      GGAGGGCACGAC CTGAGGCTGCCG AGGAAGAAGGAG ATGTCGTTCGAG TGGCACCTGTTC TCGTCCACCGTC
                                        humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Q  G  N  V   F  S  C  S   V  M  H  E   A  L  H  N   H  Y  T  Q   K  S  L  S
3385  CAGGGGAACGTC TTCTCATGCTCC GTGATGCATGAG GCTCTGCACAAC CACTACACGCAG AAGAGCCTCTCC
      GTCCCCTTGCAG AAGAGTACGAGG CACTACGTACTC CGAGACGTGTTG GTGATGTGCGTC TTCTCGGAGAGG
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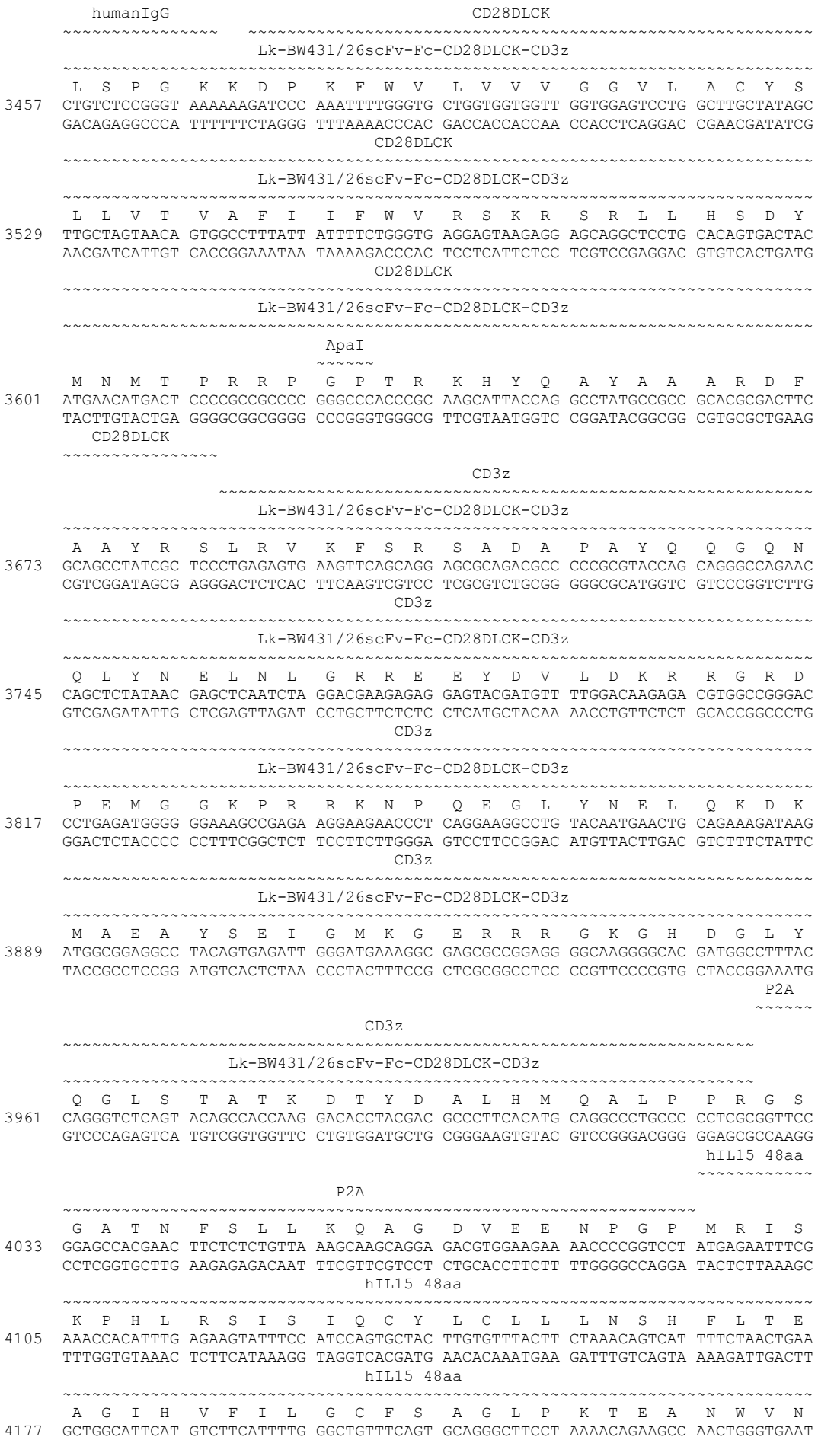
humanIgG
CD28DLCK
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
L S P G K K D P K F W V L V V V G G V L A C Y S
3457 CTGTCTCCGGGT AAAAAAGATCCC AAATTTTGGGTG CTGGTGGTGGTT GGTGGAGTCCTG GCTTGCTATAGC
GACAGAGGCCCA TTTTTTCTAGGG TTTAAAACCCAC GACCACCACCAA CCACCTCAGGAC CGAACGATATCG
CD28DLCK
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
L L V T V A F I I F W V R S K R S R L L H S D Y
3529 TTGCTAGTAACA GTGGCCTTTATT ATTTTCTGGGTG AGGAGTAAGAGG AGCAGGCTCCTG CACAGTGACTAC
AACGATCATTGT CACCGGAAATAA TAAAAGACCCAC TCCTCATTCTCC TCGTCCGAGGAC GTGTCACTGATG
CD28DLCK
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
ApaI
M N M T P R R P G P T R K H Y Q A Y A A A R D F
3601 ATGAACATGACT CCCCGCCGCCCC GGGCCCACCCGC AAGCATTACCAG GCCTATGCCGCC GCACGCGACTTC
TACTTGTACTGA GGGGCGGCGGGG CCCGGGTGGGCG TTCGTAATGGTC CGGATACGGCGG CGTGCGCTGAAG
CD28DLCK
CD3z
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
A A Y R S L R V K F S R S A D A P A Y Q Q G Q N
3673 GCAGCCTATCGC TCCCTGAGAGTG AAGTTCAGCAGG AGCGCAGACGCC CCCGCGTACCAG CAGGGCCAGAAC
CGTCGGATAGCG AGGGACTCTCAC TTCAAGTCGTCC TCGCGTCTGCGG GGGCGCATGGTC GTCCCGGTCTTG
CD3z
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
Q L Y N E L N L G R R E E Y D V L D K R R G R D
3745 CAGCTCTATAAC GAGCTCAATCTA GGACGAAGAGAG GAGTACGATGTT TTGGACAAGAGA CGTGGCCGGGAC
GTCGAGATATTG CTCGAGTTAGAT CCTGCTTCTCTC CTCATGCTACAA AACCTGTTCTCT GCACCGGCCCTG
CD3z
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
P E M G G K P R R K N P Q E G L Y N E L Q K D K
3817 CCTGAGATGGGG GGAAAGCCGAGA AGGAAGAACCCT CAGGAAGGCCTG TACAATGAACTG CAGAAAGATAAG
GGACTCTACCCC CCTTTCGGCTCT TCCTTCTTGGGA GTCCTTCCGGAC ATGTTACTTGAC GTCTTTCTATTC
CD3z
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
M A E A Y S E I G M K G E R R R G K G H D G L Y
3889 ATGGCGGAGGCC TACAGTGAGATT GGGATGAAAGGC GAGCGCCGGAGG GGCAAGGGGCAC GATGGCCTTTAC
TACCGCCTCCGG ATGTCACTCTAA CCCTACTTTCCG CTCGCGGCCTCC CCGTTCCCCGTG CTACCGGAAATG
P2A
CD3z
Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
Q G L S T A T K D T Y D A L H M Q A L P P R G S
3961 CAGGGTCTCAGT ACAGCCACCAAG GACACCTACGAC GCCCTTCACATG CAGGCCCTGCCC CCTCGCGGTTCC
GTCCCAGAGTCA TGTCGGTGGTTC CTGTGGATGCTG CGGGAAGTGTAC GTCCGGGACGGG GGAGCGCCAAGG
hIL15 48aa
P2A
G A T N F S L L K Q A G D V E E N P G P M R I S
4033 GGAGCCACGAAC TTCTCTCTGTTA AAGCAAGCAGGA GACGTGGAAGAA AACCCCGGTCCT ATGAGAATTTCG
CCTCGGTGCTTG AAGAGAGACAAT TTCGTTCGTCCT CTGCACCTTCTT TTGGGGCCAGGA TACTCTTAAAGC
hIL15 48aa
K P H L R S I S I Q C Y L C L L L N S H F L T E
4105 AAACCACATTTG AGAAGTATTTCC ATCCAGTGCTAC TTGTGTTTACTT CTAAACAGTCAT TTTCTAACTGAA
TTTGGTGTAAAC TCTTCATAAAGG TAGGTCACGATG AACACAAATGAA GATTTGTCAGTA AAAGATTGACTT
hIL15 48aa
A G I H V F I L G C F S A G L P K T E A N W V N
4177 GCTGGCATTCAT GTCTTCATTTTG GGCTGTTTCAGT GCAGGGCTTCCT AAAACAGAAGCC AACTGGGTGAAT

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      CGACCGTAAGTA CAGAAGTAAAAC CCGACAAAGTCA CGTCCCGAAGGA TTTTGTCTTCGG TTGACCCACTTA
                                   hIL15 48aa
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  I  S  D   L  K  K  I   E  D  L  I   Q  S  M  H   I  D  A  T   L  Y  T  E
4249  GTAATAAGTGAT TTGAAAAAAATT GAAGATCTTATT CAATCTATGCAT ATTGATGCTACT TTATATACGGAA
      CATTATTCACTA AACTTTTTTTAA CTTCTAGAATAA GTTAGATACGTA TAACTACGATGA AATATATGCCTT
                                   hIL15 48aa
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       S  D  V  H   P  S  C  K   V  T  A  M   K  C  F  L   L  E  L  Q   V  I  S  L
4321  AGTGATGTTCAC CCCAGTTGCAAA GTAACAGCAATG AAGTGCTTTCTC TTGGAGTTACAA GTTATTTCACTT
      TCACTACAAGTG GGGTCAACGTTT CATTGTCGTTAC TTCACGAAAGAG AACCTCAATGTT CAATAAAGTGAA
                                   hIL15 48aa
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       E  S  G  D   A  S  I  H   D  T  V  E   N  L  I  I   L  A  N  N   S  L  S  S
4393  GAGTCTGGAGAT GCAAGTATTCAT GATACAGTAGAA AATCTGATCATC CTAGCAAACAAC AGTTTGTCTTCT
      CTCAGACCTCTA CGTTCATAAGTA CTATGTCATCTT TTAGACTAGTAG GATCGTTTGTTG TCAAACAGAAGA
                                   hIL15 48aa
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       N  G  N  V   T  E  S  G   C  K  E  C   E  E  L  E   E  K  N  I   K  E  F  L
4465  AATGGGAATGTA ACAGAATCTGGA TGCAAAGAATGT GAGGAACTGGAG GAGAAGAACATT AAGGAATTTTTG
      TTACCCTTACAT TGTCTTAGACCT ACGTTTCTTACA CTCCTTGACCTC CTCTTCTTGTAA TTCCTTAAAAAC
                     hIL15 48aa
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                                 XhoI
                                                               ~~~~~~~
       Q  S  F  V   H  I  V  Q   M  F  I  N   T  S  *  L   E
4537  CAGAGTTTTGTA CATATTGTCCAA ATGTTCATCAAC ACTTCTTGACTC GAG
      GTCTCAAAACAT GTATAACAGGTT TACAAGTAGTTG TGAAGAACTGAG CTC
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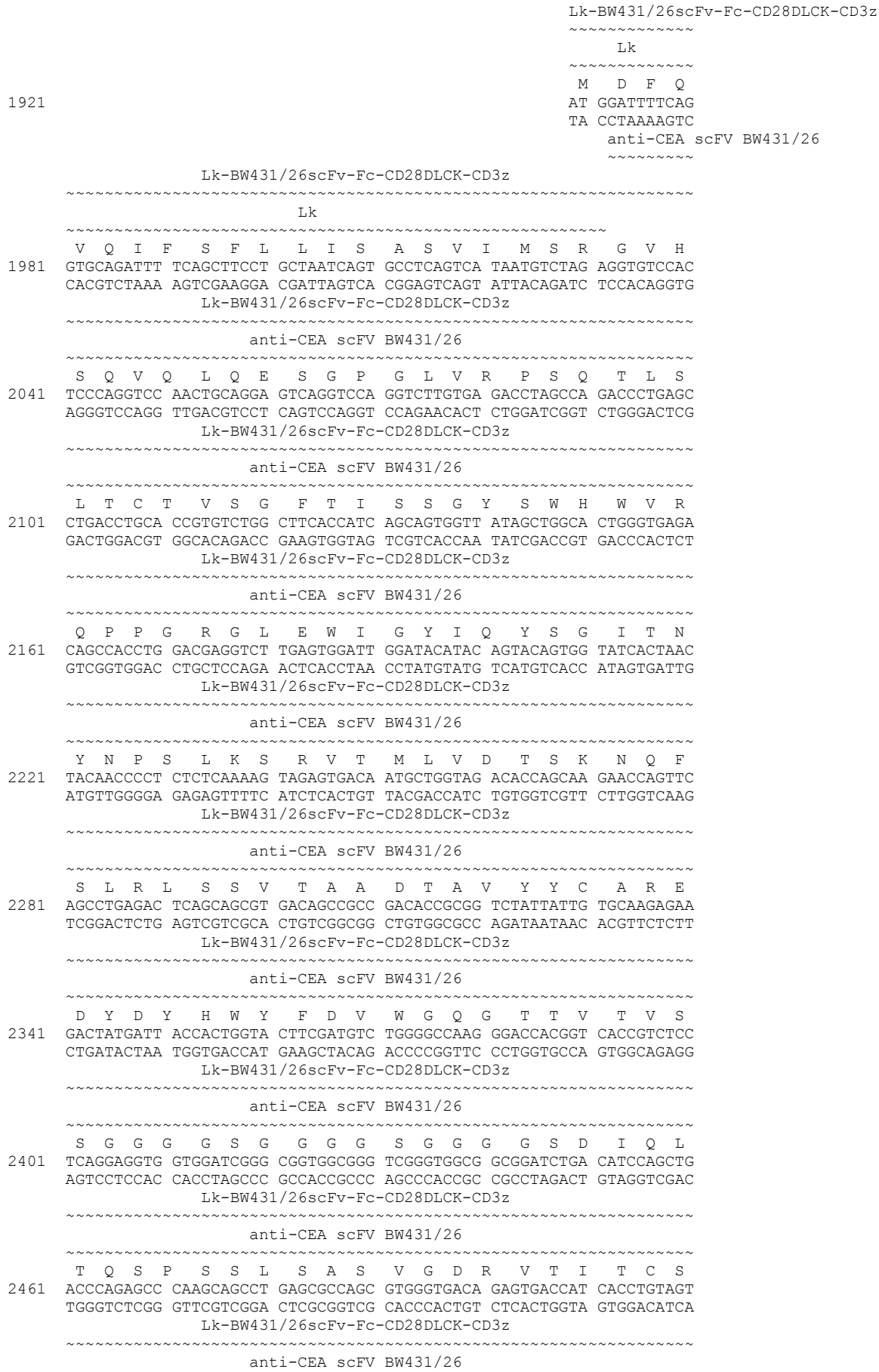
6.4 BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-IL7-T2A-IL7Rα/IL2Rβ (#1908)



```
                                  KpnI
                                 ~~~~~~~
      T  S  S  S   V  S  Y   M  H  W   Y  Q  Q  K   P  G  K   A  P  K
2521  ACCAGCTCGA GTGTAAGTTA CATGCACTGG TACCAGCAGA AGCCAGGTAA GGCTCCAAAG
      TGGTCGAGCT CACATTCAAT GTACGTGACC ATGGTCGTCT TCGGTCCATT CCGAGGTTTC
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                        anti-CEA scFV BW431/26
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      L  L  I  Y   S  T  S   N  L  A   S  G  V  P   S  R  F   S  G  S
2581  CTGCTGATCT ACAGCACATC CAACCTGGCT TCTGGTGTGC CAAGCAGATT CAGCGGTAGC
      GACGACTAGA TGTCGTGTAG GTTGGACCGA AGACCACACG GTTCGTCTAA GTCGCCATCG
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                        anti-CEA scFV BW431/26
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
             KpnI
            ~~~~~~~
      G  S  G  T   D  F  T   F  T  I   S  S  L  Q   P  E  D   I  A  T
2641  GGTAGCGGTA CCGACTTCAC CTTCACCATC AGCAGCCTCC AGCCAGAGGA CATCGCCACC
      CCATCGCCAT GGCTGAAGTG GAAGTGGTAG TCGTCGGAGG TCGGTCTCCT GTAGCGGTGG
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                        anti-CEA scFV BW431/26
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      Y  Y  C  H   Q  W  S   S  Y  P   T  F  G  Q   G  T  K   V  E  I
2701  TACTACTGCC ATCAGTGGAG TAGTTATCCC ACGTTCGGCC AAGGGACCAA GGTGGAGATC
      ATGATGACGG TAGTCACCTC ATCAATAGGG TGCAAGCCGG TTCCCTGGTT CCACCTCTAG
                              hinge
                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      anti-CEA scFV BW431/26                                    humanIgG
      ~~~~~~                                                    ~~~~~~~~~
      K  V  D  P   A  E  P   K  S  P   D  K  T  H   T  C  P   P  C  P
2761  AAAGTGGATC CCGCCGAGCC CAAATCTCCT GACAAAACTC ACACATGCCC ACCGTGCCCA
      TTTCACCTAG GGCGGCTCGG GTTTAGAGGA CTGTTTTGAG TGTGTACGGG TGGCACGGGT
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                              humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      A  P  E  L   L  G  G   P  S  V   F  L  F  P   P  K  P   K  D  T
2821  GCACCTGAAC TCCTGGGGGG ACCGTCAGTC TTCCTCTTCC CCCCAAAACC CAAGGACACC
      CGTGGACTTG AGGACCCCCC TGGCAGTCAG AAGGAGAAGG GGGGTTTTGG GTTCCTGTGG
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                              humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      L  M  I  S   R  T  P   E  V  T   C  V  V  V   D  V  S   H  E  D
2881  CTCATGATCT CCCGGACCCC TGAGGTCACA TGCGTGGTGG TGGACGTGAG CCACGAAGAC
      GAGTACTAGA GGGCCTGGGG ACTCCAGTGT ACGCACCACC ACCTGCACTC GGTGCTTCTG
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                              humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      P  E  V  K   F  N  W   Y  V  D   G  V  E  V   H  N  A   K  T  K
2941  CCTGAGGTCA AGTTCAACTG GTACGTGGAC GGCGTGGAGG TGCATAATGC CAAGACAAAG
      GGACTCCAGT TCAAGTTGAC CATGCACCTG CCGCACCTCC ACGTATTACG GTTCTGTTTC
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                              humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      P  R  E  E   Q  Y  N   S  T  Y   R  V  V  S   V  L  T   V  L  H
3001  CCGCGGGAGG AGCAGTACAA CAGCACGTAC CGTGTGGTCA GCGTCCTCAC CGTCCTGCAC
      GGCGCCCTCC TCGTCATGTT GTCGTGCATG GCACACCAGT CGCAGGAGTG GCAGGACGTG
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                              humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      Q  D  W  L   N  G  K   E  Y  K   C  K  V  S   N  K  A   L  P  A
3061  CAGGACTGGC TGAATGGCAA GGAGTACAAG TGCAAGGTCT CCAACAAAGC CCTCCCAGCC
      GTCCTGACCG ACTTACCGTT CCTCATGTTC ACGTTCCAGA GGTTGTTTCG GGAGGGTCGG
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                              humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      P  I  E  K   T  I  S   K  A  K   G  Q  P  R   E  P  Q   V  Y  T
```

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3121  CCCATCGAGA AAACCATCTC CAAAGCCAAA GGGCAGCCCC GAGAACCACA GGTGTACACC
      GGGTAGCTCT TTTGGTAGAG GTTTCGGTTT CCCGTCGGGG CTCTTGGTGT CCACATGTGG
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       L  P  P  S   R  D  E   L  T  K   N  Q  V  S   L  T  C   L  V  K
3181  CTGCCCCCAT CCCGGGATGA GCTGACCAAG AACCAGGTCA GCCTGACCTG CCTGGTCAAA
      GACGGGGGTA GGGCCCTACT CGACTGGTTC TTGGTCCAGT CGGACTGGAC GGACCAGTTT
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  F  Y  P   S  D  I   A  V  E   W  E  S  N   G  Q  P   E  N  N
3241  GGCTTCTATC CCAGCGACAT CGCCGTGGAG TGGGAGAGCA ATGGGCAGCC GGAGAACAAC
      CCGAAGATAG GGTCGCTGTA GCGGCACCTC ACCCTCTCGT TACCCGTCGG CCTCTTGTTG
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Y  K  T  T   P  P  V   L  D  S   D  G  S  F   F  L  Y   S  K  L
3301  TACAAGACCA CGCCTCCCGT GCTGGACTCC GACGGCTCCT TCTTCCTCTA CAGCAAGCTC
      ATGTTCTGGT GCGGAGGGCA CGACCTGAGG CTGCCGAGGA AGAAGGAGAT GTCGTTCGAG
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       T  V  D  K   S  R  W   Q  Q  G   N  V  F  S   C  S  V   M  H  E
3361  ACCGTGGACA AGAGCAGGTG GCAGCAGGGG AACGTCTTCT CATGCTCCGT GATGCATGAG
      TGGCACCTGT TCTCGTCCAC CGTCGTCCCC TTGCAGAAGA GTACGAGGCA CTACGTACTC
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                           humanIgG                                CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~   ~~~~~~
       A  L  H  N   H  Y  T   Q  K  S   L  S  L  S   P  G  K   K  D  P
3421  GCTCTGCACA ACCACTACAC GCAGAAGAGC CTCTCCCTGT CTCCGGGTAA AAAAGATCCC
      CGAGACGTGT TGGTGATGTG CGTCTTCTCG GAGAGGGACA GAGGCCCATT TTTTCTAGGG
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       K  F  W  V   L  V  V   V  G  G   V  L  A  C   Y  S  L   L  V  T
3481  AAATTTTGGG TGCTGGTGGT GGTTGGTGGA GTCCTGGCTT GCTATAGCTT GCTAGTAACA
      TTTAAAACCC ACGACCACCA CCAACCACCT CAGGACCGAA CGATATCGAA CGATCATTGT
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  A  F  I   I  F  W   V  R  S   K  R  S  R   L  L  H   S  D  Y
3541  GTGGCCTTTA TTATTTTCTG GGTGAGGAGT AAGAGGAGCA GGCTCCTGCA CAGTGACTAC
      CACCGGAAAT AATAAAAGAC CCACTCCTCA TTCTCCTCGT CCGAGGACGT GTCACTGATG
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       M  N  M  T   P  R  R   P  G  P   T  R  K  H   Y  Q  A   Y  A  A
3601  ATGAACATGA CTCCCCGCCG CCCCGGGCCC ACCCGCAAGC ATTACCAGGC CTATGCCGCC
      TACTTGTACT GAGGGGCGGC GGGGCCCGGG TGGGCGTTCG TAATGGTCCG GATACGGCGG
                                                CD3z
                                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
              CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  R  D  F   A  A  Y   R  S  L   R  V  K  F   S  R  S   A  D  A
3661  GCACGCGACT TCGCAGCCTA TCGCTCCCTG AGAGTGAAGT TCAGCAGGAG CGCAGACGCC
      CGTGCGCTGA AGCGTCGGAT AGCGAGGGAC TCTCACTTCA AGTCGTCCTC GCGTCTGCGG
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  A  Y  Q   Q  G  Q   N  Q  L   Y  N  E  L   N  L  G   R  R  E
3721  CCCGCGTACC AGCAGGGCCA GAACCAGCTC TATAACGAGC TCAATCTAGG ACGAAGAGAG
      GGGCGCATGG TCGTCCCGGT CTTGGTCGAG ATATTGCTCG AGTTAGATCC TGCTTCTCTC
                  Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
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      E  Y  D  V   L  D  K   R  R  G   R  D  P  E   M  G  G   K  P  R
3781  GAGTACGATG TTTTGGACAA GAGACGTGGC CGGGACCCTG AGATGGGGGG AAAGCCGAGA
      CTCATGCTAC AAAACCTGTT CTCTGCACCG GCCCTGGGAC TCTACCCCCC TTTCGGCTCT
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                    CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      R  K  N  P   Q  E  G   L  Y  N   E  L  Q  K   D  K  M   A  E  A
3841  AGGAAGAACC CTCAGGAAGG CCTGTACAAT GAACTGCAGA AAGATAAGAT GGCGGAGGCC
      TCCTTCTTGG GAGTCCTTCC GGACATGTTA CTTGACGTCT TTCTATTCTA CCGCCTCCGG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                    CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      Y  S  E  I   G  M  K   G  E  R   R  R  G  K   G  H  D   G  L  Y
3901  TACAGTGAGA TTGGGATGAA AGGCGAGCGC CGGAGGGGCA AGGGGCACGA TGGCCTTTAC
      ATGTCACTCT AACCCTACTT TCCGCTCGCG GCCTCCCCGT TCCCCGTGCT ACCGGAAATG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                    CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      Q  G  L  S   T  A  T   K  D  T   Y  D  A  L   H  M  Q   A  L  P
3961  CAGGGTCTCA GTACAGCCAC CAAGGACACC TACGACGCCC TTCACATGCA GGCCCTGCCC
      GTCCCAGAGT CATGTCGGTG GTTCCTGTGG ATGCTGCGGG AAGTGTACGT CCGGGACGGG
                                    P2A
            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~
       CD3z
      ~~~~~~
       P  R  G  S   G  A  T   N  F  S   L  L  K  Q   A  G  D   V  E  E
4021  CCTCGCGGAT CTGGAGCCAC GAACTTCTCT CTGTTAAAGC AAGCAGGAGA CGTGGAAGAA
      GGAGCGCCTA GACCTCGGTG CTTGAAGAGA GACAATTTCG TTCGTCCTCT GCACCTTCTT
          P2A
      ~~~~~~~~~~~~~
                                      mIL7
                  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                 mIL7 Signal Peptide
                  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       N  P  G  P   M  F  H   V  S  F   R  Y  I  F   G  I  P   P  L  I
4081  AACCCCGGTC CTATGTTCCA TGTTTCTTTT AGATATATCT TTGGAATTCC TCCACTGATC
      TTGGGGCCAG GATACAAGGT ACAAAGAAAA TCTATATAGA AACCTTAAGG AGGTGACTAG
                                    mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
          mIL7 Signal Peptide
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       L  V  L  L   P  V  T   S  S  E   C  H  I  K   D  K  E   G  K  A
4141  CTTGTTCTGC TGCCTGTCAC ATCATCTGAG TGCCACATTA AAGACAAAGA AGGTAAAGCA
      GAACAAGACG ACGGACAGTG TAGTAGACTC ACGGTGTAAT TTCTGTTTCT TCCATTTCGT
                                    mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                       BclI
                      ~~~~~~~
       Y  E  S  V   L  M  I   S  I  D   E  L  D  K   M  T  G   T  D  S
4201  TATGAGAGTG TACTGATGAT CAGCATCGAT GAATTGGACA AAATGACAGG AACTGATAGT
      ATACTCTCAC ATGACTACTA GTCGTAGCTA CTTAACCTGT TTTACTGTCC TTGACTATCA
                                    mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       N  C  P  N   N  E  P   N  F  F   R  K  H  V   C  D  D   T  K  E
4261  AATTGCCCGA ATAATGAACC AAACTTTTTT AGAAAACATG TATGTGATGA TACAAAGGAA
      TTAACGGGCT TATTACTTGG TTTGAAAAAA TCTTTTGTAC ATACACTACT ATGTTTCCTT
                                    mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  A  F  L   N  R  A   A  R  K   L  K  Q  F   L  K  M   N  I  S
4321  GCTGCTTTTC TAAATCGTGC TGCTCGCAAG TTGAAGCAAT TTCTTAAAAT GAATATCAGT
      CGACGAAAAG ATTTAGCACG ACGAGCGTTC AACTTCGTTA AAGAATTTTA CTTATAGTCA
                                    mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       E  E  F  N   V  H  L   L  T  V   S  Q  G  T   Q  T  L   V  N  C
4381  GAAGAATTCA ATGTCCACTT ACTAACAGTA TCACAAGGCA CACAAACACT GGTGAACTGC
      CTTCTTAAGT TACAGGTGAA TGATTGTCAT AGTGTTCCGT GTGTTTGTGA CCACTTGACG
                                    mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       T  S  K  E   E  K  N   V  K  E   Q  K  K  N   D  A  C   F  L  K
4441  ACGAGTAAGG AAGAAAAAAA CGTAAAGGAA CAGAAAAAGA ATGATGCATG TTTCCTAAAG
      TGCTCATTCC TTCTTTTTTT GCATTTCCTT GTCTTTTTCT TACTACGTAC AAAGGATTTC
                                mIL7
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
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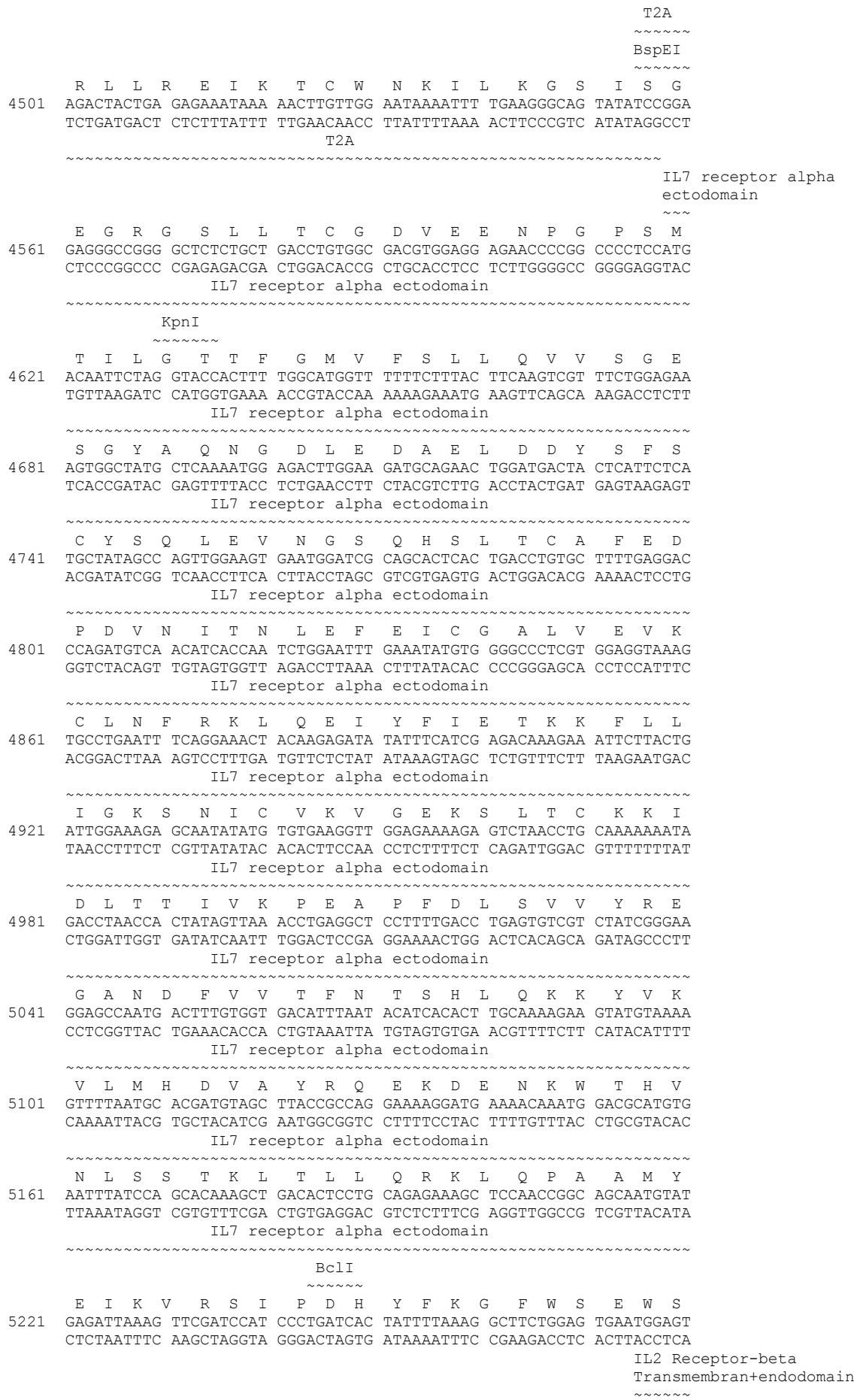
T2A
BspEI
R L L R E I K T C W N K I L K G S I S G
4501 AGACTACTGA GAGAAATAAA AACTTGTTGG AATAAAATTT TGAAGGGCAG TATATCCGGA
TCTGATGACT CTCTTTATTT TTGAACAACC TTATTTTAAA ACTTCCCGTC ATATAGGCCT
T2A
IL7 receptor alpha
ectodomain
E G R G S L L T C G D V E E N P G P S M
4561 GAGGGCCGGG GCTCTCTGCT GACCTGTGGC GACGTGGAGG AGAACCCCGG CCCCTCCATG
CTCCCGGCCC CGAGAGACGA CTGGACACCG CTGCACCTCC TCTTGGGGCC GGGGAGGTAC
IL7 receptor alpha ectodomain
KpnI
T I L G T T F G M V F S L L Q V V S G E
4621 ACAATTCTAG GTACCACTTT TGGCATGGTT TTTTCTTTAC TTCAAGTCGT TTCTGGAGAA
TGTTAAGATC CATGGTGAAA ACCGTACCAA AAAAGAAATG AAGTTCAGCA AAGACCTCTT
IL7 receptor alpha ectodomain
S G Y A Q N G D L E D A E L D D Y S F S
4681 AGTGGCTATG CTCAAAATGG AGACTTGGAA GATGCAGAAC TGGATGACTA CTCATTCTCA
TCACCGATAC GAGTTTTACC TCTGAACCTT CTACGTCTTG ACCTACTGAT GAGTAAGAGT
IL7 receptor alpha ectodomain
C Y S Q L E V N G S Q H S L T C A F E D
4741 TGCTATAGCC AGTTGGAAGT GAATGGATCG CAGCACTCAC TGACCTGTGC TTTTGAGGAC
ACGATATCGG TCAACCTTCA CTTACCTAGC GTCGTGAGTG ACTGGACACG AAAACTCCTG
IL7 receptor alpha ectodomain
P D V N I T N L E F E I C G A L V E V K
4801 CCAGATGTCA ACATCACCAA TCTGGAATTT GAAATATGTG GGGCCCTCGT GGAGGTAAAG
GGTCTACAGT TGTAGTGGTT AGACCTTAAA CTTTATACAC CCCGGGAGCA CCTCCATTTC
IL7 receptor alpha ectodomain
C L N F R K L Q E I Y F I E T K K F L L
4861 TGCCTGAATT TCAGGAAACT ACAAGAGATA TATTTCATCG AGACAAAGAA ATTCTTACTG
ACGGACTTAA AGTCCTTTGA TGTTCTCTAT ATAAAGTAGC TCTGTTTCTT TAAGAATGAC
IL7 receptor alpha ectodomain
I G K S N I C V K V G E K S L T C K K I
4921 ATTGGAAAGA GCAATATATG TGTGAAGGTT GGAGAAAAGA GTCTAACCTG CAAAAAAATA
TAACCTTTCT CGTTATATAC ACACTTCCAA CCTCTTTTCT CAGATTGGAC GTTTTTTTAT
IL7 receptor alpha ectodomain
D L T T I V K P E A P F D L S V V Y R E
4981 GACCTAACCA CTATAGTTAA ACCTGAGGCT CCTTTTGACC TGAGTGTCGT CTATCGGGAA
CTGGATTGGT GATATCAATT TGGACTCCGA GGAAAACTGG ACTCACAGCA GATAGCCCTT
IL7 receptor alpha ectodomain
G A N D F V V T F N T S H L Q K K Y V K
5041 GGAGCCAATG ACTTTGTGGT GACATTTAAT ACATCACACT TGCAAAAGAA GTATGTAAAA
CCTCGGTTAC TGAAACACCA CTGTAAATTA TGTAGTGTGA ACGTTTTCTT CATACATTTT
IL7 receptor alpha ectodomain
V L M H D V A Y R Q E K D E N K W T H V
5101 GTTTTAATGC ACGATGTAGC TTACCGCCAG GAAAAGGATG AAAACAAATG GACGCATGTG
CAAAATTACG TGCTACATCG AATGGCGGTC CTTTTCCTAC TTTTGTTTAC CTGCGTACAC
IL7 receptor alpha ectodomain
N L S S T K L T L L Q R K L Q P A A M Y
5161 AATTTATCCA GCACAAAGCT GACACTCCTG CAGAGAAAGC TCCAACCGGC AGCAATGTAT
TTAAATAGGT CGTGTTTCGA CTGTGAGGAC GTCTCTTTCG AGGTTGGCCG TCGTTACATA
IL7 receptor alpha ectodomain
BclI
E I K V R S I P D H Y F K G F W S E W S
5221 GAGATTAAAG TTCGATCCAT CCCTGATCAC TATTTTAAAG GCTTCTGGAG TGAATGGAGT
CTCTAATTTC AAGCTAGGTA GGGACTAGTG ATAAAATTTC CGAAGACCTC ACTTACCTCA
IL2 Receptor-beta
Transmembran+endodomain
IL7 receptor alpha ectodomain

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      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  S  Y  Y   F  R  T   P  E  I   N  N  S  S   G  E  M   D  I  P
5281  CCAAGTTATT ACTTCAGAAC TCCAGAGATC AATAATAGCT CAGGGGAGAT GGATATTCCG
      GGTTCAATAA TGAAGTCTTG AGGTCTCTAG TTATTATCGA GTCCCCTCTA CCTATAAGGC
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       W  L  G  H   L  L  V   G  L  S   G  A  F  G   F  I  I   L  V  Y
5341  TGGCTCGGCC ACCTCCTCGT GGGCCTCAGC GGGGCTTTTG GCTTCATCAT CTTAGTGTAC
      ACCGAGCCGG TGGAGGAGCA CCCGGAGTCG CCCCGAAAAC CGAAGTAGTA GAATCACATG
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
           BclI
          ~~~~~~~
       L  L  I  N   C  R  N   T  G  P   W  L  K  K   V  L  K   C  N  T
5401  TTGCTGATCA ACTGCAGGAA CACCGGGCCA TGGCTGAAGA AGGTCCTGAA GTGTAACACC
      AACGACTAGT TGACGTCCTT GTGGCCCGGT ACCGACTTCT TCCAGGACTT CACATTGTGG
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  D  P  S   K  F  F   S  Q  L   S  S  E  H   G  G  D   V  Q  K
5461  CCAGACCCCT CGAAGTTCTT TTCCCAGCTG AGCTCAGAGC ATGGAGGAGA CGTCCAGAAG
      GGTCTGGGGA GCTTCAAGAA AAGGGTCGAC TCGAGTCTCG TACCTCCTCT GCAGGTCTTC
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       W  L  S  S   P  F  P   S  S  S   F  S  P  G   G  L  A   P  E  I
5521  TGGCTCTCTT CGCCCTTCCC CTCATCGTCC TTCAGCCCTG GCGGCCTGGC ACCTGAGATC
      ACCGAGAGAA GCGGGAAGGG GAGTAGCAGG AAGTCGGGAC CGCCGGACCG TGGACTCTAG
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       S  P  L  E   V  L  E   R  D  K   V  T  Q  L   L  L  Q   Q  D  K
5581  TCGCCACTAG AAGTGCTGGA GAGGGACAAG GTGACGCAGC TGCTCCTGCA GCAGGACAAG
      AGCGGTGATC TTCACGACCT CTCCCTGTTC CACTGCGTCG ACGAGGACGT CGTCCTGTTC
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  P  E  P   A  S  L   S  S  N   H  S  L  T   S  C  F   T  N  Q
5641  GTGCCTGAGC CCGCATCCTT AAGCAGCAAC CACTCGCTGA CCAGCTGCTT CACCAACCAG
      CACGGACTCG GGCGTAGGAA TTCGTCGTTG GTGAGCGACT GGTCGACGAA GTGGTTGGTC
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  Y  F  F   F  H  L   P  D  A   L  E  I  E   A  C  Q   V  Y  F
5701  GGTTACTTCT TCTTCCACCT CCCGGATGCC TTGGAGATAG AGGCCTGCCA GGTGTACTTT
      CCAATGAAGA AGAAGGTGGA GGGCCTACGG AACCTCTATC TCCGGACGGT CCACATGAAA
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       T  Y  D  P   Y  S  E   E  D  P   D  E  G  V   A  G  A   P  T  G
5761  ACTTACGACC CCTACTCAGA GGAAGACCCT GATGAGGGTG TGGCCGGGGC ACCCACAGGG
      TGAATGCTGG GGATGAGTCT CCTTCTGGGA CTACTCCCAC ACCGGCCCCG TGGGTGTCCC
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       S  S  P  Q   P  L  Q   P  L  S   G  E  D  D   A  Y  C   T  F  P
5821  TCTTCCCCCC AACCCCTGCA GCCTCTGTCA GGGGAGGACG ACGCCTACTG CACCTTCCCC
      AGAAGGGGGG TTGGGGACGT CGGAGACAGT CCCCTCCTGC TGCGGATGAC GTGGAAGGGG
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       S  R  D  D   L  L  L   F  S  P   S  L  L  G   G  P  S   P  P  S
5881  TCCAGGGATG ACCTGCTGCT CTTCTCCCCC AGTCTCCTCG GTGGCCCCAG CCCCCCAAGC
      AGGTCCCTAC TGGACGACGA GAAGAGGGGG TCAGAGGAGC CACCGGGGTC GGGGGGTTCG
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       T  A  P  G   G  S  G   A  G  E   E  R  M  P   P  S  L   Q  E  R
5941  ACTGCCCCTG GGGGCAGTGG GGCCGGTGAA GAGAGGATGC CCCCTTCTTT GCAAGAAAGA
      TGACGGGGAC CCCCGTCACC CCGGCCACTT CTCTCCTACG GGGGAAGAAA CGTTCTTTCT
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  P  R  D   W  D  P   Q  P  L   G  P  P  T   P  G  V   P  D  L
6001  GTCCCCAGAG ACTGGGACCC CCAGCCCCTG GGGCCTCCCA CCCCAGGAGT CCCAGACCTG
      CAGGGGTCTC TGACCCTGGG GGTCGGGGAC CCCGGAGGGT GGGGTCCTCA GGGTCTGGAC
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  D  F  Q   P  P  P   E  L  V   L  R  E  A   G  E  E   V  P  D
6061  GTGGATTTTC AGCCACCCCC TGAGCTGGTG CTGCGAGAGG CTGGGGAGGA GGTCCCTGAC
      CACCTAAAAG TCGGTGGGGG ACTCGACCAC GACGCTCTCC GACCCCTCCT CCAGGGACTG
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  G  P  R   E  G  V   S  F  P   W  S  R  P   P  G  Q   G  E  F
6121  GCTGGCCCCA GGGAGGGAGT CAGTTTCCCC TGGTCCAGGC CTCCTGGGCA GGGGGAGTTC
      CGACCGGGGT CCCTCCCTCA GTCAAAGGGG ACCAGGTCCG GAGGACCCGT CCCCCTCAAG
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
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       R  A  L  N   A  R  L   P  L  N   T  D  A  Y   L  S  L   Q  E  L
6181   AGGGCCCTTA ATGCTCGCCT GCCCCTGAAC ACTGATGCCT ACTTGTCCCT CCAAGAACTC
       TCCCGGGAAT TACGAGCGGA CGGGGACTTG TGACTACGGA TGAACAGGGA GGTTCTTGAG
       IL2 Receptor-beta Transmembran+endodomain
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                       BspEI
                                       ~~~~~~
        Q  G  Q  D   P  T  H   L  V  *   S  G
6241   CAGGGTCAGG ACCCAACTCA CTTGGTGTAG TCCGGA
       GTCCCAGTCC TGGGTTGAGT GAACCACATC AGGCCT
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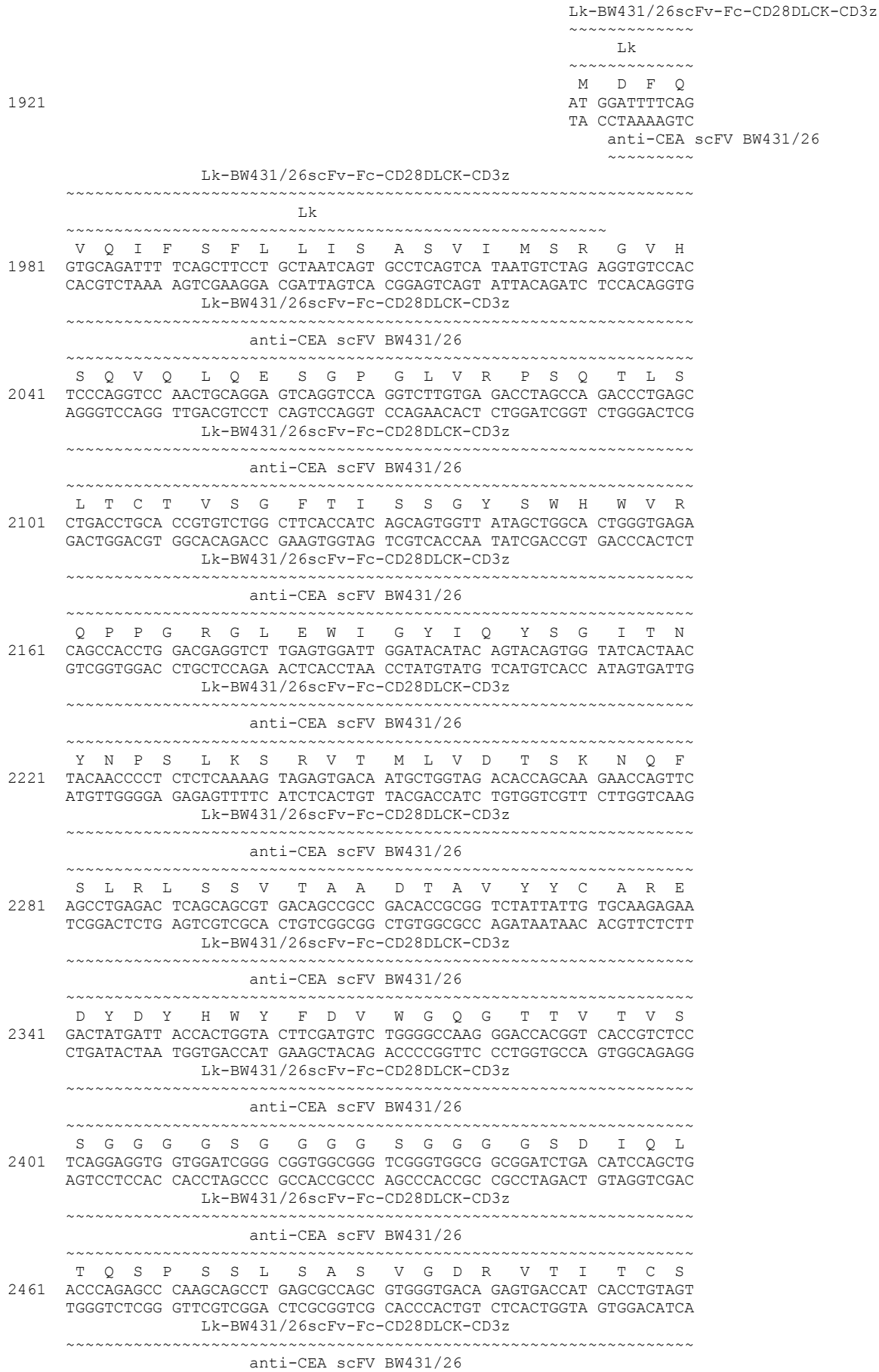
6.5 BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-T2A-IL7Rα/IL2Rβ (#1941)



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      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       T  S  S  S   V  S  Y   M  H  W   Y  Q  Q  K   P  G  K   A  P  K
2521  ACCAGCTCGA GTGTAAGTTA CATGCACTGG TACCAGCAGA AGCCAGGTAA GGCTCCAAAG
      TGGTCGAGCT CACATTCAAT GTACGTGACC ATGGTCGTCT TCGGTCCATT CCGAGGTTTC
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          anti-CEA scFV BW431/26
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       L  L  I  Y   S  T  S   N  L  A   S  G  V  P   S  R  F   S  G  S
2581  CTGCTGATCT ACAGCACATC CAACCTGGCT TCTGGTGTGC CAAGCAGATT CAGCGGTAGC
      GACGACTAGA TGTCGTGTAG GTTGGACCGA AGACCACACG GTTCGTCTAA GTCGCCATCG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          anti-CEA scFV BW431/26
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  S  G  T   D  F  T   F  T  I   S  S  L  Q   P  E  D   I  A  T
2641  GGTAGCGGTA CCGACTTCAC CTTCACCATC AGCAGCCTCC AGCCAGAGGA CATCGCCACC
      CCATCGCCAT GGCTGAAGTG GAAGTGGTAG TCGTCGGAGG TCGGTCTCCT GTAGCGGTGG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          anti-CEA scFV BW431/26
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Y  Y  C  H   Q  W  S   S  Y  P   T  F  G  Q   G  T  K   V  E  I
2701  TACTACTGCC ATCAGTGGAG TAGTTATCCC ACGTTCGGCC AAGGGACCAA GGTGGAGATC
      ATGATGACGG TAGTCACCTC ATCAATAGGG TGCAAGCCGG TTCCCTGGTT CCACCTCTAG
                                   hinge
                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      anti-CEA scFV BW431/26                                      humanIgG
      ~~~~~~                                                      ~~~~~~~~~
       K  V  D  P   A  E  P   K  S  P   D  K  T  H   T  C  P   P  C  P
2761  AAAGTGGATC CCGCCGAGCC CAAATCTCCT GACAAAACTC ACACATGCCC ACCGTGCCCA
      TTTCACCTAG GGCGGCTCGG GTTTAGAGGA CTGTTTTGAG TGTGTACGGG TGGCACGGGT
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                               humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  P  E  L   L  G  G   P  S  V   F  L  F  P   P  K  P   K  D  T
2821  GCACCTGAAC TCCTGGGGGG ACCGTCAGTC TTCCTCTTCC CCCCAAAACC CAAGGACACC
      CGTGGACTTG AGGACCCCCC TGGCAGTCAG AAGGAGAAGG GGGGTTTTGG GTTCCTGTGG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                               humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       L  M  I  S   R  T  P   E  V  T   C  V  V  V   D  V  S   H  E  D
2881  CTCATGATCT CCCGGACCCC TGAGGTCACA TGCGTGGTGG TGGACGTGAG CCACGAAGAC
      GAGTACTAGA GGGCCTGGGG ACTCCAGTGT ACGCACCACC ACCTGCACTC GGTGCTTCTG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                               humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  E  V  K   F  N  W   Y  V  D   G  V  E  V   H  N  A   K  T  K
2941  CCTGAGGTCA AGTTCAACTG GTACGTGGAC GGCGTGGAGG TGCATAATGC CAAGACAAAG
      GGACTCCAGT TCAAGTTGAC CATGCACCTG CCGCACCTCC ACGTATTACG GTTCTGTTTC
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                               humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  R  E  E   Q  Y  N   S  T  Y   R  V  V  S   V  L  T   V  L  H
3001  CCGCGGGAGG AGCAGTACAA CAGCACGTAC CGTGTGGTCA GCGTCCTCAC CGTCCTGCAC
      GGCGCCCTCC TCGTCATGTT GTCGTGCATG GCACACCAGT CGCAGGAGTG GCAGGACGTG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                               humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Q  D  W  L   N  G  K   E  Y  K   C  K  V  S   N  K  A   L  P  A
3061  CAGGACTGGC TGAATGGCAA GGAGTACAAG TGCAAGGTCT CCAACAAAGC CCTCCCAGCC
      GTCCTGACCG ACTTACCGTT CCTCATGTTC ACGTTCCAGA GGTTGTTTCG GGAGGGTCGG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                               humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  I  E  K   T  I  S   K  A  K   G  Q  P  R   E  P  Q   V  Y  T
3121  CCCATCGAGA AAACCATCTC CAAAGCCAAA GGGCAGCCCC GAGAACCACA GGTGTACACC
      GGGTAGCTCT TTTGGTAGAG GTTTCGGTTT CCCGTCGGGG CTCTTGGTGT CCACATGTGG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
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                            humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       L  P  P  S   R  D  E   L  T  K   N  Q  V  S   L  T  C   L  V  K
3181  CTGCCCCCAT CCCGGGATGA GCTGACCAAG AACCAGGTCA GCCTGACCTG CCTGGTCAAA
      GACGGGGGTA GGGCCCTACT CGACTGGTTC TTGGTCCAGT CGGACTGGAC GGACCAGTTT
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  F  Y  P   S  D  I   A  V  E   W  E  S  N   G  Q  P   E  N  N
3241  GGCTTCTATC CCAGCGACAT CGCCGTGGAG TGGGAGAGCA ATGGGCAGCC GGAGAACAAC
      CCGAAGATAG GGTCGCTGTA GCGGCACCTC ACCCTCTCGT TACCCGTCGG CCTCTTGTTG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Y  K  T  T   P  P  V   L  D  S   D  G  S  F   F  L  Y   S  K  L
3301  TACAAGACCA CGCCTCCCGT GCTGGACTCC GACGGCTCCT TCTTCCTCTA CAGCAAGCTC
      ATGTTCTGGT GCGGAGGGCA CGACCTGAGG CTGCCGAGGA AGAAGGAGAT GTCGTTCGAG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            humanIgG
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       T  V  D  K   S  R  W   Q  Q  G   N  V  F  S   C  S  V   M  H  E
3361  ACCGTGGACA AGAGCAGGTG GCAGCAGGGG AACGTCTTCT CATGCTCCGT GATGCATGAG
      TGGCACCTGT TCTCGTCCAC CGTCGTCCCC TTGCAGAAGA GTACGAGGCA CTACGTACTC
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          humanIgG                                CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~   ~~~~~~
       A  L  H  N   H  Y  T   Q  K  S   L  S  L  S   P  G  K   K  D  P
3421  GCTCTGCACA ACCACTACAC GCAGAAGAGC CTCTCCCTGT CTCCGGGTAA AAAAGATCCC
      CGAGACGTGT TGGTGATGTG CGTCTTCTCG GAGAGGGACA GAGGCCCATT TTTTCTAGGG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       K  F  W  V   L  V  V   V  G  G   V  L  A  C   Y  S  L   L  V  T
3481  AAATTTTGGG TGCTGGTGGT GGTTGGTGGA GTCCTGGCTT GCTATAGCTT GCTAGTAACA
      TTTAAAACCC ACGACCACCA CCAACCACCT CAGGACCGAA CGATATCGAA CGATCATTGT
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  A  F  I   I  F  W   V  R  S   K  R  S  R   L  L  H   S  D  Y
3541  GTGGCCTTTA TTATTTTCTG GGTGAGGAGT AAGAGGAGCA GGCTCCTGCA CAGTGACTAC
      CACCGGAAAT AATAAAAGAC CCACTCCTCA TTCTCCTCGT CCGAGGACGT GTCACTGATG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       M  N  M  T   P  R  R   P  G  P   T  R  K  H   Y  Q  A   Y  A  A
3601  ATGAACATGA CTCCCCGCCG CCCCGGGCCC ACCCGCAAGC ATTACCAGGC CTATGCCGCC
      TACTTGTACT GAGGGGCGGC GGGGCCCGGG TGGGCGTTCG TAATGGTCCG GATACGGCGG
                                                    CD3z
                                      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
            CD28DLCK
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  R  D  F   A  A  Y   R  S  L   R  V  K  F   S  R  S   A  D  A
3661  GCACGCGACT TCGCAGCCTA TCGCTCCCTG AGAGTGAAGT TCAGCAGGAG CGCAGACGCC
      CGTGCGCTGA AGCGTCGGAT AGCGAGGGAC TCTCACTTCA AGTCGTCCTC GCGTCTGCGG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                              CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       P  A  Y  Q   Q  G  Q   N  Q  L   Y  N  E  L   N  L  G   R  R  E
3721  CCCGCGTACC AGCAGGGCCA GAACCAGCTC TATAACGAGC TCAATCTAGG ACGAAGAGAG
      GGGCGCATGG TCGTCCCGGT CTTGGTCGAG ATATTGCTCG AGTTAGATCC TGCTTCTCTC
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                              CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                  SanDI
                                 ~~~~~~~
       E  Y  D  V   L  D  K   R  R  G   R  D  P  E   M  G  G   K  P  R
3781  GAGTACGATG TTTTGGACAA GAGACGTGGC CGGGACCCTG AGATGGGGGG AAAGCCGAGA
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      CTCATGCTAC AAAACCTGTT CTCTGCACCG GCCCTGGGAC TCTACCCCCC TTTCGGCTCT
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                  CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       R  K  N  P   Q  E  G   L  Y  N   E  L  Q  K   D  K  M   A  E  A
3841  AGGAAGAACC CTCAGGAAGG CCTGTACAAT GAACTGCAGA AAGATAAGAT GGCGGAGGCC
      TCCTTCTTGG GAGTCCTTCC GGACATGTTA CTTGACGTCT TTCTATTCTA CCGCCTCCGG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                  CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Y  S  E  I   G  M  K   G  E  R   R  R  G  K   G  H  D   G  L  Y
3901  TACAGTGAGA TTGGGATGAA AGGCGAGCGC CGGAGGGGCA AGGGGCACGA TGGCCTTTAC
      ATGTCACTCT AACCCTACTT TCCGCTCGCG GCCTCCCCGT TCCCCGTGCT ACCGGAAATG
                    Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                  CD3z
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Q  G  L  S   T  A  T   K  D  T   Y  D  A  L   H  M  Q   A  L  P
3961  CAGGGTCTCA GTACAGCCAC CAAGGACACC TACGACGCCC TTCACATGCA GGCCCTGCCC
      GTCCCAGAGT CATGTCGGTG GTTCCTGTGG ATGCTGCGGG AAGTGTACGT CCGGGACGGG
      Lk-BW431/26scFv-Fc-CD28DLCK-CD3z
      ~~~~~~
                                    T2A
            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       CD3z
      ~~~~~~
            BspEI
            ~~~~~~~
       P  R  S  G   E  G  R   G  S  L   L  T  C  G   D  V  E   E  N  P
4021  CCTCGCTCCG GAGAGGGCCG GGGCTCTCTG CTGACCTGTG GCGACGTGGA GGAGAACCCC
      GGAGCGAGGC CTCTCCCGGC CCCGAGAGAC GACTGGACAC CGCTGCACCT CCTCTTGGGG
         T2A
      ~~~~~~~~~
                         IL7 receptor alpha ectodomain
               ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       G  P  S  M   T  I  L   G  T  T   F  G  M  V   F  S  L   L  Q  V
4081  GGCCCCTCCA TGACAATTCT AGGTACCACT TTTGGCATGG TTTTTTCTTT ACTTCAAGTC
      CCGGGGAGGT ACTGTTAAGA TCCATGGTGA AAACCGTACC AAAAAAGAAA TGAAGTTCAG
                    IL7 receptor alpha ectodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  S  G  E   S  G  Y   A  Q  N   G  D  L  E   D  A  E   L  D  D
4141  GTTTCTGGAG AAAGTGGCTA TGCTCAAAAT GGAGACTTGG AAGATGCAGA ACTGGATGAC
      CAAAGACCTC TTTCACCGAT ACGAGTTTTA CCTCTGAACC TTCTACGTCT TGACCTACTG
                    IL7 receptor alpha ectodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Y  S  F  S   C  Y  S   Q  L  E   V  N  G  S   Q  H  S   L  T  C
4201  TACTCATTCT CATGCTATAG CCAGTTGGAA GTGAATGGAT CGCAGCACTC ACTGACCTGT
      ATGAGTAAGA GTACGATATC GGTCAACCTT CACTTACCTA GCGTCGTGAG TGACTGGACA
                    IL7 receptor alpha ectodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  F  E  D   P  D  V   N  I  T   N  L  E  F   E  I  C   G  A  L
4261  GCTTTTGAGG ACCCAGATGT CAACATCACC AATCTGGAAT TTGAAATATG TGGGGCCCTC
      CGAAAACTCC TGGGTCTACA GTTGTAGTGG TTAGACCTTA AACTTTATAC ACCCCGGGAG
                    IL7 receptor alpha ectodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  E  V  K   C  L  N   F  R  K   L  Q  E  I   Y  F  I   E  T  K
4321  GTGGAGGTAA AGTGCCTGAA TTTCAGGAAA CTACAAGAGA TATATTTCAT CGAGACAAAG
      CACCTCCATT TCACGGACTT AAAGTCCTTT GATGTTCTCT ATATAAAGTA GCTCTGTTTC
                    IL7 receptor alpha ectodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       K  F  L  L   I  G  K   S  N  I   C  V  K  V   G  E  K   S  L  T
4381  AAATTCTTAC TGATTGGAAA GAGCAATATA TGTGTGAAGG TTGGAGAAAA GAGTCTAACC
      TTTAAGAATG ACTAACCTTT CTCGTTATAT ACACACTTCC AACCTCTTTT CTCAGATTGG
                    IL7 receptor alpha ectodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       C  K  K  I   D  L  T   T  I  V   K  P  E  A   P  F  D   L  S  V
4441  TGCAAAAAAA TAGACCTAAC CACTATAGTT AAACCTGAGG CTCCTTTTGA CCTGAGTGTC
      ACGTTTTTTT ATCTGGATTG GTGATATCAA TTTGGACTCC GAGGAAAACT GGACTCACAG
                    IL7 receptor alpha ectodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       V  Y  R  E   G  A  N   D  F  V   V  T  F  N   T  S  H   L  Q  K
4501  GTCTATCGGG AAGGAGCCAA TGACTTTGTG GTGACATTTA ATACATCACA CTTGCAAAAG
      CAGATAGCCC TTCCTCGGTT ACTGAAACAC CACTGTAAAT TATGTAGTGT GAACGTTTTC
                    IL7 receptor alpha ectodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       K  Y  V  K   V  L  M   H  D  V   A  Y  R  Q   E  K  D   E  N  K
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4561  AAGTATGTAA AAGTTTTAAT GCACGATGTA GCTTACCGCC AGGAAAAGGA TGAAAACAAA
      TTCATACATT TTCAAAATTA CGTGCTACAT CGAATGGCGG TCCTTTTCCT ACTTTTGTTT
                    IL7 receptor alpha ectodomain
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       W  T  H  V   N  L  S   S  T  K   L  T  L  L   Q  R  K   L  Q  P
4621  TGGACGCATG TGAATTTATC CAGCACAAAG CTGACACTCC TGCAGAGAAA GCTCCAACCG
      ACCTGCGTAC ACTTAAATAG GTCGTGTTTC GACTGTGAGG ACGTCTCTTT CGAGGTTGGC
                    IL7 receptor alpha ectodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  A  M  Y   E  I  K   V  R  S   I  P  D  H   Y  F  K   G  F  W
4681  GCAGCAATGT ATGAGATTAA AGTTCGATCC ATCCCTGATC ACTATTTTAA AGGCTTCTGG
      CGTCGTTACA TACTCTAATT TCAAGCTAGG TAGGGACTAG TGATAAAATT TCCGAAGACC
                    IL7 receptor alpha ectodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       S  E  W  S   P  S  Y   Y  F  R   T  P  E  I   N  N  S   S  G  E
4741  AGTGAATGGA GTCCAAGTTA TTACTTCAGA ACTCCAGAGA TCAATAATAG CTCAGGGGAG
      TCACTTACCT CAGGTTCAAT AATGAAGTCT TGAGGTCTCT AGTTATTATC GAGTCCCCTC
                  IL2 Receptor-beta Transmembran+endodomain
            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
      IL7 receptor alpha ectodomain
      ~~~~~~
       M  D  I  P   W  L  G   H  L  L   V  G  L  S   G  A  F   G  F  I
4801  ATGGATATTC CGTGGCTCGG CCACCTCCTC GTGGGCCTCA GCGGGGCTTT TGGCTTCATC
      TACCTATAAG GCACCGAGCC GGTGGAGGAG CACCCGGAGT CGCCCCGAAA ACCGAAGTAG
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       I  L  V  Y   L  L  I   N  C  R   N  T  G  P   W  L  K   K  V  L
4861  ATCTTAGTGT ACTTGCTGAT CAACTGCAGG AACACCGGGC CATGGCTGAA GAAGGTCCTG
      TAGAATCACA TGAACGACTA GTTGACGTCC TTGTGGCCCG GTACCGACTT CTTCCAGGAC
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       K  C  N  T   P  D  P   S  K  F   F  S  Q  L   S  S  E   H  G  G
4921  AAGTGTAACA CCCCAGACCC CTCGAAGTTC TTTTCCCAGC TGAGCTCAGA GCATGGAGGA
      TTCACATTGT GGGGTCTGGG GAGCTTCAAG AAAAGGGTCG ACTCGAGTCT CGTACCTCCT
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       D  V  Q  K   W  L  S   S  P  F   P  S  S  S   F  S  P   G  G  L
4981  GACGTCCAGA AGTGGCTCTC TTCGCCCTTC CCCTCATCGT CCTTCAGCCC TGGCGGCCTG
      CTGCAGGTCT TCACCGAGAG AAGCGGGAAG GGGAGTAGCA GGAAGTCGGG ACCGCCGGAC
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  P  E  I   S  P  L   E  V  L   E  R  D  K   V  T  Q   L  L  L
5041  GCACCTGAGA TCTCGCCACT AGAAGTGCTG GAGAGGGACA AGGTGACGCA GCTGCTCCTG
      CGTGGACTCT AGAGCGGTGA TCTTCACGAC CTCTCCCTGT TCCACTGCGT CGACGAGGAC
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Q  Q  D  K   V  P  E   P  A  S   L  S  S  N   H  S  L   T  S  C
5101  CAGCAGGACA AGGTGCCTGA GCCCGCATCC TTAAGCAGCA ACCACTCGCT GACCAGCTGC
      GTCGTCCTGT TCCACGGACT CGGGCGTAGG AATTCGTCGT TGGTGAGCGA CTGGTCGACG
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       F  T  N  Q   G  Y  F   F  F  H   L  P  D  A   L  E  I   E  A  C
5161  TTCACCAACC AGGGTTACTT CTTCTTCCAC CTCCCGGATG CCTTGGAGAT AGAGGCCTGC
      AAGTGGTTGG TCCCAATGAA GAAGAAGGTG GAGGGCCTAC GGAACCTCTA TCTCCGGACG
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       Q  V  Y  F   T  Y  D   P  Y  S   E  E  D  P   D  E  G   V  A  G
5221  CAGGTGTACT TTACTTACGA CCCCTACTCA GAGGAAGACC CTGATGAGGG TGTGGCCGGG
      GTCCACATGA AATGAATGCT GGGGATGAGT CTCCTTCTGG GACTACTCCC ACACCGGCCC
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       A  P  T  G   S  S  P   Q  P  L   Q  P  L  S   G  E  D   D  A  Y
5281  GCACCCACAG GGTCTTCCCC CCAACCCCTG CAGCCTCTGT CAGGGGAGGA CGACGCCTAC
      CGTGGGTGTC CCAGAAGGGG GGTTGGGGAC GTCGGAGACA GTCCCCTCCT GCTGCGGATG
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       C  T  F  P   S  R  D   D  L  L   L  F  S  P   S  L  L   G  G  P
5341  TGCACCTTCC CCTCCAGGGA TGACCTGCTG CTCTTCTCCC CCAGTCTCCT CGGTGGCCCC
      ACGTGGAAGG GGAGGTCCCT ACTGGACGAC GAGAAGAGGG GGTCAGAGGA GCCACCGGGG
               IL2 Receptor-beta Transmembran+endodomain
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       S  P  P  S   T  A  P   G  G  S   G  A  G  E   E  R  M   P  P  S
5401  AGCCCCCCAA GCACTGCCCC TGGGGGCAGT GGGGCCGGTG AAGAGAGGAT GCCCCCTTCT
      TCGGGGGGTT CGTGACGGGG ACCCCCGTCA CCCCGGCCAC TTCTCTCCTA CGGGGGAAGA
               IL2 Receptor-beta Transmembran+endodomain
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       L  Q  E  R   V  P  R   D  W  D   P  Q  P  L   G  P  P   T  P  G
5461   TTGCAAGAAA GAGTCCCCAG AGACTGGGAC CCCCAGCCCC TGGGGCCTCC CACCCCAGGA
       AACGTTCTTT CTCAGGGGTC TCTGACCCTG GGGGTCGGGG ACCCCGGAGG GTGGGGTCCT
                IL2 Receptor-beta Transmembran+endodomain
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
        V  P  D  L   V  D  F   Q  P  P   P  E  L  V   L  R  E   A  G  E
5521   GTCCCAGACC TGGTGGATTT TCAGCCACCC CCTGAGCTGG TGCTGCGAGA GGCTGGGGAG
       CAGGGTCTGG ACCACCTAAA AGTCGGTGGG GGACTCGACC ACGACGCTCT CCGACCCCTC
                IL2 Receptor-beta Transmembran+endodomain
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
        E  V  P  D   A  G  P   R  E  G   V  S  F  P   W  S  R   P  P  G
5581   GAGGTCCCTG ACGCTGGCCC CAGGGAGGGA GTCAGTTTCC CCTGGTCCAG GCCTCCTGGG
       CTCCAGGGAC TGCGACCGGG GTCCCTCCCT CAGTCAAAGG GGACCAGGTC CGGAGGACCC
                IL2 Receptor-beta Transmembran+endodomain
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
        Q  G  E  F   R  A  L   N  A  R   L  P  L  N   T  D  A   Y  L  S
5641   CAGGGGGAGT TCAGGGCCCT TAATGCTCGC CTGCCCCTGA ACACTGATGC CTACTTGTCC
       GTCCCCCTCA AGTCCCGGGA ATTACGAGCG GACGGGGACT TGTGACTACG GATGAACAGG
       IL2 Receptor-beta Transmembran+endodomain
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                          BspEI
                                                          ~~~~~~
        L  Q  E  L   Q  G  Q   D  P  T   H  L  V  *   S  G
5701   CTCCAAGAAC TCCAGGGTCA GGACCCAACT CACTTGGTGT AGTCCGGA
       GAGGTTCTTG AGGTCCCAGT CCTGGGTTGA GTGAACCACA TCAGGCCT
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8 PUBLICATIONS

Faitschuk, E, **Nagy, V**, Hombach, AA and Abken, H (2016). A dual chain chimeric antigen receptor (CAR) in the native antibody format for targeting immune cells towards cancer cells without the need of an scFv. *Gene Ther.* 23: 718–726.

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Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. med. Hinrich Abken betreut worden.

Übersicht der Publikationen:

1. Faitschuk, E, **Nagy, V**, Hombach, AA and Abken, H (2016). A dual chain chimeric antigen receptor (CAR) in the native antibody format for targeting immune cells towards cancer cells without the need of an scFv. *Gene Ther*. 23: 718–726.

2. **Golumba-Nagy, V**, Kuehle, J and Abken, H (2017). Genetic Modification of T Cells with Chimeric Antigen Receptors: A Laboratory Manual. *Hum. Gene Ther. Methods* 28: 302–309.

3. **Golumba-Nagy V**, Kuehle J, Hombach AA, Abken H (2018) CD28-ζ CAR T Cells Resist TGF-β Repression through IL-2 Signaling, Which Can Be Mimicked by an Engineered IL-7 Autocrine Loop. Mol Ther. 5;26(9):2218-2230

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