

A chimeric switch receptor with IL-2 receptor- β signalling converts TGF- β resistance to 4-1BB CAR T cells



DISSERTATION ZUR ERLANGUNG DES DOKTOGRADES DER
NATURWISSENSCHAFTEN (DR. RER. NAT.) DER FAKULTÄT FÜR BIOLOGIE UND
VORKLINISCHE MEDIZIN DER UNIVERSITÄT REGENSBURG

vorgelegt von

Jordan Hartley

aus

Burnley, England, United Kingdom

Im Jahr

2023

Das Promotionsgesuch wurde eingereicht am:

06.04.2023

Die Arbeit wurde angeleitet von:

Prof. Dr. Hinrich Abken

Unterschrift: 

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to Prof. Dr. Hinrich Abken for his trust, patience and above all, his priceless guidance and scientific support throughout my PhD. Through your infectious scientific enthusiasm along with a calming personality, you have provided me with invaluable knowledge and understanding to allow me to move forward with my career in therapeutic research. I would also like to show appreciation for giving me the opportunity to work, live and experience such a scenic city like Regensburg.

I would like to thank Dr. Astrid Holzinger for her warm welcome from the beginning along with continuous support and guidance along the way. Words cannot describe how much of an incredible team you and Hinrich are.

I would like to extend my thanks to my colleagues in AG Abken, for their help, scientific support but most importantly for creating a great working atmosphere. I would also like to show appreciation to all my colleagues in the LIT for such a positively welcoming scientific community.

A special thanks also goes out to my parents, close family, and friends. This chapter in my life wouldn't have been possible without your support.

Finally, I would like to wholeheartedly say thank you to my girlfriend Clare, your support and patience through this stage in my career is a testament to how amazing you truly are.

ABSTRACT

The success of CAR T cell therapy towards solid tumours is so far limited due to the immunosuppressive tumour environment. Transforming growth factor- β (TGF- β) is highly abundant and one of the main causes of an unsuccessful CAR T cell response due to its T cell suppressive properties. We have previously identified that CAR T cell signalling through the CD28 costimulatory domain provides resistance to TGF- β by high amounts of IL-2 released by the stimulated T cells whereas 4-1BB does not. However, accumulating IL-2 in the targeted tumour stimulates regulatory T cells, contributing further to immunosuppression. Therefore, we have developed a Switch Receptor (SWR) that is composed of the TGF- β receptor-II (TGF- β RII) binding domain and the intracellular IL-2 receptor- β chain (IL-2R β) mimicking IL-2 signalling upon TGF- β engagement. The TGF- β RII-IL-2R β SWR converted TGF- β resistance to 4-1BB CAR T cells with increased T cell amplification, cytolytic properties and tumour cell cytotoxicity. The SWR also converted resistance to the OX40 CAR and an IL-2 deficient variant CD28 Δ LCK CAR, which were all previously sensitive to TGF- β mediated suppression. Moreover, the SWR showed superiority over the currently used dominant-negative (DN) TGF- β receptor in amplification and tumour eradication after multiple rounds of tumour re-challenging. Signalling through IL-2R β , without the presence of the IL-2R γ chain, induced pSTAT5 and this was sufficient enough to convert TGF- β resistance. This study shows that CAR T cells equipped with a TGF- β RII-IL2-R β SWR are resistant to TGF- β mediated suppression therefore leading to an enhanced therapeutic effect in the fight against solid tumours.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	2
ABSTRACT	3
TABLE OF CONTENTS	4
LIST OF FIGURES	7
LIST OF TABLES	8
LIST OF ABBREVIATIONS	9
1 INTRODUCTION	12
1.1 Immune Response to Cancer	12
1.2 Immunotherapy	12
1.3 Adoptive Cell Therapy	13
1.4 Chimeric Antigen Receptors (CARs)	16
1.5 The limitations of CAR T cells targeting solid tumours	20
1.6 Transforming Growth Factor- β (TGF- β)	22
1.7 CAR T cells against TGF- β	23
1.8 Aims	25
2 MATERIALS	27
2.1 Chemicals and Reagents	27
2.2 Kits	28
2.3 Medium used for culture of cells	28
2.4 Buffers	28
2.5 Antibodies	29
2.5.1 Unconjugated antibodies	29
2.5.1 Conjugated antibodies	29
2.6 Cytokines	30
2.7 Plasmid Vectors	30
2.8 Bacterial Strains	32
2.9 Cell Lines	32
2.10 Primary Cells	32
2.11 Software	33
3 METHODS	34
3.1 Bacteria	34
3.1.1 Bacteria Culture	34

3.1.2	Heat-shock transformation of bacteria	34
3.2	Working with DNA.....	34
3.2.1	Plasmid isolation	34
3.2.2	Quantification of DNA	35
3.3	Working with cells	35
3.3.1	Cell culture conditions.....	35
3.3.2	Passaging of adherent cells	35
3.3.3	Passaging of suspension cells.....	35
3.3.4	Freezing and thawing of cells.....	36
3.3.5	Determination of cell counts.....	36
3.3.6	Isolation of human peripheral blood mononuclear cells (PBMCs)	36
3.3.7	Transfection of HEK 293T cells	37
3.3.8	Retroviral transduction of human T cells.....	38
3.3.9	Lentiviral transduction of N87 tumour cells	38
3.3.10	Fluorescence-activated cell sorting (FACS)	39
3.3.11	Detection of cell surface markers	39
3.3.11.1	<i>Detection of CD107a</i>	39
3.3.11.2	<i>Detection of memory phenotype markers</i>	40
3.3.12	Intracellular Staining	40
3.3.12.1	<i>Detection of Phosphorylated STAT5</i>	40
3.3.12.2	<i>Detection of Perforin</i>	40
3.4	Assays	41
3.4.1	Cytokine Secretion	41
3.4.2	Proliferation Assay	41
3.4.3	Enzyme-linked Immunosorbent Assay (ELISA)	41
3.4.4	Repetitive killing (stress test) Assay	42
4	RESULTS.....	43
4.1	CD28 co-stimulatory domain provides resistance to TGF- β mediated suppression due to IL-2 mediated signalling.....	43
4.1.1	<i>The effect of TGF-β on T cell proliferation triggered by various 2nd generation CARs...</i>	43
4.1.2	<i>CD28-ζ CAR T cells secrete significantly higher amount of IL-2 in comparison to other CAR T cells.</i>	44
4.2	Converting TGF- β binding into a stimulatory IL-2 receptor signal	45
4.2.1	<i>The generation of Switch Receptor CAR T cells</i>	45
4.2.2	<i>4-1BB-ζ-SWR CAR is expressed on human CD3⁺ T cells</i>	46
4.2.3	<i>CAR T cells maintain their memory phenotype after engineering with the SWR</i>	47

4.2.4	<i>4-1BB-ζ-SWR CAR T cells overcome TGF-β mediated repression in T cell amplification</i>	48
4.2.5	<i>Switch Receptor CAR T cells display better cytolytic and pro-inflammatory properties in the presence of TGF-β</i>	49
4.2.6	<i>Switch Receptor CAR T cells induce phosphorylated STAT5 through IL-2Rβ chain</i>	51
4.3	<i>Utilizing the Switch Receptor to convert resistance to other CARs sensitive to TGF-β</i>	52
4.3.1	<i>Switch Receptor rescues T cell amplification for IL-2 deficient CD28 CAR in the presence of TGF-β</i>	52
4.3.2	<i>OX40-ζ CAR T cells benefit from the switch receptor under high concentrations of TGF-β</i>	54
4.4	<i>Switch Receptor shows superiority compared to state-of-the-art Dominant Negative Receptor</i>	56
4.4.1	<i>Switch Receptor out competes Dominant Negative Receptor by maintaining amplification properties under high concentration of TGF-β</i>	56
4.4.2	<i>Demonstration of STAT5 phosphorylation in SWR CAR T cells in comparison to DNR CAR T cells</i>	58
4.4.3	<i>Switch Receptor CAR T cells show better TGF-β resistance in tumour cell elimination and proliferation under repetitive stimulatory conditions</i>	59
4.5	<i>Engineering N87 tumour cells to express TGF-β</i>	61
4.5.1	<i>Transduction of TGF-β1 expression vector on N87 tumour cells</i>	61
4.5.2	<i>Switch receptor CAR T cells show superior activity against N87-TGF-β⁺ cells</i>	62
5	DISCUSSION	63
6	APPENDIX	69
6.1	BW431/26scFv-Fc-CD28-CD3ζ (#607)	69
6.2	BW431/26scFv-Fc-CD3ζ-OX40 (#848)	72
6.3	BW431/26scFv-Fc-4-1BB-CD3ζ (#908)	75
6.4	BW431/26scFv-Fc-CD28ΔLCK-CD3ζ (#946)	78
6.5	BW431/26scFv-Fc-4-1BB-CD3ζ-P2A-TGF-βRII-PDGFRβTM-IL-2Rβ (#2356)	81
6.6	BW431/26scFv-Fc-CD28ΔLCK-CD3ζ-P2A-TGF-βRII-PDGFRβTM-IL-2Rβ (#2357)	86
6.7	BW431/26scFv-Fc-CD3ζ-OX40-P2A-TGF-βRII-PDGFRβTM-IL-2Rβ (#2362)	91
6.8	BW431/26scFv-Fc-4-1BB-CD3ζ-P2A-TGF-βRII-PDGFRβTM (#2410)	96
6.9	pLV[Exp]-mCherry-EF1A-TGF-β1 (#2561)	100
7	REFERENCES	106

LIST OF FIGURES

Figure 1. Schematic diagram to show the process of adoptive cell therapy.	15
Figure 2. Evolution of CAR generations and switch receptors.	20
Figure 3. CD28 overcomes the inhibitory effect of TGF- β on T cell amplification whereas 4-1BB and OX40 do not.	43
Figure 4. CD28- ζ CAR T cells secrete higher amounts of IL-2 upon stimulation.	44
Figure 5. Schematic Illustration and function of switch receptor CAR T cells	45
Figure 6. Expression of CARs on human CD3 ⁺ T cells.....	46
Figure 7. Central memory T cell subset after CAR T cell manufacturing	47
Figure 8. Switch receptor rescues CAR T cell amplification in the presence of TGF- β	48
Figure 9. Switch Receptor CAR T cells display better cytolytic properties in the presence of TGF- β	50
Figure 10. SWR CAR T cells show increased levels of pSTAT5.....	51
Figure 11. Switch Receptor rescues proliferation of IL-2 deficient CD28 Δ LCK CAR	53
Figure 12. OX40- ζ CAR T cells benefit from switch receptor signalling under high concentrations of TGF- β	55
Figure 13. SWR CAR T cells maintain better proliferative capabilities under high concentrations of TGF- β	57
Figure 14. SWR CAR T cells show increased levels of pSTAT5.....	58
Figure 15. SWR CAR T cells display better resistance to TGF- β during repetitive rounds of stimulation	60
Figure 16. Transduction of N87 tumour cells with human TGF- β 1 expression vector	61
Figure 17. Switch receptor CAR T cells show better TGF- β resistance with a higher cytotoxic effect against TGF- β ⁺ tumour cells	62
Figure 18. Concept of SWR CAR T cells overcoming TGF- β suppression.....	68

LIST OF TABLES

Table 1. Comparison of different co-stimulatory domains used in chimeric antigen receptors.	18
Table 2. Table containing chemicals, reagents and their origin used in this study	27
Table 3. Table containing the kits and their company origin used in this study	28
Table 4. Table containing the mediums and their company origin used in this study	28
Table 5. Table containing buffers and their composition used in this study	28
Table 6. Table containing the antibodies and their source used in this study	29
Table 7. Table of conjugated antibodies and their relevant information used in this study	29
Table 8. Table of cytokines and their source used in this study	30
Table 9. Table of plasmids and relevant information used in this study	31
Table 10. Table of bacterial strains used in this study	32
Table 11. Table of cell lines used in this study	32
Table 12. Table of software used in this study	33
Table 13. Overview of transfection scheme	37
Table 14. Plasmids used for retroviral transfection	37
Table 15. Plasmids used for Lentiviral transfection	38
Table 16. Overview of components used for the detection of IL-2 and IFN- γ via ELISA	42

LIST OF ABBREVIATIONS

4-1BB	Tumour necrosis factor receptor superfamily member (CD137)
Ab	Antibody
ABECMA	A B-cell maturation antigen (BCMA-directed)
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ACT	Adoptive Cell Therapy
Akt	Ak strain transforming
ALL	Acute lymphoblastic leukaemia
Amp	Ampicillin
APC	Allophycocyanin
ATCC	American type culture collection
Bcl-2	B cell lymphoma 2
BCMA	B-cell maturation antigen
Bcl-x	B cell lymphoma-extra large
BSA	Bovine serum albumin
BV421	Brilliant Violet 421
BV605	Brilliant Violet 605
CAR	Chimeric Antigen Receptor
CAF	Cancer associated fibroblast
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CH2-CH3	Constant region 2/3 of heavy chain
CO₂	Carbon dioxide
CRISPR-CAS9	Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9
CSRs	Cytokine Switch Receptor
CTLA-4	Cytotoxic T Lymphocyte-associated protein 4
CTLs	Cytotoxic T lymphocytes
DAP	DNAX-activating protein
DC	Dendritic cells
DLBCL	Diffuse large B cell lymphoma
DMEM	Dubecco's Modified Eagles Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DNTGFβ	Dominant-negative TGF-β Receptor II
E. coli	<i>Escherichia coli</i>
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FACS	Fluorescence-activated cell sorting
FAP	Fibroblast activation protein
Fc	Fragment crystallizable
FcεRIg	Fc epsilon receptor I
FcγRs	IgG Fc gamma receptor

LIST OF ABBREVIATIONS

FCS	Fetal calf serum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
g	Gravitational acceleration
GALV	Gamma-retroviral envelope protein
GD	Disialoganglioside
H₂O_{dd}	Water (deionised)
HEK	Human Embryonic Kidney (cell)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER	Human epidermal growth factor receptor
ICOS	Inducible costimulator CD278
ICR	Inverted cytokine receptor
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interlukin
JAK	Janus Kinase
KIR	Killer-cell immunoglobulin-like receptor
LAKs	Lymphokine-activated killer cells
LB	Lysogeny broth
LCK	Lymphocyte-specific protein tyrosine kinase
mAb	Monoclonal antibody
MAGE	Melanoma-associated antigen
MDSCs	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
Min	Minute
mTOR	Mammalian target of rapamycin
MUC	Mucin
NK	Natural killer
NY-ESO-1	Cancer testis antigen
OD	Optical density
OX40	Tumour necrosis factor receptor superfamily, member 4 (CD134)
p	Phosphorylated
P2A	“self-cleaving” peptide
PBLs	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline tween
PD-1	Programmed cell death protein 1
PDL	Poly-D-lysine
PE	Phycoerythrin
Penstrep	Penicillin Streptomycin mixture
pH	Lat.: <i>potentia Hydrogenii</i>
PI3K	Phosphatidylinositide 3-kinases
POD	Peroxidase
PSCA	Prostate stem cell antigen
RNA	Ribonucleic acid

LIST OF ABBREVIATIONS

rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute (Medium)
S.D	Standard deviation
scFv	Single chain variable fragment
STAT	Signal transducer and activator of transcription
TAA	Tumour associated antigen
TAMs	Tumour associated macrophages
TANs	Tumour associated neutrophils
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	Type helper
TILs	Tumour infiltrating lymphocytes
TM	Transmembrane domain
TME	Tumour microenvironment
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
TRUCKs	T cells re-directed for universal cytokine mediated killing
U	Unit
V_H	Variable region of the immunoglobulin heavy chain
V_L	Variable region of the immunoglobulin light chain
v/v	Volume concentration (volume/volume)
w/o	Without
w/v	Mass concentration (weight/volume)
WHO	World health organisation

1 INTRODUCTION

1.1 Immune Response to Cancer

Cancer is described as a disease within the genome and is characterised as genome instability in which the process of point mutations accumulate and structural alterations occur known as tumour progression (Gonzalez et al., 2018). These mutations have the possibility to lead to tumour antigens, such antigens may be recognised by the immune system and initiate immune cellular responses (Jin et al., 2019). One vital role of the immune system is immunosurveillance, in which, immune cells of both innate and adaptive immune systems infiltrate into the tumour microenvironment (TME), upon migration, such cells contribute to preventing progression of the tumour (Chang & Beatty, 2020).

Cells such as, natural killer (NK), basophils, phagocytic, eosinophils, monocytes, macrophages, neutrophils, and dendritic cells (DCs), are all characterised as innate immune cells, these cells play a role in suppressing the tumour by direct tumour cell elimination or triggering neighbour adaptive cells to initiate an adaptive immune response (Gajewski et al., 2013). The adaptive immune system is composed of lymphocytes that include B cells and T cells. B cells are responsible for a humoral response and T cells are associated with a cell mediated response (Ribas, 2015). A successful immune response is considered when malignant cells are either eliminated or their phenotype and function has been affected (Adam et al., 2003). Unfortunately, cancer cells have developed mechanisms capable of escaping immune surveillance, such as recruitment of immune suppressive cells, antigen loss and the regulation of negative pathways. All contributing to a less effective anti-tumour immune response (Beatty & Gladney, 2015).

1.2 Immunotherapy

Immunotherapy, a strategy involving using the patient's immune system to eradicate malignant cells is a significant step towards cancer treatment. Immunotherapy has shown promising effectiveness for multiple cancer types with clinical response being achieved. Although with response rates being limited and the uncertainty of mechanisms, the breakthrough of immunotherapy is considered a revolutionary step in the treatment of cancer. Immune cells are the major focus, having the ability to understand their migration and infiltration into the TME is vital towards improving response rates but also critical for the development of new therapeutic strategies for the treatment of cancer (Waldman et al., 2020).

There are several types of immunotherapies such as, monoclonal antibodies, cytokine therapy or adoptive cell transfer. The use of monoclonal antibodies was one of the first forms of immunotherapy to be approved by the Food and Drug Administration (FDA) in

2011. This was achieved by successfully binding antibodies against the regulatory molecule CTLA-4 (cytotoxic T lymphocyte-associated protein 4) and preventing the inhibitory signal on the T cell, allowing for a more effective elimination of cancer cells by the CTLs (cytotoxic T lymphocytes) (Fife & Bluestone, 2008). In recent years the focus has polarised towards the understanding of T cell characterization and modification, with efforts drawn towards adoptive T cell therapy, a promising strategy in which a patient's own T cells are utilised for the treatment of cancer (Rosenberg et al., 1988).

1.3 Adoptive Cell Therapy

Adoptive cell therapy is a process in which a patient's own T cells are collected, modified and/or expanded ex vivo and then reinfused back into the patient aiming to eliminate the cancer. One of the main advantages to ACT is that transferred cells have the specificity to target and eliminate cancer cells with limited risk of targeting normal tissue (Rohaan et al., 2019).

One of the first approaches to ACT was the re-infusion of isolated T cells activated and amplified in vitro in a specific fashion. Prior to the re-infusion process, lymphokine-activated killer cells (LAKs) were generated in vitro from the patient's blood T cells, stimulated by interleukin-2 (IL-2) in vitro then re-administered back into the patient (Rosenberg et al., 1986). The activated LAKs were then able to recognise and eliminate cancer cells without the risk of effecting normal cells. The approach of using stimulated LAKs was effective in mice and humans. However, the use of this therapy was limited due to its lack of specificity towards other cancers and this therapy in combination with systemic IL-2 lead to toxic side effects in patients (Glassman, 1989; Rosenberg et al., 1986).

The adoptive transfer of tumour-infiltrating lymphocytes (TILs) is another form of ACT. The benefit to using TIL therapy is, TILs are endogenous lymphocytes found within the tumours, once expanded ex vivo and re-infused back into the patient, these cells possess a higher therapeutic effect in comparison to LAKs (Zhao et al., 2022). Rosenberg group discovered that, prior to lymphodepletion, the combination of systemic IL-2 and TIL therapy, most of all mice suffering from colon adenocarcinoma recovered (Rosenberg, 2014). However, the therapeutic response rate of TIL adoptive cell therapy is restricted by certain factors, such as, the presence of immunosuppressive cell types within the TME, low persistence and failure of adoptive transferred cells and the initial number of tumour-infiltrating T cells. TIL therapy also requires simultaneous high amounts of IL-2, Rosenberg also highlighted the administration of high doses of IL-2 in cancer patients contributes to toxic side effects (Rosenberg, 2014). However, on the contrary to this, the administration of TIL therapy with low-dose IL-2 was successful in the treatment of metastatic melanoma in the context of a single institution phase II protocol (ClinicalTrials.gov identifier NCT01883323.). The trial included 12 patients and all but one had previously progressed after treatment. The primary end point was response rate and the second was safety and feasibility. The trial concluded that low-dose IL-2 following adoptive cell transfer of TIL is feasible and clinically active (Nguyen et al., 2019).

The application of adoptive cell therapy progressed significantly when it was discovered that human T cells can be engineered to express the TCR of known specificity or an artificial receptor, armouring the T cell with known specificity for targeting tumour antigens (Rohaani et al., 2019). Modifying T cells to express either a Chimeric Antigen Receptor (CAR) or $\alpha\beta$ T cell receptor (TCR) and re-infusing them back into the patient with defined specificity is considered to be a major development in the field of ACT. Both types of therapy have their advantages and disadvantages, however, a direct comparison between the two is difficult due to several parameters such as affinity, ligand density and ligand structure.

CAR T cell therapy has shown promising results, especially in targeting haematological cancer (Porter et al., 2011), unfortunately the success is yet to be applied for the treatment of solid cancer. Regarding the current success, the CD19 specific CAR targeting B cell malignancies is the most successful CAR currently in the clinic. In 2017, the FDA approved the first two CAR T cell therapies, the Tisagenlecleucel CAR (Kymriah®) (Maude et al., 2018) and the Axicabtagene ciloeucel CAR (Yescarta®) - (Neelapu et al., 2017). Both CD19-targeting, with the product Kymriah® used for the treatment of children and young adults up to 25 years old suffering from B-cell ALL who do not respond to treatment or have relapsed twice (ClinicalTrials.gov numbers, NCT01626495 and NCT01029366). The product from Yescarta®, used for the treatment of adults with relapsed or refractory DLBCL. Furthermore, In 2018 this also led to both products Yescarta® and Kymriah® being approved by the European Medicines Agency (EMA) for the treatment of relapsed/refractory B-cell ALL, DLBCL or PMBCL after two or more lines of therapy (Styczyński, 2020).

Regarding the success of TCRs in clinic, TCRs have shown promising results in both solid cancers and haematological, however, unfortunately severe side effects were observed. One example of this is targeting the tumour antigen MAGE-A4 in myeloma and melanoma (Shafer et al., 2022). TCR therapy towards this antigen induced off-target toxicity leading to damage in non-cancerous tissue in which the patient unfortunately died. On the other hand, targeting NY-ESO-1 which is a cancer-testis antigen appeared to be successful with no off-target toxicity reported. Although the limitation with TCR therapy is that each TCR is specific for a certain MHC-peptide (major histocompatibility complex) which therefore means its only suitable for patients with matching MHC-tumours, the advantage of this therapy is intracellular and cell surface proteins can be targeted if they are sufficiently presented on the MHC (Chen et al., 1997; Yakirevich et al., 2003).

To summarise, while both strategies have common aspects and clinical success, as mentioned above. Their differences dictate the purpose they serve. The main difference being the receptors engineered into the cell, CAR T cells have receptors targeting naturally occurring antigens, whereas TCRs rely on the MHC to mark cancer cells with recognisable antigens.

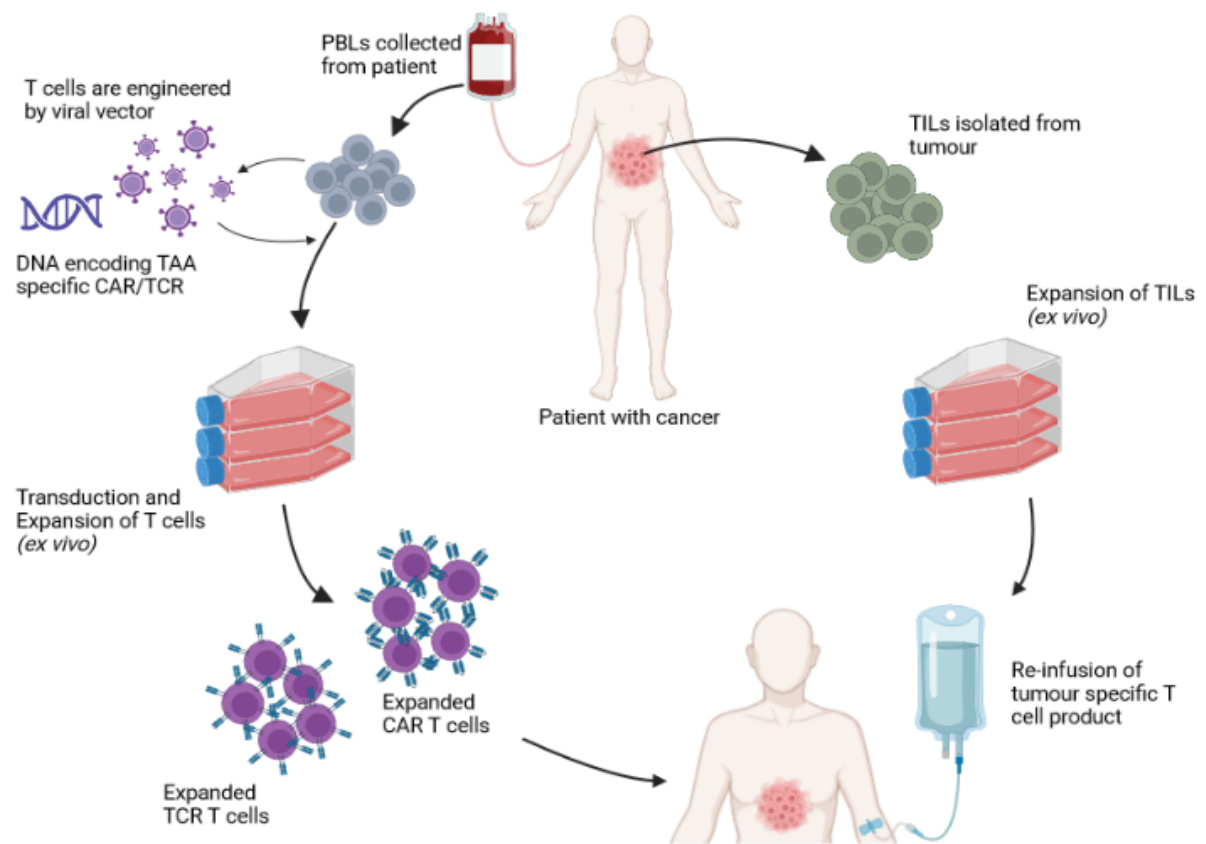


Figure 1. Schematic diagram to show the process of adoptive cell therapy.

1.4 Chimeric Antigen Receptors (CARs)

CAR T cell therapy is when a patient's own T cells are engineered to express a Chimeric Antigen Receptor and then adoptively transferred back into the patient, this type of ACT is considered one of the most promising advancements in the field of immunotherapy. The structure of a classical CAR is made up of an antigen binding domain, a hinge domain, transmembrane domain and finally one or multiple signalling domains.

Equipping T cells with a receptor to recognise antigens dates back to 1987 & 1989, with studies from Kuwana Y et al and Gross G et al, in which these chimeric receptors are referred to as the first CAR prototypes (Gross et al., 1989; Kuwana et al., 1987). The introduction of CD3 ζ signalling came when it was fused to an antigen binding domain in 1993 by Eshhar Z et al in which showed T cell specific activation can be achieved. Below we address each component of a CAR, their importance and how they impact functionality of engineered T cells (Eshhar et al., 1993).

The antigen-binding domain is mostly a single chain fragment of variable region (scFv) of an antibody, this allows for specific recognition of tumour-associated antigens (TAAs) on target cells. Present is the antigen recognizing variable regions of an immunoglobulin heavy chain (V_H) and a immunoglobulin light chain (V_L), both chains are connected together with a peptide linker that is short and flexible (Welschof et al., 1997). The scFv usually has high binding affinity and impacts binding of the CAR which therefore carries importance in overall functionality of the CAR T cell. Chmielewski et al in 2004 showed that in order to target cells which express an antigen on a low level, a scFv with high binding affinity is required. However, it was shown that when the binding affinity is above a certain threshold, overall T cell activation is not affected (Chmielewski et al., 2004). On the other hand, if the antigen being targeted is expressed on a high level, then a scFv with lower binding affinity is suggested. In conclusion it is recommended when designing a CAR to take into consideration the expression levels of the TAA you are targeting, whether it, may be expressed in healthy tissues or not and the antigen binding affinity of the scFv, as this importantly contributes to overall functionality of the CAR T cell.

Downstream of the scFv is what's known as the hinge region or spacer domain. The importance of this domain is to bring stability or homodimerization to the receptor for better expression on the T cell surface but also allows for flexibility of the single chain for targeting the required antigen (Jayaraman et al., 2020). Common spacer domains include CD4, CD8 α and CD28 however, the immunoglobulin CH2-CH3 (Fc) domain from the constant region of immunoglobulin G (IgG) is often used. The Ig domain also carries importance for the effector function of the CAR, when targeting certain antigens. The length of the spacer has to be optimal to allow sufficient and effective antigen binding to allow for CAR T cell activation (Fujiwara et al., 2020). In a study conducted by Fujiwara et al in 2020, they found that although a difference in transmembrane domain effected expression level and stability on the

T cell, however, CARs with a CD8a or CD28 derived hinge domain as spacer showed significant differences in CAR T cell function, despite having similar expression levels.

Therefore, concluding that CAR T cell signalling intensity is not only affected by the expression but also by the hinge domain. On the other hand, removing the hinge domain can also reduce the expansion properties of the CAR, along with reducing the antitumour potential. This was demonstrated targeting CD19, mesothelin, PSCA, MUC1 and HER2 (Qin et al., 2017).

The transmembrane domain, the function of the transmembrane domain is to not only serve as an anchor for the receptor to the membrane of the T cell, but it allows for transducing ligand recognition signals to the intracellular cytoplasmic domain. Common transmembrane domains used in CARs include CD3 ζ , CD4, CD8 α , CD28, OX40, ICOS and Fc ϵ R1 γ . It was reported that first generation CD19 specific CARs containing the CD3 ζ transmembrane domain were less stable on the T cell surface in comparison to 2nd generation CARs containing the CD28 transmembrane domain (Dotti et al., 2014; Savoldo et al., 2011). Additionally, the use of coupled transmembrane domains to form transmembrane mediated interactions has also been used. Referred to as a “split CAR”, this novel design can deliver trans-cytoplasmic domain signalling. This was demonstrated targeting Mesothelin and CD19 using an scFv-linked KIR domain and a DAP12 domain (E. Wang et al., 2015). Further novel transmembrane domain designs involve “trans” signalling. This was demonstrated using the Fc ϵ R1 γ TM domain, which consists of 3 subunits α β γ , all 3 contribute to TM interactions. In order to create the “trans” signalling CAR, the γ and β cytoplasmic subunit was replaced with 4-1BB and CD3 ζ and the extracellular α domain was replaced with a scFv targeting CD19 (Cappello et al., 2016). Designs as such allow for the development of CARs with different functionality.

Lastly, are the cytoplasmic domains or often referred to as intracellular signalling domains. In T cells, the intracellular domain of the TCR-CD3 complex is important for the primary signal which is necessary for beginning the signal cascade. The secondary signal, often referred to as a co-stimulatory signal is necessary for providing a sustained signal leading to T cell activation and proliferation (Jayaraman et al., 2020). Co-stimulatory receptors include CD28, 4-1BB, OX40 and ICOS. In the context of CAR T cells, these co-stimulatory signals are coupled with CD3 ζ to allow for an in-cis signalling effect. As previously described, first generation CARs were typically designed only consisting of a primary signal providing the activating signal upon antigen engagement, usually this was the CD3 domain. However, research showed that CAR T cells engineered only with the CD3 ζ domain showed a lack of persistence and proliferation in vivo (Weinkove et al., 2019). Moving forward, next came the 2nd Generation CAR T cells. These included a co-stimulatory domain and, in most cases, would consist of either CD28 or 4-1BB. Although both function differently, the addition of a secondary signal provided functionalities such as long term persistence and increased efficacy (Imai et al., 2004; Kalos et al., 2011; Savoldo et al., 2011), all of which led to the clinical approval of CAR T cell therapy with Kymriah and Yescarta (Lu & Jiang, 2022). Below is a table to summarize the different co-stimulatory molecules and their function in CAR T cells.

Co-stimulatory Domain	Receptor Family	Physiological function during T cell maturation	Kinetics/Characteristics
CD28	Ig Superfamily	Effector Memory	Potent cytotoxic function, High IL-2 production, faster and higher signal intensity, PI3K/Akt/mTOR pathway activation, resistant to TGF- β
4-1BB	TNF-R Superfamily	Central Memory	Greater persistence compared to CD28, Central memory polarization, less chance of exhaustion, Wnt/ β -catenin pathway activation
ICOS	Ig Superfamily	Th1 & Th17 Polarization	Member of the Ig superfamily, favours Th1 and Th17 polarization, reinforces Th1 & Th2 cytokines such as IL-4, IL-10, IL-21 & IFN- γ
OX40	TNF-R Superfamily	Central Memory	Promotes sustained T cell survival and expansion, suppress Treg development, less susceptible to exhaustion, acts via both PI3K/Akt and TRAF pathways
CD27	TNF-R Superfamily	Central Memory	Member of TNF-R superfamily, favours CAR T cell persistence due to upregulated Bcl-X expression, promotes effector differentiation

Table 1. Comparison of different co-stimulatory domains used in chimeric antigen receptors.

The rationale to use two co-stimulatory domains in a CAR along with the primary signal from CD3 ζ , paved the way for the introduction of 3rd generation CARs. 3rd Gen CAR T cells utilizing CD28 & 4-1BB have been tested targeting CD19, PSMA, mesothelin and GD2 (Quintarelli et al., 2018; Ramos et al., 2018; Z. Zhao et al., 2015). Preclinical studies of 3rd gen CAR T cells targeting PSMA and mesothelin showed a superior anti-tumour effect and increased persistence in comparison to 2nd generation CAR T cells. Equally, the combination of 4-1BB and ICOS in a third generation CAR T cell format has also been used targeting mesothelin, results from this study also showed an increase in persistence and anti-tumour potency (Guedan et al., 2018; Zhong et al., 2010). However, the conclusion that third generation CARs are better than second is a matter of debate. For example, a study conducted by Abate-Daga et al investigated the comparison of 2nd generation CD28 CAR T cells and 3rd generation CD28/4-1BB CAR T cells targeting prostate stem cell antigen (PSCA). It was reported that although third generation CARs exhibited an improved persistence in a pancreatic xenograft model, the second generation CARs outperformed in the context of anti-tumour potency (Abate-Daga et al., 2014).

In recent years, the introduction of the 4th generation of CAR T cells or T cells redirected for universal cytokine-mediated killing (TRUCKs) have emerged. TRUCKs are unique in the way they have the potential to modulate the immune regulatory milieu within the tumour tissue. This is done by arming the CAR T cell with a transgenic cytokine that is released upon CAR specific antigen engagement. The release of such cytokines allows to activate other neighbouring T cells and innate immune cells within the environment, this strategy also aids to overcome the insufficient production of pro-inflammatory cytokines by T cells present within the tumour. Introducing this new strategy of a cell product with the ability to deliver a desired payload opened the way for the possible treatment of solid tumours, as positively impacting the tumour milieu could allow for a better therapeutic CAR T cell effect (Chmielewski & Abken, 2015).

To improve the effectiveness of CAR T cell therapy towards solid tumours, the strategy of using chimeric switch receptors (CSRs) in recent years has been introduced. CSRs are aimed to reverse suppressive inhibitory signals into a positive stimulatory signal. This is done by fusing the binding ligand of a suppressive inhibitory molecule to a T cell stimulatory domain and co-expressing this receptor with a CAR. In doing this, the CSR out competes the endogenous inhibitory receptor and thus provides the T cells with a positive stimulatory signal. One example of this is targeting IL-4 that is present at elevated levels within solid tumours. To target this, the extracellular domain of the IL-4 receptor was fused to the intracellular domain of the IL-7 or IL-21 receptor, creating the IL-4/IL-7 & IL-4/IL-21 ICR (Y. Wang et al., 2019). Research showed that CAR T cells equipped with the IL-4/IL-7 or IL-4/IL-21 ICR showed enhanced cytolytic function and proliferative capabilities upon exposure to both IL-4 and respective antigen. It was also reported that T cell polarization difference was also shown, with 4/7 CAR ICR maintaining a Th1 phenotype whereas 4/21 CAR ICR displayed a Th17 phenotype. This concept has also been applied to protect CAR T cells from immune checkpoint inhibitors. CAR T cells co-expressing a PD-1/CD28 switch receptor increased resistance to inhibitory signals from PD-L1, allowing for a better antitumour response (Liu et al., 2016). One other example is CTLA-4/CD28 switch receptor. It was reported that T cells over expressing CTLA-4/CD28 switch receptor significantly enhanced T cell reactivity. However, this effect was observed predominantly in CD4 T cells (Lin et al., 2021).

The introduction of a switch receptors provides the T cells with protection from inhibitory molecules present in the TME. Furthermore, the additional benefit of this is having the capability to convert the suppressive signal into a positive stimulatory signal to allow for a more efficient antitumour response.

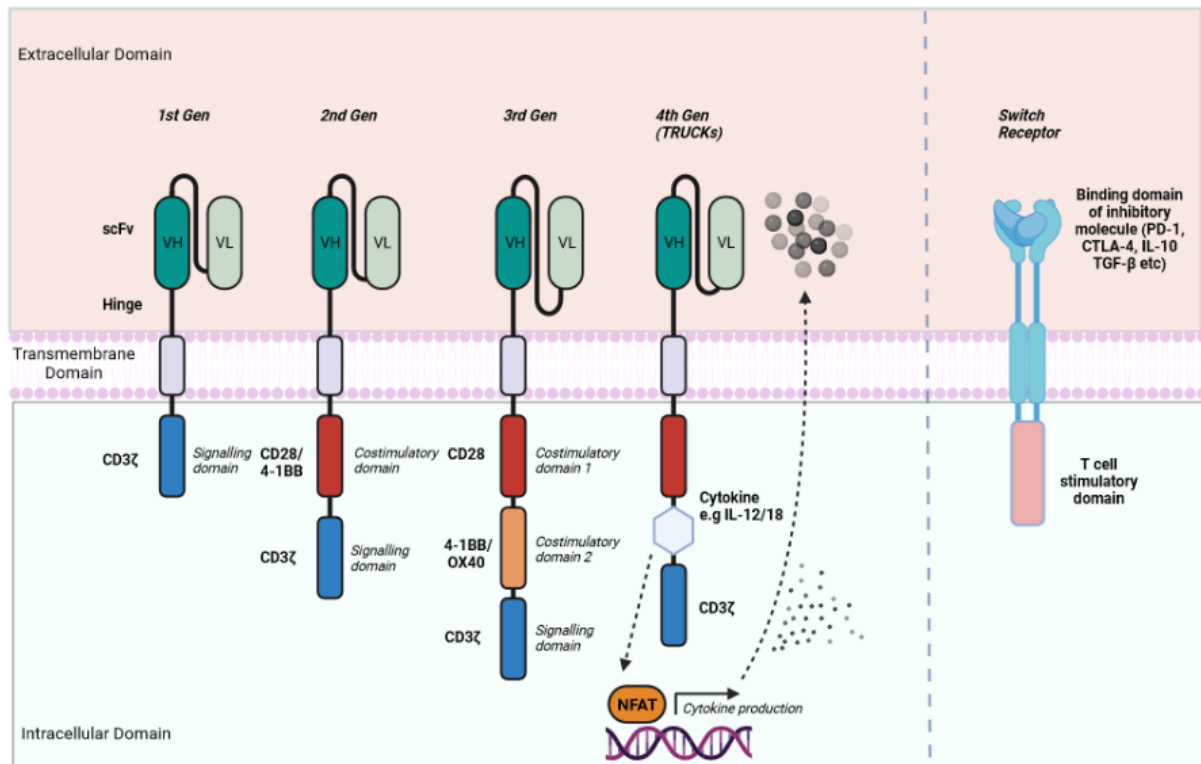


Figure 2. Evolution of CAR generations and switch receptors.

Schematic to show the evolution of CARs, highlighting the addition of signalling domains and cytokines for advanced effects. Illustration to show the design and function of a switch receptor.

1.5 The limitations of CAR T cells targeting solid tumours

The potential of CAR T cell therapy is widely recognised as remarkable, due to its FDA approval and success in the treatment of haematological malignancies such as leukaemia and lymphoma. The current clinically approved CAR T cell therapies are Kymriah for the treatment of ALL and DLBCL, Yescarta® for DLBCL and follicular lymphoma, BREYANYI for DLBCL and follicular lymphoma, ABECMA for multiple myeloma and TECARTUS for mantle cell lymphoma. Although clinical success has been reported, such CAR T cell therapies have also been subject to clinically manageable adverse side effects such as cytokine release syndrome and neurotoxicity (Z. Zhao et al., 2018; Zheng et al., 2018). However, the next challenge for CAR T cell therapy is to be effective against solid tumours. The difference compared to haematological cancer is the CARs must migrate and locate the tumour in which they face a number of challenges, a brief overview of these challenges is listed below. (Alnefaie et al., 2022).

Tumour Antigen Escape, this is a problem for single antigen targeting CARs. CAR T cells face the problem of tumour resistance after the first phase of a high response. The decrease in a successful response is caused by a complete or partial antigen expression loss. Tumour cells have the potential to avoid eradication by mutations in their antigen coding genes, this results in the down regulation of antigen expression. One way to overcome this obstacle is to use a bi-specific or split CAR, containing two scFv's targeting two different TAA's (Majzner & Mackall, 2018).

In the context of toxicities, one limitation is on-target off-tumour effects. This occurs when the antigen the CAR is targeting is also expressed in healthy tissues, resulting in a response from the CAR against normal tissues therefore leading to toxic effects. To combat this, CAR-T cell administration directly to the disease site is one suggestion. Another is to design CARs with high affinity targeting properties. With the hope that such CARs only recognise tumour cells with an increased density of target antigen. Whereas normal tissues expressing the antigen on a low level will not be affected (Sternier & Sternier, 2021; Sun et al., 2018).

Trafficking and infiltration of CAR T cells into the tumour is also considered to be a hurdle for CAR T therapy. This is caused by the immunosuppressive environment of the tumour along with physical barriers such as the stroma restrain mobility and overall diffusion of CAR T cells (Alnefaie et al., 2022) . One approach to overcome the trafficking issue is to use local administration as the delivery method for the therapy, whereas, to target the physical barrier of the stroma cells, CAR T cells engineered with heparinase expression has been shown to degrade heparin sulphate proteoglycan which is the primary component within the extracellular matrix of the stroma barrier. Research conducted by Caruna et al., found that this method led to better tumour infiltration and eradication (Caruana et al., 2015). Similarly, Wang et al engineered CAR T cells to target the fibroblast activation protein (FAP) in animal models, in conclusion they reported an increased cytotoxic function along with a significant reduction in fibroblasts present (S. Wang et al., 2014).

The immunosuppressive TME is considered one of the most challenging limitations regarding CAR T cell therapy against solid tumours. Present within the TME are multiple tumour-infiltrating cells contributing towards a suppressive environment such as, regulatory T cells (Tregs), myeloid derived suppressor cells (MDSCs), tumour associated macrophages (TAMs), or tumour associated neutrophils (TANs) (Alnefaie et al., 2022). Once these cells migrate to the tumour, they produce tumour promoting growth factors, cytokines, and chemokines, and immune suppressive factors, therefore, immunity against the tumour is reduced. One example is the presence of immune checkpoint inhibitors such as CTLA-4 and PD-1. Research into CAR T cells targeting the TME have shown poor T cell expansion and persistence indicating an exhausted phenotype. One approach to combat PD-1 in the TME was the combination of CAR T cell therapy with a checkpoint blocking for the PD-1/PDL1 interaction. Combining both strategies allowed for penetration of CAR T cells followed by enhanced resistance and T cell function (Grosser et al., 2019; June et al., 2018). Recently, efforts have been drawn to develop CAR T cells to overcome mediated suppression caused

by Transforming Growth Factor- β (TGF- β). Secreted by many cells present within the TME such as, tumour cells, CAF, Tregs, M2 TAMs, N2 TANs and MDSCs, the presence of this inhibitory cytokine is responsible for suppressing an effective CAR T cell response and is therefore considered a key target in overcoming CAR T therapy against solid cancer.

1.6 Transforming Growth Factor- β (TGF- β)

Transforming Growth Factor- β is a multifunctioning cytokine with vital roles in both the development of the embryo and homeostasis of the adult tissues. Involved in processes such as, differentiation, apoptosis, migration, wound healing and regulation of cell proliferation, TGF- β has paradoxical effects in which can either be for or against the progression of tumours (Ahmadi et al., 2019). The anti-tumour effects of TGF- β are through its proapoptotic and cytostatic properties reducing cell growth, however, on the contrary to this, a loss in this effect can lead to increased amplification properties and cancer (Siegel & Massagué, 2003). In the context of solid tumours, TGF- β is overproduced creating an immunosuppressive environment utilized for tumour growth, survival and to escape immune surveillance.

There are three known isoforms of TGF- β : TGF- β 1, TGF- β 2 & TGF- β 3, with their respective receptors (TGF- β RI, TGF- β RII & TGF- β RIII). These isoforms are also evolutionary conserved proteins and share around 80% of identical amino acid sequences (Ahmadi et al., 2019). TGF- β is found in the blood of cancer patients in vast amounts, it is known that a dysregulation within the signalling pathway is responsible for the progression of cancer. More specifically, the TGF- β superfamily is highly dependent on the epigenetics and microRNAs for their regulation and in the context of cancer, one type of cancer epigenetic contribution could exceed microRNA and vice versa. Genetic changes within the TGF- β signalling such as, DNA methylation, deletion, mutation and amplification all contribute to cancer progression and it is estimated that alterations within the genome of TGF- β signalling accounts for about 40% of cancers, making it a suitable candidate to target to improve immunotherapy (Han et al., 2018; Korkut et al., 2018).

Solutions for the treatment against TGF- β include, monoclonal antibodies, small molecule inhibitors and antisense oligonucleotides. However, it is vital to know that a complete block against TGF- β can lead to fatal outcomes. This was shown in mice, more than 50% of TGF- β KO mouse had inflammatory diseases and embryonic lethality, whereas a complete loss of TGF- β 2 and TGF- β 3 in mice resulted in 100% embryonic lethality (Kaartinen et al., 1995; Kulkarni et al., 1993; Sanford et al., 1997). However, inhibiting the production of the protein on a molecular level was successful. This was done with an antisense oligonucleotide against TGF- β 2, this treatment showed promising results in clinical I/II trials treating grade IV astrocytoma (Fakhrai et al., 2006; Schlingensiepen et al., 2005). Furthermore, the use of a soluble TGF- β receptor II was successful in the treatment of human breast cancer. The soluble receptor could bind to TGF- β 1 and inhibit signalling associated with in CAFs activation.

TGF- β promotes tumour growth by altering the metabolic reprogramming of CAFs, inhibiting this self-sustaining autocrine signalling reduces the rise to tumour promoting CAF myofibroblasts during tumour progression. (Huang et al., 2021).

In addition, the use of a Dominant Negative Receptor DNR (dnTGF- β RII) has also been used for treatment against TGF- β . This is composed of over expressing the TGF- β RII without downstream signalling moieties, however in a decoy format in which no negative signal is given to the cell, this receptor acts as a sink to trap TGF- β in the environment. This approach showed better anti-tumour effects due to a greater abundance of CD8⁺ cytotoxic T cells in transgenic mice. In a recent clinical trial using the dominant negative receptor, eight patients suffering from Epstein-Barr virus (EBV) positive lymphoma all received treatment with DNR expressing T cells with specificity for EBV-derived tumour antigens such as Latent membrane protein (LMP)-1 and LMP-2. Remarkably, 4 of 8 patients achieved clinical response, with one being a complete response. It was also recorded that during the 4 years of treatment no patient suffered from toxicity or uncontrollable T cell amplification (Bollard et al., 2002, 2018).

The abundance of TGF- β in cancerous environments, coupled with its paradoxical properties to promote tumour progression and escape immunosurveillance make it a suitable candidate to target for the improvement of immunotherapy against cancer.

1.7 CAR T cells against TGF- β

To improve CAR T cell therapy against solid tumours, recent strategies have involved engineering CARs to target TGF- β that is present within the suppressive tumour microenvironment. One of the most successful approaches is combining the dominant negative TGF- β receptor with a CAR, in a co-expression format. Targeting prostate-specific membrane antigen (PSMA), CAR T cells equipped with the dnTGF- β RII display a significantly better amplification capacity along with the ability to eliminate advanced PSMA expressing tumours in aggressive human prostate cancer mouse models (Kloss et al., 2018). In addition to this, the PSMA-dnTGF- β RII targeting CAR was also used in a clinical trial. Findings from the trial show that on average the TGF- β present in the patient's serum levels decreased to approximately 30% in treated patients (Narayan et al., 2022). It was also reported that although there was some tumour infiltration within the first two weeks of treatment, it was not as consistently high as expected in all patients. Furthermore, the trial demonstrated the safety and feasibility of delivering a CAR T cell product capable of showing resistance to TGF- β .

Another approach to protect CAR T cells from TGF- β mediated suppression was to develop the TGFRII KO CAR T cells. This strategy involved using the CRISPR/Cas9 system to knock out the TGFRII gene in T cells, aiming at inhibiting the chance for TGF- β to bind and provide a negative suppressive signal to the T cell. It was shown that TGFRII KO CARs were able to effectively eliminate antigen positive tumour cells and were able to overcome the anti-proliferative effect caused by TGF- β in vitro. Interestingly, it was also shown that TGFRII KO

CAR T cells were able to overcome the suppressive effect caused by Tregs in vitro, once again highlighting the importance of targeting TGF- β in the context of active immune repression (Alishah et al., 2021).

Altering the TGF- β signalling pathway either by a decoy or a knockout has not been the only way to overcome the suppressive effect on CAR T cells, the introduction of cytokines and cytokine signalling is also another strategy to combat TGF- β . It has been shown CAR T cells with the CD28 co-stimulatory domain are resistant to TGF- β . This is due to CD28's high IL-2 induction properties through Lymphocyte-Specific Protein Tyrosine Kinase (Lck) signalling. Abolishment of the Lck-binding motif of the CD28 signalling endodomain, completely reducing the IL-2 production resulted in mediated suppression for CD28 CAR T cells (Golomba-Nagy et al., 2018). However, re-adding IL-2 in a constitutive format reversed this effect, clearly demonstrating that IL-2 and not another function triggered by LCK is promoting TGF- β resistance. From here it was discovered that cytokines such as IL-2, IL-7 & IL-15, all sharing the common chain, were able to convert resistant to an IL-2 deficient CAR. However, in the context of targeting a TME constitutively expressing cytokines could impact neighbouring cells resulting in tumour progression, for example IL-2 and IL-15 sustain Treg activation. Therefore, this study engineered a CAR T cell with an IL-7 autocrine loop, signalling via the intracellular domain of IL-2R β . From that we draw the concept that providing CAR T cells with additional intracellular signalling provides a real advantage for targeted therapy with minimal impact within the TME, providing focused signalling and driving the T cells towards a certain phenotype could be a potential strategy for improving CAR T cells against solid cancer.

1.8 Aims

CAR T cell therapy has shown promising effects for the treatment of haematological malignancies, however, development of this therapy for solid tumours produced disappointingly poor results so far. One main limitation is the suppressive tumour microenvironment, even if migration to the tumour lesion is successful, CAR T cells turn dysfunctional due to inhibitory and suppressive signals present within the TME. TGF- β is highly abundant within the TME and considered to be a dominant suppressive cytokine. Its immune suppressive and tumour promoting properties allow for tumour cells to evade an immune response thus promoting tumour progression and to suppress a productive anti-tumour T cell response.

Research conducted previously by our group showed CAR T cells with the CD28 co-stimulatory domain are resistant to TGF- β mediated suppression. The driving factor for this is high IL-2 production due to Lck signalling triggered through the CD28 domain (Golumba-Nagy et al., 2018). However, high IL-2 induction stimulated regulatory T cells which contribute towards the suppressive effect in the TME. This was shown in an *in vivo* model, mice bearing tumour cells were treated with CD28- ζ and CD28 Δ LCK- ζ CAR T cells, the latter deficient in CAR induced IL-2 release, along with a dose of Tregs (Kofler et al., 2011). The Treg population significantly increased in the mice treated with CD28- ζ CAR T cells, whereas in the mice treated with the CD28 Δ LCK- ζ CAR with abolished IL-2 properties, the number of Tregs present did not increase. To reduce the chance of stimulating Tregs in the TME, work previously done in our group incorporated an IL-7 autocrine loop compromised of IL-7 signalling, followed by IL-7R ectodomain and IL-2R β intracellular domain, providing the CAR T cell with an intracellular IL-2 signal to combat TGF- β resistance (Golumba-Nagy et al., 2018).

To further improve this concept from a translational and structural point of view, we designed a Switch Receptor capable of converting TGF- β resistance to 4-1BB- ζ CAR T cells. The design is comprised of a co-expressed receptor built up with the binding domain of TGF- β receptor-II (TGF- β RII) extracellularly and fused to the IL-2R β signalling chain intracellularly. The T cell will receive full activation via 4-1BB co-stimulation and CD3 ζ primary signal upon antigen engagement via the CAR; however, in the presence of TGF- β , the co-expressed SWR is aimed at outcompeting the endogenous TGF- β receptor and convert the negative suppressive signal, into an intracellular stimulatory signal through the IL-2R β signalling chain.

The 4-1BB- ζ CAR is sensitive to TGF- β mediated suppression, highly effective against leukaemia/lymphoma and is also FDA approved and widely used in clinical application for the treatment of haematological malignancies. However, 4-1BB- ζ CAR T cells did not produce efficacy against solid tumours so far. Additionally, it has very low IL-2 secretory properties and therefore we believe it is the best model for proving the concept of a TGF- β -IL2R switch receptor for a more effective CAR T cell response against the suppressive TME.

Throughout this project we addressed the following questions:

- Can a Switch Receptor bind TGF- β and transmit an IL-2R signal to the engineered T cell?
- Can the Switch Receptor convert TGF- β resistance to 4-1BB- ζ CAR T cells?
- How does the SWR compare to the current most successful mechanism used for TGF- β resistance, i.e., the Dominant Negative Receptor?

2 MATERIALS

2.1 Chemicals and Reagents

Chemicals & Reagents	Company
ABTS	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
ABTS Buffer	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
AccuCheck Counting Beads	ThermoFisher Scientific, Waltham, MA, USA
Ampicillin	Merck/CalBiochem, Darmstadt, Germany
Bacto Agar	Carl Roth GmbH, Karlsruhe, Germany
BSA	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Cell Proliferation Dye (eFluor™ 450)	Invitrogen/ThermoFisher Scientific, Waltham, MA, USA
DMSO	Carl Roth GmbH, Karlsruhe, Germany
eBioscience™ Fixable Viability Dye™ 780	eBioscience/ThermoFisher Scientific, Waltham, MA, USA
eBioscience™ Protein Transport Inhibitor Cocktail (500x)	eBioscience/ThermoFisher Scientific, Waltham, MA, USA
Ethanol	Carl Roth GmbH, Karlsruhe, Germany
Glycerin	Merck/CalBiochem, Darmstadt, Germany
HEPES	GIBCO/ThermoFisher Scientific, Waltham, MA, USA
Isopropanol	Carl Roth GmbH, Karlsruhe, Germany
Pancoll	PAN-Biotech GmbH, Aidenback, Germany
PBS 10x	GIBCO/ThermoFisher Scientific, Waltham, MA, USA
PEIpro® Transfection Reagent	Polyplus transfection, Illkirch, France
PenStrep	PAN-Biotech GmbH, Aidenback, Germany
Streptavidine-Peroxidase conjugate	Roche Diagnostics, Rotkreuz, Switzerland
Trypan Blue	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Trypsin/EDTA	PAN-Biotech GmbH, Aidenback, Germany
Tween®20	Merck, Darmstadt, Germany

Table 2. Table containing chemicals, reagents and their origin used in this study

2.2 Kits

Kits	Company
Fixation/Permeabilization Kit	BD Bioscience, San Jose, CA, USA
NucleBond® Xtra Midi Kit	Macherey Nagel GmbH, Düren, Germany
Transcription Factor Phospho Buffer Set	BD Bioscience, San Jose, CA, USA

Table 3. Table containing the kits and their company origin used in this study

2.3 Medium used for culture of cells

Medium	Company
D-MEM, High Glucose, GlutaMAX™ supplement	GIBCO/ThermoFisher Scientific, Waltham, MA, USA
Heat-Inactivated (HI) FCS (56°C, 30 minutes)	PAN-Biotech GmbH, Aidenback, Germany
RPMI1640 GlutaMAX™ supplement	GIBCO/ThermoFisher Scientific, Waltham, MA, USA

Table 4. Table containing the mediums and their company origin used in this study

2.4 Buffers

Buffers	Composition
Blocking buffer (ELISA)	1% BSA (w/v), 1x PBS
Coating buffer (ELISA)	0.1M Na ₂ HPO ₄ , pH 9
LB agar	11 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 ₂ HPO ₄ , 20 mM KH ₂ PO ₄ , ddH ₂ O; pH 7.4
PBS/Tween (PBS-T)	1x PBS, 0.1% (v/v) Tween®-20

Table 5. Table containing buffers and their composition used in this study

2.5 Antibodies

2.5.1 Unconjugated antibodies

Antibody	Species/Isotype	Company/Source
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., 1988)
Anti-human BW431/26scFv, Clone BW2064/36	Mouse IgG1	Hybridoma cell line, (Bosslet et al., 1985)

Table 6. Table containing the antibodies and their source used in this study

2.5.1 Conjugated antibodies

Antibody	Species/Isotype	Conjugate	Company
Anti-human CD107a (LAMP-1), Clone H4A3	Mouse IgG1k	VioBlue	Miltenyi Biotec, Bergisch Gladbach, Germany
Anti-human CD3, Clone OKT3	Mouse IgG2ak	APC	BioLegend®, CA, USA
Anti-human CD3, Clone UCHT1	Mouse IgG1k	BV421	BioLegend®, CA, USA
Anti-human CD4, Clone M-T466	Mouse IgG1k	APC	Miltenyi Biotec, Bergisch Gladbach, Germany
Anti-human CD45RO, Clone UCHL1	Mouse BALB/c IgG2a, κ	BV605	BD Bioscience, San Jose, CA, USA
Anti-human CD62L, Clone DREG-56	Mouse IgG1k	BV421	BioLegend®, CA, USA
Anti-human CD8, Clone BW135/80	Mouse IgG2ak	FITC	Miltenyi Biotec, Bergisch Gladbach, Germany
Anti-human hIgG F(ab') Clone	Goat F(ab') ₂ IgG	PE	Southern Biotechnology, AL, USA
Anti-human Perforin, Clone B-D48	Mouse IgG1k	APC	BioLegend®, CA, USA
Mouse Anti-STAT5, Clone 47/stat5(pY694)	Mouse IgG1k	BV421	BD Bioscience, San Jose, CA, USA
Anti-human TGF-β Receptor II	Rat IgG2b, κ	APC	BioLegend®, CA, USA
Anti-human IL-2, Clone B33.2	Mouse IgG1	Biotin	BD Pharmingen™, Hamburg, Germany
Anti-human IFN-γ, Clone 42.B3	Mouse IgG1	Biotin	BD Pharmingen™, Hamburg, Germany

Table 7. Table of conjugated antibodies and their relevant information used in this study

2.6 Cytokines

Cytokines	Company
Imukin® (IFN- γ standard)	Boehringer Ingelheim, Ingelheim, Germany
Proleukin® (IL-2 standard)	Novartis Pharma GmbH, Basel, Switzerland
Recombinant Human TGF- β 1	Miltenyi Biotec, Bergisch Gladbach, Germany

Table 8. Table of cytokines and their source used in this study

2.7 Plasmid Vectors

ID	Plasmid	Characteristics/Reference
#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)
#607	pBullet-Lk-BW431/26scFv-Fc-CD28-CD3 ζ	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-CD28-CD3 ζ (Hombach et al., 2001)
#848	pBullet-Lk-BW431/26scFv-Fc-OX40-CD3 ζ	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-OX40-CD3 ζ
#908	pBullet-Lk-BW431/26scFv-Fc-4-1BB-CD3 ζ	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-4-1BB-CD3 ζ (Hombach et al., 2011)
#946	pBullet-Lk-BW431/26scFv-Fc-CD28 Δ LCK-CD3 ζ	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-CD28 Δ LCK-CD3 ζ (Kofler et al., 2011)

ID	Plasmid	Characteristics/Reference
#2299	pcDNA.3HIV1.GP.4xCTE	Lentiviral vector containing the expression cassette of the HIV-1 GAG/POL Protein. Precursor structural protein of the lentiviral particle containing Matrix, Capsid, and Nucleocapsid components. Precursor protein containing Reverse Transcriptase and Integrase components.
#2300	P.MD2.G (VSVg)	Lentiviral vector containing the expression cassette of the VSV-G Protein. (Vesicular stomatitis virus G glycoprotein)
#2301	P.RSV.REV	Lentiviral vector containing the expression cassette of the REV Protein. Binds to the Rev Response Element (RRE) within unspliced and partially spliced transcripts to facilitate nuclear export.
#2356	pBullet-Lk-BW431/26scFv-Fc-4-1BB-CD3 ζ -TGF- β RII-IL2R β	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-4-1BB-CD3 ζ -TGF- β RII-IL-2R β
#2357	pBullet-Lk-BW431/26scFv-Fc-CD28 Δ LCK-CD3 ζ -TGF- β RII-IL2R β	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-CD28 Δ LCK-CD3 ζ -TGF- β RII-IL-2R β
#2362	pBullet-Lk-BW431/26scFv-Fc-OX40-CD3 ζ -TGF- β RII-IL2R β	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-OX40-CD3 ζ -TGF- β RII-IL-2R β
#2410	pBullet-Lk-BW431/26scFv-Fc-4-1BB-CD3 ζ -TGF- β RII	contains the mammalian expression cassette of the CEA-specific scFv CAR BW431/26scFv-Fc-4-1BB-CD3 ζ -TGF- β RII
#2561	pLV[Exp]-mCherry-EF1A-hTGF- β 1	Contains the mammalian gene expression vector mCherry-EF1A-hTGFB1 (VectorBuilder)

Table 9. Table of plasmids and relevant information used in this study

2.8 Bacterial Strains

Bacteria Strains	Description
E.Coli DH5 α	F-, end A1, hsd R17 (rk-, mk-) sup E44, thi-1, lambda-, recA1, gyrA96, Φ 80 d lacZ δ M15

Table 10. Table of bacterial strains used in this study

2.9 Cell Lines

Cell Line	Characteristics	Reference
HEK 293T	human embryonic kidney 293 cell line (ATCC CRL-3216™) stably expressing the SV40 large T antigen; neomycin resistant	(DuBridge et al., 1987)
NCI-N87 (CRL-5822)	Human Gastric Carcinoma isolated from the stomach of a patient in 1976. Cell line provided by Dr. Arpad Szoor, University of Debrecen Hungary.	ATCC CRL-5822
N87-TGF- β 1-mCherry	N87 cell line transduced with human TGF- β 1 expression vector (#2561)	

Table 11. Table of cell lines used in this study

2.10 Primary Cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood from the platelet and leukocyte fraction of a blood donation from healthy donors. Samples were provided and approved by the Universitätsklinikum Regensburg (Ethics Vote no 21-2224-101).

2.11 Software

Software	Company
BioRender	BioRender
FACS	BD Bioscience
FlowJo	FlowJo LLC
GraphPad Prism	GraphPad Software, Inc
Microsoft Excel	Microsoft
Miscrosoft Powerpoint	Microsoft
Microsoft Word	Microsoft
SnapGene Viewer	SnapGene

Table 12. Table of software used in this study

3 METHODS

3.1 Bacteria

All bacterial cells were handled in a security level S2 gene technology laboratory.

3.1.1 Bacteria Culture

Bacterial cells *Escherichia coli* (*E. coli*) DH5 α were cultured overnight at 37°C and shaking at 200 rpm in liquid LB medium or on a LB plate. 100 μ g/ml Ampicillin (Amp) was added to select the growth of genetically modified bacteria carrying the Amp-resistant gene. For long term storage, 700 μ l bacteria culture was mixed with 300 μ l of 87% (v/v) glycerol and stored at -80°C.

3.1.2 Heat-shock transformation of bacteria

DH5 α competent cells (stored at -80°C) were defrosted on ice and 1-100 ng plasmid DNA was added. Plasmid DNA and bacteria mix were heat-pulsed at 42°C for 90 seconds then immediately cooled on ice for 2 minutes. For antibiotic resistance, the bacteria were incubated in 900 μ l LB medium (without antibiotic) at 37°C, shaking at 200 rpm for 60 minutes. The suspension was then centrifuged for 5 minutes at 5000 x g, resuspended in 100 μ l and spread on a LB-agar plate with ampicillin using a plating spatula and stored at 37°C overnight.

3.2 Working with DNA

3.2.1 Plasmid isolation

For plasmid DNA preparation, the “NucleBond® Xtra Midi Kit” (Macherey Nagel GmbH) (for DNA amounts up to 400 μ g) were used according to the manufacturer’s instructions. Prior to DNA preparation, 100 ml of bacteria suspension was prepared. Finally, the eluted plasmid DNA was diluted in sterile H₂O_{dd} and stored at -20°C.

3.2.2 Quantification of DNA

Before storage of DNA at -20°C , the concentration of the DNA was measured spectrophotometrically using the NanoDrop™ 1000 spectrophotometer (ThermoFisher Scientific). To do so, 1 μl DNA sample is applied onto the NanoDrop™ pedestal, the DNA concentration is measured at a wavelength of 260 nm, whereas the protein concentration is measured at 280 nm. Purity from protein contaminations of the preparation is defined by the ratio O.D. 260 nm / abs 280 nm. A 1.8 ratio indicates high purity of DNA, below this value indicates contaminations with proteins and aromatic substances. Higher ratios suggest RNA contamination.

3.3 Working with cells

3.3.1 Cell culture conditions

All cell culture work was carried out in a Biosafety Level S2 laboratory using a laminar flow hood (Class II) (HERASAFE 2030i, ThermoFisher Scientific). 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 used were DMEM and RPMI1640 with GlutaMAX™ and supplemented with 10% (v/v) FCS, 1 M HEPES, 10 mg/ml streptomycin and 10,000 U/ml penicillin. Prior to use, media and supplements were pre-warmed up to 37°C .

3.3.2 Passaging of adherent cells

The medium was removed from the flask containing the adherent cell line, 5 ml of pre-warmed 1x PBS was added to wash cells. Trypsin/EDTA (3-5 ml) was added to cover the entire surface of the flask and the cells were incubated at 37°C for 5-10 minutes. 5-10 ml fresh culture medium was added to stop the effect of Trypsin/EDTA, cells were transferred to a 15/50 ml falcon and centrifuged at $500 \times g$ for 5 minutes. The supernatant was discarded, cells were resuspended in fresh culture medium and transferred back into a flask in a ratio of 1:10 or 1:20 depending on growth characteristics of the cell line.

3.3.3 Passaging of suspension cells

Suspension cells were transferred to a 15/50 ml falcon and centrifuged at $300 \times g$ for 5 minutes. The supernatant was discarded, cells were resuspended in fresh culture medium and transferred into a culture flask in a ratio of 1:5 or 1:10 depending on growth

characteristics. In the case of peripheral blood lymphocytes, IL-2 was added in a concentration of 100-250 U/ml and cells were resuspending at 1×10^6 /ml.

3.3.4 Freezing and thawing of cells

For long term storage of cells, cells ($1-150 \times 10^6$) were centrifuged at $300 \times g$ for 5 minutes. The supernatant was discarded, cells were resuspending in 500 μ l freezing medium A (60% FCS + 40% RPMI 1640) and 500 μ l freezing medium B (80% FCS + 20% DMSO) and pipetted into a cryovial. Cryovials were placed in a “Mr Frosty™ Freezing Container” (ThermoFisher Scientific) and transferred to a -80°C freezer. To thaw cells, cryovials were taken from the -80°C freezer and re-suspended in 1 ml pre-warmed culture medium and immediately transferred into a 15 ml falcon containing 9 ml pre-warmed culture medium. Cells were washed and centrifuged at $300 \times g$ for 5 minutes to remove DMSO. Finally, the cells were resuspended in fresh culture medium and stored in hully humidified 5% or 10% (v/v) CO_2 incubator at 37°C .

3.3.5 Determination of cell counts

The cell count and viability were recorded using the Vi-CELL BLU cell viability analyzer device (Beckman Coulter). For the measurement, 200 μ l of sample was added to a Vi-CELL Blu sample vial and inserted into the chamber of the device. Alternatively, cells were counted manually. Cells were diluted (1:10) with trypan blue (to exclude dead cells), 10 μ l of mixture was loaded onto a “Neubauer” counting chamber (Celeromics). The total number of cells was counted in each of the 4 squares. The calculation to determine cell number is: Total cells / $0.4 \times$ (dilution) $\times 1000$ = total number of cells /ml.

3.3.6 Isolation of human peripheral blood mononuclear cells (PBMCs)

Human PBMCs were isolated from fresh blood of healthy donors provided by the “Transfusionmedizin” department from the Universitätsklinikum Regensburg. The isolation was done via density gradient centrifugation. The separation procedure is based on a polysaccharide solution with a density of 1.077 g/ml. Mononuclear cells have a lower density than erythrocytes and granulocytes, therefore they don’t sediment during the centrifugation process. Firstly, 1x PBS is added to the blood sample to bring the volume up to 50 ml. Next, 25 ml of the diluted blood is carefully layered on top of 15 ml Human Pancoll Solution (PAN Biotech, Aidenbach, Germany) in a 50 ml falcon tube. The mixture is centrifuged at 2000 rpm with a brake speed of 3 at 20°C for 20 minutes. After centrifugation, using a Pasteur pipette the lymphocytes are collected, transferred to a 50 ml falcon, washed with 30ml 1x PBS three times (1400 rpm, 20°C , 10 minutes). The cells were then resuspended in RPMI1640 medium supplemented with 10% FCS (v/v), 1 M

HEPES, 10 mg/ml streptomycin and 10,000 U/ml penicillin in a cell density of 1×10^6 cell/ml. Cells were also supplemented with 1000 IU/ml human IL-2, 200 ng/ml OKT3 (anti-CD3 mAb) and 50 ng/ml 15E8 (anti-CD28 mAb).

3.3.7 Transfection of HEK 293T cells

Transfection of HEK 293T cells was done using the transfection reagent PEIpro® (Polyplus transfection) according to the manufacturer's instructions. On the day before transfection, HEK 293T cells were seeded into a 10 cm petri dish cultured in DMEM supplemented with 10% FCS (v/v), 1 M HEPES, 10 mg/ml streptomycin and 10,000 U/ml penicillin. On the day of transfection, the desired confluency of cells should be around 70-80%. Construct DNA was mixed with helper plasmid DNA in DMEM medium without supplements. The DNA/Helper solution was mixed with the transfection reagent solution, vortexed and incubated for 15 minutes at room temperature. During this time, the supernatant of the HEK 293T cells was exchanged with RPMI1640+ GlutaMax™ supplemented with 1 M HEPES, 10 mg/ml streptomycin and 10,000 U/ml penicillin. After incubation, the DNA/Transfection reagent mixture was added dropwise to the HEK 293T cells, the cells were then incubated in 5% (v/v) CO₂ incubator at 37°C overnight. The following transfection process was followed.

Culture vessel	Number of HEK 293T cell	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 HEK 293T cells
10 cm petri dish	3×10^6	10 ml	20 µg (Retro) 25 µg (Lenti)	250 µl	20 µl	250 µl	540 µl (Retro) 545 µl (Lenti)

Table 13. Overview of transfection scheme

DNA	Name	Volume
Helper plasmid	pHIT60	5 µg (5 µl)
Helper plasmid	pCOLT-GALV	5 µg (5 µl)
Encoding vector		10 µg (10 µl)

Table 14. Plasmids used for retroviral transfection

DNA	Name	Volume
Helper plasmid	pcDNA.3HIV1.GP.4xCTE	5 µg (5 µl)
Helper plasmid	P.MD2.G (VSVg)	5 µg (5 µl)
Helper plasmid	P.RSV.REV	5 µg (5 µl)
Encoding vector		10 µg (10 µl)

Table 15. Plasmids used for Lentiviral transfection

3.3.8 Retroviral transduction of human T cells

Virus supernatant was produced by retroviral transfection, prior to harvesting the virus supernatant, 1 ml of FCS was added to the transfected HEK 293T cells. After collection, 10 ml fresh medium (RPMI1640+ GlutaMax™, 10% FCS (v/v), with 1 M HEPES, 10 mg/ml streptomycin and 10,000 U/ml penicillin) was added to the transfected HEK 293T cells for a second harvest the day after. 5×10^6 PBLs were resuspended in 10 ml virus supernatant and transferred to a T25 cell culture flask which had been coated with poly-D-lysine (PDL, 10 µg/ml in 2 ml sterile distilled H₂O) 24 hours prior. 200 U/ml IL-2 was added and the flask containing the cells and virus supernatant were centrifuged at 1600 x g for 1 hour at 32°C. After centrifugation, the cells were incubated for 24 hours at 37°C in a 5% (v/v) CO₂ incubator. The next day, this process was repeated again with the freshly produced virus supernatant.

3.3.9 Lentiviral transduction of N87 tumour cells

Virus supernatant was produced by lentiviral transduction, after harvesting the virus supernatant 10 ml fresh medium (RPMI1640+ GlutaMax™, 10% FCS (v/v), with 1 M HEPES, 10 mg/ml streptomycin and 10,000 U/ml penicillin) was added to the transfected HEK 293T cells for a second harvest the day after. N87 (2×10^6) tumour cells were resuspended in 10ml virus supernatant and transferred to a T25 cell culture flask which had been coated with poly-D-lysine (PDL, 10 µg/ml in 2 ml sterile distilled H₂O) 24 hours prior. The cells and virus supernatant were centrifuged at 1600 x g for 1 hour at 32°C. After centrifugation, the cells were incubated for 24 hours at 37°C in a 5% (v/v) CO₂ incubator. The next day, this process was repeated again with the freshly produced virus supernatant.

3.3.10 Fluorescence-activated cell sorting (FACS)

To identify the presence of surface receptors such as a chimeric antigen receptor, cell surface molecules and other intracellular components, the cells were stained with a fluorochrome-conjugated antibody for Flow Cytometry analysis.

3.3.11 Detection of cell surface markers

Transduced T cells ($1-3 \times 10^5$) were used for FACS staining. Cells were transferred to a 5 ml round bottom polystyrene tube (Falcon®) and washed twice with 2 ml 1x PBS (centrifuge at $500 \times g$ for 4 minutes). Supernatant was discarded and the cell pellet was resuspended in 100 μ l 1x PBS. The specific fluorochrome-conjugated antibody was used according to the manufacturer's instructions. In general, cells were incubated with the fluorochrome conjugated antibody for 20 minutes at 4°C in the dark. After incubation, cells were washed twice with 2 ml 1x PBS and resuspended in 350 μ l 1x PBS containing 2% FCS. CAR detection was done with the PE-conjugated goat F(ab')₂ anti-human IgG antibody, T cell labelling was done using the BV421-conjugated or APC-conjugated anti-human CD3 antibody and the TGF- β RII was identified using the APC-conjugated anti-human TGF- β RII antibody. Data was recorded using the BD FACSLyric™ Flow Cytometry System and the data was analysed using the FlowJo v10.8.1 software (FlowJo LLC).

3.3.11.1 Detection of CD107a

To detect CD107a, 5×10^4 (cells/well) anti-CEA CAR modified T cells were stimulated with cognate immobilized BW2064/36 antibody, CAR T cells incubated with the "Protein Transport Inhibitor Cocktail", VioBlue-conjugated CD107a (LAMP-1) mAb in the presence and absence of TGF- β 1 (100 ng/ml) for 24 hours. After incubation and to detect CAR T cells, cells were harvested, washed twice with 1x PBS, and stained with PE-conjugated goat F(ab')₂ anti-human IgG antibody and APC-conjugated anti-human CD3 antibody and incubated in the dark at 4°C for 20 minutes. After incubation, cells were washed twice, centrifuged at $500 \times g$ for 4 minutes, resuspended in 350 μ l 1x PBS containing 2% FCS and transferred to a 5 ml round bottom polystyrene tube for FACS analysis.

3.3.11.2 Detection of memory phenotype markers

To identify memory phenotype markers, $1-3 \times 10^5$ CAR-modified T cells were transferred to a 5 ml round bottom polystyrene tube and washed twice with 1x PBS (centrifuge at 500 x g for 4 minutes). After washing, cells were stained with APC-conjugated anti-human CD4, FITC-conjugated anti-human CD8, PE-conjugated goat F(ab')₂ anti-human IgG, BV421-conjugated anti-human CD62L, BV605-conjugated anti-human CD45^{RO} and incubated in the dark at 4°C for 20 minutes. After incubation, cells were washed twice, centrifuged at 500 x g for 4 minutes, resuspended in 350 µl 1x PBS containing 2% FCS.

3.3.12 Intracellular Staining

3.3.12.1 Detection of Phosphorylated STAT5

For the detection of pSTAT5, 1×10^5 (cells/well) anti-CEA CAR-modified T cells were stimulated with cognate immobilized BW2064/36 antibody for 3 days in the presence and absence of TGF-β1 (10 ng/ml). For the detection of phosphorylated epitopes, cells were fixed and permeabilized according to the “Transcription Factor Phospho Buffer Set” manufacturers protocol. The procedure was done on a 96-well plate. Cells were stained with BV421-conjugated Mouse Anti-STAT5 (pY694), PE-conjugated goat F(ab')₂ anti-human IgG antibody and APC-conjugated anti-human CD3 antibody for 40 minutes at 4°C in the dark. After incubation, cells were washed twice, centrifuged at 500 x g for 4 minutes, resuspended in 350 µl 1x PBS containing 2% FCS and transferred to a 5 ml round bottom polystyrene tube for FACS analysis.

3.3.12.2 Detection of Perforin

To detect perforin, 5×10^4 (cells/well) anti-CEA CAR modified T cells were stimulated with cognate immobilized BW2064/36 antibody and incubated with the “Protein Transport Inhibitor Cocktail”, in the presence and absence of TGF-β1 (100 ng/ml) for 24 hours. Cells were harvested, fixed and permeabilized using the BD “Cytofix/Cytoperm” Kit according to manufacturer’s instructions. Detection of CAR T cells was done using the PE-conjugated F(ab')₂ anti-IgG1 antibody and BV421 conjugated anti-CD3 mAb. Perforin was detected using APC-conjugated anti-human Perforin antibody. Cells were stained and incubated in the dark for 20 minutes at 4°C. After incubation, cells were washed twice, centrifuged at 500 x g for 4 minutes, resuspended in 350 µl 1x PBS containing 2% FCS and transferred to a 5 ml round bottom polystyrene tube for FACS analysis.

3.4 Assays

3.4.1 Cytokine Secretion

For cytokine secretion, anti-CEA CAR modified T cells (2.5×10^4 cells/well) were incubated on a 96-well plate coated with cognate immobilized BW2064/36 antigen. After 48 hours cell culture supernatant was taken and analysed for IL-2 by ELISA.

3.4.2 Proliferation Assay

To monitor CAR T cell amplification, T cells were washed twice with 1x PBS and stained with 10 μ M “Cell Proliferation Dye eFluor 450” for 10 minutes at 37°C. Staining was stopped by the addition of cold RPMI containing 10% (v/v) serum and incubated on ice for 5 minutes. The cells were then washed 3 times with complete cell culture medium before subject to assay. CAR modified and proliferation dye labelled (1×10^5 cells/well) T cells were transferred to 96-well plate pre-coated with BW2064/36 immobilized antigen. Cells were in a total volume of 200 μ l and incubated in the presence and absence of TGF- β 1 for 5 days. After 5 days, cells were harvested and stained for the CAR with the PE-conjugated F(ab')₂ anti-IgG1 antibody and BV421 conjugated anti-CD3 mAb. Dilution of the dye was recorded using Flow Cytometry.

3.4.3 Enzyme-linked Immunosorbent Assay (ELISA)

MaxiSorp™ microtitre plates were coated with capture antibodies (50 μ l/well in coating buffer) and left to incubate overnight at 4°C. The following day, the plates were then blocked to prevent unspecific binding using 1% (w/v) BSA dissolved in 1x PBS (200 μ l/well) for 2 hours at room temperature on a plate shaker at 300 rpm. Plates were then washed three times with PBS-T (200 μ l/well) before adding supernatant (50 μ l/well) and standard samples (100 μ l/well), plates were placed on the plate shaker at 300 rpm at room temperature for 2 hours. After incubation, plates were washed four times with PBS-T (200 μ l/well) and biotinylated antibodies were added in 1% (w/v) BSA in PBS-T (50 μ l/well), the plates were left to incubate at room temperature on a plate shaker at 300 rpm for 1 hour. Plates were washed four times with PBS-T (200 μ l/well) and Streptavidin-POD (1:10,000 in 1% (w/v) BSA in PBS-T, 50 μ l/well) was added, plates were incubated for 30 minutes at room temperature on a plate shaker at 300 rpm. Finally, plates were washed five times with PBS-T (200 μ l/well), before the addition of ABTS (10 mg/ml in 1 ml ABTS 10x buffer and 9 ml ddH₂O, 50 μ l/well). Analysis with a Tecan Spark 20M Microplate Reader (absorption 405 nm, 1-ref 405 – 490 nm). 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 was used for ELISA:

	IL-2 ELISA	IFN- γ ELISA
Capture Antibody	1 μ g/ml mouse anti-human IL-2	1 μ g/ml mouse anti-human IFN- γ
Detection Antibody	1 μ g/ml biotin mouse anti-human IL-2	0.5 μ g/ml biotin mouse anti-human IFN- γ
Standard	1:900 of stock solution Proleukin® equals 2000 U/ml IL-2 in in 1% (w/v) BSA in PBS-T	1:10,000 of stock solution Imukin® 20 ng/ml IFN- γ in 1% (w/v) BSA in PBS-T

Table 16. Overview of components used for the detection of IL-2 and IFN- γ via ELISA

3.4.4 Repetitive killing (stress test) Assay

For reading repetitive killing (“stress test”), CAR T cells (1×10^5) were co-incubated with N87 tumour cells (1×10^5) in 12-well flat bottom plates in the presence and absence of TGF- β 1 (10 ng/ml). After 3 days cells were harvested and re-plated to a fresh plate with seeded tumour cells, this process was repeated for 3 rounds. During each harvest, cell culture supernatant was taken to analyse IFN- γ by ELISA and also a sample was taken for flow cytometry to determine CAR T cell and tumour cell count using “AccuCheck Counting Beads”. CAR T cells were identified with PE-conjugated F(ab')₂ anti-IgG1 antibody, that identifies the CAR, and BV421 conjugated anti-CD3 mAb. Tumour cells were identified by antigen-negative back gating. Cell viability was determined with eBioscience “Fixable Viability Dye eFluor 780”. Calculation for cell count; $\frac{\text{Number of cell counted}}{\text{Total number of beads counted}} \times \text{Number of AccuCheck Counting Beads per } \mu\text{l} = \text{Absolute Count (cells/}\mu\text{l)}$.

4 RESULTS

4.1 CD28 co-stimulatory domain provides resistance to TGF- β mediated suppression due to IL-2 mediated signalling.

4.1.1 The effect of TGF- β on T cell proliferation triggered by various 2nd generation CARs.

In this study we investigated the effect of TGF- β mediated suppression on various 2nd generation CAR T cells. CAR T cell amplification upon antigen stimulation was recorded in the presence and absence of TGF- β for 2nd generation CARs containing the co-stimulatory domain; CD28, CD28 Δ LCK, 4-1BB or OX40. All constructs also have the CD3 ζ domain for delivering the primary signal.

Human T cells were engineered with carcinoembryonic antigen (CEA)-specific CARs and activated upon engagement of immobilized cognate antigen, the BW2064/36 antibody, which is an anti-idiotypic antibody against the anti-CEA single-chain fragment variable region (scFv) of the CAR and serves as surrogate antigen for CEA. CAR T cells were labelled with a proliferation dye and amplification in the presence and absence of TGF- β was recorded after 5 days by flow cytometry measuring the dilution of dye. As shown in Figure 3, T cell amplification triggered by 4-1BB- ζ and OX40- ζ CAR was repressed in the presence of TGF- β whereas T cells with CD28- ζ CAR were not.

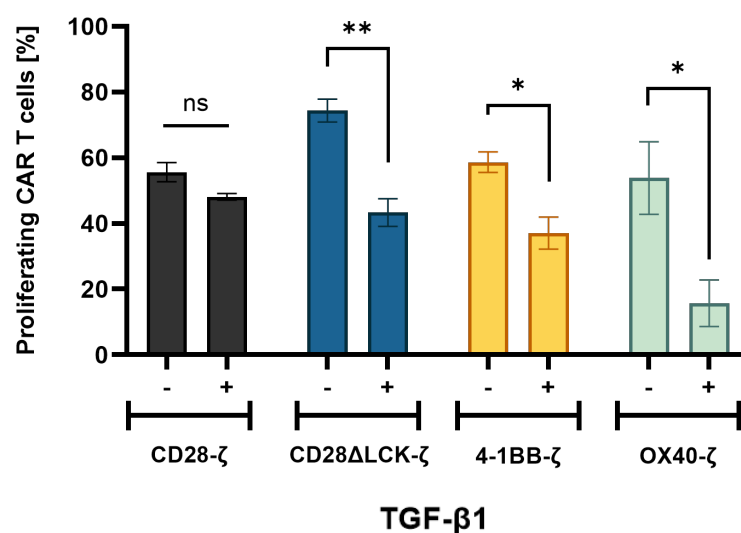


Figure 3. CD28 overcomes the inhibitory effect of TGF- β on T cell amplification whereas 4-1BB and OX40 do not.

Human T cells were engineered with CD28- ζ , CD28 Δ LCK- ζ , 4-1BB- ζ or OX40- ζ CAR and labelled with “Cell Proliferation Dye” eFluor 450. CAR T cells (1×10^5) were incubated for 5 days on a 96 well plate coated with the anti-idiotypic mAb BW2064/36 (1.5 μ g/ml) in the presence and absence of TGF- β 1 (10 ng/ml). CAR T cell amplification was recorded via flow cytometry measuring the dilution of the cell proliferation dye. CAR T cells were identified by staining with the PE-conjugated anti-IgG antibody which binds to the extracellular IgG₁ spacer domain of the CAR. Data represent the mean of triplicates $\pm$ SEM. The assay was repeated with three donor T cells. Statistical analyses were performed using a two-tailed t test (*p < 0.05; **p < 0.005, ns., not significant).

4.1.2 CD28- ζ CAR T cells secrete significantly higher amount of IL-2 in comparison to other CAR T cells.

To further investigate the mechanism of why CD28- ζ CAR T cells show resistance to TGF- β mediated suppression, we analysed the IL-2 release upon CAR stimulation. CAR T cells were activated through immobilized antigen, supernatant was collected after 2 days, the concentration of IL-2 released by CAR T cells was measured by ELISA.

CAR T cells stimulated by CD28- ζ CAR released substantial amounts of IL-2 whereas stimulation through 4-1BB- ζ and OX40- ζ CAR secrete significantly less. We assume that IL-2 is involved in mediating TGF- β resistance. Since IL-2 induction is mediated through LCK activation, we deleted the LCK binding site in the CD28 endodomain by substituting proline P187 and P190 by alanine, generating the CD28 Δ LCK- ζ CAR. The abolished mutation of the LCK binding site resulting in significant reduction of IL-2 production upon CAR stimulation (Fig. 4). CD28 Δ LCK- ζ CAR T cells are now susceptible to TGF- β mediated suppression whereas non-modified are not (Fig. 3).

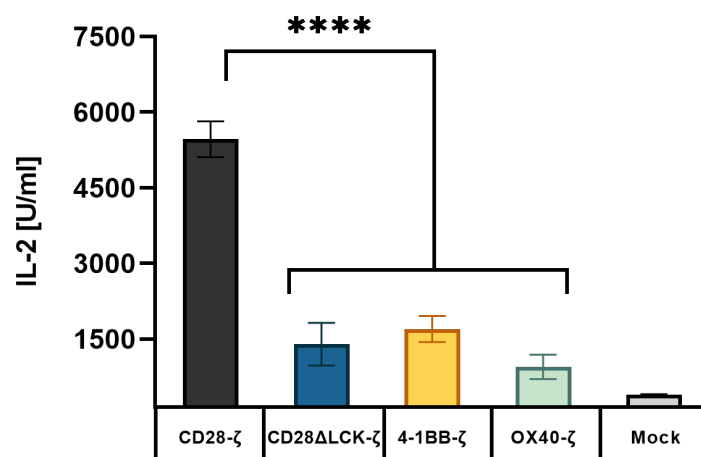


Figure 4. CD28- ζ CAR T cells secrete higher amounts of IL-2 upon stimulation.

CAR T cells with different co-stimulatory domains were incubated on 96 well plates (2.5×10^4 CAR T cell per well) coated with anti-idiotypic mAb BW2064/36 (1.5 μ g/ml) for 2 days. Mock transduced T cells were taken as a control. Secreted IL-2 concentration was recorded in the culture supernatants via ELISA. The assay was repeated with three donor T cells, data represents the mean of triplicates $\pm$ SEM. Statistical analysis was carried out using one-way ANOVA test. (****p < 0.0001).

4.2 Converting TGF- β binding into a stimulatory IL-2 receptor signal

4.2.1 The generation of Switch Receptor CAR T cells

In the context of solid tumours, high amounts of IL-2 can stimulate Regulatory T cells (Tregs) in the tumour tissue. Tregs as well as TGF- β contribute to the suppression of the therapeutic efficacy of CAR T cells. Therefore, we introduced intracellular IL-2 signalling into the 4-1BB- ζ CAR T cell to provide TGF- β resistance and avoid Treg stimulation by the release of IL-2. To do this, we designed a composite “switch receptor” (SWR) that comprises the extracellular domain of TGF- β RII from 1 to 159 amino acid residue, which is responsible for binding all three isoforms of TGF- β (TGF- β 1, TGF- β 2 & TGF- β 3). Anchored on the cell membrane with PDGFR β TM and intracellularly the IL-2R β chain (Fig. 5A). This receptor is aimed at converting the repressive signal that is physiologically provided upon TGF- β binding, into a positive stimulatory signal mediated by the IL-2R β chain mimicking the presence of IL-2 upon TGF- β binding (Fig. 5B).

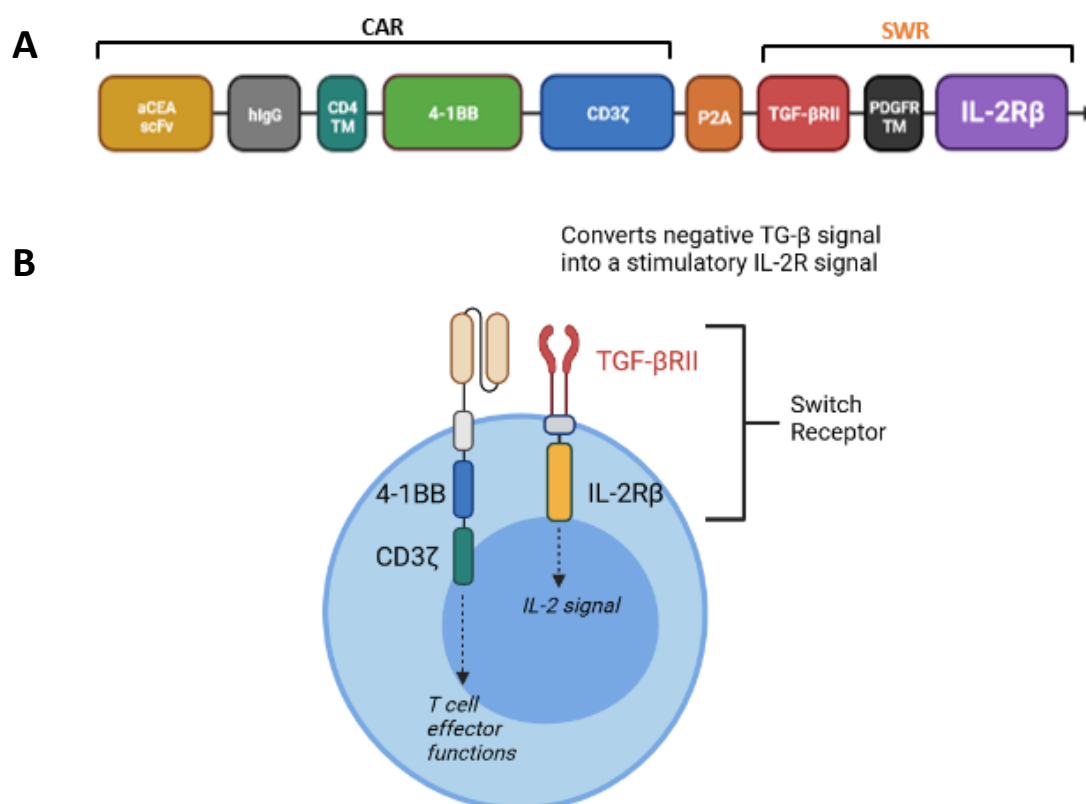


Figure 5. Schematic Illustration and function of switch receptor CAR T cells

(A) Illustration of gene structure for 4-1BB- ζ -SWR CAR T cells. **(B)** Graphical diagram to show human CD3⁺ T cells equipped with 4-1BB- ζ -SWR CAR T cells with intended function.

4.2.2 4-1BB- ζ -SWR CAR is expressed on human CD3⁺ T cells

Human T cells were retrovirally transduced with the 4-1BB- ζ CAR along with the SWR via spinfection. T cells were also transduced with the 4-1BB- ζ CAR without SWR for comparative analysis. Expression was detected via flow cytometry. Fig. 6A shows expression of the CAR on human CD3⁺ T cells via staining CAR hinge domain. For detection of the co-expressed switch receptor, an antibody against TGF- β RII was used to identify the SWR; the CAR was recorded by staining the hinge domain. Double positive cells indicate co-expression of CAR and SWR. Fig. 6B shows a higher expression of TGF- β RII on 4-1BB- ζ -SWR CAR T cells, verifying that both the CAR and Switch Receptor is present on the T cell.

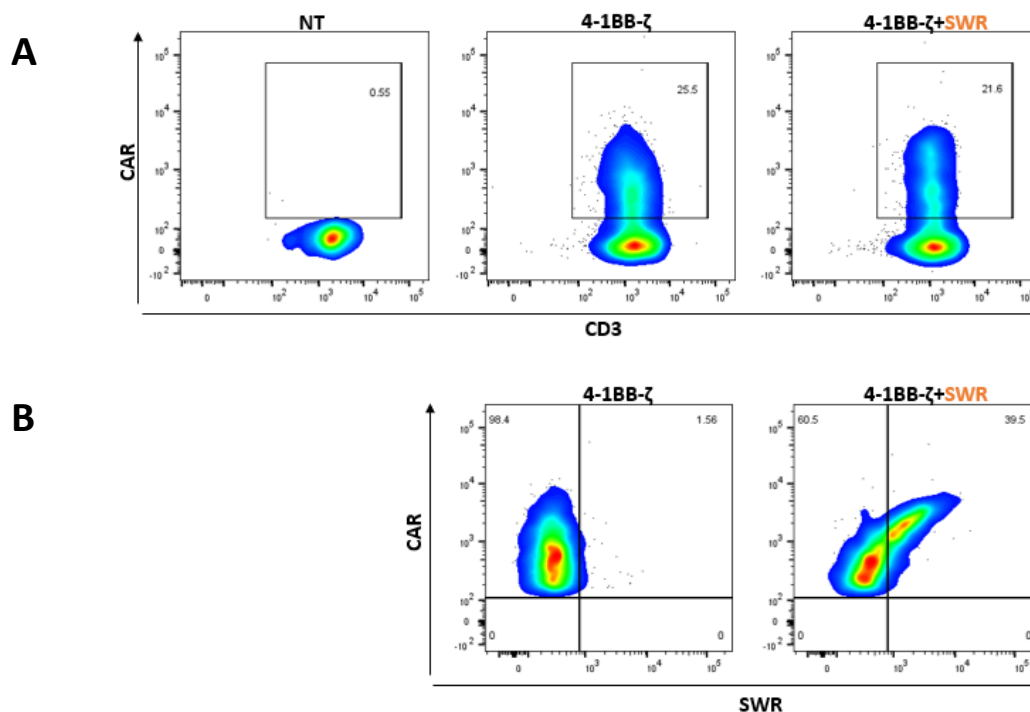


Figure 6. Expression of CARs on human CD3⁺ T cells

(A) 4-1BB- ζ and 4-1BB- ζ -SWR CAR was expressed on human CD3⁺ T cell surface. Human T cells were retrovirally transduced with respective CAR by spinfection. CAR expression was detected by flow cytometry using the PE-conjugated anti hlgG antibody and for the T cells the BV421-conjugated anti CD3 antibody was used. The percentage indicates the total number of CAR⁺ T cells. **(B)** SWR expression after gating on 4-1BB- ζ CAR T cells. SWR was detected by staining for TGF- β RII using APC-conjugated anti-human TGF- β RII antibody.

4.2.3 CAR T cells maintain their memory phenotype after engineering with the SWR

After the CAR T cell manufacturing process, the effector/memory phenotype of the cells was monitored. This was to investigate whether or not the addition of the intracellular IL-2R β domain affected a predetermined T cell state. To do so, T cells were pre-divided into CD4/CD8 populations via flow cytometry, percentage of CARs expressed on CD4⁺ and CD8⁺ T cells is shown in Figure 7A. Figure 7B shows the percentage of T cell subsets represented in a pie chart for each CAR. To identify memory T cell subsets, the combination of CD62L and CD45^{RO} were used. The percentage of T naïve / stem cell memory cells within the CD8 population appears to be slightly higher in CAR T cells equipped with the switch receptor

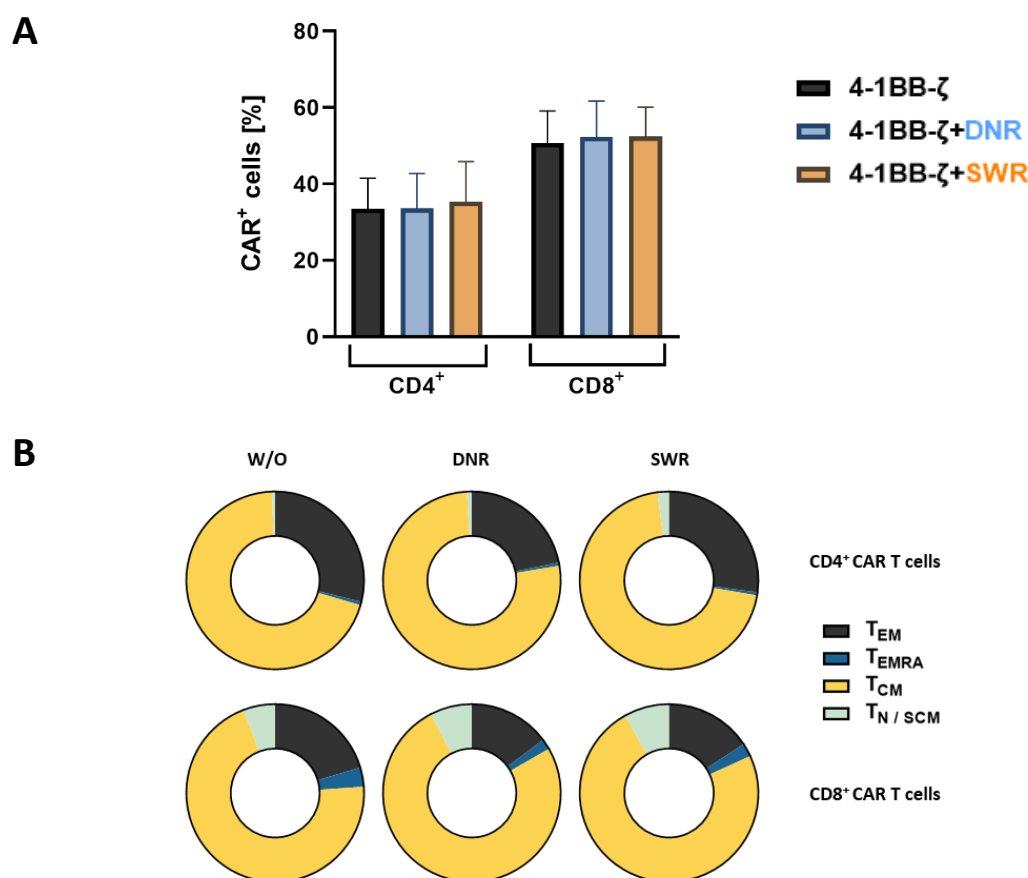


Figure 7. Central memory T cell subset after CAR T cell manufacturing

(A) Percentage of CAR⁺ CD4/CD8 T cells. **(B)** Distribution of central memory phenotype populations after CAR T cell manufacturing. The upper line represents phenotypes within the CD4⁺ population of CAR T cells, the lower line represents the phenotypes within the CD8⁺ population of CAR T cells. W/O stands for 4-1BB- ζ CAR T cells without additional receptor, DNR stands for 4-1BB- ζ CAR T cells equipped with dominant negative receptor, and SWR are 4-1BB- ζ CAR T cells with the switch receptor. (T_{EM} = T effector memory, T_{EMRA} = terminally differentiated effector memory cells, T_{CM} = T central memory and T_N / T_{SCM} = T Naïve, T stem cell central memory) The experiment was repeated three times, data represents the mean $\pm$ SEM.

4.2.4 4-1BB- ζ -SWR CAR T cells overcome TGF- β mediated repression in T cell amplification

We addressed if the switch receptor can rescue CAR T cell amplification upon antigen engagement of cognate target in the presence of TGF- β . T cells were engineered with the 4-1BB- ζ CAR with or without the switch receptor, labelled with a cell trace proliferation dye and activated by cognate antigen in the presence or absence of TGF- β for 5 days. Switch receptor CAR T cells showed resistance against TGF- β mediated suppression at concentrations up to 100 ng/ml, as shown in Fig. 8A. Whereas as expected, 4-1BB- ζ CAR T cells amplification is suppressed in the presence of TGF- β . Figure 8B shows the dilution of dye for after gating on CAR⁺ T cells, successful labelling of the cells is verified by observing no dilution in the unstimulated control.

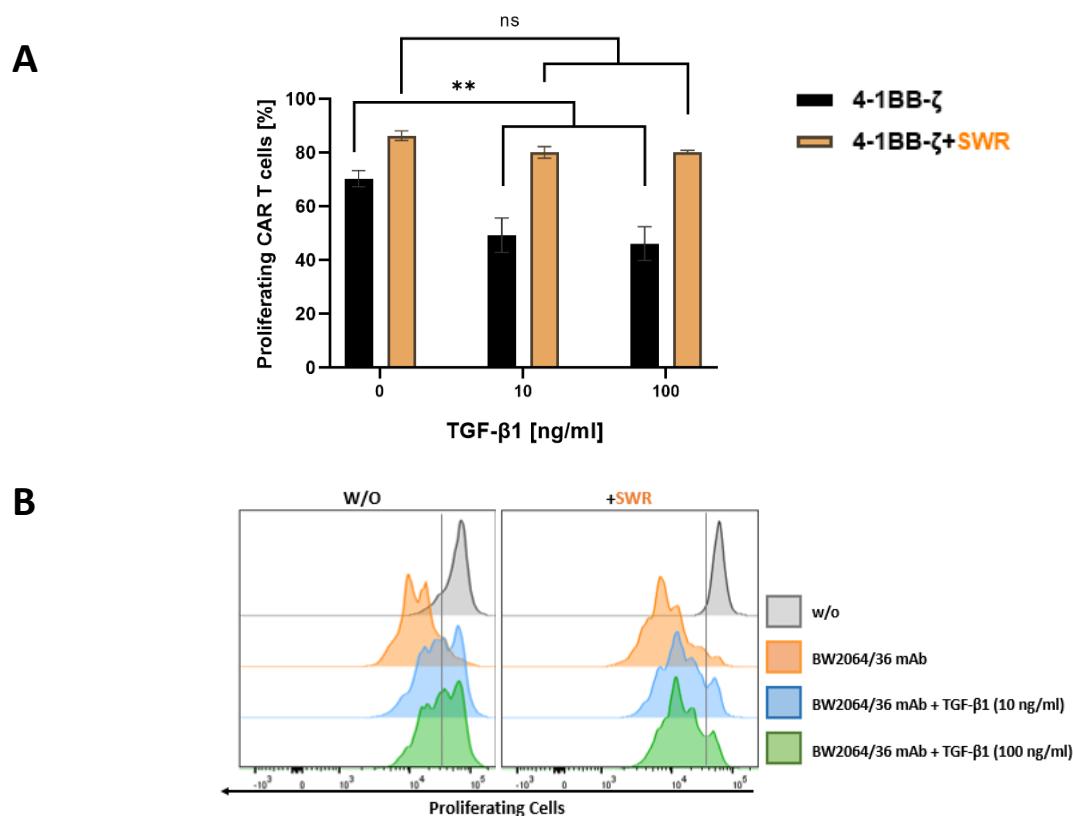


Figure 8. Switch receptor rescues CAR T cell amplification in the presence of TGF- β

(A) Human T cells were engineered to express 4-1BB- ζ CAR with and without switch receptor (W/O) and labelled with Cell Proliferation Dye eFluor 450 (Invitrogen). CAR T cells (1×10^5) were incubated on a 96-well plate coated with anti-idiotypic mAb BW2064/36 (1.5 μ g/ml) in the presence and absence of TGF- β 1 (10 or 100 ng/ml) for 5 days. Proliferation was recorded by flow cytometry, PE-conjugated anti-IgG antibody was used to detect CARs and dilution of the dye measured CAR T cell proliferation. **(B)** Representative histogram from 3 donors of CAR⁺ dye dilution under different stimulatory conditions. Data represent the mean of triplicates $\pm$ SEM. The assay was repeated three times. Statistical analyses were performed using a two-way ANOVA test (** $p < 0.005$; ns., not significant).

4.2.5 Switch Receptor CAR T cells display better cytolytic and pro-inflammatory properties in the presence of TGF- β

We investigated the suppression on cytolytic capacities in regard to perforin and CD107a, which serves as a marker for T cell granulation. To do so, T cells engineered with 4-1BB- ζ CAR with and without switch receptor were activated through immobilized antigen BW2064/26 antibody for 24 hours in the presence and absence of TGF- β . After 24 hours, CAR T cells were harvested and stained for respected cytolytic markers and analysed by flow cytometry.

High concentrations of TGF- β decreased the perforin levels for 4-1BB- ζ CAR T cells. Whereas CAR T cells equipped with the switch receptor maintained their perforin levels as shown in Figure 9A and 9B. T cell degranulation after activation by cognate antigen in the presence and absence of TGF- β was recorded by staining for CD107a, 4-1BB- ζ CAR T cells exhibited no suppression in degranulation as a result of TGF- β (Fig. 9C,D) However, CAR T cells with the switch receptor showed a higher staining for CD107a, indicating superior cytolytic capacities compared to CARs without the switch receptor.

IFN- γ is a pro-inflammatory cytokine and a result of downstream activation of the signalling pathways. Therefore, we monitored the secretion of IFN- γ in the supernatant after CAR T cells with and without switch receptor that were incubated with CEA antigen positive N87 tumour cells in the presence and absence of TGF- β , non-modified T cells were used as a control. After 3 days, cell culture supernatant was collected and IFN- γ was detected via ELISA. CAR T cells equipped with a switch receptor secrete higher amounts of IFN- γ upon antigen engagement via tumour cell interaction in the presence of TGF- β as opposed to CAR T cells without switch receptor (Fig. 9E).

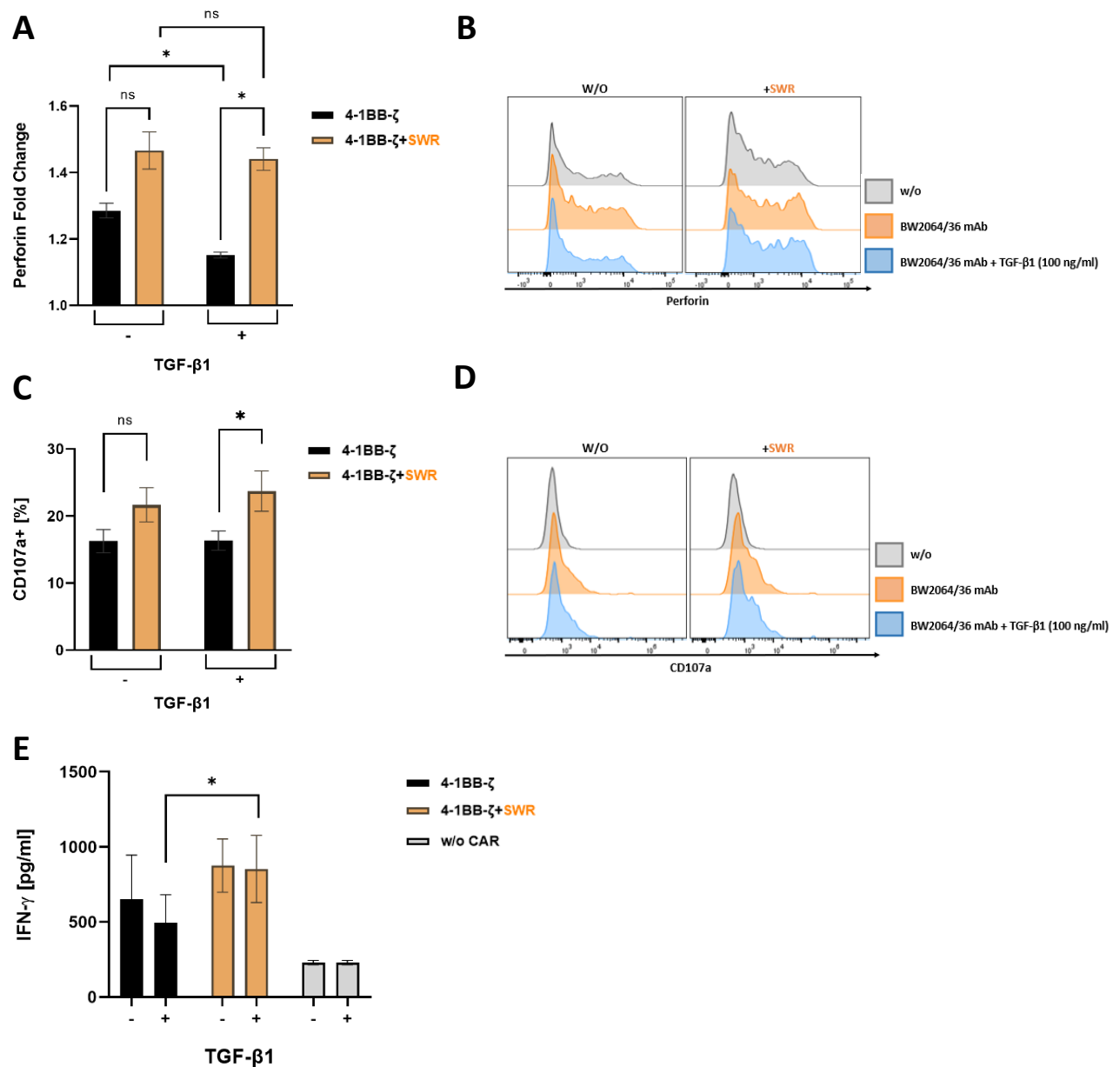


Figure 9. Switch Receptor CAR T cells display better cytolytic properties in the presence of TGF-β

4-1BB-ζ CAR T cells with and without switch receptor (5×10^4 cells) were incubated in the presence and absence of TGF-β1 (100 ng/ml) on a 96-well plate coated with anti-idiotypic mAb BW2064/36 (1.5 μg/ml) for 24 hours. CAR T cells were detected by staining with the PE-conjugated anti-IgG antibody. **(A)** Perforin MFI Fold change of CAR T cells, perforin was detected using APC-conjugated anti-perforin antibody. **(B)** Representative histogram from 3 donors showing perforin expression in CAR T cells. **(C)** CD107a expression of activated CAR T cells, CD107a was detected by a VioBlue-conjugated anti-CD107a (LAMP-1) antibody. **(D)** Representative histogram from 3 donors showing CD107a expression on CAR T cells. **(E)** 4-1BB-ζ CAR T cells with and without switch receptor (2×10^4 cells) were incubated in a 1:1 ratio (E:T) with CEA⁺ N87 tumour cells in the presence and absence of TGF-β1. Supernatant was collected after 3 days and IFNγ was detected by ELISA. Data represents the mean of triplicates ± SEM. Statistical analyses were performed using an unpaired t test * $p < 0.05$; ns., not significant).

4.2.6 Switch Receptor CAR T cells induce phosphorylated STAT5 through IL-2R β chain

To further understand the mechanism of the SWR we explored the downstream signalling of IL-2R β chain. Literature states that IL-2 signalling induces the JAK-STAT pathway, more specifically IL-2R β phosphorylates STAT5 (Mahmud et al., 2013). 4-1BB- ζ CAR T cells with and without SWR were stimulated through immobilized anti-CEA antigen (BW2064/36) in the presence and absence of TGF- β for 3 days. SWR CAR T cells display a greater shift in phosphorylated STAT5 when activated through immobilized antigen, this shift is greater when TGF- β is added. In conclusion, the IL-2R β chain in the switch receptor upregulated pSTAT5 upon antigen engagement and is elevated when TGF- β is present (Fig. 10).

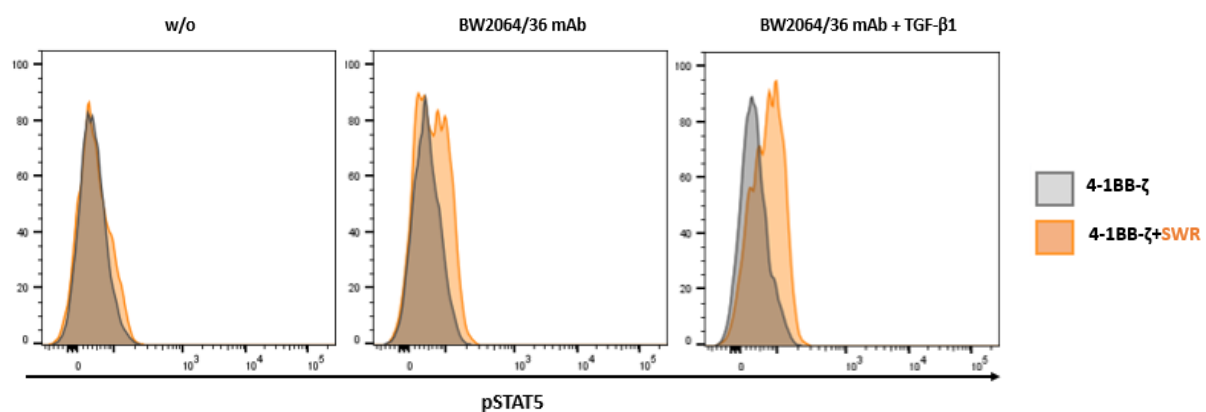


Figure 10. SWR CAR T cells show increased levels of pSTAT5

4-1BB- ζ CAR T cells with and without switch receptor (1×10^5) were incubated on a 96-well plate coated with anti-idiotypic mAb BW2064/36 (1.5 μ g/ml) in the presence and absence of TGF- β 1 (10 ng/ml). After 3 days cells were harvested and stained with BV421-conjugated anti-human pSTAT5 antibody. CAR T cells were detected by staining with the PE-conjugated anti-IgG antibody. The histogram demonstrates pSTAT5 after gating on CAR⁺ T cells. This assay was repeated with three donors, the data shows a representative assay.

4.3 Utilizing the Switch Receptor to convert resistance to other CARs sensitive to TGF- β

4.3.1 *Switch Receptor rescues T cell amplification for IL-2 deficient CD28 CAR in the presence of TGF- β*

CD28 Δ LCK- ζ CAR T cells also suffer from TGF- β mediated suppression due to the mutation within the CD28 domain which reduces the production of IL-2, as shown in Figure 3 & 4. Therefore, we wanted to test the universal capacities of the switch receptor to investigate whether co-expressing the switch receptor with CD28 Δ LCK- ζ CAR T cells would rescue the amplification capacity of these CAR T cells.

First, the generation and validation of CD28 Δ LCK- ζ -SWR CAR T cells was done by retroviral transduction and expression of CAR T cells was verified by flow cytometry. Figure 11B shows expression of CD28 Δ LCK- ζ and CD28 Δ LCK- ζ -SWR CARs on human CD3⁺ T cells. CAR detection was done via antibody staining against the hinge region of the CAR, for the detection of the co-expression switch receptor, the TGF- β RII antibody was used. Fig 11C shows an expected higher expression of TGF- β RII for the CD28 Δ LCK- ζ -SWR CAR T cells.

T cells engineered with CD28 Δ LCK- ζ CAR with and without switch receptor were labelled with a proliferation dye and activated by cognate antigen in the presence and absence of TGF- β for 5 days. After 5 days the proliferation of CAR T cells was analysed by flow cytometry, recording the dilution of dye. While the proliferation of CD28 Δ LCK- ζ CAR T cells was reduced in the presence of TGF- β , we concluded the addition of the switch receptor to CD28 Δ LCK- ζ CAR T cells rescues the proliferative capacity even after the reduction of IL-2 production due to selective mutation (Fig.11 D,E). Furthermore, CD28 Δ LCK- ζ CAR equipped with a switch receptor hold an advantaged therapeutic effect towards solid tumours due to TGF- β resistance and less potential to stimulate nearby Tregs because of low IL-2 production.

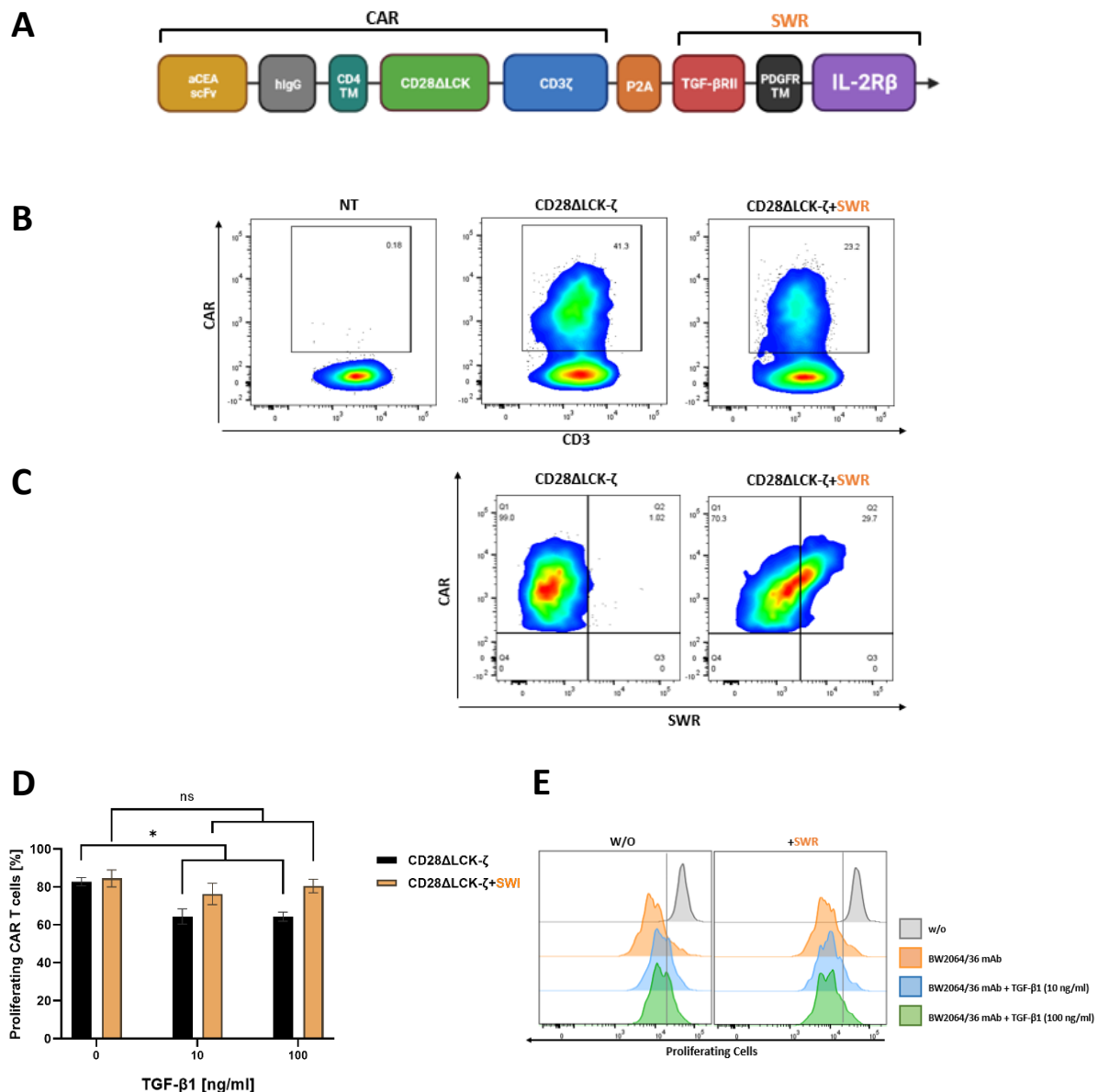


Figure 11. Switch Receptor rescues proliferation of IL-2 deficient CD28ΔLCK CAR

(A) Illustration of gene structure for CD28ΔLCK-ζ-SWR CAR T cells. Expression of CD28ΔLCK-ζ and CD28ΔLCK-ζ-SWR CARs on human CD3⁺ T cells. **(B)** Human T cells were retrovirally transduced with respective CAR by spinfection. CAR expression was detected by flow cytometry using the PE-conjugated anti-hlgG antibody and for the T cells the BV421-conjugated anti CD3 antibody was used. The percentage indicates the total number of CAR⁺ T cells. **(C)** Expression of switch receptor on CAR⁺ T cells. SWR was detected using APC-conjugated anti-human TGF-βRII antibody. T cells engineered with CD28ΔLCK-ζ CAR with and without switch receptor (1×10^5) were labelled with Cell Proliferation Dye eFluor 450 (Invitrogen) and incubated on a 96-well plate coated with anti-idiotypic mAb BW2064/36 (1.5 μg/ml) in the presence and absence of TGF-β1 (10 & 100 ng/ml) for 5 days. **(D)** Percentage of CAR T cell proliferation. **(E)** Representative histogram from 3 donors of CAR⁺ dye dilution under different stimulatory conditions. Data represent the mean of triplicates $\pm$ SEM. The assay was repeated with three T cell donors. Statistical analyses were performed using a two-way ANOVA test (*p<0.05; ns., not significant).

4.3.2 OX40- ζ CAR T cells benefit from the switch receptor under high concentrations of TGF- β

TGF- β mediated suppression also effects OX40- ζ CAR T cells (Fig. 3), dampening their amplification. To further explore the universal capacity of the switch receptor, we engineered the OX40- ζ -SWR CAR and tested to see if co-expressing the switch receptor could rescue T cell amplification.

Human T cells were engineered to express OX40- ζ or OX40- ζ -SWR CARs. Expression was verified using flow cytometry (Fig. 12A). To detect the switch receptor, an antibody against TGF- β RII was used, expression of the switch receptor was confirmed after gating on CAR⁺ T cells (Fig 12B). OX40- ζ or OX40- ζ -SWR CAR T cells were labelled with a cell proliferation dye and activated in the presence and absence of TGF- β . By introducing the switch receptor into OX40- ζ CARs, T cell amplification is increased in the presence of TGF- β . We observed no significant suppression under high concentration of TGF- β (Fig. 12C). Therefore, demonstrating the rescue effect of applying the switch receptor to CAR T cells suffering from TGF- β mediated suppression.

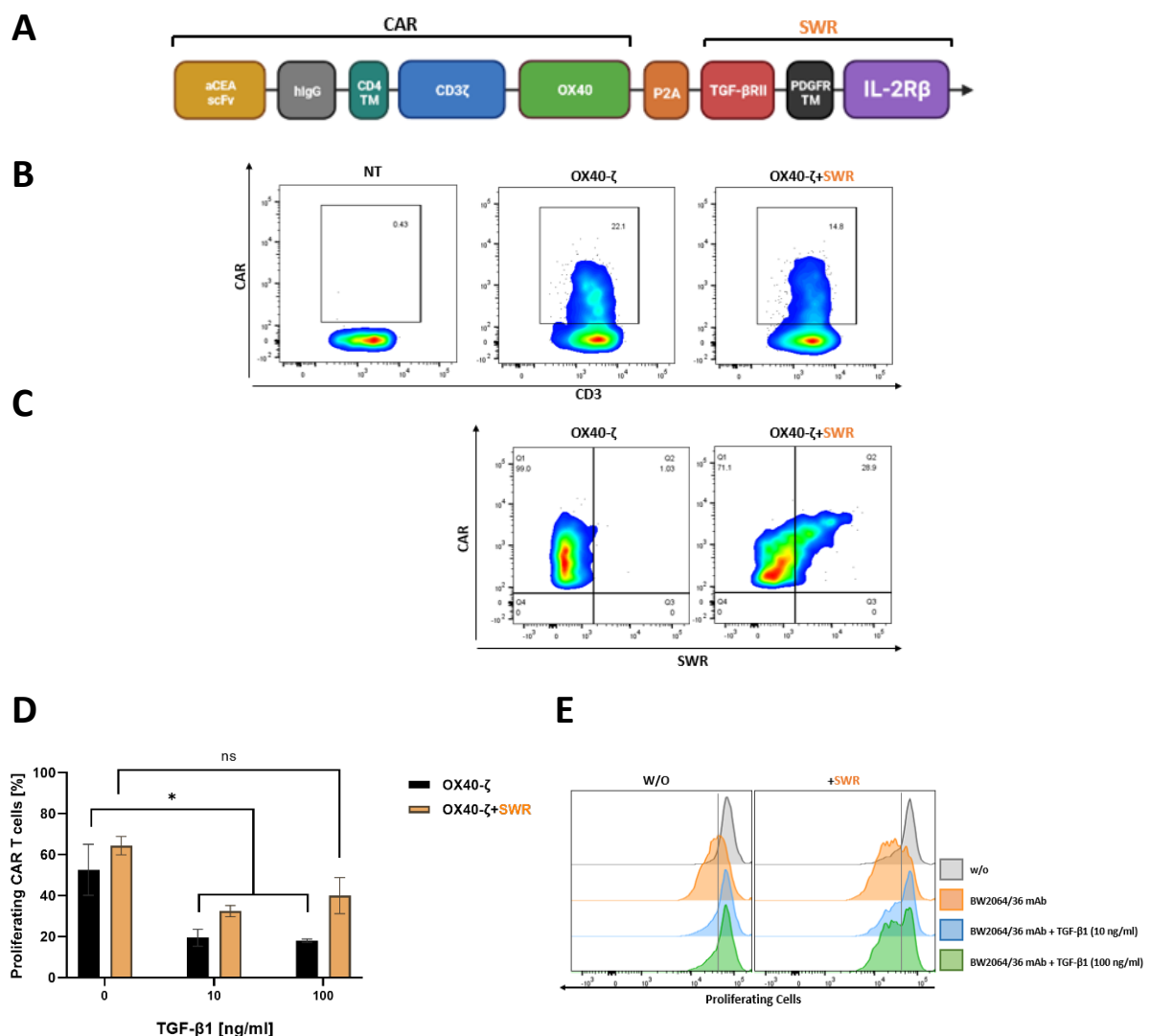


Figure 12. OX40- ζ CAR T cells benefit from switch receptor signalling under high concentrations of TGF- β

(A) Illustration of gene structure for OX40- ζ -SWR CAR T cells. Expression of OX40- ζ and OX40- ζ -SWR CARs on human CD3⁺ T cells. **(B)** Human T cells were engineered with respective CAR by spinfection. CAR expression was detected by flow cytometry using the PE-conjugated anti-IgG antibody and for the T cells the BV421-conjugated anti CD3 antibody was used. The percentage indicates the total number of CAR⁺ T cells. **(C)** Expression of switch receptor on CAR⁺ T cells. SWR was detected using APC-conjugated anti TGF- β RII antibody. T cells engineered with OX40- ζ CAR with and without switch receptor (1×10^5) were labelled with Cell Proliferation Dye eFluor 450 (Invitrogen) and incubated on a 96-well plate coated with anti-idiotypic mAb BW2064/36 (1.5 μ g/ml) in the presence and absence of TGF- β 1 (10 & 100 ng/ml) for 5 days. **(D)** Percentage of CAR T cell proliferation. **(E)** Representative histogram from 3 donors of CAR⁺ dye dilution under different stimulatory conditions. Data represent the mean of triplicates $\pm$ SEM. The assay was repeated three times. Statistical analyses were performed using a two-way ANOVA test (* $p < 0.05$; ns., not significant).

4.4 Switch Receptor shows superiority compared to state-of-the-art Dominant Negative Receptor

4.4.1 *Switch Receptor out competes Dominant Negative Receptor by maintaining amplification properties under high concentration of TGF- β*

The dominant negative receptor (DNR) is very successful in converting resistance to TGF- β . Therefore, as a state-of-the-art technique we wanted to further test the SWR and compare whether the additional signal provided by the SWR has a better effect than a decoy provided by the DNR.

Human T cells were engineered to express either 4-1BB- ζ -SWR or 4-1BB- ζ -DNR CAR. CAR expression on T cell surface was verified using flow cytometry. Both the co-expressed Switch Receptor and Dominant Negative Receptor was done by staining for TGF- β RII and checking for expression on CD3⁺ CAR⁺ T cells (Fig. 13B).

4-1BB- ζ -SWR and 4-1BB- ζ -DNR CAR T cells were labelled with a cell trace proliferation dye and activated via cognate plate bound coated antigen in the presence and absence of TGF- β 1 at concentrations of 10 & 100 ng/ml. As expected, the DNR showed resistance to mediated suppression and the proliferative capacity was not affected. The SWR also demonstrated resistance to TGF- β , however, the proliferative capability was substantially higher, especially in high concentrations of TGF- β (Fig. 13C).

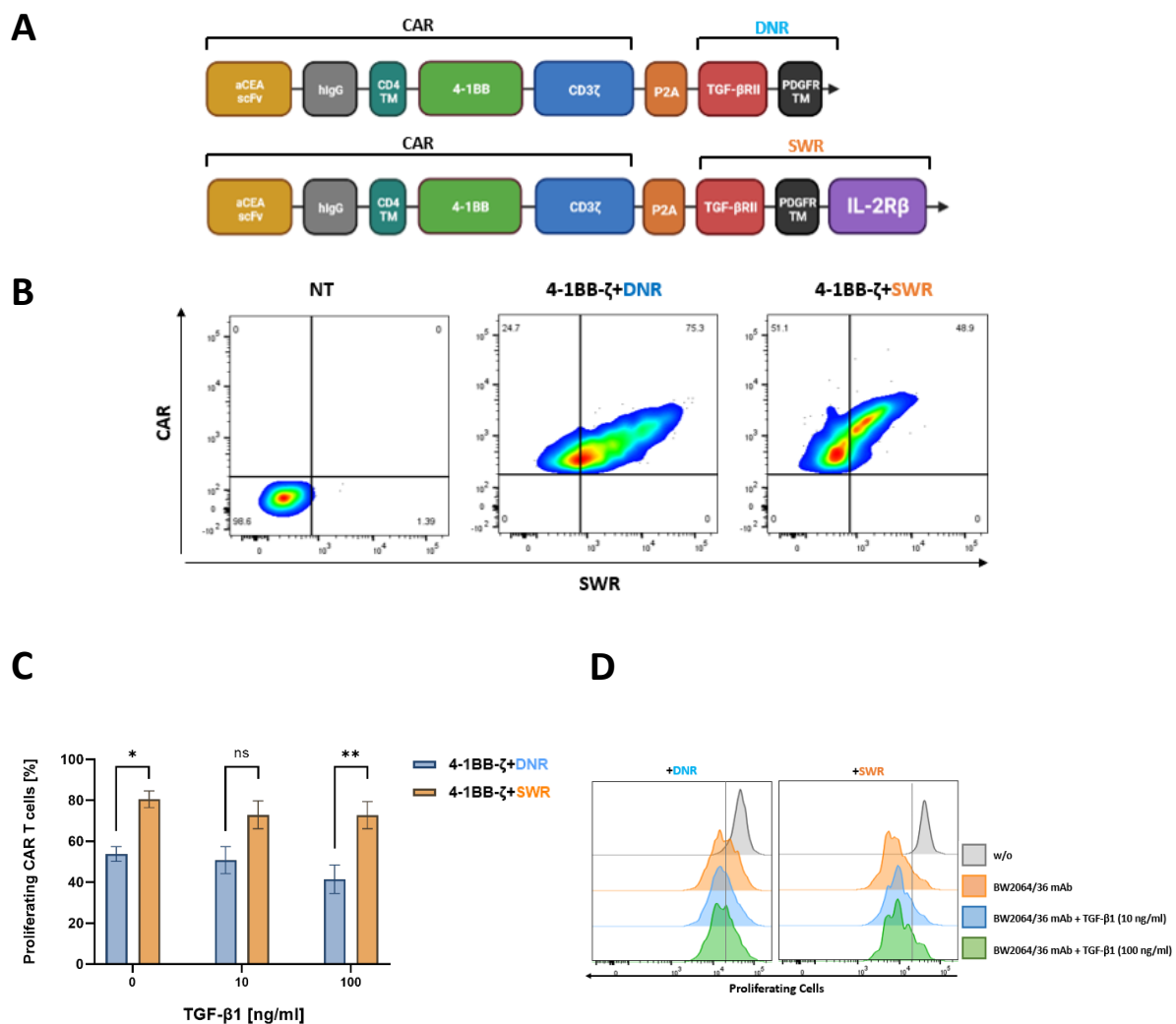


Figure 13. SWR CAR T cells maintain better proliferative capabilities under high concentrations of TGF- β

(A) Illustration of gene structure for 4-1BB- ζ -DNR & 4-1BB- ζ -SWR CAR T cells Expression of 4-1BB- ζ -SWR or 4-1BB- ζ -DNR CARs on human CD3⁺ T cells after retroviral transduction by spinfection. **(B)** CAR expression was detected by flow cytometry using the PE-conjugated anti-hlgG antibody and for the T cells the BV421-conjugated anti CD3 antibody was used. Expression of SWR & DNR on CAR⁺ T cells. Co-expressed receptors were detected using APC-conjugated anti TGF- β RII antibody. 4-1BB- ζ -SWR or 4-1BB- ζ -DNR CARs (1×10^5) were labelled with Cell Proliferation Dye eFluor 450 (Invitrogen) and incubated on a 96-well plate coated with anti-idiotypic mAb BW2064/36 (1.5 μ g/ml) in the presence and absence of TGF- β 1 (10 & 100 ng/ml) for 5 days. **(C)** Percentage of CAR T cell proliferation. **(D)** Representative histogram from 3 donors of CAR⁺ T cell dye dilution under different stimulatory conditions. Data represent the mean of triplicates $\pm$ SEM. The assay was repeated with three T cell donors. Statistical analyses were performed using a two-way ANOVA test (* $p < 0.05$, ** $p < 0.005$; ns., not significant).

4.4.2 Demonstration of STAT5 phosphorylation in SWR CAR T cells in comparison to DNR CAR T cells

To further highlight the functional difference between a decoy mechanism and providing an additional signal, we demonstrated the upregulation of pSTAT5 as a consequence of IL-2R β signalling in switch receptor CAR T cells. T cells were engineered with a 4-1BB- ζ CAR co-expressing either the DNR or SWR. Cells were activated by cognate antigen through the BW2064/36 antibody in the presence and absence of TGF- β for 3 days. CAR T cells co-expressing the switch receptor demonstrate a higher shift in pSTAT5 upon antigen recognition, this shift is further enhanced in the presence of TGF- β . As expected, CAR T cells co-expressing the DNR exhibit no shift in pSTAT5. (Fig. 14).

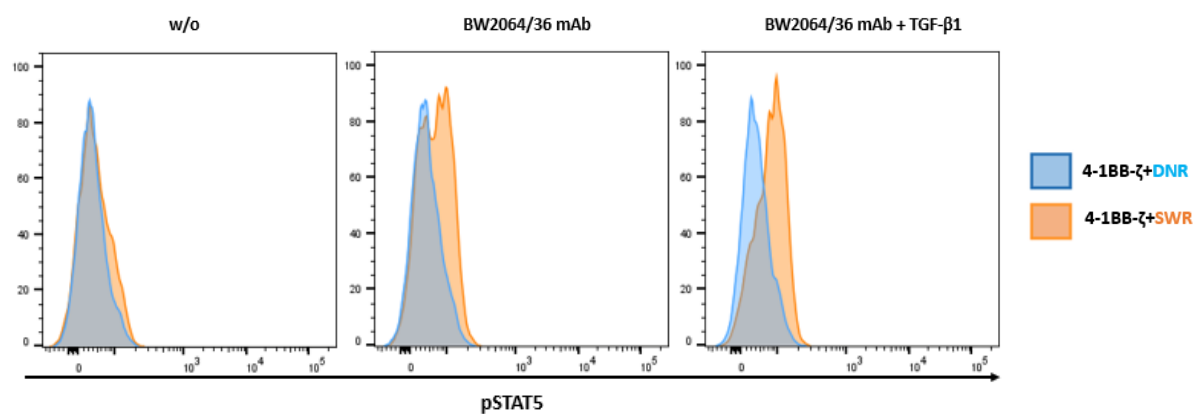


Figure 14. SWR CAR T cells show increased levels of pSTAT5

4-1BB- ζ CAR T cells co-expressing either the DNR or SWR (1×10^5) were incubated on a 96-well plate coated with anti-idiotypic mAb BW2064/36 (1.5 μ g/ml) in the presence and absence of TGF- β 1 (10 ng/ml). After 3 days cells were harvested and stained with BV421-conjugated anti pSTAT5 antibody. CAR T cells were detected by staining with the PE-conjugated anti-IgG antibody. The histogram demonstrates pSTAT5 after gating on CAR $^+$ T cells. This assay was repeated with three T cell donors, the data shows a representative assay.

4.4.3 Switch Receptor CAR T cells show better TGF- β resistance in tumour cell elimination and proliferation under repetitive stimulatory conditions

To further test the advantages of providing an additional signal when converting resistance to TGF- β , we addressed whether under “stress conditions” of repetitive stimulation, can CAR T cells with the SWR maintain their cytotoxic and proliferative capabilities in the presence of TGF- β . Therefore, CAR T cells were incubated with CEA⁺ N87 cells in the presence or absence of TGF- β . After 3 days, cells were re-plated onto freshly seeded N87 cells. This process was repeated for 3 rounds and during each harvest both CAR T cells and tumour cells were counted (Fig. 15B,C).

When CAR T cells were repetitively challenged with N87 tumour cells in the presence of TGF- β , the number of SWR CAR T cells increased in rounds 2 and 3 compared to CAR T cells with and without the DNR (Fig. 15B). Both SWR and DNR CAR T cells effectively eliminated tumour cells repetitively, however, in rounds 2 and 3 SWR CAR T cells were more efficacious (Fig. 15C). No difference in CAR T cell count or tumour cell elimination was observed when no TGF- β was added. Therefore, we concluded the 4-1BB- ζ CAR T cells co-expressing the SWR have a better proliferative and cytotoxic capacity compared to CAR T cells with the DNR in the presence of TGF- β when re-challenged with tumour cells. The additional signalling by the SWR provides more of a positive stimulatory effect, proving to be more advantageous than a decoy effect, enabling for a better CAR T cell response in the presence of TGF- β .

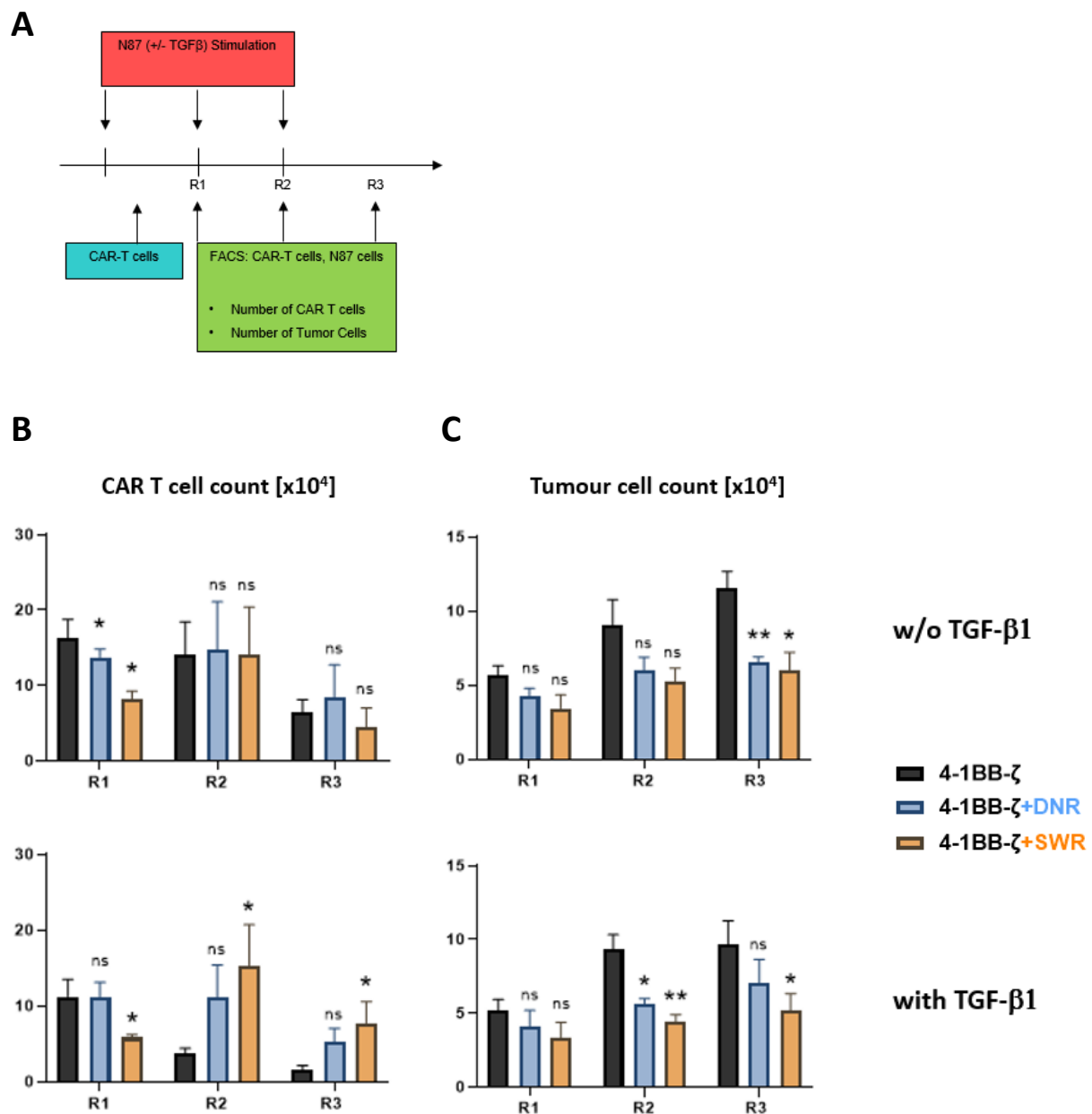


Figure 15. SWR CAR T cells display better resistance to TGF- β during repetitive rounds of stimulation

(A) Experimental overview of tumour re-challenging assay. **(B)** CAR T cell count after each round of tumour re-challenging. CAR T cells (1×10^5) were incubated with CEA⁺ N87 cells (1×10^5) in the presence or absence of TGF- β 1 (10ng/ml). After 3 days all cells were harvested and added to freshly seeded N87 cells (1×10^5). The process was repeated for 3 rounds. During each harvest, cell counts determined via flow cytometry using AccuCheck Counting Beads (ThermoFisher). Living CAR T cells were identified using PE-conjugated anti-IgG antibody, BV421-conjugated anti-human CD3 and Fixable Viability Dye eFluor 780 (ThermoFisher). **(C)** Tumour cells count after each round of re-challenging. N87 cell counts determined via flow cytometry using AccuCheck Counting Beads, Fixable Viability Dye eFluor 780 and antigen negative back gating. Data represent the mean of triplicates $\pm$ SEM. The assay was repeated 3 times. Statistical analyses were performed using an unpaired t test (*p<0.05; **p<0.005 ns., not significant).

4.5 Engineering N87 tumour cells to express TGF- β

4.5.1 Transduction of TGF- β 1 expression vector on N87 tumour cells

With the goal to test switch receptor CAR T cells *in vivo* we engineered the N87 cell line with a human TGF- β 1 expression vector to allow for constitutive release of high amounts of TGF- β .

N87 Tumour cells were lentiviral transduced with a commercial human TGF- β 1-mCherry expression vector (Fig. 16A). Expression was verified by flow cytometry through the constitutively fluorescent protein mCherry in the vector (Fig. 16B).

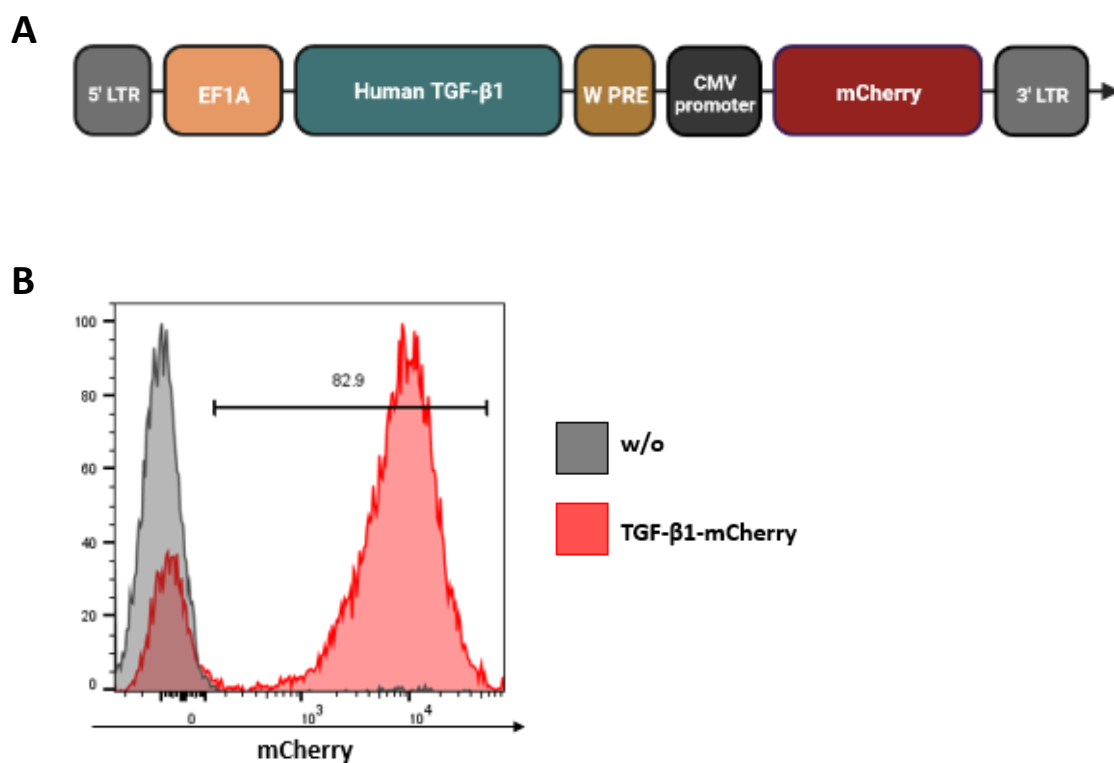


Figure 16. Transduction of N87 tumour cells with human TGF- β 1 expression vector

(A) Schematic illustration of domain structure for the hTGF- β 1-mCherry expression vector. **(B)** Flow cytometry verification of the expression of hTGF- β 1-mCherry on N87 tumour cells. Non-transduced tumour cells represented in grey (w/o) and Tumour cells transduced with respective vector in red.

4.5.2 Switch receptor CAR T cells show superior activity against N87-TGF- β^+ cells

After successful transduction of tumour cells, we wanted to test the cytotoxic capacity of CAR T cells after co-culture. To do so, 4-1BB- ζ CAR T cells with and without the DNR or SWR were incubated with CEA⁺ N87-TGF- β^+ cells for 3 days. Cells were harvested after 3 days and analysed by flow cytometry.

The number of tumour cells was significantly lower when incubating with CAR T cells co-expressing the switch receptor, thus indicating that CAR T cells equipped with a SWR are more efficacious when challenged by antigen positive tumour cells with high TGF- β expression compared to tumour cells with low or no TGF- β (Fig. 17). No difference in tumour cell count was seen between 4-1BB- ζ CARs with and without the dominant negative receptor. Moving forward, we would see this cell line as a candidate to further test the switch receptors capacity to overcome TGF- β mediated suppression in a growing tumour *in vivo*.

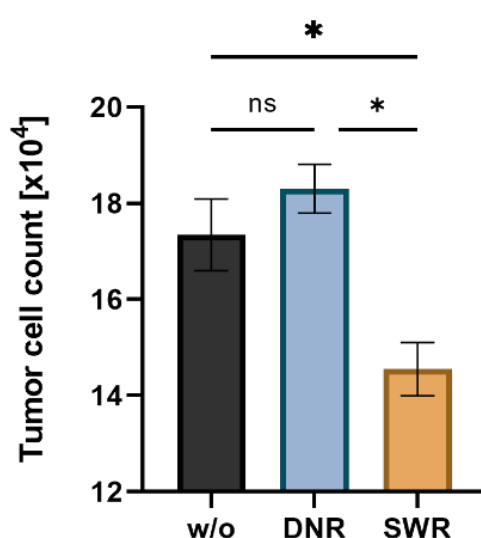


Figure 17. Switch receptor CAR T cells show better TGF- β resistance with a higher cytotoxic effect against TGF- β^+ tumour cells

CAR T cells (1×10^5) were incubated with N87-TGF- β^+ (1×10^5). After 3 days the cells were harvested and analysed by flow cytometry. Tumour cell count was determined using AccuCheck Counting Beads, Fixable Viability Dye eFluor 780 and antigen negative back gating. (w/o = 4-1BB- ζ without co-expressed receptor, DNR = 4-1BB- ζ co-expressing Dominant Negative Receptor, SWR = 4-1BB- ζ co-expressing Switch Receptor). Data represents the mean $\pm$ SEM. Statistical analyses were performed using an unpaired t test (* $p < 0.05$; ns., not significant).

5 DISCUSSION

The success of CAR T cell therapy against solid tumours is limited compared to the remarkable efficacy in the treatment of haematological malignancies. The complex tumour environment compromising an immune response is considered to be one of the biggest hurdles in preventing success against solid cancer (Sternier & Sternier, 2021). Once CAR T cells are adoptively transferred, they migrate to the tumour where they are heavily impacted by anti-immune cell activities upon encountering both the tumour stroma and the tumour site, resulting in reduced anti-tumour efficacy (Rodriguez-Garcia et al., 2020). A major consequence of this is local immunosuppression, in particular through immunosuppressive cytokines such as TGF- β . TGF- β is a frequent tumour resident suppressive cytokine, which in the majority of cases is responsible for the decreased T cell activity within the tumour milieu (Massagué, 2008). Our previous research demonstrated that CD28 co-stimulation makes CAR T cells resistant to TGF- β mediated suppression, whereas 4-1BB co-stimulation does not (Golumba-Nagy et al., 2018). CD28 mediates LCK activation and in consequence T cells releases high amounts of IL-2; IL-2 mediated autocrine signalling is the key driver to induce this resistant effect. However, although the CD28-induced autocrine IL-2 loop is effective, the strategy to use CD28- ζ CAR T cells as a therapy for the treatment of solid tumours is limited, due to the risk of recruiting tumour-infiltrating regulatory T cells through accumulating IL-2 in the tumour tissue (Kofler et al., 2011). To further explore the suppressive capabilities of TGF- β against 2nd generation CAR T cells, we monitored T cell amplification in the presence and absence of TGF- β . Figure 3 highlights that T cell amplification upon activation is not suppressed when CAR T cells signal through CD28 co-stimulatory domain. However, CAR T cell's signalling through 4-1BB and OX40 suffers from TGF- β mediated suppression. This data correlates with our previous research as in Figure 4, the IL-2 secretion between these CAR T cells is strikingly different, further supporting the hypothesis that IL-2 signalling is the key driver in overcoming TGF- β mediated suppression.

To combat this issue, we combined IL-2 receptor signalling, recognised as the key component for TGF- β resistance, with the binding domain for TGF- β , to create a Switch Receptor (SWR) that provides the T cell with intracellular IL-2R β signalling when TGF- β is present (Fig. 5A, B). In this scenario, the T cell becomes activated upon recognition of tumour antigen by the CAR, but, when TGF- β is present, the SWR binds TGF- β and converts the naturally suppressive signal into a stimulatory IL-2 receptor signal, solely intracellularly from the IL-2R β chain, not only protecting CAR T cells from TGF- β mediated suppression but providing the T cell with an additional stimulatory signal. By armouring 4-1BB- ζ CAR T cells with a SWR, we ensure the accumulation of IL-2 is significantly reduced compared to CD28- ζ CAR T cells whilst maintaining the 4-1BB- ζ CAR T cells well known characteristics of prolonged survival, persistence and being protected from TGF- β mediated suppression.

After CAR T cell manufacturing, we observed a similar expression level between 4-1BB- ζ CAR T cells with and without the SWR on human CD3⁺ T cells (Fig. 6A). Furthermore, we saw no significant changes in the memory phenotype of the CAR T cells after manufacturing (Fig. 7B). T cells equipped with the SWR maintained a central memory phenotype although the additional IL-2 receptor signal is present, T cells possessing this phenotype have the function of memory stem cells, with the advantage of having stronger immune replacement abilities and stronger survival abilities (Li et al., 2021). This was reassuring as the function of SWR CAR T cells is depending on antigen engagement and TGF- β , therefore carrying no threat for a predefined antigen independent effector state, which is important for the translation to clinical application.

Accordingly, we tested the SWR's ability in overcoming TGF- β mediated suppression. As we know that T cells amplification is suppressed, the proliferative capacity of CAR T cells with the switch receptor was analysed in the presence and absence of TGF- β . We found that, 4-1BB- ζ CAR T cells equipped with the SWR were able to maintain their amplification abilities even in rising concentrations of TGF- β (Fig. 8). In respect to cytotoxic T cell functions, it was reported that TGF- β specifically inhibits cytolytic gene products such as perforin (Thomas & Massagué, 2005). Activated CTLs, which are predominantly responsible for antigen-specific clearance of tumour cells, typically function through two contact-dependant pathways in order to kill the target cell. One is the granule exocytosis pathway, in which activated CTLs recognise a tumour cell and the membrane-pore-forming protein known as perforin initiates a delivery of apoptosis inducing proteases into the target cell (Trapani & Smyth, 2002). The Fas-Fas ligand pathway is the second contact dependant mechanism, this is responsible for activating target cell death by cytochrome release and activation of caspases (Russell & Ley, 2002). As this mechanism is vital for CAR T cell therapy in order to eliminate tumour cells, we monitored whether the switch receptor can rescue the inhibition of cytotoxic functions caused by TGF- β , such as perforin. To do so, 4-1BB- ζ CAR T cells with and without the switch receptor were activated in the presence and absence of TGF- β , perforin was recorded via intracellular flow cytometry staining (Fig. 9A,B). We identified that CAR T cells co-expressing the switch receptor did not suffer from perforin repression in the presence of TGF- β , unlike CAR T cells without the switch receptor. It is also demonstrated that 4-1BB- ζ CAR T cells equipped with the switch receptor induce higher perforin levels in the absence of TGF- β , although it is maintained, compared to 4-1BB- ζ without the switch receptor. It is known that IL-2 receptor signalling is an important regulator of perforin and activated T cells lacking IL-2R β show a reduced perforin mRNA expression (Malek et al., 2001; Pipkin et al., 2010). Therefore, we believe the elevated perforin levels are a consequence of signalling through the IL-2R β chain in the switch receptor, highlighting the advantage of not only providing resistance to TGF- β but an additional stimulatory signal.

Cytolytic activities of T cells go along with degranulation; CD107a is recognised as a marker for degranulation of NK activated CD8⁺ T cells. Degranulation is a cellular process that releases antimicrobial cytotoxic molecules from secretory vesicles, as degranulation occurs secretory lysosomes are released and the lysosome-associated membrane protein-1 (LAMP-1, CD107a) is transported to the surface (Alter et al., 2004), allowing for detection. As it is known that IL-2 signalling leads to an increase of CD107a expression (Aktas et al., 2009), we investigated the CD107a expression of switch receptor CAR T cells in the presence of TGF- β (Fig. 9 C/D). We observed that although CAR T cells without the switch receptor maintain CD107a expression in the presence of TGF- β , CAR T cells with the switch receptor express higher amounts of CD107a in the presence of TGF- β , displaying a higher degranulation profile. This along with the finding from the perforin experiments conclude that 4-1BB- ζ CAR T cells equipped with the switch receptor do not suffer from cytolytic inhibition caused by TGF- β but show an enhanced cytolytic profile compared to 4-1BB- ζ CAR T cells that don't have the switch receptor.

Previous research has shown that TGF- β also inhibits the cytokine production of immune cells (Yoshimura et al., 2010). In regard to CAR T cells, the IL-2 and IFN- γ production was inhibited for CARs signalling through either the CD28 or 4-1BB co-stimulatory domain, in the presence of TGF- β (Golumba-Nagy et al., 2018). IFN- γ is predominantly secreted by activated lymphocytes such as CD4 T helper-1 cells, cytotoxic CD8 T cells and plays a vital role in adaptive immunity (Castro et al., 2018). Therefore, we tested whether 4-1BB- ζ CAR T cells armoured with the switch receptor were able to prevent the inhibition of IFN- γ production in presence of TGF- β . To do so, CAR T cells with and without the SWR were incubated with antigen positive tumour cells in the presence and absence of TGF- β and the IFN- γ production was analysed in the supernatant (Fig. 9E). We observed that 4-1BB- ζ CAR T cells armoured with the SWR show no inhibition and are able to secrete higher amounts of IFN- γ in the presence of TGF- β compared to 4-1BB- ζ without the switch receptor.

Mechanistically, we explored intracellular IL-2 signalling. IL-2 exhibits is an essential regulator of numerous signalling pathways, including those underlying cell survival, proliferation, differentiation and effector functions (Jones et al., 2020). Although IL-2 has been shown to activate several STAT family members, including STAT1, STAT3 and STAT5, STAT5 is the predominant signalling molecule (Hou et al., 1995). Therefore, we believed phosphorylated STAT5 was the best read out to validate the SWR functionally and mechanistically. In Figure 10, SWR CAR T cells show an upregulation of pSTAT5 upon antigen recognition, and this is elevated when TGF- β is present. 4-1BB- ζ CAR T cells without the SWR show no levels of pSTAT5. Interestingly, converting resistance to TGF- β does not require both IL-2 receptor β and γ chains for a high affinity signal, the IL-2R β signalling alone is sufficient enough to provide the T cell with pSTAT5 downstream signalling providing resistance to TGF- β .

To test the universal capacity of the SWR we armoured knowingly sensitive OX40- ζ CARs and a previously described IL-2 deficient CD28 Δ LCK- ζ CAR (Golumba-Nagy et al., 2018) with the SWR and tested whether these CARs are now resistant to TGF- β mediated suppression. Our concept to provide an IL-2 signal to convert TGF- β resistance was further validated when suppressed amplification of the IL-2 deficient CD28 Δ LCK- ζ CAR T cells in the presence of TGF- β were rescued by adding the SWR (Fig. 11 D,E). This also correlates with our previous findings that re-introducing transgenic IL-2 release to the CD28 Δ LCK- ζ CAR restores resistance to TGF- β (Golumba-Nagy et al., 2018). In regard to OX40- ζ CARs equipped with the SWR, we observed an increase in T cell amplification in the presence of TGF- β compared to CARs without the SWR, this was shown in higher concentrations of TGF- β (Fig. 12 D/E) highlighting the universal capabilities of armouring CAR T cells with the SWR to convert resistance to TGF- β .

Alternative strategies to overcome the suppressive effect of TGF- β include the expression of the dominant negative TGF- β receptor (Bollard et al., 2018; Kloss et al., 2018; Narayan et al., 2022). This approach utilizes the co-expression of the TGF- β binding receptor (TGF- β RII) and acts as a sink, out competing the endogenous receptor and trapping TGF- β in the environment. The DNR is effective at protecting T cells from TGF- β mediated suppression however, limitations such as a sustained trapping effect over a longer period time hinder the potential of a lasted CAR T cell effect. To explore this, first we monitored T cell amplification in different concentrations of TGF- β (Fig. 13). The DNR performs as expected in displaying suppression in the presence of TGF- β . However, CAR T cells with the SWR show higher T cell amplification properties when T cells are challenged with higher amounts of TGF- β . Furthermore, we observed both amplification and cytotoxicity in a tumour re-challenging assay in the presence and absence of TGF- β (Fig. 15). We found that over multiple rounds, CAR T cells equipped with a SWR were able to sustain CAR T cell amplification and show better cytotoxic abilities compared to the DNR over multiple rounds of tumour re-challenging in the presence of TGF- β . We believe mechanistically, the SWR is advantageous over the DNR because not only are T cells protected from suppression caused by a trapping effect but, T cells are also given an additional stimulatory signal through the SWR's IL-2R β chain, highlighted through pSTAT5.

The dominant negative receptor is widely recognised as the most effective strategy to overcome TGF- β mediated suppression and has shown to be effective in clinical trials, with some patients showing a complete response (Bollard et al., 2018). Here we have demonstrated that adding of the IL-2R β stimulatory signal by the Switch Receptor can further improve CAR T cell efficacy in the context of TGF- β rich tumours. The additional signal providing the T cell with a stimulatory effect is reinforced when challenged in high TGF- β conditions, this is a clear benefit over the dominant negative strategy that exploits exclusively a decoy effect. Therefore, we believe that the high TGF- β abundance in tumour tissues further enhances the CAR T cell functionality whilst providing protection; the strategy has the capacity to improve CAR T cell efficacy towards the treatment of solid tumours.

Our research has shown that TGF- β causes T cell mediated suppression and IL-2 signalling is able to overcome this suppressive effect. Interestingly we have demonstrated that IL-2R β signalling alone is sufficient to compensate this suppressive effect. Therefore, we assume there is a crossover between the TGF- β and IL-2R signalling pathway. The suppressive effect by TGF- β is caused by the inhibition of several mechanisms for example, the upregulation of cell cycle regulators such as cyclin-dependent kinase inhibitors (CKIs), or the downregulation of cell cycle promoters such as c-Myc, cyclin D2, CDK2 and cyclin E (Ruegemer et al., 1990) whereas IL-2 is responsible for the accumulation of the c-Myc protein in activation T cells. Following T cell activation, the upregulation of amino acids and glucose concentrations is required in order to sustain all T cell functions necessary to be an effector T cell (Chou et al., 2014). During this metabolic reprogramming the transcriptional regulator c-Myc plays an important role. The regulation of c-Myc expression is driven by TCR stimulation and pro-inflammatory cytokines such as IL-2, IL-7, and IL-15. Interestingly, these cytokines signal through the JAK-STAT(5) signalling pathway, however, whilst TCR stimulation is associated with turning on c-Myc synthesis, IL-2 signalling is supporting long-term accumulation of the c-Myc protein (Preston et al., 2015).

Mechanistically c-Myc acts as a central modulator in the regulation of T cell proliferation, TGF- β mediated suppression causes the downregulation of this process, whereas IL-2 signalling causes upregulation. Taking this into consideration we applied the concept of a Switch Receptor to reduce the down regulation of c-Myc by binding TGF- β and out-competing the endogenous receptor, but more importantly utilizing TGF- β to provide an IL-2 signal through the IL-2R β chain to upregulate c-Myc synthesis, shifting the balance to support T cell proliferation. Using the intracellular components of the IL-2R β chain we were able to mimic IL-2R signalling as shown with pSTAT5 to mediate enhanced proliferation through its conventional role as a transcription factor upregulating proliferative and survival genes such as c-Myc, bcl-2 and bcl-x (Miyazaki et al., 1995). To support this, research conducted by Lord *et al.*, showed that mutations within the IL-2R β chain such as cytoplasmic tyrosine deletions led to the retained ability to induce STAT5 activation and proliferation in T cells. On the other-hand, the over expression of STAT5a and STAT5b induced the expression of c-Myc, bcl-2 and bcl-x (Lord et al., 2000).

The current approaches to overcome TGF- β mediated suppression mainly focus on the TGF- β signalling pathway in an attempt to inhibit regulation, although effective it is important to point out that the role of TGF- β in the immune homeostasis is essential (Sanjabi et al., 2017). This was shown in TGF- β 1 KO mice, which led to embryonic lethality and inflammatory diseases (Kulkarni et al., 1993). In contrary to this, our strategy doesn't solely disrupt the signalling but by utilizing IL-2R β signalling we are converting what is normally a suppressive signal into a positive stimulatory signal, providing the CAR T cell with not only protection from TGF- β , but additional functions to allow for better tumour efficacy in TGF- β rich environments.

This thesis highlights the use of a new approach for the development of CAR T cell therapy against solid tumours. Transforming growth factor- β is highly abundant within the TME, it creates an immunosuppressive environment utilized for tumour growth, survival and allows for the escape of immune surveillance. The development of our strategy enables CAR T cells to utilize this overproduced cytokine as a stimulatory benefit to counteract what is normally a suppressive signal. Therefore, our CAR-engineered T cells are not only protected against immunosuppression caused by TGF- β but boosted for a more anti-tumour response when challenged in the suppressive TGF- β rich tumour microenvironment.

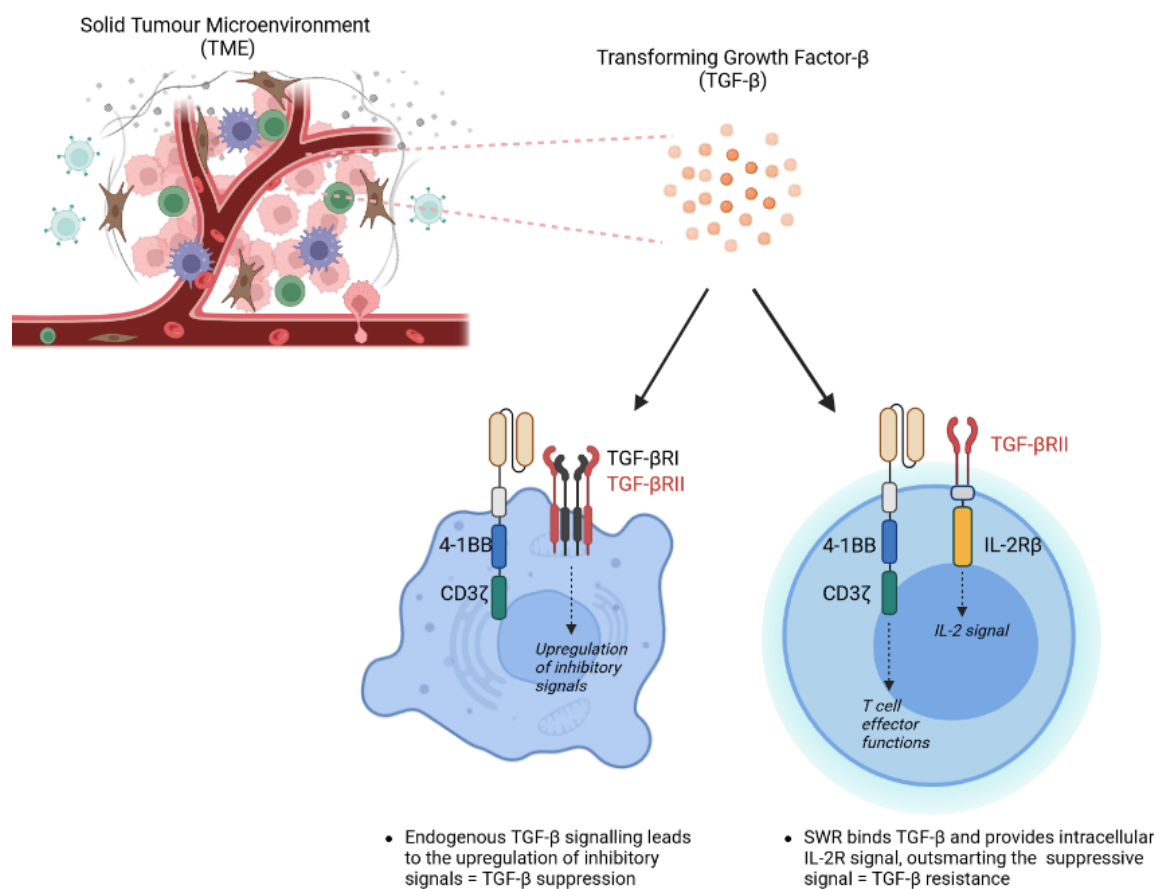


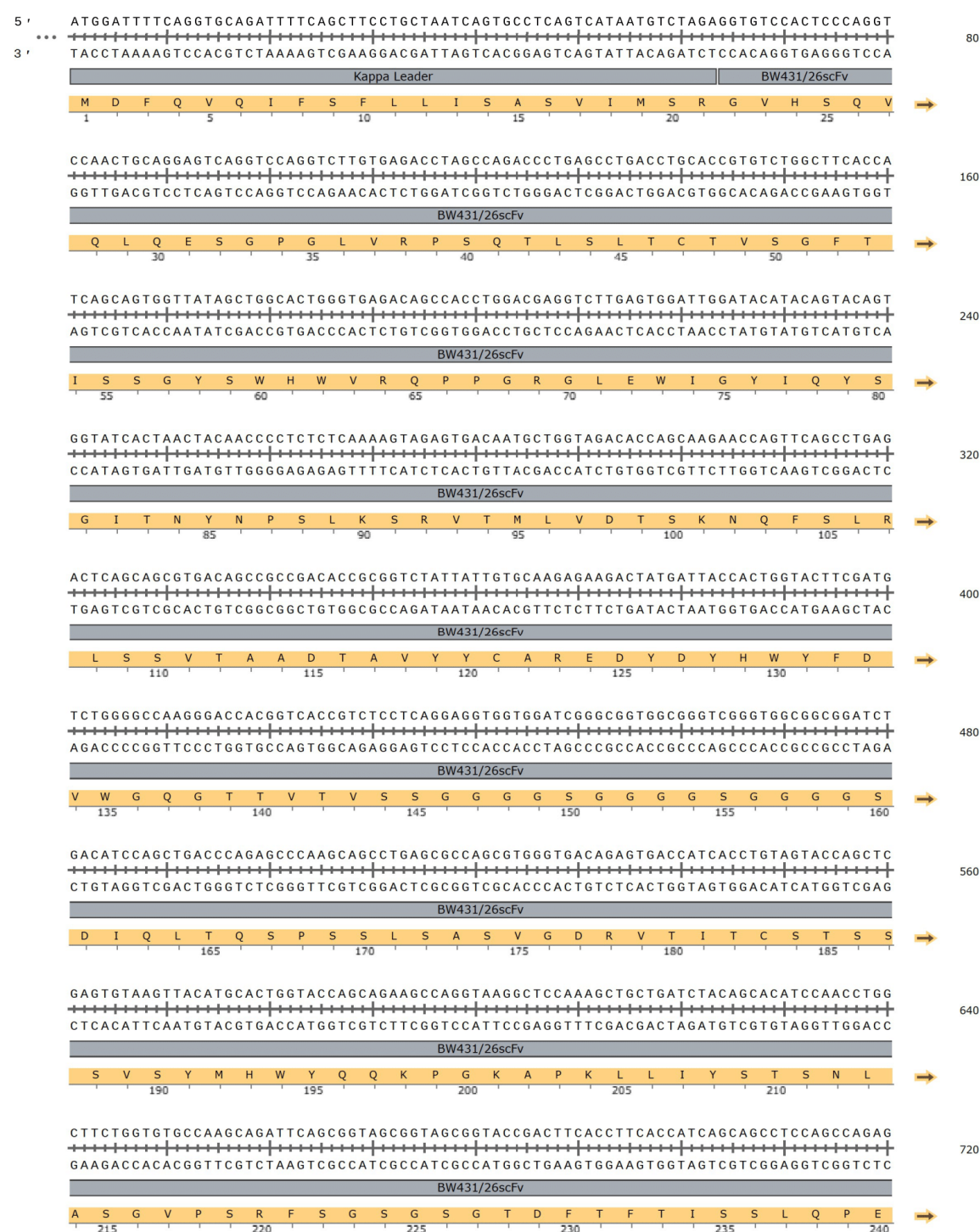
Figure 18. Concept of SWR CAR T cells overcoming TGF- β suppression

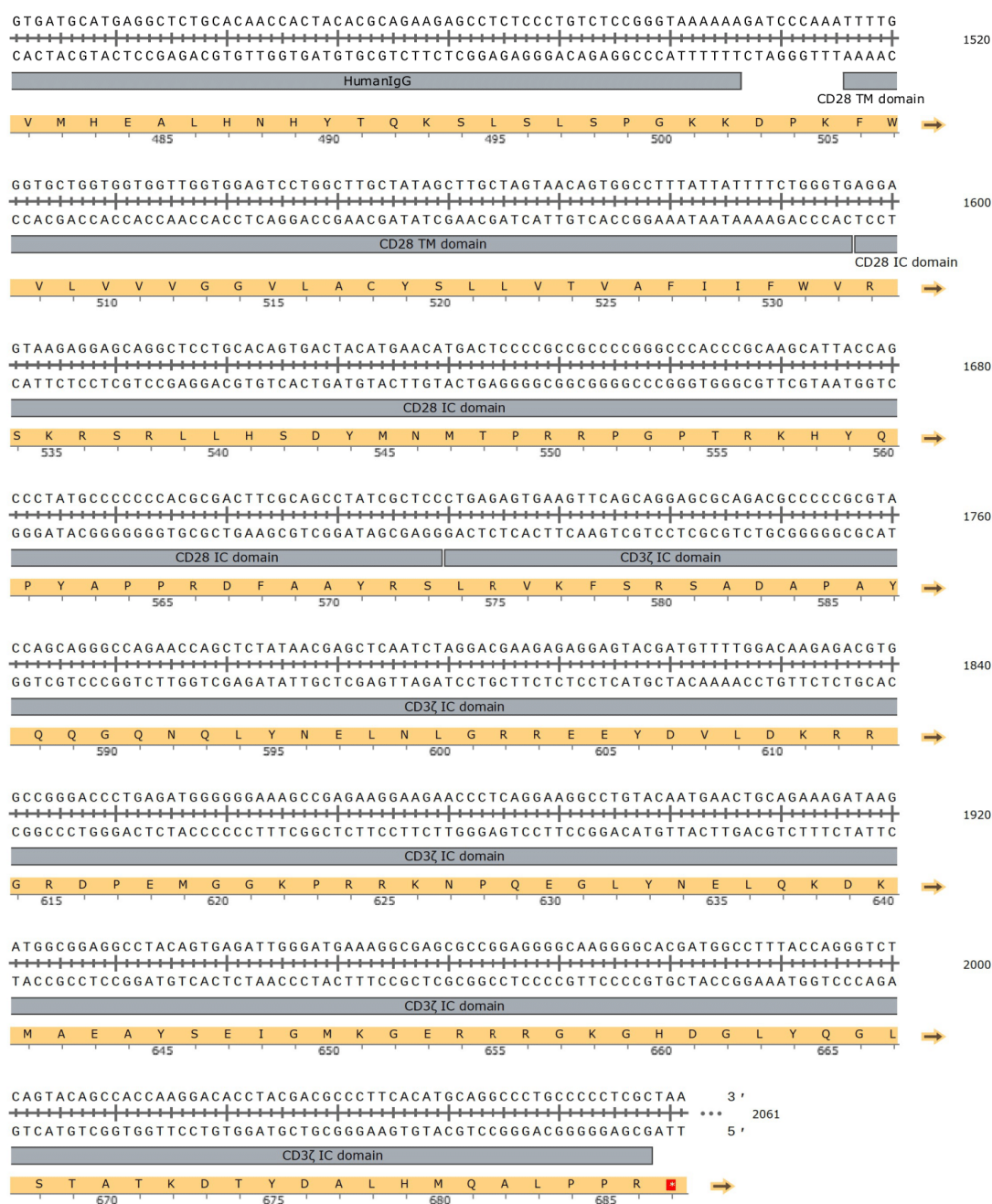
A summary to visualize the effect of TGF- β on 4-1BB- ζ CAR T cells with and without the Switch Receptor upon activation.

6 APPENDIX

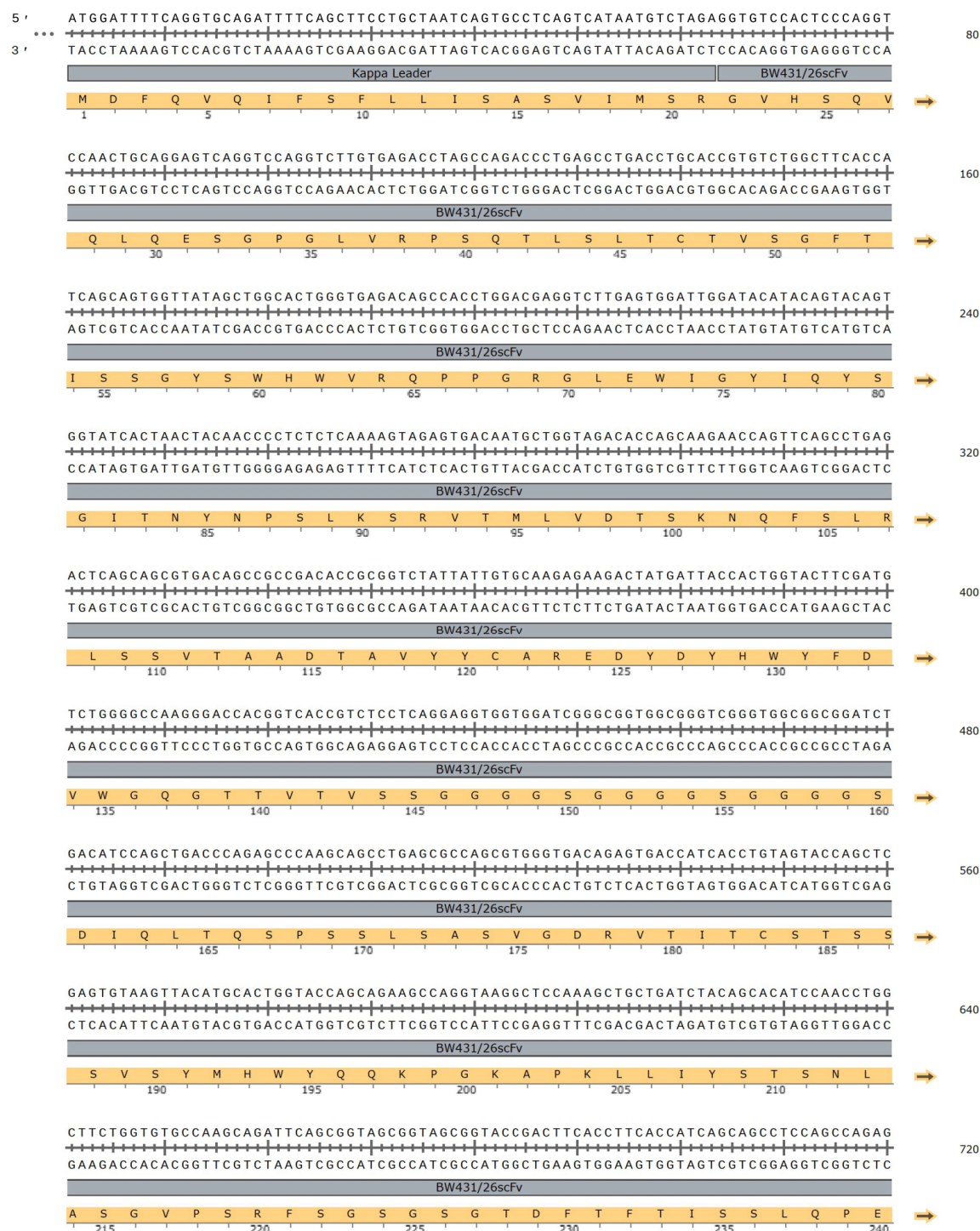
The DNA and amino acid sequence of each Chimeric Antigen Receptor and Switch Receptor used in this study along with the commercially bought human TGF- β 1 expression Vector. Generated by SnapGene Viewer.

6.1 BW431/26scFv-Fc-CD28-CD3 ζ (#607)

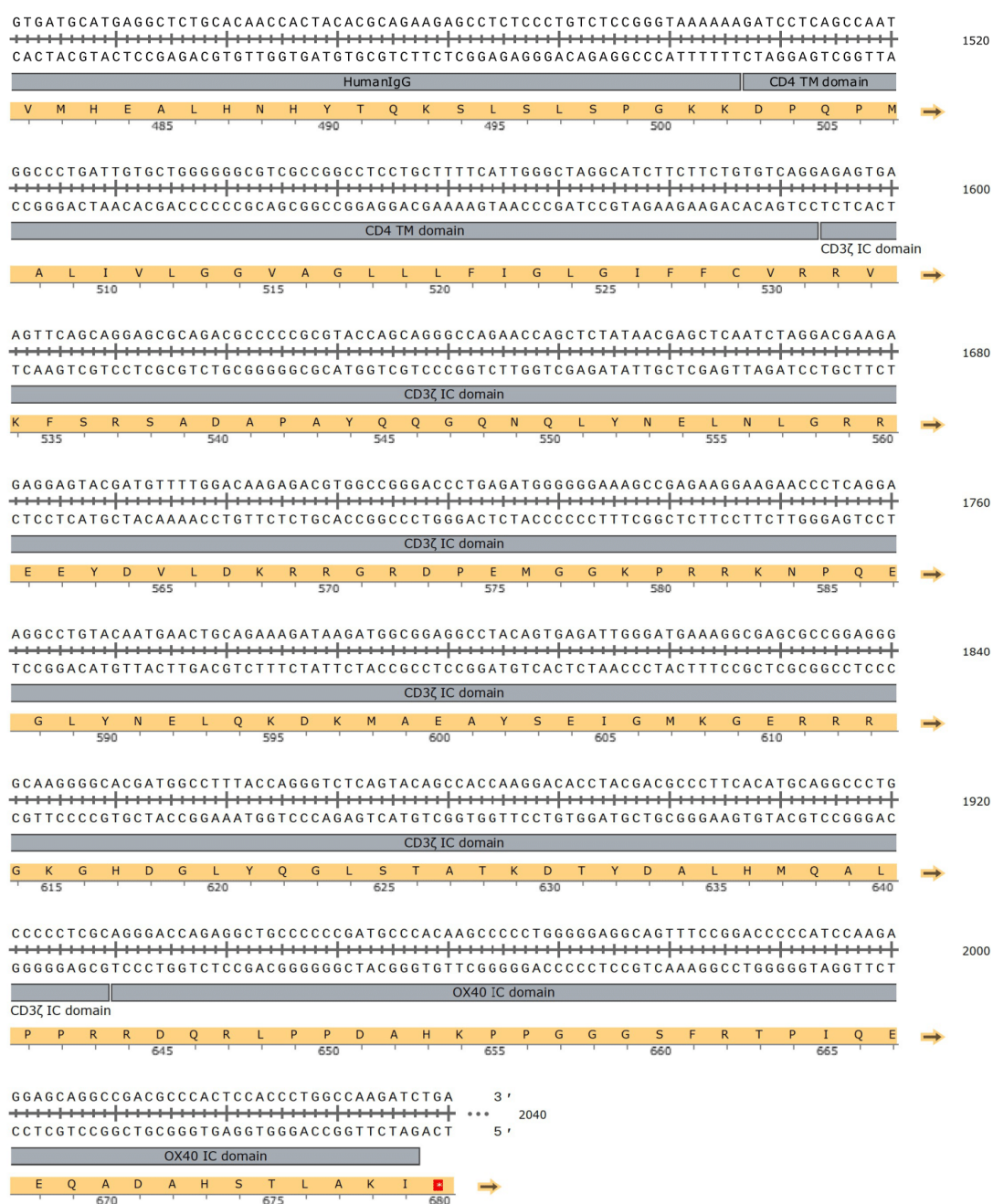




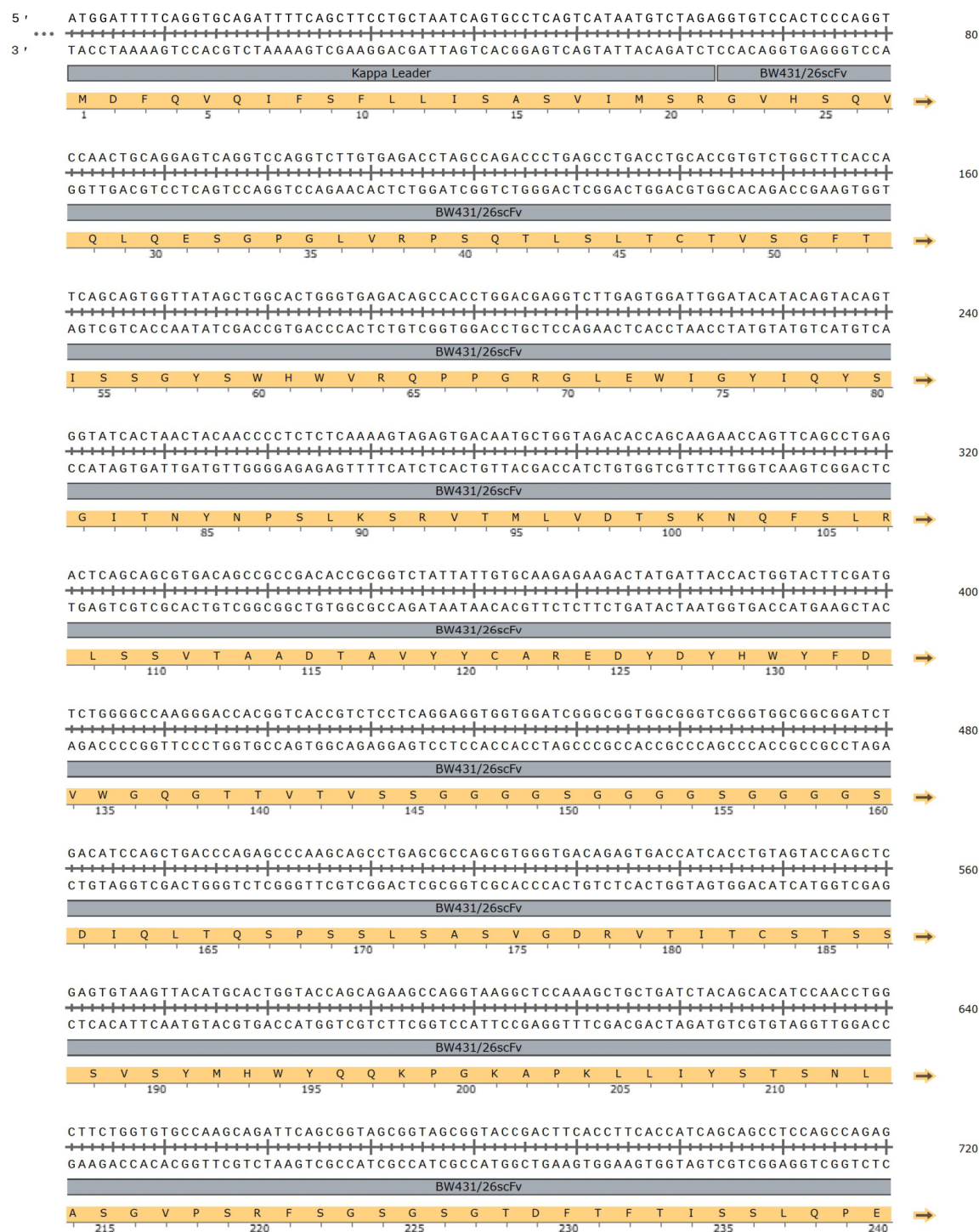
6.2 BW431/26scFv-Fc-CD3ζ-OX40 (#848)

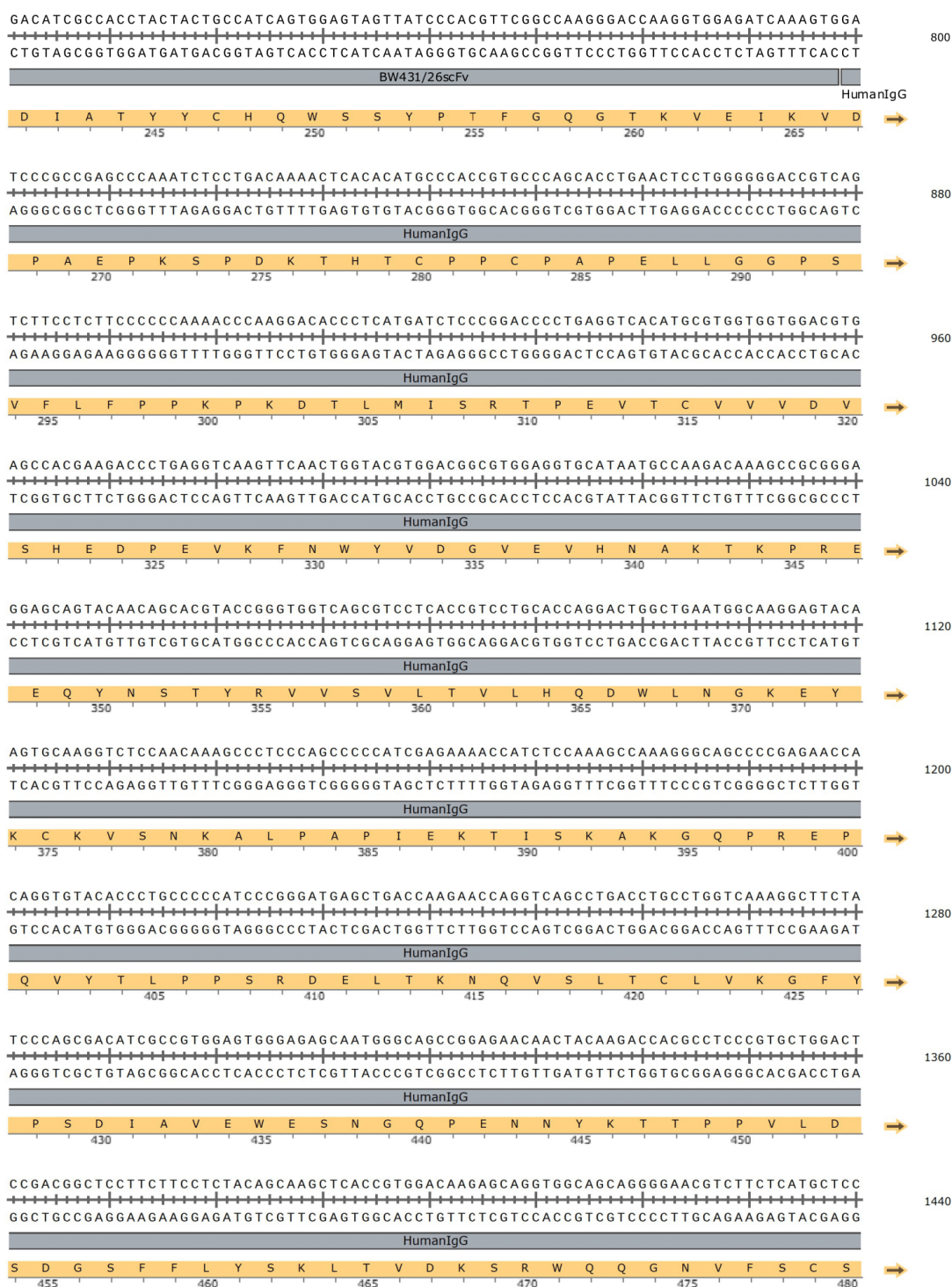


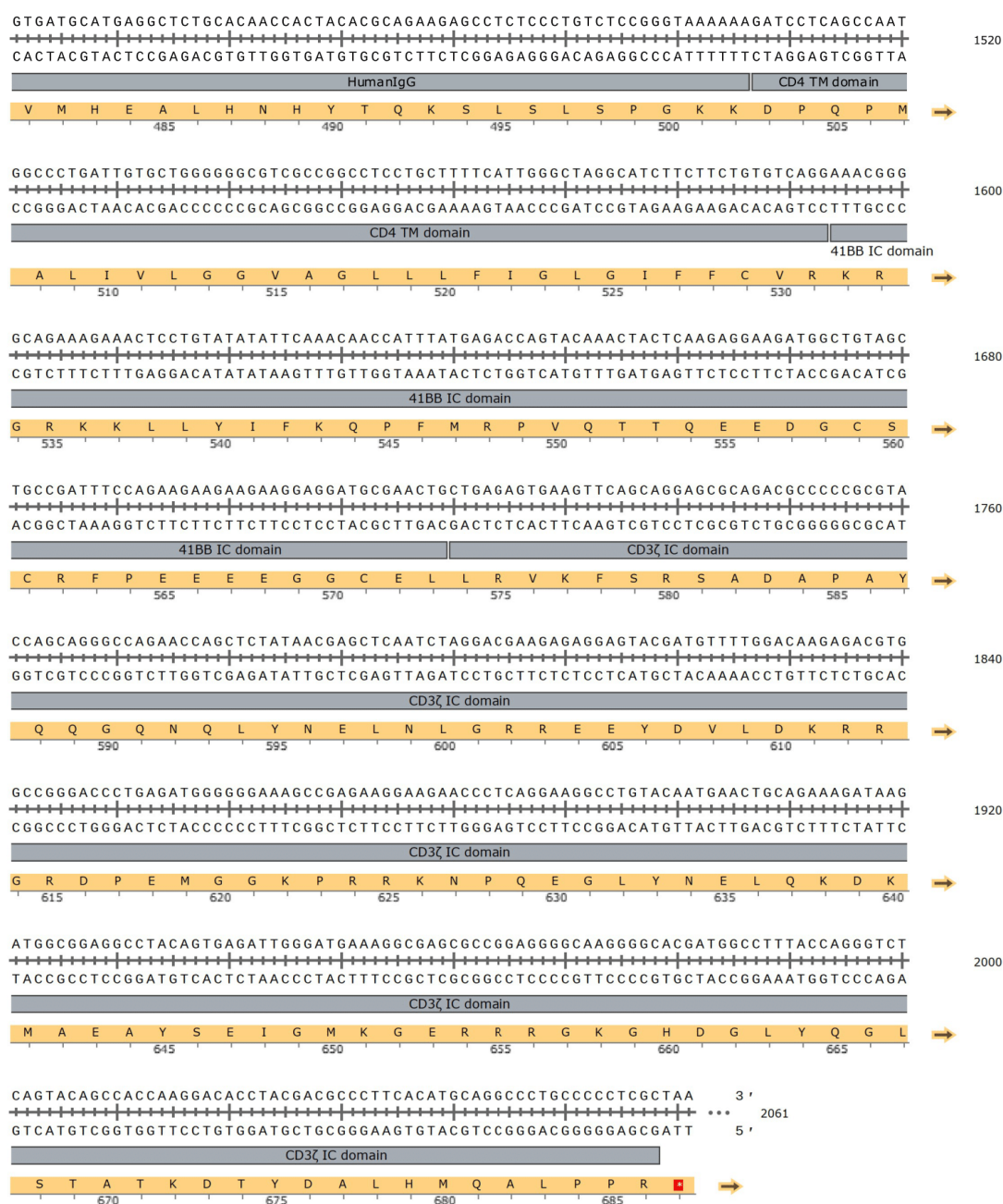
GACATCGCCACCTACTACTGCCATCAGTGGAGTAGTTATCCACGTTTCGGCCAAGGGACCAAGGTGGAGATCAAAGTGGA CTGTAGCGGTGGATGATGACGGTAGTCACCTCATCAATAGGGTGCAAGCCGGTTCCTGGTTCCACCTCTAGTTTACCT	800
BW431/26scFv	
D I A T Y Y C H Q W S S Y P T F G Q G T K V E I K V D	→
245 250 255 260 265	
TCCCGCCGAGCCCAAATCTCCTGACAAAACCTCACACATGCCACCGTGCCAGCACCTGAACTCCTGGGGGGACCGTCAG AGGGCGGCTCGGGTTTAGAGGACTGTTTTGAGTGTGTACGGTGGCAGGGTCGTGGACTTGAGGACCCCTGGCAGTC	880
HumanIgG	
P A E P K S P D K T H T C P P C P A P E L L G G P S	→
270 275 280 285 290	
TCTTCTCTTCCCCCAAACCCAAGGACACCCCTCATGATCTCCCGACCCCTGAGGTTCACATGCGTGGTGGTGGACGTG AGAAGGAGAAGGGGGTTTTGGGTTCTGTGGGAGTACTAGAGGCCCTGGGGACTCCAGTGTACGCACCACCACCTGCAC	960
HumanIgG	
V F L F P P K P K D T L M I S R T P E V T C V V V D V	→
295 300 305 310 315 320	
AGCCACGAAGACCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGA TCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTACGGTTCTGTTTCGGGCGCCT	1040
HumanIgG	
S H E D P E V K F N W Y V D G V E V H N A K T K P R E	→
325 330 335 340 345	
GGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCCTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACA CCTCGTCATGTTGTCGTGCATGGCACACCAAGTCGACGAGGAGTGGCAGGACGTGGTCTGACCGACTTACCGTTCTCATGT	1120
HumanIgG	
E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y	→
350 355 360 365 370	
AGTGCAAGGTCTCCAACAAAGCCCTCCAGCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA TCACGTTCCAGAGGTTGTTTCGGGAGGGTCGGGGGTAGCTCTTTTGGTAGAGGTTTCGGTTTCCCGTCGGGGCTCTTGGT	1200
HumanIgG	
K C K V S N K A L P A P I E K T I S K A K G Q P R E P	→
375 380 385 390 395 400	
CAGGTGTACACCCTGCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTACGCTGACCTGCCTGGTCAAAGGCTTCTA GTCCACATGTGGGACGGGGGTAGGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGACGGACCAAGTTCCGAAGAT	1280
HumanIgG	
Q V Y T L P P S R D E L T K N Q V S L T C L V K G F Y	→
405 410 415 420 425	
TCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACT AGGGTCGCTGTAGCGGCACCTCACCTCTCGTTACCCGTGGCCTCTTGTGATGTTCTGGTGGCAGGGGACGACCTGA	1360
HumanIgG	
P S D I A V E W E S N G Q P E N N Y K T T P P V L D	→
430 435 440 445 450	
CCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGCTTCTCATGCTCC GGCTGCCGAGGAAGAAGGAGATGTCGTTTCGAGTGGCACCTGTTCTCTGTCACCGTCGTCCTTGCAGAAGAGTACGAGG	1440
HumanIgG	
S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S	→
455 460 465 470 475 480	



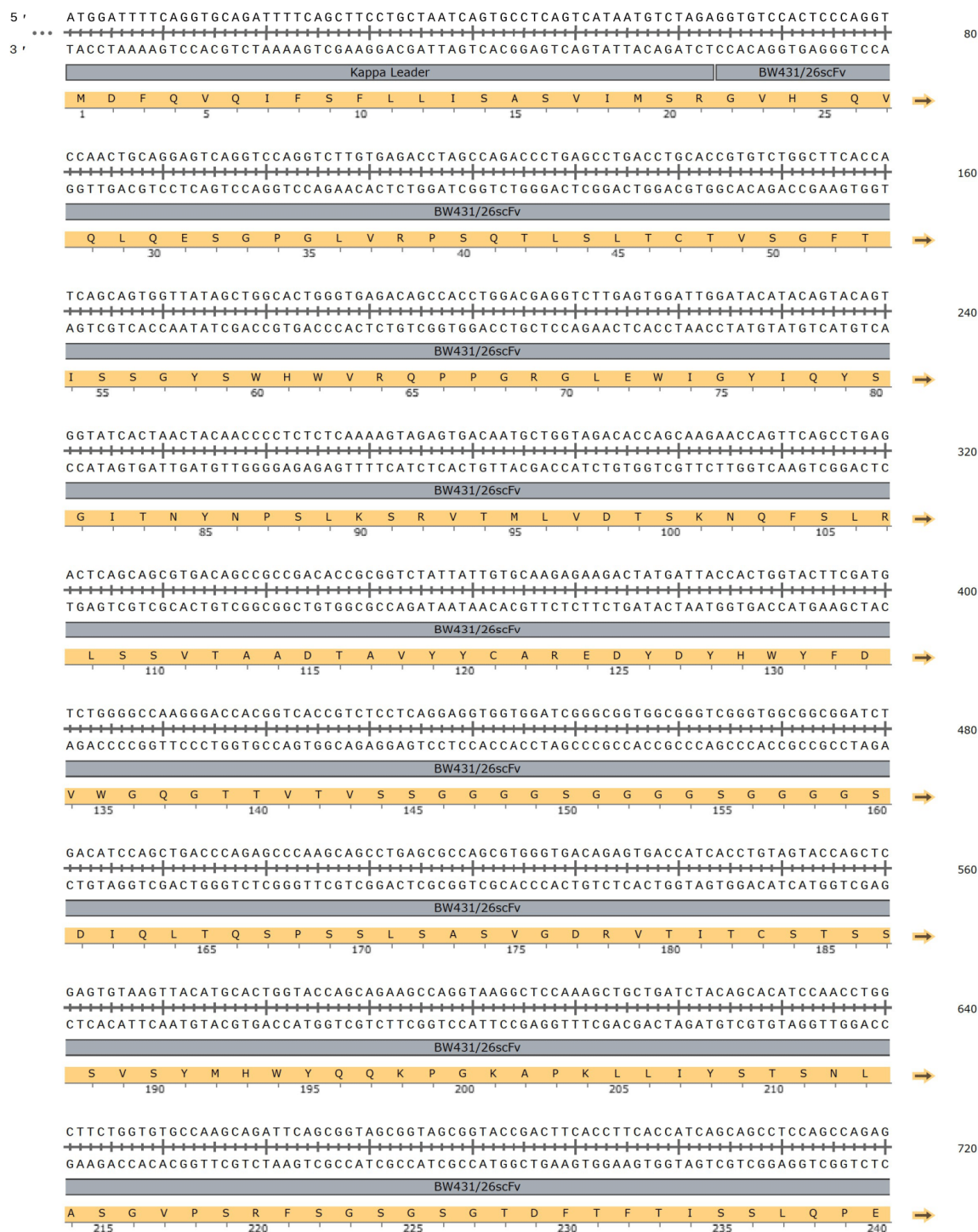
6.3 BW431/26scFv-Fc-4-1BB-CD3ζ (#908)

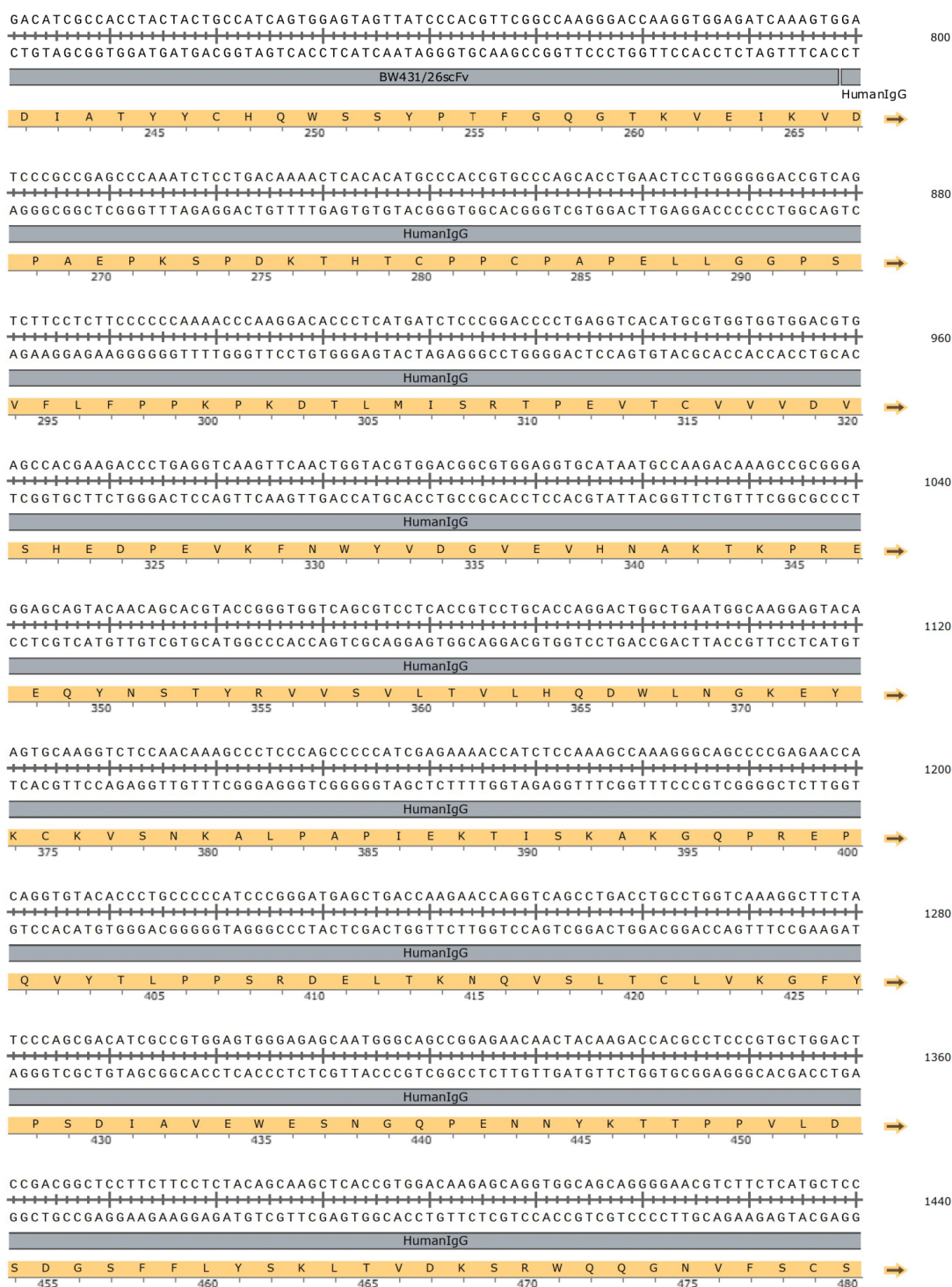






6.4 BW431/26scFv-Fc-CD28ΔLCK-CD3ζ (#946)







6.5 BW431/26scFv-Fc-4-1BB-CD3 ζ -P2A-TGF- β RII-PDGFR β TM-IL-2R β (#2356)

5'	CCATGGATTTCAGGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCCTCAGTCATAATGTCTAGAGGTGTCCACTCCAG	80
3'	GGTACCTAAAAGTCCACGTCTAAAAGTCGAAGGACGATTAGTCACGGAGTCAGTATTACAGATCTCCACAGGTGAGGGTC	
	Kappa Leader BW431/26scfv	
	M D F Q V Q I F S F L L I S A S V I M S R G V H S Q	→
	GTCCAACTGCAGGAGTCAGGTCCAGGTCTTGTGAGACCTAGCCAGACCTGAGCCTGACCTGCACCGTGTCTGGCTTCAC	160
	CAGGTTGACGTCTCAGTCCAGGTCCAGAACACTCTGGATCGGTCTGGGACTCGGACTGGACGTGGCACAGACCGAAGTG	
	BW431/26scfv	
	V Q L Q E S G P G L V R P S Q T L S L T C T V S G F T	→
	CATCAGCAGTGGTTATAGCTGGCACTGGGTGAGACAGCCACCTGGACGAGGTCTTGAGTGGATTGGATACATACAGTACA	240
	GTAGTCGTACCAATATCGACCGTGACCCACTCTGTGGTGGACCTGCTCCAGAACTCACCTAACCTATGTATGTCATGT	
	BW431/26scfv	
	I S S G Y S W H W V R Q P P G R G L E W I G Y I Q Y	→
	GTGGTATCACTAACTACAACCCCTCTCTCAAAAGTAGAGTGACAATGCTGGTAGACACCAAGCAAGAACAGTTTCAGCCTG	320
	CACCATAGTGATTGATGTTGGGGAGAGAGTTTCATCTCACTGTTACGACCATCTGTGGTCTGTTGTTGCTCAAGTCGGAC	
	BW431/26scfv	
	S G I T N Y N P S L K S R V T M L V D T S K N Q F S L	→
	AGACTCAGCAGCGTGACAGCCGCGACACCGCGGTCTATTATTGTGCAAGAGAAGACTATGATTACCACTGGTACTTTCGA	400
	TCTGAGTCGTGCACTGTGCGCGGTGTGGCGCCAGATAATAACAGTTCTCTTCTGATACTAATGGTGACCATGAAGCT	
	BW431/26scfv	
	R L S S V T A A D T A V Y Y C A R E D Y D Y H W Y F D	→
	TGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGGAGGTGGTGGATCGGGCGGTGGCGGGTCGGGTGGCGGGGAT	480
	ACAGACCCCGGTTCCCTGGTGCCAGTGGCAGAGGAGTCTCCACCACCTAGCCCGCCACCGCCAGCCACCGCCGCGCTA	
	BW431/26scfv	
	V W G Q G T T V T V S S G G G G S G G G G S G G G G	→
	CTGACATCCAGCTGACCCAGAGCCCAAGCAGCCTGAGCGCCAGCGTGGGTGACAGAGTGACCATCACCTGTAGTACCAGC	560
	GACTGTAGGTCGACTGGGTCTCGGGTTCGTGGGACTCGCGGTGCGACCCACTGTCTCACTGGTAGTGGACATCATGGTCTG	
	BW431/26scfv	
	S D I Q L T Q S P S S L S A S V G D R V T I T C S T S	→
	TCAAGTGTAAGTTACATGCACTGGTACCAGCAGAAGCCAGGTAAGGCTCCAAAGCTGCTGATCTACAGCACATCCAACCT	640
	AGTTCACATTCAATGTACGTGACCATGGTCTCTCGGTCCATTCCGAGGTTTCGACGACTAGATGTCGTGAGGTTGGA	
	BW431/26scfv	
	S S V S Y M H W Y Q Q K P G K A P K L L I Y S T S N L	→
	GGCTTCTGGTGTGCCAAGCAGATTACGCGGTAGCGGTAGCGGTACCGACTTCACCTTCACCATCAGCAGCCTCCAGCCAG	720
	CCGAAGACCACACGGTTCGTCTAAGTCGCCATCGCCATCGCCATGGCTGAAGTGAAGTGGTAGTCGTGGAGGTCGGTC	
	BW431/26scfv	
	A S G V P S R F S G S G S G T D F T F T I S S L Q P	→
	AGGACATCGCCACCTACTACTGCCATCAGTGGAGTAGTTATCCACGTTTCGGCCAAAGGACCAAGGTGGAGATCAAAGTG	800
	TCCTGTAGCGGTGGATGATGACGGTAGTCACCTCATCAATAGGGTGCAAGCCGGTTCCTGGTTCACCTCTAGTTTCAC	
	BW431/26scfv	
	E D I A T Y Y C H Q W S S Y P T F G Q G T K V E I K V	→

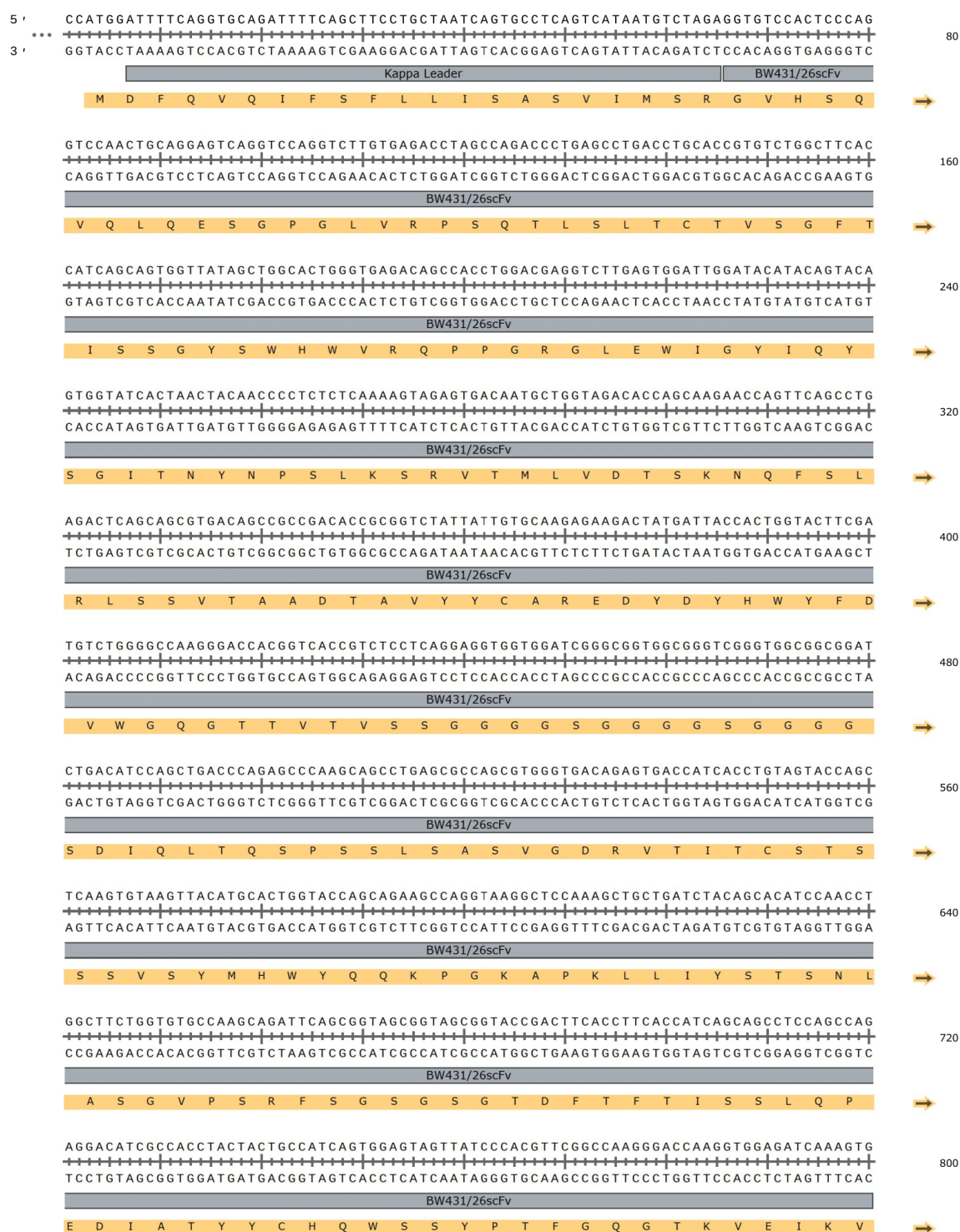
GGATCCGATCCCGCCGAGCCCAATCTCCTGACAAAACCTCACACATGCCACCGTGCCACGACCTGAACTCCTGGGGGG	880
CCTAGGCTAGGGCGGCTCGGGTTTAGAGGACTGTTTGAAGTGTGTACGGGTGGCACGGGTCGTGGACTTGAGGACCCCC	
HumanIgG	
G S D P A E P K S P D K T H T C P P C P A P E L L G G	→
V H G G H G A G S S R P P	
ACCGTCAGTCTTCTCTTCCCCCAAAACCAAGGACACCCCTCATGATCTCCCGGACCCCTGAGGTACACATGCGTGGTGG	960
TGGCAGTCAGAAGGAGAAGGGGGTTTTGGGTTCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTGTACGCCACCAC	
HumanIgG	
P S V F L F P P K P K D T L M I S R T P E V T C V V	→
G D T K R K G G F G L S V R M I E R V G S T V H T T T	
TGGACGTGAGCCACGAAGACCCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG	1040
ACCTGCACCTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTACGGTTCTGTTTC	
HumanIgG	
V D V S H E D P E V K F N W Y V D G V E V H N A K T K	→
S T L W S S G S T L N L Q Y T S P T S T C L A L V F	
CCGCGGGAGGAGCAGTACAACAGCACGTACCGGGTGGTCAGCGTCCTCACCCTGCTGCACAGGACTGGCTGAATGGCAA	1120
GGCGCCCTCCTCGTCATGTTGTCGTGCATGGCCACCACTCGCAGGAGTGGCAGGACGTGGTCTTGACCGACTTACCGTT	
HumanIgG	
P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K	→
G R S S C Y L L V Y R T T L T R V T R C W S Q S F P L	
GGAGTACAAGTGAAGGTCTCCAACAAAGCCCTCCCAGCCCCATCGAGAAAACCATCTCCAAGCCAAAGGGCAGCCCC	1200
CCTCATGTTACAGTTCAGAGGTTGTTTCGGGAGGGTGGGGGTAGCTCTTTGGTAGAGGTTTCGGTTTCCCGTCGGGG	
HumanIgG	
E Y K C K V S N K A L P A P I E K T I S K A K G Q P	→
S Y L H L T E L L A R G A G M S F V M	
GAGAACCACAGGTGTACACCCTGCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTACGCTGACCTGCCTGGTCAAA	1280
CTCTTGGTGTCCACATGTGGGACGGGGGTAGGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGACGGACCAAGTTT	
HumanIgG	
R E P Q V Y T L P P S R D E L T K N Q V S L T C L V K	→
GGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCAGCCTCCCGT	1360
CCGAAGATAGGGTCGCTGTAGCGGCACCTCACCTCTCGTTACCCGTGGCCCTCTTGTGATGTTCTGGTGGGAGGGCA	
HumanIgG	
G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V	→
GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCT	1440
CGACCTGAGGCTGCCGAGGAAGAAGGAGATGTGCTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTCCCTTGACAGAAGA	
HumanIgG	
L D S D G S F F L Y S K L T V D K S R W Q Q G N V F	→
CATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAAAAGCCGGC	1520
GTACGAGGCACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCATTTTTTCGGCCG	
HumanIgG	
S C S V M H E A L H N H Y T Q K S L S L S P G K K A G	→
GATCCTCAGCCAATGGCCCTGATTGTGCTGGGGGGCGTCGCCGGCCTCTGCTTTTCATTGGGCTAGGCATCTTCTTCTG	1600
CTAGGAGTCGGTTACCGGGACTAACACGACCCCGCAGCGGGCGGAGGACGAAAAGTAACCCGATCCGTAGAAGAAGAC	
CD4 TM domain	
D P Q P M A L I V L G G V A G L L L F I G L G I F F C	→

	TGTCAGGAAACGGGGCAGAAAGAACTCCTGTATATATTCAAACAACCATTTATGAGACCAGTACAACTACTCAAGAGG ACAGTCCTTTGCCCGTCTTTCTTTGAGGACATATATAAGTTTGTGGTAAATACTCTGGTCATGTTTGATGAGTTCTCC	1680
CD4 TM domain	41BB IC domain V R K R G R K K L L Y I F K Q P F M R P V Q T T Q E	→
	AAGATGGCTGTAGCTGCCGATTTCAGAGAAGAAGAAGGAGGATGCGAACTGCAATTGCTGAGAGTGAAGTTCAGCAGG TTCTACCGACATCGACGGCTAAAGGCTTTCTTCTTCTTCTCTACGCTTGACGTTAACGACTCTCACTTCAAGTCGTCC	1760
	41BB IC domain CD3ζ IC domain E D G C S C R F P E E E E G G C E L Q L L R V K F S R	→
	AGCGCAGACGCCCCGCGTACCAGCAGGGCCAGAACCAAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGA TCGCGTCTGCGGGGCGCATGGTCGTCGCCGCTTGGTCGAGATATTGCTCGAGTTAGATCCTGCTTCTCTCTCATGCT	1840
	CD3ζ IC domain S A D A P A Y Q Q G Q N Q L Y N E L N L G R R E E Y D	→
	TGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGAAAGCCGAGAAGGAAGAACCTCAGGAAGGCCTGTACA ACAAAACCTGTTCTCTGCACCGGCCCTGGGACTCTACCCCTTTCGGCTCTTCTTCTTGGGAGTCCTCCGGACATGT	1920
	CD3ζ IC domain V L D K R R G R D P E M G G K P R R K N P Q E G L Y	→
	ATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCAC TACTTGACGTCTTTCTATTCTACCGCTCCGGATGTCACCTTAACCCTACTTTCGGCTCGCGGCTCCCGGTTCCCGTG	2000
	CD3ζ IC domain N E L Q K D K M A E A Y S E I G M K G E R R R G K G H	→
	GATGGCCTTTACAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCCCCCTCGCGT CTACCGGAAATGGTCCCAGAGTCATGTCGGTGGTTCTGTGGATGCTGCGGGAAGTGACGTCCGGGACGGGGAGCGCA	2080
	CD3ζ IC domain D G L Y Q G L S T A T K D T Y D A L H M Q A L P P R V M C A R G G R T	→
	CGACAGCGCTGGATCTGGAGCCACGAACCTTCTCTGTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCTTAAGC GCTGTGCGACCTAGACCTCGGTGCTTGAAGAGAGACAATTCGTTCTGCTCTGCACCTTCTTTGGGGCCAGGATTCG	2160
	P2A D S A G S G A T N F S L L K Q A G D V E E N P G P K S L A P D P A V F K E R N F C A P S T S S F G P G L S	→
	TTATGGGCAAGGGCCTGCTGAGGGGCTGTGGCCCTGCACATCGTGCTGTGGACCAGGATCGCCAGCACCATCCCCC AATACCCGTCCCCGGACGACTCCCCGGACACCGGGGACGTGTAGCACGACACCTGGTCCTAGCGGTCTGTTAGGGGGG	2240
	TGFβRII EC domain L M G R G L L R G L W P L H I V L W T R I A S T I P P I P L P R S L P R H G R C M T S H V L I A L V M G G	→
	CACGTGCAGAAGAGCGTGAACAACGACATGATCGTGACCGACAACAACGGCGCCGTGAAGTTCCTCCAGCTGTGCAAGTT GTGCACGTCTTCTCGCACTTGTGTCTGTACTAGCACTGGCTGTTGTTGCCGGGCACTTCAAGGGGTCGACACGTTCAA	2320
	TGFβRII EC domain H V Q K S V N N D M I V T D N N G A V K F P Q L C K F W T C F L T F L S M I T V S L L P A T F N G W S H L N	→
	CTGCGACGTGAGGTTTCAGCACCTGCGACAACCAAGAGCTGCATGAGCAACTGCAGCATCACCAGCATCTGCGAGAAGC GACGCTGCACTCCAAGTCGTGGACGCTGTTGGTCTTCTCGACGTAAGTTCGTTGACGTCGTAAGTGGTCGTAGACGCTCTTCG	2400
	TGFβRII EC domain C D V R F S T C D N Q K S C M S N C S I T S I C E K Q S T L N L V Q S L W F L Q M L L Q L M V L M Q S F G	→

84

CCCAGCCTGCTGGGCGGGCCCCAGCCCCCCCAGCACCGCCCCGGCGGGCAGCGGCCGGCGAGGAGAGGATGCCCCCAG	
GGGTCGGACGACCCGCCGGGTCGGGGGGGTCGTGGCGGGGGCCGCCGTCGCCGCGGCCGCTCCTCTCTACGGGGGGTC	3280
IL-2R β IC domain	
P S L L G G P S P P S T A P G G S G A G E E R M P P S	→
CCTGCAGGAGAGGGTGCCAGGGACTGGGACCCCCAGCCCTGGGCCCCCCACCCCCGGCGTGCCCGACCTGGTGGACT	
GGACGTCCTCTCCACGGGTCCCTGACCCTGGGGGTCGGGGACCCGGGGGGGTGGGGGCCGCACGGGCTGGACCACCTGA	3360
IL-2R β IC domain	
L Q E R V P R D W D P Q P L G P P T P G V P D L V D	→
TCCAGCCCCCCCCGAGCTGGTGCTGAGGGAGGCCGGCGAGGAGGTGCCCGACGCCGGCCCCAGGGAGGGCGTGAGCTTC	
AGGTCGGGGGGGGGCTCGACCACGACTCCCTCCGGCCGCTCTCCACGGGCTGCGGGCCGGGGTCCCTCCCGCACTCGAAG	3440
IL-2R β IC domain	
F Q P P P E L V L R E A G E E V P D A G P R E G V S F	→
CCCTGGAGCAGGCCCCCCGGCCAGGGCGAGTTCAAGGGCCCTGAACGCCAGGCTGCCCTGAACACCGACGCCTACCTGAG	
GGGACCTCGTCCGGGGGGCGGTCCCGCTCAAGTCCCGGGACTTGCGGTCCGACGGGGACTTGTGGCTGCGGATGGACTC	3520
IL-2R β IC domain	
P W S R P P G Q G E F R A L N A R L P L N T D A Y L S	→
CCTGCAGGAGCTGCAGGGCCAGGACCCACCCACCTGGTGTGCGGATAG	3'
GGACGTCCTCGACGTCCCGGTCTGGGGTGGGTGGACCACAGCGCTATC	5' 3569
IL-2R β IC domain	
L Q E L Q G Q D P T H L V S R	→

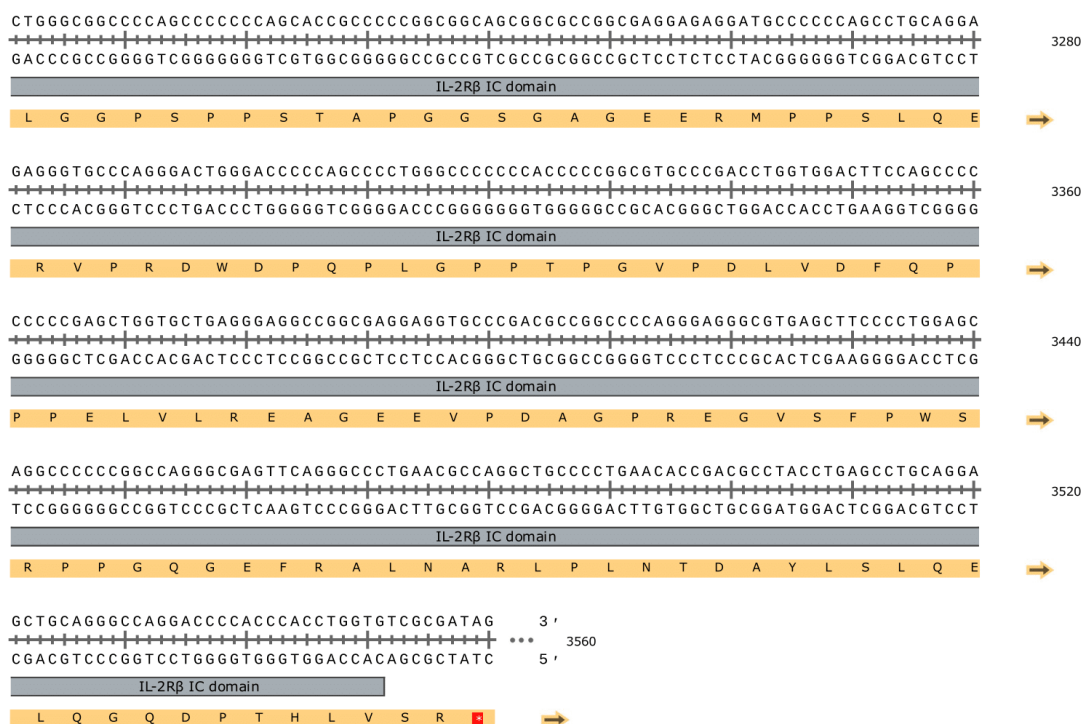
6.6 BW431/26scFv-Fc- CD28ΔLCK -CD3ζ-P2A-TGF-βRII-PDGFRβTM-IL-2Rβ (#2357)



GGATCCGATCCCGCCGAGCCCAATCTCTGACAAAACCTCACACATGCCACCGTGCCACGACCTGAACTCCTGGGGGG	880
CCTAGGCTAGGGCGGCTCGGGTTTAGAGGACTGTTTGAAGTGTGTACGGGTGGCACGGGTGCTGGACTTGAGGACCCCC	
HumanIgG	
G S D P A E P K S P D K T H T C P P C P A P E L L G G	→
V H G G H G A G S S R P P	
ACCGTCAGTCTTCTCTTCCCCCAAAACCAAGGACACCCCTCATGATCTCCCGGACCCCTGAGGTACATGCGTGGTGG	960
TGGCAGTCAGAAGGAGAAGGGGGTTTTGGGTTCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTGTACGCCACCAC	
HumanIgG	
P S V F L F P P K P K D T L M I S R T P E V T C V V	→
G D T K R K G G F G L S V R M I E R V G S T V H T T T	
TGGACGTGAGCCACGAAGACCCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG	1040
ACCTGCACTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTACGGTTCTGTTTC	
HumanIgG	
V D V S H E D P E V K F N W Y V D G V E V H N A K T K	→
S T L W S S G S T L N L Q Y T S P T S T C L A L V F	
CCGCGGGAGGAGCAGTACAACAGCACGTACCGGGTGGTCAGCGTCCTCACCCTGCTGCACCAGGACTGGCTGAATGGCAA	1120
GGCGCCCTCCTCGTCATGTTGTCGTGCATGGCCACCAGTCGCAGGAGTGGCAGGACGTGGTCTTGACCGACTTACCGTT	
HumanIgG	
P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K	→
G R S S C Y L L V Y R T T L T R V T R C W S Q S F P L	
GGAGTACAAGTGAAGGTCTCCAACAAAGCCCTCCCAGCCCCATCGAGAAAACCATCTCCAAGCCAAAGGGCAGCCCC	1200
CCTCATGTTACAGTTCAGAGGTTGTTTCGGGAGGGTGGGGGTAGCTCTTTGGTAGAGGTTTCGGTTTCCGTCGGGG	
HumanIgG	
E Y K C K V S N K A L P A P I E K T I S K A K G Q P	→
S Y L H L T E L L A R G A G M S F V M	
GAGAACCACAGGTGTACACCTGCCCCCATCCGGGATGAGCTGACCAAGAACAGGTCAGCCTGACCTGCCTGGTCAAA	1280
CTCTTGGTGTCCACATGTGGGACGGGGTAGGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGACGGACCAAGTTT	
HumanIgG	
R E P Q V Y T L P P S R D E L T K N Q V S L T C L V K	→
GGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCAGCCTCCCGT	1360
CCGAAGATAGGGTCGCTGTAGCGGCACCTCACCTCTCGTTACCCGTGGCCCTCTTGTGATGTTCTGGTGGGAGGGCA	
HumanIgG	
G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V	→
GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCT	1440
CGACCTGAGGCTGCCGAGGAAGAAGGAGATGTCGTTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTCCCTTGCAGAAGA	
HumanIgG	
L D S D G S F F L Y S K L T V D K S R W Q Q G N V F	→
CATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACAGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAAAAGCCGGC	1520
GTACGAGGCACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCATTTTTTCGGCCG	
HumanIgG	
S C S V M H E A L H N H Y T Q K S L S L S P G K K A G	→
TTTTGGGTGCTGGTGGTGGTGGTGGAGTCTGGCTTGCTATAGCTTGTAGTAACAGTGGCCTTTATTATTTCTGGGT	1600
AAAACCCACGACCACCAACCACCTCAGGACCGAAGCATATCGAAGCATATTGTACCGGAAATAATAAAGACCCA	
CD28ALCK TD/IC domain	
F W V L V V V G G V L A C Y S L L V T V A F I I F W V	→

GAGGAGTAAGAGGAGCAGGCTCTGCACAGTGACTACATGAACATGACTCCCCGCCGCCCGGGCCACCCGCAAGCATT CTCCTCATTTCTCTCGTCCGAGGACGTGTCACTGATGTACTTGTACTGAGGGGCGGCGGGGCCCGGGTGGGCGTTTCGTAA	1680
CD28ΔLCK TD/IC domain	
R S K R S R L L H S D Y M N M T P R R P G P T R K H	→
ACCAGGCCTATGCCGCCGCACGCGACTTCGCAGCCTATCGCTCCCAATTGCTGAGAGTGAAGTTCAGCAGGAGCGCAGAC TGGTCCGGATACGGCGGCGTGCCTGAAGCGTCGGATAGCGAGGGTTAACGACTCTCACTTCAAGTCGTCCTCGCGTCTG	1760
CD28ΔLCK TD/IC domain	
P2A	
Y Q A Y A A A R D F A A Y R S Q L L R V K F S R S A D	→
GCCCCCGCTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTGGGA CGGGGCGGCATGGTCTGCCGGTCTTGGTCGAGATATTGCTCGAGTTAGATCCTGCTTCTCTCTCATGCTACAAAACCT	1840
P2A	
A P A Y Q Q G Q N Q L Y N E L N L G R R E E Y D V L D	→
CAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAGCCGAGAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAAGTGC GTTCTCTGCACCGGCCCTGGGACTCTACCCCTTTTCGGCTCTTCTCTTCTTGGGAGTCTTCCGGACATGTTACTTGACG	1920
P2A	
K R R G R D P E M G G K P R R K N P Q E G L Y N E L	→
AGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTT TCTTTCTATTCTACCGCTCCGGATGTCACTCTAACCTACTTTCGGCTCGCGGGCTCCCGTTCCTCCGCTGCTACCGGAA	2000
P2A	
Q K D K M A E A Y S E I G M K G E R R R G K G H D G L	→
TACCAAGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCGCTGCCCTCTCGCTCGACAGCGC ATGGTCCCAGAGTCATGTCGGTGGTTCCTGTGGATGCTCGGGAAAGTGACGTCCGGGACGGGGGAGCGCAGCTGTGCGG	2080
P2A	
Y Q G L S T A T K D T Y D A L H M Q A L P P R V D S A	→
M C A R G G R T S L A	→
TGGATCTGGAGCCACGAACCTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGTCTAAGCTTATGGGCA ACCTAGACCTCGGTGCTTGAAGAGAGACAATTTCTGTTCTCTGACCTTCTTTTGGGGCCAGGATTCTGAATACCCGT	2160
TGFβRII EC domain	
G S G A T N F S L L K Q A G D V E E N P G P K L M G	→
P D P A V F K E R N F C A P S T S S F G P G L S I P L	→
GGGGCTGTGAGGGGCTGTGGCCCTGCACATCGTGCTGTGGACCAGGATCGCCAGCACCATCCCCCCCCACGTGCAG CCCCGGACGACTCCCGGACACGGGGACGTGTAGCACGACCTGGTCTTAGCGGTCTGTTAGGGGGGGGTGCAGGTC	2240
TGFβRII EC domain	
R G L L R G L W P L H I V L W T R I A S T I P P H V Q	→
P R S L P R H G R C M T S H V L I A L V M G G W T C	→
AAGAGCGTGAACAACGACATGATCGTGACCGACAACAACGGCGCCGTGAAGTTCCCCAGCTGTGCAAGTTCTGCGACGT TTCTCGCACTTGTGCTGTACTAGCACTGGCTGTTGTTGCCGCGGCACCTTCAAGGGGTCGACACGTTCAAGACGCTGCA	2320
TGFβRII EC domain	
K S V N N D M I V T D N N G A V K F P Q L C K F C D V	→
F L T F L S M I T V S L L P A T F N G W S H L N Q S T	→
GAGGTTTCAGCACCTGCGACAACCAGAAGAGCTGCATGAGCAACTGCAGCATCACCAGCATCTGCGAGAAGCCCCAGGAGG CTCCAAGTCGTGGACGCTGTTGGTCTTCTCGACGTACTCGTTGACGTCGTAGTGGTCTGTAAGACGCTCTTCGGGGTCTCTC	2400
TGFβRII EC domain	
R F S T C D N Q K S C M S N C S I T S I C E K P Q E	→
L N L V Q S L W F L Q M L L Q L M V L M Q S F G W S T	→

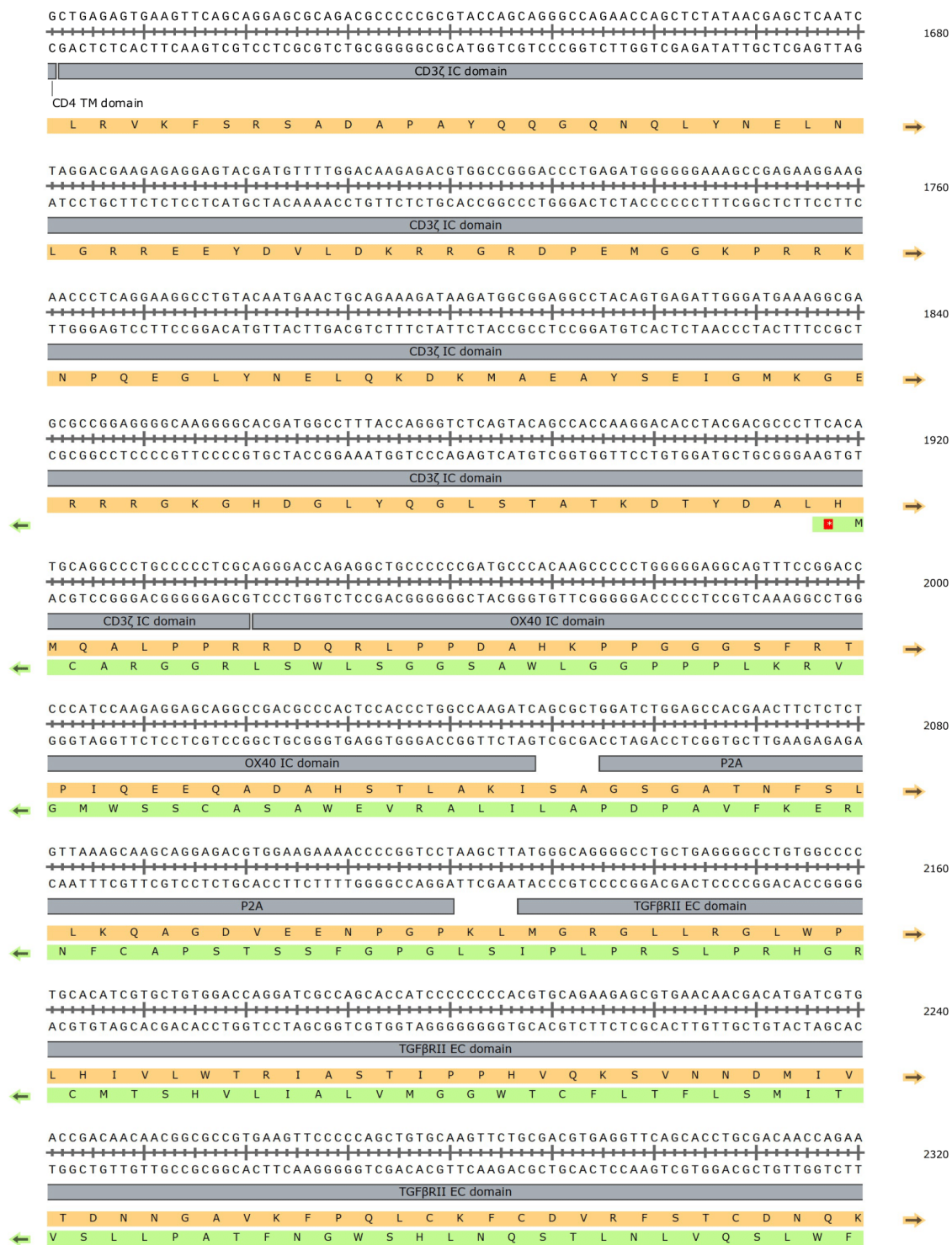
TGTGCGTGGCCGTGTGGAGGAAGAACGACGAGAACATCACCTGGAGACCGTGTGCCACGACCCCAAGCTGCCCTACCAC ACACGCACCGGCACACCTCCTTCTTGCTGCTCTTGTAGTGGGACCTCTGGCACACGGTGCTGGGGTTTCGACGGGATGGTG	2480
TGFβRII EC domain	
V C V A V W R K N D E N I T L E T V C H D P K L P Y H H T A T H L F F S S F M	→
GACTTCATCCTGGAGGACGCCGCCAGCCCCAAGTGCATCATGAAGGAGAAGAAGAAGCCCGGCGAGACCTTCTTCATGTG CTGAAGTAGGACCTCCTGCGGCGGTCTGGGGTTACGCTAGTACTTCTCTTCTTCTCGGGCCGCTCTGGAAGAAGTACAC	2560
TGFβRII EC domain	
D F I L E D A A S P K C I M K E K K K P G E T F F M C	→
CAGCTGCAGCAGCGACGAGTGCAACGACAACATCATCTTCAGCGAGGAGTACAACACCGCAACCCCGACGTGGTGATCA GTCGACGTCGCTGCTGCTACGTTGCTGTTGTAGTAGAAGTCGCTCCTCATGTTGTGGTCGTTGGGGCTGCACCACTAGT	2640
TGFβRII EC domain	
PDGFRβ TM domain	
S C S S D E C N D N I I F S E E Y N T S N P D V V I	→
GCGCCATCCTGGCCCTGGTGGTGCTGACCATCATCAGCCTGATCATCTGATCAACTGCAGGAACACCGGCCCTGGCTG CGCGGTAGGACCGGGACCAACGACTGGTAGTAGTCGGACTAGTAGGACTAGTTGACGTCCTTGTGGCCGGGGACCGAC	2720
PDGFRβ TM domain	
IL-2Rβ IC domain	
S A I L A L V V L T I I S L I I L I N C R N T G P W L	→
AAGAAGGTGCTGAAGTGCAACACCCCGACCCGACGCAAGTTCTTCAGCCAGCTGAGCAGCGAGCACGGCGGCACGTGCA TTCTTCCACGACTTCACGTTGTGGGGCTGGGGTCGTTCAAGAAGTCGGTCGACTCGTCGCTCGTGCCGCGCTGCACGT	2800
IL-2Rβ IC domain	
K K V L K C N T P D P S K F F S Q L S S E H G G D V Q	→
GAAGTGGCTGAGCAGCCCTTCCCCAGCAGCAGCTTCAGCCCCGGCGGCTGGCCCCGAGATCAGCCCTGGAGGTGC CTTCACCGACTCGTCGGGGAAGGGGTCTGTCGTCGAAGTCGGGGCCGCCGGGACCGGGGCTCTAGTCGGGGGACCTCCACG	2880
IL-2Rβ IC domain	
K W L S S P F P S S S F S P G G L A P E I S P L E V	→
TGGAGAGGGACAAGGTGACCCAGCTGCTGCTGTCAGCAGGACAAGGTGCCGAGCCCGCCAGCCTGAGCAGCAACCACAGC ACCTCTCCCTGTTCCACTGGGTGACGACGACGTCGTCCTGTTCCACGGGCTCGGGCGGTCGGACTCGTCGTTGGTGTCG	2960
IL-2Rβ IC domain	
L E R D K V T Q L L L Q Q D K V P E P A S L S S N H S	→
CTGACCAGCTGCTTCACCAACCAGGGCTACTTCTTCTTCACCTGCCCGACGCCCTGGAGATCGAGGCCTGCCAGGTGTA GACTGGTCGACGAAGTGGTGGTCCCGATGAAGAAGAAGGTGGACGGGCTCGGGACCTCTAGCTCCGGACGGTCCACAT	3040
IL-2Rβ IC domain	
L T S C F T N Q G Y F F F H L P D A L E I E A C Q V Y	→
CTTCACCTACGACCCCTACAGCGAGGAGGACCCCGACGAGGGCGTGCGCGGCGCCCCACCGGACGACGCCCCAGCCCC GAAGTGGATGCTGGGGATGTCGCTCCTCTGGGGCTGCTCCCGCACCGGCGCGGGGTTGGCGTCTGTCGGGGTCTGGGG	3120
IL-2Rβ IC domain	
F T Y D P Y S E E D P D E G V A G A P T G S S P Q P	→
TGCAGCCCTGAGCGGGCAGGACGACGCCTACTGCACCTTCCCCAGCAGGGACGACCTGCTGCTGTTACGCCACGCTG ACGTCGGGACTCGCCGCTCCTGCTGCGGATGACGTGGAAGGGGTCTGCCCTGCTGGACGACGACAAAGTCGGGGTCTGGAC	3200
IL-2Rβ IC domain	
L Q P L S G E D D A Y C T F P S R D D L L L F S P S L	→



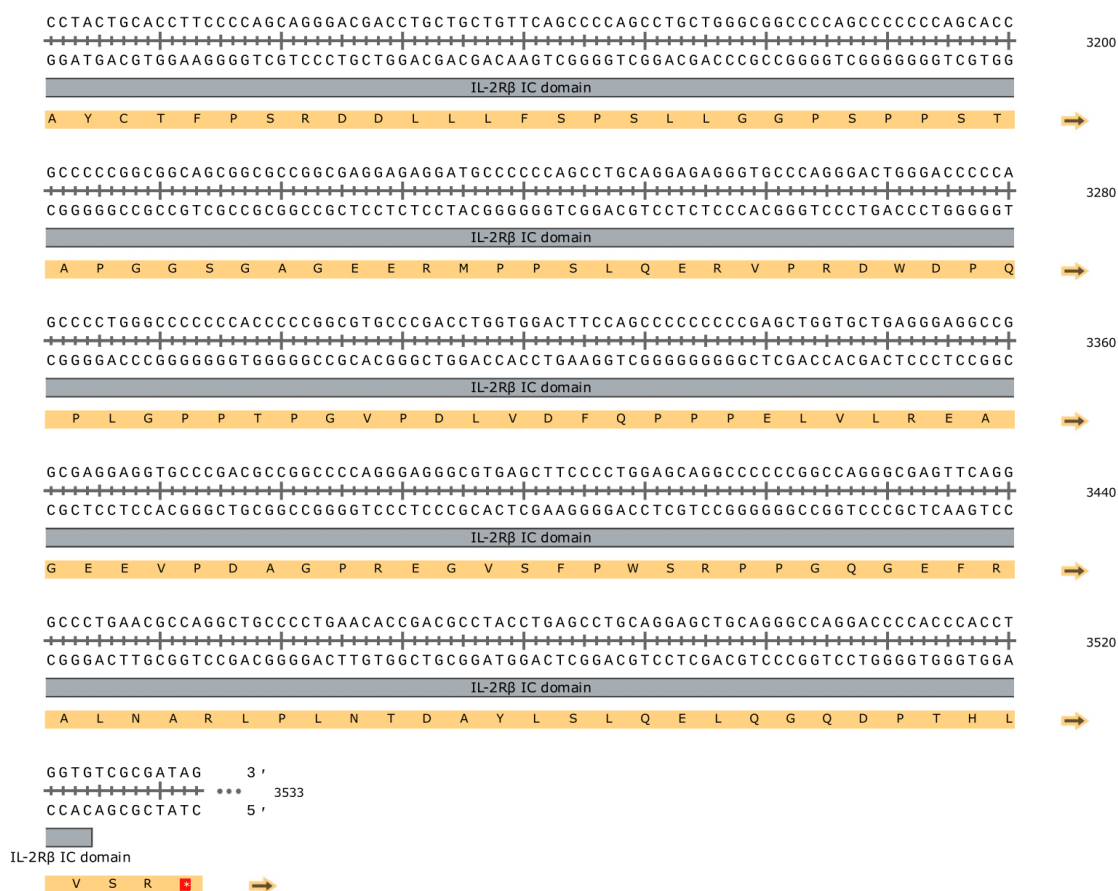
6.7 BW431/26scFv-Fc-CD3 ζ -OX40-P2A-TGF- β RII-PDGFR β TM-IL-2R β (#2362)

5'	CCATGGATTTCAGGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCCTCAGTCATAATGTCTAGAGGTGCCACTCCAG	80
3'	GGTACCTAAAGTCCACGTCCTAAAGTCGAAGGACGATTAGTCACGGAGTCAGTATTACAGATCTCCACAGGTGAGGGTC	
	Kappa Leader BW431/26scFv	
	M D F Q V Q I F S F L L I S A S V I M S R G V H S Q	→
	GTCCAAC TGCAGGAGTCAGGTCCAGGTCTTGTGAGACCTAGCCAGACCC T GAGCCTGACCTGCACCGTGTCTGGCTTCAC	160
	CAGGTTGACGTCCTCAGTCCAGGTCCAGAACACTCTGGATCGGTCTGGGACTCGGACTGGACGTGGCACAGACCGAAGTG	
	BW431/26scFv	
	V Q L Q E S G P G L V R P S Q T L S L T C T V S G F T	→
	CATCAGCAGTGGTTATAGCTGGCACTGGGTGAGACAGCCACCTGGACGAGGTCTTGAGTGGATTGGATACATACAGTACA	240
	GTAGTCGTCACCAATATCGACCGTGACCCACTCTGTCTGGTGGACCTGCTCCAGAAGTCACTAACCTATGTATGTCTATGT	
	BW431/26scFv	
	I S S G Y S W H W V R Q P P G R G L E W I G Y I Q Y	→
	GTGGTATCACTAACTACAACCCCTCTCTCAAAAGTAGAGTGACAATGCTGGTAGACACCAGCAAGAACCAGTTTCAGCCTG	320
	CACCATAGTGATTGATGTTGGGGAGAGAGTTTTCATCTCACTGTTACGACCATCTGTGGTCTGTTCTTGGTCAAGTCGGAC	
	BW431/26scFv	
	S G I T N Y N P S L K S R V T M L V D T S K N Q F S L	→
	AGACTCAGCAGCGTGACAGCCGCCGACACCGCGGTCTATTATTGTGCAAGAGAAGACTATGATTACCACTGGTACTTTCGA	400
	TCTGAGTCGTCGCACTGTCGGCGGCTGTGGCGCCAGATAATAACACGTTCTCTTCTGATACTAATGGTGACCATGAAGCT	
	BW431/26scFv	
	R L S S V T A A D T A V Y Y C A R E D Y D Y H W Y F D	→
	TGTCTGGGGCCAAAGGGACACGGTCACCGTCTCCTCAGGAGGTGGTGGATCGGGCGGTGGCGGGTCGGGTGGCGGGCGGAT	480
	ACAGACCCCGGTTCCCTGGTGCCAGTGGCAGAGGAGTCTCCACCACCTAGCCCGCCACCGCCAGCCACCGCCCGCTA	
	BW431/26scFv	
	V W G Q G T T V T V S S G G G G S G G G G S G G G G	→
	CTGACATCCAGCTGACCCAGAGCCCAAGCAGCCTGAGCGCCAGCGTGGGTGACAGAGTGACCATCACCTGTAGTACCAGC	560
	GACTGTAGGTCGACTGGGTCTCGGGTTCGTGGGACTCGCGGTTCGCACCCACTGTCTCACTGGTAGTGACATCATGGTCTG	
	BW431/26scFv	
	S D I Q L T Q S P S S L S A S V G D R V T I T C S T S	→
	TCAAGTGTAAGTTACATGCACTGGTACCAGCAGAAGCCAGGTAAGGCTCCAAAGCTGCTGATCTACAGCACATCCAACCT	640
	AGTTACATTCAATGTACGTGACCATGGTCGTCTTCGGTCCATTCCGAGGTTTCGACGACTAGATGTCGTGTAGGTTGGA	
	BW431/26scFv	
	S S V S Y M H W Y Q Q K P G K A P K L L I Y S T S N L	→
	GGCTTCTGGTGTGCCAAGCAGATTACGCGGTAGCGGTAGCGGTACCGACTTCACCTTCACCATCAGCAGCCTCCAGCCAG	720
	CCGAAGACCACACGGTTCGTCTAAGTCGCCATCGCCATCGCCATGGCTGAAGTGAAGTGGTAGTCGTGGAGGTGGGTC	
	BW431/26scFv	
	A S G V P S R F S G S G S G T D F T F T I S S L Q P	→
	AGGACATCGCCACCTACTACTGCCATCAGTGGAGTAGTTATCCACGTTTCGGCCAAAGGACCAAGGTGGAGATCAAAGTG	800
	TCCTGTAGCGGTGGATGATGACGGTAGTCACCTCATCAATAGGGTGCAAGCGGTTCCCTGGTTCCACCTCTAGTTTCAC	
	BW431/26scFv	
	E D I A T Y Y C H Q W S S Y P T F G Q G T K V E I K V	→

GGATCCGATCCCGCCGAGCCCAATCTCCTGACAAAACCTCACACATGCCACCGTGCCCAAGCACCTGAACTCCTGGGGGG CCTAGGCTAGGGCGGCTCGGGTTTAGAGGACTGTTTGAAGTGTGTACGGGTGGCACGGGTCGTGGACTTGAGGACCCCC	880
HumanIgG	
G S D P A E P K S P D K T H T C P P C P A P E L L G G V H G G H G A G S S R P P	
ACCGTCAGTCTTCTCTTCCCCCAAAACCAAGGACACCCCTCATGATCTCCCGGACCCCTGAGGTACATGCGTGGTGG TGGCAGTCAGAAGGAGAAGGGGGTTTGGGTTCTGTGGGAGTACTAGAGGGCCTGGGACTCCAGTGTACGCACCACC	960
HumanIgG	
P S V F L F P P K P K D T L M I S R T P E V T C V V G D T K R K G G F G L S V R M I E R V G S T V H T T T	
TGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAAG ACCTGCACTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTACGGTTCTGTTTC	1040
HumanIgG	
V D V S H E D P E V K F N W Y V D G V E V H N A K T K S T L W S S G S T L N L Q Y T S P T S T C L A L V F	
CCGCGGGAGGAGCAGTACAACAGCACGTACCGGGTGGTCAGCGTCCTCACCGTCTGCACCAGGACTGGCTGAATGGCAA GGCGCCCTCCTCGTCATGTTGTCTGTCATGGCCACCAAGTCGACGAGGTGGCAGGACGTGGTCTCTGACCGACTTACCGTT	1120
HumanIgG	
P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K G R S S C Y L L V Y R T T L T R V T R C W S Q S F P L	
GGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAGCCAAAGGGCAGCCCC CCTCATGTTACAGTTCAGAGGTTGTTTCGGGAGGGTTCGGGGTAGCTCTTTGGTAGAGGTTTCGGTTTCCCGTCGGGG	1200
HumanIgG	
E Y K C K V S N K A L P A P I E K T I S K A K G Q P S Y L H L T E L L A R G A G M S F V M	
GAGAACCACAGGTGTACACCCTGCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTACGCCTGACCTGCCTGGTCAAA CTCTTGGTGTCCACATGTGGGACGGGGTAGGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGACGGACCAAGTTT	1280
HumanIgG	
R E P Q V Y T L P P S R D E L T K N Q V S L T C L V K	
GGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACCTACAAGACCACGCTCCCGT CCGAAGATAGGGTCGCTGTAGCGGCACCTCACCTCTCGTTACCCGTCGGCCTCTTGTGATGTTCTGGTGCAGGAGGCA	1360
HumanIgG	
G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V	
GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCT CGACCTGAGGCTGCCAGGAAGAAGGAGATGTCGTTGAGTGGCACCTGTTCTCGTCCACCGTCGTCCCTTGCAGAAGA	1440
HumanIgG	
L D S D G S F F L Y S K L T V D K S R W Q Q G N V F	
CATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAAGAAGCCTCTCCCTGTCTCCGGGTAAAAAAGATCCT GTACGAGGCACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCATTTTTTCTAGGA	1520
HumanIgG	
CD4 TM domain S C S V M H E A L H N H Y T Q K S L S L S P G K K D P	
CAGCCAATGGCCCTGATTGTGCTGGGGGGCGTCGCCGGCCTCCTGCTTTTCATTGGGCTAGGCATCTTCTTCTGTGTCA GTCGGTTACCGGGAATAACACGACCCCCGACGGCCGGAGGACGAAAAGTAACCCGATCCGTAGAAGAAGACACAGTC	1600
CD4 TM domain	
Q P M A L I V L G G V A G L L L F I G L G I F F C V R	



←	GAGCTGCATGAGCAACTGCAGCATCACCAGCATCTGCGAGAAGCCCCAGGAGGTGTGCGTGGCCGTGTGGAGGAAGAACG	2400
	CTCGACGTACTCGTTGACGTCGTAGTGGTCGTAGACGCTCTTCGGGGTCCTCCACACGCACCGGCACACCTCCTTCTTGC	
	TGFβRII EC domain	
←	S C M S N C S I T S I C E K P Q E V C V A V W R K N	→
	L Q M L L Q L M V L M Q S F G W S T H T A T H L F F S	
←	ACGAGAACATCACCCTGGAGACCGTGTGCCACGACCCCAAGCTGCCCTACCACGACTTCATCCTGGAGGACGCCGCCAGC	2480
	TGCTCTTGTAGTGGGACCTCTGGCACACGGTGCTGGGGTTCGACGGGATGGTGCTGAAGTAGGACCTCCTGCGGCGGTCTG	
	TGFβRII EC domain	
←	D E N I T L E T V C H D P K L P Y H D F I L E D A A S	→
	S F M	
←	CCCAAGTGCATCATGAAGGAGAAGAAGAAGCCCGGCGAGACCTTCTTCATGTGCAGCTGCAGCAGCGACGAGTGCAACGA	2560
	GGGTTTCACGTAGTACTTCTCTTCTTCTTCGGGCGCTCTGGAAGAAGTACACGTCGACGTCGTGCTGCTCACGTTGCT	
	TGFβRII EC domain	
	P K C I M K E K K K P G E T F F M C S C S S D E C N D	→
←	CAACATCATCTTCAGCGAGGAGTACAACACCAAGCAACCCCGACGTGGTGATCAGCGCCATCCTGGCCCTGGTGGTGCTGA	2640
	GTTGTAGTAGAAGTCGCTCCTCATGTTGTGGTCGTTGGGGCTGCACCACTAGTCGCGGTAGGACCGGACCAACCAAGCT	
	TGFβRII EC domain PDGFRβ TM domain	
	N I I F S E E Y N T S N P D V V I S A I L A L V V L	→
←	CCATCATCAGCCTGATCATCCTGATCAACTGCAGGAACACCGGCCCTGGCTGAAGAAGGTGCTGAAGTGCAACACCCCC	2720
	GGTAGTAGTCGGACTAGTAGGACTAGTTGACGTCCTTGTGGCCGGGACCGACTTCTTCCACGACTTCACGTTGTGGGGG	
	PDGFRβ TM domain IL-2Rβ IC domain	
	T I I S L I I L I N C R N T G P W L K K V L K C N T P	→
←	GACCCAGCAAGTTCTTCAGCCAGCTGAGCAGCGAGCACGGCGGCGACGTGCAGAAGTGGCTGAGCAGCCCTTCCCCAG	2800
	CTGGGGTCGTTCAAGAAGTCGGTCGACTCGTCGCTCGTGCCGCGCTGCACGTCTTCACCGACTCGTCGGGGAAGGGGTC	
	IL-2Rβ IC domain	
	D P S K F F S Q L S S E H G G D V Q K W L S S P F P S	→
←	CAGCAGCTTCAGCCCCGGCGGCTGCCCCCGAGATCAGCCCCCTGGAGGTGCTGGAGAGGGACAAGGTGACCCAGCTGC	2880
	GTCGTCGAAGTCGGGGCCGCCGGACCGGGGCTCTAGTCGGGGACCTCCACGACCTCTCCCTGTTCCACTGGGTCGACG	
	IL-2Rβ IC domain	
	S S F S P G G L A P E I S P L E V L E R D K V T Q L	→
←	TGCTGCAGCAGGACAAGGTGCCCGAGCCCGCCAGCCTGAGCAGCAACACAGCCTGACCAGCTGCTTCACCAACCAAGGGC	2960
	ACGACGTCGTCCTGTTCCACGGGCTCGGGCGGTGCGACTCGTCGTTGGTGTCGGACTGGTCGACGAAGTGGTTGGTGCCG	
	IL-2Rβ IC domain	
	L L Q Q D K V P E P A S L S S N H S L T S C F T N Q G	→
←	TACTTCTTCTTCACCTGCCCGACGCCCTGGAGATCGAGGCCTGCCAGGTGTACTTCACCTACGACCCCTACAGCGAGGA	3040
	ATGAAGAAGAAGGTGGACGGGCTGCGGGACCTCTAGCTCCGGACGGTCCACATGAAGTGGATGCTGGGGATGTCGCTCCT	
	IL-2Rβ IC domain	
	Y F F F H L P D A L E I E A C Q V Y F T Y D P Y S E E	→
←	GGACCCCGACGAGGGCGTGGCCGGCGCCCCACCGGCAGCAGCCCCAGCCCTGACGCCCTGAGCGGCGAGGACGACG	3120
	CCTGGGGCTGCTCCCGACCGGCGGGGGTGGCCGTCGTCGGGGTGGGGACGTCGGGGACTCGCCGCTCCTGCTGC	
	IL-2Rβ IC domain	
	D P D E G V A G A P T G S S P Q P L Q P L S G E D D	→



6.8 BW431/26scFv-Fc-4-1BB-CD3 ζ -P2A-TGF- β RII-PDGFR β TM (#2410)

5'	CCATGGATTTCAGGTGCAGATTTTCAGCTTCTGCTAATCAGTGCCTCAGTCATAATGCTAGAGGTGTCCACTCCAG	80
3'	GGTACCTAAAGTCCACGTCCTAAAGTCGAAGGACGATTAGTCACGGAGTCAGTATTACAGATCTCCACAGGTGAGGGTC	
	Kappa Leader	BW431/26scFv
	M D F Q V Q I F S F L L I S A S V I M S R G V H S Q	→
	GTCCAAC TGCAGGAGTCAGGTCCAGGTCTTGTGAGACCTAGCCAGACCC T GAGCCTGACCTGCACCGTGTCTGGCTTCAC	160
	CAGGTTGACGTCCTCAGTCCAGGTCCAGAACACTCTGGATCGGTCTGGGACTCGGACTGGACGTGGCACAGACCGAAGTG	
	BW431/26scFv	
	V Q L Q E S G P G L V R P S Q T L S L T C T V S G F T	→
	CATCAGCAGTGGTTATAGCTGGCACTGGGTGAGACAGCCACCTGGACGAGGTCTTGAGTGGATTGGATACATACAGTACA	240
	GTAGTCGTCACCAATATCGACCGTGACCCACTCTGTCGGTGGACCTGCTCCAGAACTCACCTAACCTATGTATGTCTATGT	
	BW431/26scFv	
	I S S G Y S W H W V R Q P P G R G L E W I G Y I Q Y	→
	GTGGTATCACTAACTACAACCCCTCTCTCAAAAGTAGAGTGACAATGCTGGTAGACACCAGCAAGAACCAGTTTCAGCCTG	320
	CACCATAGTGATTGATGTTGGGGAGAGAGTTTTCATCTCACTGTTACGACCATCTGTGGTGGTCTTGGTCAAGTCGGAC	
	BW431/26scFv	
	S G I T N Y N P S L K S R V T M L V D T S K N Q F S L	→
	AGACTCAGCAGCGTGACAGCCGCCGACACCGCGGTCTATTATTGTGCAAGAGAAGACTATGATTACCACTGGTACTTTCGA	400
	TCTGAGTCGTCGCACTGTCGGCGGCTGTGGCGCCAGATAATAACACGTTCTCTTCTGATACTAATGGTGACCATGAAGCT	
	BW431/26scFv	
	R L S S V T A A D T A V Y Y C A R E D Y D Y H W Y F D	→
	TGTCTGGGGCCAAAGGGACCACGGTCACCGTCTCCTCAGGAGGTGGTGGATCGGGCGGTGGCGGGTCGGGTGGCGGGCGGAT	480
	ACAGACCCCGGTTCCCTGGTGCCAGTGGCAGAGGAGTCTCCACCACCTAGCCCGCCACCGCCAGCCACCGCCGCTA	
	BW431/26scFv	
	V W G Q G T T V T V S S G G G G S G G G G S G G G G	→
	CTGACATCCAGCTGACCCAGAGCCCAAGCAGCCTGAGCGCCAGCGTGGGTGACAGAGTGACCATCACCTGTAGTACCAGC	560
	GACTGTAGGTCGACTGGGTCTCGGGTTCGTGGGACTCGCGGTTCGCACCCACTGTCTCACTGGTAGTGACATCATGGTCTG	
	BW431/26scFv	
	S D I Q L T Q S P S S L S A S V G D R V T I T C S T S	→
	TCAAGTGTAAGTTACATGCACTGGTACCAGCAGAAGCCAGGTAAGGCTCCAAAGCTGCTGATCTACAGCACATCCAACCT	640
	AGTTACATTCAATGTACGTGACCATGGTCGTCTTCGGTCCATTCCGAGGTTTCGACGACTAGATGTCGTGTAGGTTGGA	
	BW431/26scFv	
	S S V S Y M H W Y Q Q K P G K A P K L L I Y S T S N L	→
	GGCTTCTGGTGTGCCAAGCAGATTACGCGGTAGCGGTAGCGGTACCGACTTCACCTTCACCATCAGCAGCCTCCAGCCAG	720
	CCGAAGACCACACGGTTCGTCTAAGTCGCCATCGCCATCGCCATGGCTGAAGTGAAGTGGTAGTCGTGGAGGTGGGTC	
	BW431/26scFv	
	A S G V P S R F S G S G S G T D F T F T I S S L Q P	→
	AGGACATCGCCACCTACTACTGCCATCAGTGGAGTAGTTATCCACGTTTCGGGCCAAGGGACCAAGGTGGAGATCAAAGTG	800
	TCCTGTAGCGGTGGATGATGACGGTAGTCACCTCATCAATAGGGTGCAAGCGGTTCCCTGGTTCACCTCTAGTTTCAC	
	BW431/26scFv	
	E D I A T Y Y C H Q W S S Y P T F G Q G T K V E I K V	→

GGATCCGATCCCGCCGAGCCCAAATCTCCTGACAAAACCTCACACATGCCACCGTGCCGACGACCTGAACTCCTGGGGGG	
CCTAGGCTAGGGCGGCTCGGGTTTAGAGGACTGTTTGAAGTGTGTACGGGTGGCACGGGTCGTGGACTTGAGGACCCCC	880
HumanIgG	
G S D P A E P K S P D K T H T C P P C P A P E L L G G	→
V H G G H G A G S S R P P	
ACCGTCAGTCTTCTCTTCCCCCAAAACCAAGGACACCCCTCATGATCTCCGGGACCCCTGAGGTACATGCGTGGTGG	
TGGCAGTCAGAGGAGAAGGGGGGTTTGGGTTCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTGTACGCACCCACC	960
HumanIgG	
P S V F L F P P K P K D T L M I S R T P E V T C V V	→
G D T K R K G G F G L S V R M I E R V G S T V H T T T	
TGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG	
ACCTGCACCTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTACGGTTCTGTTTC	1040
HumanIgG	
V D V S H E D P E V K F N W Y V D G V E V H N A K T K	→
S T L W S S G S T L N L Q Y T S P T S T C L A L V F	
CCGCGGGAGGAGCAGTACAACAGCACGTACCGGGTGGTCAGCGTCCTCACCGTCCTGCACGAGGACTGGCTGAATGGCAA	
GGCGCCCTCCTCGTCATGTTGTCGTGCATGGCCCAACAGTCGACAGGAGTGGCAGGACGTGGTCTGACCGACTTACCGTT	1120
HumanIgG	
P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K	→
G R S S S C Y L L V Y R T T L T R V T R C W S Q S F P L	
GGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCAGCCCCATCGAGAAAACCATCTCCAAGCCAAAGGGCAGCCCC	
CCTCATGTTACAGTTCAGAGGTTGTTTCGGGAGGGTCGGGGTAGCTCTTTGGTAGAGGTTTCGGTTTCCGCTCGGGG	1200
HumanIgG	
E Y K C K V S N K A L P A P I E K T I S K A K G Q P	→
S Y L H L T E L L A R G A G M S F V M	
GAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTACGCTGACCTGCCTGGTCAAA	
CTCTTGGTGTCCACATGTGGGACGGGGGTAGGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGACGGACCAAGTTT	1280
HumanIgG	
R E P Q V Y T L P P S R D E L T K N Q V S L T C L V K	→
GGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCGGAGAGCAACTACAAGACCACGCTCCCGT	
CCGAAGATAGGGTCGCTGTAGCGGCACCTCACCTCTCGTTACCCGTCGGCCTCTTGTGATGTTCTGGTGGGAGGGCA	1360
HumanIgG	
G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V	→
GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCT	
CGACCTGAGGCTGCCGAGGAAGAAGGAGATGTGCTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTCCCTTGCAGAAGA	1440
HumanIgG	
L D S D G S F F L Y S K L T V D K S R W Q Q G N V F	→
CATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAAGAGCCTCTCCCTGTCTCCGGGTAAAAAGCCGGC	
GTACGAGGCACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCATTTTTTCGGCCG	1520
HumanIgG	
S C S V M H E A L H N H Y T Q K S L S L S P G K K A G	→
GATCCTCAGCCAATGGCCCTGATTGTGCTGGGGGGCGTCGCCGGCCTCCTGCTTTTCATTGGGCTAGGCATCTTCTCTG	
CTAGGAGTCGGTTACCGGGACTAACACGACCCCCCGCAGCGCCGAGGACGAAAAGTAACCCGATCCGTAGAAGAAGAC	1600
CD4 TM domain	
D P Q P M A L I V L G G V A G L L L F I G L G I F F C	→

	TGTCAGGAAACGGGGCAGAAAGAACTCCTGTATATATTCAAACAACCATTTATGAGACCAGTACAACTACTCAAGAGG ACAGTCCTTTGCCCCGCTTTCTTTGAGGACATATATAAGTTTGGTAAATACTCTGGTCATGTTTGATGAGTTCTCC	1680
	CD4 TM domain	
	V R K R G R K K L L Y I F K Q P F M R P V Q T T Q E	→
	AAGATGGCTGTAGCTGCCGATTTCAGAAGAAGAAGGAGGATGCGAACTGCAATTGCTGAGAGTGAAGTTCAGCAGG TTCTACCGACATCGACGGCTAAAGGCTTCTTCTTCTCTACGCTTGACGTTAACGACTCTCACTTCAAGTCGTCC	1760
	41BB IC domain	
	E D G C S C R F P E E E E G G C E L Q L L R V K F S R	→
	AGCGCAGACGCCCCGCGTACCAGCAGGGCCAGAACAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGA TCGCGTCTGCGGGGGCGCATGTCGTCGCCGCTTGGTCGAGATATTGCTCGAGTTAGATCCTGCTTCTCTCCTCATGCT	1840
	CD3ζ IC domain	
	S A D A P A Y Q Q G Q N Q L Y N E L N L G R R E E Y D	→
	TGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAGCCGAGAAGGAAGAACCCCTCAGGAAGGCCTGTACA ACAAACCTGTTCTCTGACCGGCCCTGGGACTCTACCCCTTTCGGCTTCTCTTCTGGGAGTCTTCCGGACATGT	1920
	CD3ζ IC domain	
	V L D K R R G R D P E M G G K P R R K N P Q E G L Y	→
	ATGAACTGCAGAAAGATAAGATGGCGGAGGCCCTACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCAC TACTTGACGTCTTCTATTCTACCGCCTCCGGATGTCACTCTAACCTACTTTCGGCTCGCGGCCCTCCCGGTTCCCGGTG	2000
	CD3ζ IC domain	
	N E L Q K D K M A E A Y S E I G M K G E R R R G K G H	→
	GATGGCCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCCCTCTCGCGT CTACCGGAAATGGTCCCAGAGTCATGTCGGTGTTCTGTGGATGCTGCGGGAAGTGACGTCCGGGACGGGGAGCGCA	2080
	CD3ζ IC domain	
←	D G L Y Q G L S T A T K D T Y D A L H M Q A L P P R V	→
	M C A R G G R T	
	CGACAGCGCTGGATCTGGAGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCTAAGC GCTGTGCGACCTAGACCTCGGTGCTTGAAGAGAGACAATTTGCTTCTGCTCTGACCTTCTTTTGGGGCCAGGATTCTG	2160
	P2A	
←	D S A G S G A T N F S L L K Q A G D V E E N P G P K	→
	S L A P D P A V F K E R N F C A P S T S S F G P G L S	
	TTATGGGCAGGGGCTGCTGAGGGGCTGTGGCCCTGCACATCGTGCTGTGGACCAGGATGCCAGCACCATCCCCC AATACCCGTCGCCGACGACTCCCCGGACACCGGGGACGTGTAGCAGACACCTGGTCCTAGCGGTCTGTGGTAGGGGGG	2240
	TGFβRII EC domain	
←	L M G R G L L R G L W P L H I V L W T R I A S T I P P	→
	I P L P R S L P R H G R C M T S H V L I A L V M G G	
	CACGTGCAGAAGAGCGTGAACAACGACATGATCGTGACCGACAACAACGGCGCCGTGAAGTTCCTCCAGCTGTGCAAGTT GTGCACGTCTTCTCGCACTTGTGTGCTGTACTAGCACTGGCTGTTGTTGCCGGGCACTTCAAGGGGGTCGACACGTTCAA	2320
	TGFβRII EC domain	
←	H V Q K S V N N D M I V T D N N G A V K F P Q L C K F	→
	W T C F L T F L S M I T V S L L P A T F N G W S H L N	
	CTGCGACGTGAGGTTTCAGCACCTGCGACAACCAGAAGAGCTGCATGAGCAACTGCAGCATCACCAGCATCTGCGAGAAGC GACGCTGCACTCCAAGTCGTGGACGCTGTTGGTCTTCTCGACGTACTCGTTGACGTCGTAGTGCTCGTAGACGCTCTTCG	2400
	TGFβRII EC domain	
←	C D V R F S T C D N Q K S C M S N C S I T S I C E K	→
	Q S T L N L V Q S L W F L Q M L L Q L M V L M Q S F G	

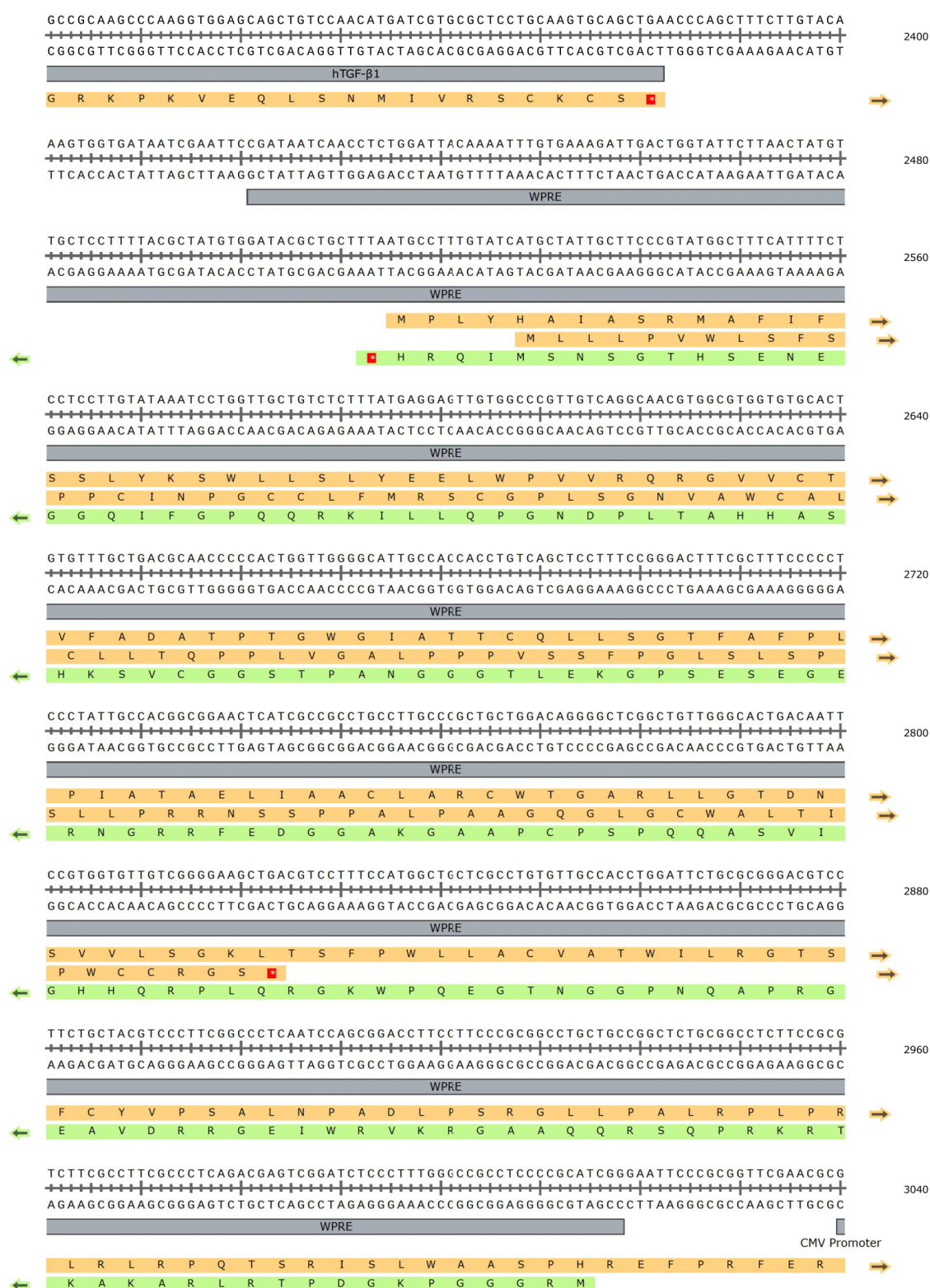


6.9 pLV[Exp]-mCherry-EF1A-TGF- β 1 (#2561)

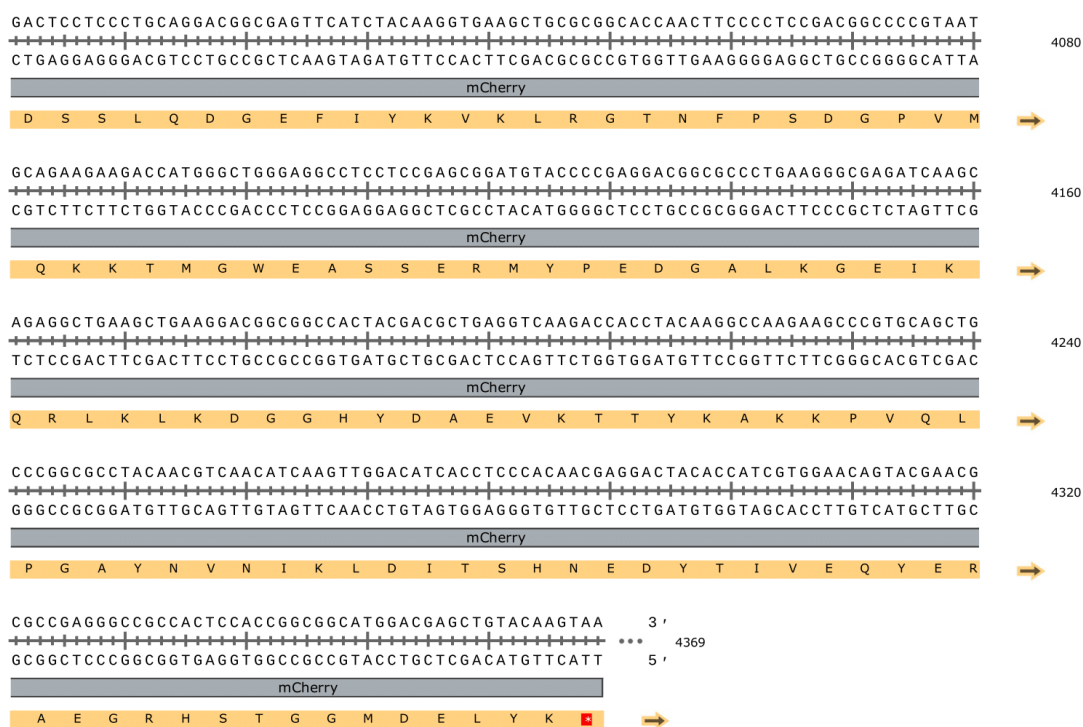




TATATATGTTCTTCAACACATCAGAGCTCCGAGAAGCGGTACCTGAACCCGTTGCTCTCCCGGGCAGAGCTGCGTCTG ATATATACAAGAAGTTGTGTAGTCTCGAGGCTCTTCGCCATGGACTTGGGCACAACGAGAGGGGCCGCTCTCGACGCAAGC	1680
hTGF-β1	
I Y M F F N T S E L R E A V P E P V L L S R A E L R L Y I N K L V D S S R S A T G S G T N S E R A S S R R	→
CTGAGGCTCAAGTTAAAAGTGGAGCAGCAGTGGAGCTGTACCAGAAATACAGCAACAATTCTGGCGATACCTCAGCAA GACTCCGAGTTCAATTTTCACCTCGTCGTGCACCTCGACATGGTCTTTATGTCGTTGTTAAGGACCGCTATGGAGTCGTT	1760
hTGF-β1	
L R L K L K V E Q H V E L Y Q K Y S N N S W R Y L S N S L S L N F T S C C T S S Y W F Y L L L E Q R Y R L L	→
CCGGCTGCTGGCAGCCAGCGACTCGCCAGAGTGGTTATCTTTTGATGTCACCGGAGTTGTGCGGCAGTGGTTGAGCCGTG GGCCGACGACCGTGGGTGCTGAGCGGTCTCACCAATAGAAAACTACAGTGGCCTCAACACGCCGTCACCAACTCGGCAC	1840
hTGF-β1	
R L L A P S D S P E W L S F D V T G V V R Q W L S R R S S A G L S E G S H N D K S T V P T T R C H N L R P R K I D G S N H P L P Q A T	→
GAGGGGAAATTGAGGGCTTTTCGCCTTAGCGCCCACTGCTCCTGTGACAGCAGGGATAACACACTGCAAGTGGACATCAAC CTCCCTTTAACTCCCGAAAGCGGAATCGCGGGTGACGAGGACACTGTCGTCCTATTGTGTGACGTTACCTGTAGTTG	1920
hTGF-β1	
G G E I E G F R L S A H C S C D S R D N T L Q V D I N P S I S P K R R L A W Q E Q S L L S L V S C T S M L S P F N L A K A K A G V A G T V A P I V C Q L H V D V	→
GGGTTCACTACCGGCCGCGGAGGTGACCTGGCCACCATTCATGGCATGAACCGGCCCTTTCTGCTTCTCATGGCCACCCC CCCAAGTGATGGCGGGCGGCTCCACTGGACCGGTGGTAAGTACCGTACTTGCCGGAAAGGACGAAGAGTACCGGTGGGG	2000
hTGF-β1	
G F T T G R R G D L A T I H G M N R P F L L L M A T P P N V V P R R P S R A V M P E S G A A S T V Q G G N M A H V P R E Q K E H G G R	→
GCTGGAGAGGGCCAGCATCTGCAAAGCTCCCGGCACCGCCGAGCCCTGGACACCAACTATTGCTTCAGCTCCACGGAGA CGACCTCTCCCGGGTCTGACGTTTCGAGGGCCGTGGCGGCTCGGGACCTGTGGTTGATAACGAAGTCGAGGTGCCTCT	2080
hTGF-β1	
L E R A Q H L Q S S R H R R A L D T N Y C F S S T E Q L P G L M Q L A G P V A S G Q V G V I A E A G R L	→
AGAACTGCTGCGTGGCGCAGCTGTACATTGACTTCCGCAAGGACCTCGGCTGGAAGTGGAATCCACGAGCCCAAGGGCTAC TCTTGACGACGACGCCGTCGACATGTAAGTGAAGGCGTTCTGGAGCCGACCTTCACCTAGGTGCTCGGGTTCCCGATG	2160
hTGF-β1	
K N C C V R Q L Y I D F R K D L G W K W I H E P K G Y L V A A H P L Q V N V E A L V E A P L P D V L G L A V	→
CATGCCAACTTCTGCCTCGGGCCCTGCCCTACATTTGGAGCCTGGACACGACGAGTACAGCAAGGTCCTGGCCCTGTACAA GTACGGTTGAAGACGGAGCCCGGGACGGGATGTAAACCTCGGACCTGTGCGTCATGTCGTTCCAGGACCGGGACATGTT	2240
hTGF-β1	
H A N F C L G P C P Y I W S L D T Q Y S K V L A L Y N M G V E A E P G A G V N P A Q V R L V A L D Q G Q V V	→
CCAGCATAACCCGGGCGCCTCGGGCGGCGCTGCTGCGTGCCGACGGCGCTGGAGCCGCTGCCATCGTGTAATACTACGTGG GGTCTGATTGGGGCCGCGGAGCCGCGCGGACGACGACGCGCTCCGCGACCTCGGGCAGGGGTAGCACATGATGCACC	2320
hTGF-β1	
Q H N P G A S A A P C C V P Q A L E P L P I V Y Y V L M	→



TTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTTCATAGCCCATATATGGAGTTCCGCG AACTGTAACATAAAGTATCAATAATTATCATTAGTTAATGCCCCAGTAATCAAGTATCGGGTATATACCTCAAGGCGC CMV Promoter V D I D Y	3120
TTACATAACTTACGGTAAATGGCCCGCTGGCTGACCGCCCAACGACCCCGCCATTGACGTCAATAATGACGTATGTT AATGTATTGAATGCCATTTACGGGGCGGACCGACTGGCGGGTGGCTGGGGCGGGTAACGTCAGTTATTACTGCATACAA CMV Promoter	3200
CCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA GGGTATCATTGCGGTTATCCCTGAAAGGTAAGTGCAGTTACCCACCTCATAAATGCCATTTGACGGGTGAACCGTCATGT CMV Promoter	3280
TCAAGTGATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCTGGCATTATGCCCAGTACA AGTTCACATAGTATACGGTTCATGCGGGGGATAACTGCAGTTACTGCCATTACCGGGCGGACCGTAATACGGGTCATGT CMV Promoter	3360
TGACCTTATGGGACTTTCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAG ACTGGAATACCCTGAAAGGATGAACCGTCATGTAGATGCATAATCAGTAGCGATAATGGTACCACTACGCCAAACCGTC CMV Promoter	3440
TACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCGAAGTCTCCACCCCAATTGACGTCAATGGGAGTTTGT ATGTAGTTACCCGCACCTATCGCCAAACTGAGTGCCCCCTAAAGGTTTCAGAGGTGGGGTAACTGCAGTTACCCCTCAAACAA CMV Promoter	3520
TTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACCTCGCCCCATTGACGCAAAATGGGCGGTAGGCGTGTAC AACCGTGGTTTTAGTTGCCCTGAAAGGTTTTACAGCATTGTTGAGGCGGGGTAACGCGTTTTACCGCCATCCGCACATG CMV Promoter	3600
GGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCGCCACCATGGTGAGCAAGGGCGAGGAGG CCACCCTCCAGATATATTCGCTCGAGAGACCGATTGATCTCTTGGGTGACGCGGTGGTACCACTCGTTCCCGCTCTCTCC CMV Promoter mCherry M V S K G E E	3680
ATAACATGGCCATCATCAAGGAGTTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTTCGAGATC TATTGTACCGGTAGTAGTTCTCAAGTACGCGAAGTTCCACGTGTACCTCCGAGGCACCTGCGGCTGCTCAAGCTCTAG mCherry D N M A I I K E F M R F K V H M E G S V N G H E F E I	3760
GAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCACCCAGACCGCAAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTT CTCCCGCTCCGCTCCCGGGCGGGATGCTCCCGTGGGTCTGGCGGTTGCACTTCCACTGGTTCCACCGGGGACGGGAA mCherry E G E G E G R P Y E G T Q T A K L K V T K G G P L P F	3840
CGCCTGGGACATCCTGTCCCCTCAGTTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACT GCGGACCTGTAGGACAGGGGAGTCAAGTACATGCCGAGGTTCCGGATGCACTTCGTGGGGCGGCTGTAGGGGCTGATGA mCherry A W D I L S P Q F M Y G S K A Y V K H P A D I P D Y	3920
TGAAGCTGTCTTCCCCGAGGGCTTCAAAGTGGGAGCGCGTGATGAACCTTCGAGGACGGCGGCTGGTGACCGTGACCCAG ACTTCGACAGGAAGGGGCTCCCGAAGTTACCCCTCGCGCACTACTTGAAGCTCCTGCCGCGCACCACTGGCACTGGGTC mCherry L K L S F P E G F K W E R V M N F E D G G V V T V T Q	4000



7 REFERENCES

- Abate-Daga, D., Lagisetty, K. H., Tran, E., Zheng, Z., Gattinoni, L., Yu, Z., Burns, W. R., Miermont, A. M., Teper, Y., Rudloff, U., Restifo, N. P., Feldman, S. A., Rosenberg, S. A., & Morgan, R. A. (2014). A Novel Chimeric Antigen Receptor Against Prostate Stem Cell Antigen Mediates Tumor Destruction in a Humanized Mouse Model of Pancreatic Cancer. *Human Gene Therapy*, 25(12), 1003–1012. <https://doi.org/10.1089/hum.2013.209>
- Adam, J. K., Odhav, B., & Bhoola, K. D. (2003). Immune responses in cancer. *Pharmacology & Therapeutics*, 99(1), 113–132. [https://doi.org/10.1016/S0163-7258\(03\)00056-1](https://doi.org/10.1016/S0163-7258(03)00056-1)
- Ahmadi, A., Najafi, M., Farhood, B., & Mortezaee, K. (2019). Transforming growth factor- β signaling: Tumorigenesis and targeting for cancer therapy. *Journal of Cellular Physiology*, 234(8), 12173–12187. <https://doi.org/10.1002/jcp.27955>
- Aktas, E., Kucuksezer, U. C., Bilgic, S., Erten, G., & Deniz, G. (2009). Relationship between CD107a expression and cytotoxic activity. *Cellular Immunology*, 254(2), 149–154. <https://doi.org/10.1016/j.cellimm.2008.08.007>
- Alishah, K., Birtel, M., Masoumi, E., Jafarzadeh, L., Mirzaee, H. R., Hadjati, J., Voss, R.-H., Diken, M., & Asad, S. (2021). CRISPR/Cas9-mediated TGF β RII disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells in vitro. *Journal of Translational Medicine*, 19(1), 482. <https://doi.org/10.1186/s12967-021-03146-0>
- Alnefaie, A., Albogami, S., Asiri, Y., Ahmad, T., Alotaibi, S. S., Al-Sanea, M. M., & Althobaiti, H. (2022). Chimeric Antigen Receptor T-Cells: An Overview of Concepts, Applications, Limitations, and Proposed Solutions. *Frontiers in Bioengineering and Biotechnology*, 10, 797440. <https://doi.org/10.3389/fbioe.2022.797440>
- Alter, G., Malenfant, J. M., & Altfeld, M. (2004). CD107a as a functional marker for the identification of natural killer cell activity. *Journal of Immunological Methods*, 294(1–2), 15–22. <https://doi.org/10.1016/j.jim.2004.08.008>
- Beatty, G. L., & Gladney, W. L. (2015). Immune Escape Mechanisms as a Guide for Cancer Immunotherapy. *Clinical Cancer Research*, 21(4), 687–692. <https://doi.org/10.1158/1078-0432.CCR-14-1860>
- Bollard, C. M., Rössig, C., Calonge, M. J., Huls, M. H., Wagner, H.-J., Massague, J., Brenner, M. K., Heslop, H. E., & Rooney, C. M. (2002). Adapting a transforming growth factor β -related tumor protection strategy to enhance antitumor immunity. *Blood*, 99(9), 3179–3187. <https://doi.org/10.1182/blood.V99.9.3179>
- Bollard, C. M., Tripic, T., Cruz, C. R., Dotti, G., Gottschalk, S., Torrano, V., Dakhova, O., Carrum, G., Ramos, C. A., Liu, H., Wu, M.-F., Marcogliese, A. N., Barese, C., Zu, Y., Lee, D. Y., O'Connor, O., Gee, A. P., Brenner, M. K., Heslop, H. E., & Rooney, C. M. (2018). Tumor-Specific T-Cells Engineered to Overcome Tumor Immune Evasion Induce Clinical Responses in Patients With Relapsed Hodgkin Lymphoma. *Journal of Clinical Oncology*, 36(11), 1128–1139. <https://doi.org/10.1200/JCO.2017.74.3179>

- Cappello, V., Marchetti, L., Parlanti, P., Landi, S., Tonazzini, I., Cecchini, M., Piazza, V., & Gemmi, M. (2016). Ultrastructural Characterization of the Lower Motor System in a Mouse Model of Krabbe Disease. *Scientific Reports*, 6(1), 1. <https://doi.org/10.1038/s41598-016-0001-8>
- Caruana, I., Savoldo, B., Hoyos, V., Weber, G., Liu, H., Kim, E. S., Ittmann, M. M., Marchetti, D., & Dotti, G. (2015). Heparanase promotes tumor infiltration and antitumor activity of CAR-redirected T lymphocytes. *Nature Medicine*, 21(5), 524–529. <https://doi.org/10.1038/nm.3833>
- Castro, F., Cardoso, A. P., Gonçalves, R. M., Serre, K., & Oliveira, M. J. (2018). Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. *Frontiers in Immunology*, 9, 847. <https://doi.org/10.3389/fimmu.2018.00847>
- Chang, R. B., & Beatty, G. L. (2020). The interplay between innate and adaptive immunity in cancer shapes the productivity of cancer immunosurveillance. *Journal of Leukocyte Biology*, 108(1), 363–376. <https://doi.org/10.1002/JLB.3MIR0320-475R>
- Chen, Y.-T., Scanlan, M. J., Sahin, U., Türeci, Ö., Gure, A. O., Tsang, S., Williamson, B., Stockert, E., Pfreundschuh, M., & Old, L. J. (1997). A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proceedings of the National Academy of Sciences*, 94(5), 1914–1918. <https://doi.org/10.1073/pnas.94.5.1914>
- Chmielewski, M., & Abken, H. (2015). TRUCKs: The fourth generation of CARs. *Expert Opinion on Biological Therapy*, 15(8), 1145–1154. <https://doi.org/10.1517/14712598.2015.1046430>
- Chmielewski, M., Hombach, A., Heuser, C., Adams, G. P., & Abken, H. (2004). T Cell Activation by Antibody-Like Immunoreceptors: Increase in Affinity of the Single-Chain Fragment Domain above Threshold Does Not Increase T Cell Activation against Antigen-Positive Target Cells but Decreases Selectivity. *The Journal of Immunology*, 173(12), 7647–7653. <https://doi.org/10.4049/jimmunol.173.12.7647>
- Chou, C., Pinto, A. K., Curtis, J. D., Persaud, S. P., Cella, M., Lin, C.-C., Edelson, B. T., Allen, P. M., Colonna, M., Pearce, E. L., Diamond, M. S., & Egawa, T. (2014). C-Myc-induced transcription factor AP4 is required for host protection mediated by CD8+ T cells. *Nature Immunology*, 15(9), 884–893. <https://doi.org/10.1038/ni.2943>
- Dotti, G., Gottschalk, S., Savoldo, B., & Brenner, M. K. (2014). Design and development of therapies using chimeric antigen receptor-expressing T cells. *Immunological Reviews*, 257(1), 107–126. <https://doi.org/10.1111/imr.12131>
- Eshhar, Z., Waks, T., Gross, G., & Schindler, D. G. (1993). Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proceedings of the National Academy of Sciences*, 90(2), 720–724. <https://doi.org/10.1073/pnas.90.2.720>
- Fakhrai, H., Mantil, J. C., Liu, L., Nicholson, G. L., Murphy-Satter, C. S., Ruppert, J., & Shawler, D. L. (2006). Phase I clinical trial of a TGF- β antisense-modified tumor cell vaccine in patients with advanced glioma. *Cancer Gene Therapy*, 13(12), 1052–1060. <https://doi.org/10.1038/sj.cgt.7700975>
- Fife, B. T., & Bluestone, J. A. (2008). Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunological Reviews*, 224(1), 166–182. <https://doi.org/10.1111/j.1600-065X.2008.00662.x>

- Fujiwara, K., Tsuneji, A., Kusabuka, H., Ogaki, E., Tachibana, M., & Okada, N. (2020). Hinge and Transmembrane Domains of Chimeric Antigen Receptor Regulate Receptor Expression and Signaling Threshold. *Cells*, 9(5), 1182. <https://doi.org/10.3390/cells9051182>
- Gajewski, T. F., Schreiber, H., & Fu, Y.-X. (2013). Innate and adaptive immune cells in the tumor microenvironment. *Nature Immunology*, 14(10), 1014–1022. <https://doi.org/10.1038/ni.2703>
- Glassman, A. B. (1989). Interleukin-2 and lymphokine activated killer cells: Promises and cautions. *Annals of Clinical and Laboratory Science*, 19(1), 51–55.
- Golumba-Nagy, V., Kuehle, J., Hombach, A. A., & 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. *Molecular Therapy*, 26(9), 2218–2230. <https://doi.org/10.1016/j.ymthe.2018.07.005>
- Gonzalez, H., Hagerling, C., & Werb, Z. (2018). Roles of the immune system in cancer: From tumor initiation to metastatic progression. *Genes & Development*, 32(19–20), 1267–1284. <https://doi.org/10.1101/gad.314617.118>
- Gross, G., Waks, T., & Eshhar, Z. (1989). Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proceedings of the National Academy of Sciences*, 86(24), 10024–10028. <https://doi.org/10.1073/pnas.86.24.10024>
- Grosser, R., Cherkassky, L., Chintala, N., & Adusumilli, P. S. (2019). Combination Immunotherapy with CAR T Cells and Checkpoint Blockade for the Treatment of Solid Tumors. *Cancer Cell*, 36(5), 471–482. <https://doi.org/10.1016/j.ccell.2019.09.006>
- Guedan, S., Posey, A. D., Shaw, C., Wing, A., Da, T., Patel, P. R., McGettigan, S. E., Casado-Medrano, V., Kawalekar, O. U., Uribe-Herranz, M., Song, D., Melenhorst, J. J., Lacey, S. F., Scholler, J., Keith, B., Young, R. M., & June, C. H. (2018). Enhancing CAR T cell persistence through ICOS and 4-1BB costimulation. *JCI Insight*, 3(1), e96976. <https://doi.org/10.1172/jci.insight.96976>
- Han, Y., Liu, Q., Hou, J., Gu, Y., Zhang, Y., Chen, Z., Fan, J., Zhou, W., Qiu, S., Zhang, Y., Dong, T., Li, N., Jiang, Z., Zhu, H., Zhang, Q., Ma, Y., Zhang, L., Wang, Q., Yu, Y., ... Cao, X. (2018). Tumor-Induced Generation of Splenic Erythroblast-like Ter-Cells Promotes Tumor Progression. *Cell*, 173(3), 634–648.e12. <https://doi.org/10.1016/j.cell.2018.02.061>
- Hou, J., Schindler, U., Henzel, W. J., Wong, S. C., & McKnight, S. L. (1995). Identification and purification of human stat proteins activated in response to interleukin-2. *Immunity*, 2(4), 321–329. [https://doi.org/10.1016/1074-7613\(95\)90140-X](https://doi.org/10.1016/1074-7613(95)90140-X)
- Huang, C.-Y., Chung, C.-L., Hu, T.-H., Chen, J.-J., Liu, P.-F., & Chen, C.-L. (2021). Recent progress in TGF-β inhibitors for cancer therapy. *Biomedicine & Pharmacotherapy*, 134, 111046. <https://doi.org/10.1016/j.biopha.2020.111046>
- Imai, C., Mihara, K., Andreansky, M., Nicholson, I. C., Pui, C.-H., Geiger, T. L., & Campana, D. (2004). Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. *Leukemia*, 18(4), 676–684. <https://doi.org/10.1038/sj.leu.2403302>
- Jayaraman, J., Mellody, M. P., Hou, A. J., Desai, R. P., Fung, A. W., Pham, A. H. T., Chen, Y. Y., & Zhao, W. (2020). CAR-T design: Elements and their synergistic function. *EBioMedicine*, 58, 102931. <https://doi.org/10.1016/j.ebiom.2020.102931>

- Jin, J., Wu, X., Yin, J., Li, M., Shen, J., Li, J., Zhao, Y., Zhao, Q., Wu, J., Wen, Q., Cho, C. H., Yi, T., Xiao, Z., & Qu, L. (2019). Identification of Genetic Mutations in Cancer: Challenge and Opportunity in the New Era of Targeted Therapy. *Frontiers in Oncology*, 9, 263. <https://doi.org/10.3389/fonc.2019.00263>
- Jones, D. M., Read, K. A., & Oestreich, K. J. (2020). Dynamic Roles for IL-2–STAT5 Signaling in Effector and Regulatory CD4+ T Cell Populations. *The Journal of Immunology*, 205(7), 1721–1730. <https://doi.org/10.4049/jimmunol.2000612>
- June, C. H., O'Connor, R. S., Kawalekar, O. U., Ghassemi, S., & Milone, M. C. (2018). CAR T cell immunotherapy for human cancer. *Science*, 359(6382), 1361–1365. <https://doi.org/10.1126/science.aar6711>
- Kaartinen, V., Voncken, J. W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N., & Groffen, J. (1995). Abnormal lung development and cleft palate in mice lacking TGF- β 3 indicates defects of epithelial–mesenchymal interaction. *Nature Genetics*, 11(4), 415–421. <https://doi.org/10.1038/ng1295-415>
- Kalos, M., Levine, B. L., Porter, D. L., Katz, S., Grupp, S. A., Bagg, A., & June, C. H. (2011). T Cells with Chimeric Antigen Receptors Have Potent Antitumor Effects and Can Establish Memory in Patients with Advanced Leukemia. *Science Translational Medicine*, 3(95). <https://doi.org/10.1126/scitranslmed.3002842>
- Kloss, C. C., Lee, J., Zhang, A., Chen, F., Melenhorst, J. J., Lacey, S. F., Maus, M. V., Fraietta, J. A., Zhao, Y., & June, C. H. (2018). Dominant-Negative TGF- β Receptor Enhances PSMA-Targeted Human CAR T Cell Proliferation And Augments Prostate Cancer Eradication. *Molecular Therapy*, 26(7), 1855–1866. <https://doi.org/10.1016/j.ymthe.2018.05.003>
- Kofler, D. M., Chmielewski, M., Rappl, G., Hombach, A., Riet, T., Schmidt, A., Hombach, A. A., Wendtner, C.-M., & Abken, H. (2011). CD28 Costimulation Impairs the Efficacy of a Redirected T-cell Antitumor Attack in the Presence of Regulatory T cells Which Can Be Overcome by Preventing Lck Activation. *Molecular Therapy*, 19(4), 760–767. <https://doi.org/10.1038/mt.2011.9>
- Korkut, A., Zaidi, S., Kanchi, R. S., Rao, S., Gough, N. R., Schultz, A., Li, X., Lorenzi, P. L., Berger, A. C., Robertson, G., Kwong, L. N., Datto, M., Roszik, J., Ling, S., Ravikumar, V., Manyam, G., Rao, A., Shelley, S., Liu, Y., ... Mariamidze, A. (2018). A Pan-Cancer Analysis Reveals High-Frequency Genetic Alterations in Mediators of Signaling by the TGF- β Superfamily. *Cell Systems*, 7(4), 422–437.e7. <https://doi.org/10.1016/j.cels.2018.08.010>
- Kulkarni, A. B., Huh, C. G., Becker, D., Geiser, A., Lyght, M., Flanders, K. C., Roberts, A. B., Sporn, M. B., Ward, J. M., & Karlsson, S. (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proceedings of the National Academy of Sciences*, 90(2), 770–774. <https://doi.org/10.1073/pnas.90.2.770>
- Kuwana, Y., Asakura, Y., Utsunomiya, N., Nakanishi, M., Arata, Y., Itoh, S., Nagase, F., & Kurosawa, Y. (1987). Expression of chimeric receptor composed of immunoglobulin-derived V regions and T-cell receptor-derived C regions. *Biochemical and Biophysical Research Communications*, 149(3), 960–968. [https://doi.org/10.1016/0006-291X\(87\)90502-X](https://doi.org/10.1016/0006-291X(87)90502-X)
- Li, Y., Wu, D., Yang, X., & Zhou, S. (2021). Immunotherapeutic Potential of T Memory Stem Cells. *Frontiers in Oncology*, 11, 723888. <https://doi.org/10.3389/fonc.2021.723888>

- Lin, S., Cheng, L., Ye, W., Li, S., Zheng, D., Qin, L., Wu, Q., Long, Y., Lin, S., Wang, S., Huang, G., Li, P., Yao, Y., & Sun, X. (2021). Chimeric CTLA4-CD28-CD3z T Cells Potentiate Antitumor Activity Against CD80/CD86-Positive B Cell Malignancies. *Frontiers in Immunology*, 12, 642528. <https://doi.org/10.3389/fimmu.2021.642528>
- Liu, X., Ranganathan, R., Jiang, S., Fang, C., Sun, J., Kim, S., Newick, K., Lo, A., June, C. H., Zhao, Y., & Moon, E. K. (2016). A Chimeric Switch-Receptor Targeting PD1 Augments the Efficacy of Second-Generation CAR T Cells in Advanced Solid Tumors. *Cancer Research*, 76(6), 1578–1590. <https://doi.org/10.1158/0008-5472.CAN-15-2524>
- Lord, J. D., McIntosh, B. C., Greenberg, P. D., & Nelson, B. H. (2000). The IL-2 Receptor Promotes Lymphocyte Proliferation and Induction of the *c-myc*, *bcl-2*, and *bcl-x* Genes Through the *trans*-Activation Domain of Stat5. *The Journal of Immunology*, 164(5), 2533–2541. <https://doi.org/10.4049/jimmunol.164.5.2533>
- Lu, J., & Jiang, G. (2022). The journey of CAR-T therapy in hematological malignancies. *Molecular Cancer*, 21(1), 194. <https://doi.org/10.1186/s12943-022-01663-0>
- Mahmud, S. A., Manlove, L. S., & Farrar, M. A. (2013). Interleukin-2 and STAT5 in regulatory T cell development and function. *JAK-STAT*, 2(1), e23154. <https://doi.org/10.4161/jkst.23154>
- Majzner, R. G., & Mackall, C. L. (2018). Tumor Antigen Escape from CAR T-cell Therapy. *Cancer Discovery*, 8(10), 1219–1226. <https://doi.org/10.1158/2159-8290.CD-18-0442>
- Malek, T. R., Yu, A., Scibelli, P., Lichtenheld, M. G., & Codias, E. K. (2001). Broad Programming by IL-2 Receptor Signaling for Extended Growth to Multiple Cytokines and Functional Maturation of Antigen-Activated T Cells. *The Journal of Immunology*, 166(3), 1675–1683. <https://doi.org/10.4049/jimmunol.166.3.1675>
- Massagué, J. (2008). TGFβ in Cancer. *Cell*, 134(2), 215–230. <https://doi.org/10.1016/j.cell.2008.07.001>
- Maude, S. L., Laetsch, T. W., Buechner, J., Rives, S., Boyer, M., Bittencourt, H., Bader, P., Verneris, M. R., Stefanski, H. E., Myers, G. D., Qayed, M., De Moerloose, B., Hiramatsu, H., Schlis, K., Davis, K. L., Martin, P. L., Nemecek, E. R., Yanik, G. A., Peters, C., ... Grupp, S. A. (2018). Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. *New England Journal of Medicine*, 378(5), 439–448. <https://doi.org/10.1056/NEJMoa1709866>
- Miyazaki, T., Liu, Z.-J., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barsoumian, E. L., Perlmutter, R. M., & Taniguchi, T. (1995). Three distinct IL-2 signaling pathways mediated by *bcl-2*, *c-myc*, and *lck* cooperate in hematopoietic cell proliferation. *Cell*, 81(2), 223–231. [https://doi.org/10.1016/0092-8674\(95\)90332-1](https://doi.org/10.1016/0092-8674(95)90332-1)
- Narayan, V., Barber-Rotenberg, J. S., Jung, I.-Y., Lacey, S. F., Rech, A. J., Davis, M. M., Hwang, W.-T., Lal, P., Carpenter, E. L., Maude, S. L., Plesa, G., Vapiwala, N., Chew, A., Moniak, M., Sebro, R. A., Farwell, M. D., Marshall, A., Gilmore, J., Lledo, L., ... Haas, N. B. (2022). PSMA-targeting TGFβ-insensitive armored CAR T cells in metastatic castration-resistant prostate cancer: A phase 1 trial. *Nature Medicine*, 28(4), 724–734. <https://doi.org/10.1038/s41591-022-01726-1>

- Neelapu, S. S., Locke, F. L., Bartlett, N. L., Lekakis, L. J., Miklos, D. B., Jacobson, C. A., Braunschweig, I., Oluwole, O. O., Siddiqi, T., Lin, Y., Timmerman, J. M., Stiff, P. J., Friedberg, J. W., Flinn, I. W., Goy, A., Hill, B. T., Smith, M. R., Deol, A., Farooq, U., ... Go, W. Y. (2017). Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *New England Journal of Medicine*, 377(26), 2531–2544. <https://doi.org/10.1056/NEJMoa1707447>
- Nguyen, L. T., Saibil, S. D., Sotov, V., Le, M. X., Khoja, L., Ghazarian, D., Bonilla, L., Majeed, H., Hogg, D., Joshua, A. M., Crump, M., Franke, N., Spreafico, A., Hansen, A., Al-Habeeb, A., Leong, W., Easson, A., Reedijk, M., Goldstein, D. P., ... Butler, M. O. (2019). Phase II clinical trial of adoptive cell therapy for patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and low-dose interleukin-2. *Cancer Immunology, Immunotherapy*, 68(5), 773–785. <https://doi.org/10.1007/s00262-019-02307-x>
- Pipkin, M. E., Rao, A., & Lichtenheld, M. G. (2010). The transcriptional control of the perforin locus: Perforin transcription. *Immunological Reviews*, 235(1), 55–72. <https://doi.org/10.1111/j.0105-2896.2010.00905.x>
- Porter, D. L., Levine, B. L., Kalos, M., Bagg, A., & June, C. H. (2011). Chimeric Antigen Receptor–Modified T Cells in Chronic Lymphoid Leukemia. *New England Journal of Medicine*, 365(8), 725–733. <https://doi.org/10.1056/NEJMoa1103849>
- Preston, G. C., Sinclair, L. V., Kaskar, A., Hukelmann, J. L., Navarro, M. N., Ferrero, I., MacDonald, H. R., Cowling, V. H., & Cantrell, D. A. (2015). Single cell tuning of Myc expression by antigen receptor signal strength and interleukin-2 in T lymphocytes. *The EMBO Journal*, 34(15), 2008–2024. <https://doi.org/10.15252/embj.201490252>
- Qin, L., Lai, Y., Zhao, R., Wei, X., Weng, J., Lai, P., Li, B., Lin, S., Wang, S., Wu, Q., Liang, Q., Li, Y., Zhang, X., Wu, Y., Liu, P., Yao, Y., Pei, D., Du, X., & Li, P. (2017). Incorporation of a hinge domain improves the expansion of chimeric antigen receptor T cells. *Journal of Hematology & Oncology*, 10(1), 68. <https://doi.org/10.1186/s13045-017-0437-8>
- Quintarelli, C., Orlando, D., Boffa, I., Guercio, M., Polito, V. A., Petretto, A., Lavarello, C., Sinibaldi, M., Weber, G., Del Bufalo, F., Giorda, E., Scarsella, M., Petrini, S., Pagliara, D., Locatelli, F., De Angelis, B., & Caruana, I. (2018). Choice of costimulatory domains and of cytokines determines CAR T-cell activity in neuroblastoma. *Onc Immunology*, 7(6), e1433518. <https://doi.org/10.1080/2162402X.2018.1433518>
- Ramos, C. A., Rouce, R., Robertson, C. S., Reyna, A., Narala, N., Vyas, G., Mehta, B., Zhang, H., Dakhova, O., Carrum, G., Kamble, R. T., Gee, A. P., Mei, Z., Wu, M.-F., Liu, H., Grilley, B., Rooney, C. M., Heslop, H. E., Brenner, M. K., ... Dotti, G. (2018). In Vivo Fate and Activity of Second- versus Third-Generation CD19-Specific CAR-T Cells in B Cell Non-Hodgkin's Lymphomas. *Molecular Therapy*, 26(12), 2727–2737. <https://doi.org/10.1016/j.ymthe.2018.09.009>
- Ribas, A. (2015). Adaptive Immune Resistance: How Cancer Protects from Immune Attack. *Cancer Discovery*, 5(9), 915–919. <https://doi.org/10.1158/2159-8290.CD-15-0563>
- Rodriguez-Garcia, A., Palazon, A., Noguera-Ortega, E., Powell, D. J., & Guedan, S. (2020). CAR-T Cells Hit the Tumor Microenvironment: Strategies to Overcome Tumor Escape. *Frontiers in Immunology*, 11, 1109. <https://doi.org/10.3389/fimmu.2020.01109>

- Rohaani, M. W., Wilgenhof, S., & Haanen, J. B. A. G. (2019). Adoptive cellular therapies: The current landscape. *Virchows Archiv*, 474(4), 449–461. <https://doi.org/10.1007/s00428-018-2484-0>
- Rosenberg, S. A. (2014). IL-2: The First Effective Immunotherapy for Human Cancer. *The Journal of Immunology*, 192(12), 5451–5458. <https://doi.org/10.4049/jimmunol.1490019>
- Rosenberg, S. A., Packard, B. S., Aebbersold, P. M., Solomon, D., Topalian, S. L., Toy, S. T., Simon, P., Lotze, M. T., Yang, J. C., Seipp, C. A., Simpson, C., Carter, C., Bock, S., Schwartzentruber, D., Wei, J. P., & White, D. E. (1988). Use of Tumor-Infiltrating Lymphocytes and Interleukin-2 in the Immunotherapy of Patients with Metastatic Melanoma. *New England Journal of Medicine*, 319(25), 1676–1680. <https://doi.org/10.1056/NEJM19881223192527>
- Rosenberg, S. A., Spiess, P., & Lafreniere, R. (1986). A New Approach to the Adoptive Immunotherapy of Cancer with Tumor-Infiltrating Lymphocytes. *Science*, 233(4770), 1318–1321. <https://doi.org/10.1126/science.3489291>
- Ruegemer, J. J., Ho, S. N., Augustine, J. A., Schlager, J. W., Bell, M. P., McKean, D. J., & Abraham, R. T. (1990). Regulatory effects of transforming growth factor-beta on IL-2- and IL-4-dependent T cell-cycle progression. *Journal of Immunology (Baltimore, Md.: 1950)*, 144(5), 1767–1776.
- Russell, J. H., & Ley, T. J. (2002). Lymphocyte-Mediated Cytotoxicity. *Annual Review of Immunology*, 20(1), 323–370. <https://doi.org/10.1146/annurev.immunol.20.100201.131730>
- Sanford, L. P., Ormsby, I., Gittenberger-de Groot, A. C., Sariola, H., Friedman, R., Boivin, G. P., Cardell, E. L., & Doetschman, T. (1997). TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development*, 124(13), 2659–2670. <https://doi.org/10.1242/dev.124.13.2659>
- Sanjabi, S., Oh, S. A., & Li, M. O. (2017). Regulation of the Immune Response by TGF-β: From Conception to Autoimmunity and Infection. *Cold Spring Harbor Perspectives in Biology*, 9(6), a022236. <https://doi.org/10.1101/cshperspect.a022236>
- Savoldo, B., Ramos, C. A., Liu, E., Mims, M. P., Keating, M. J., Carrum, G., Kamble, R. T., Bollard, C. M., Gee, A. P., Mei, Z., Liu, H., Grilley, B., Rooney, C. M., Heslop, H. E., Brenner, M. K., & Dotti, G. (2011). CD28 costimulation improves expansion and persistence of chimeric antigen receptor–modified T cells in lymphoma patients. *Journal of Clinical Investigation*, 121(5), 1822–1826. <https://doi.org/10.1172/JCI46110>
- Schlingensiepen, R., Goldbrunner, M., Szyrach, M. N. I., Stauder, G., Jachimczak, P., Bogdahn, U., Schulmeyer, F., Hau, P., & Schlingensiepen, K.-H. (2005). Intracerebral and Intrathecal Infusion of the TGF-β2-Specific Antisense Phosphorothioate Oligonucleotide AP 12009 in Rabbits and Primates: Toxicology and Safety. *Oligonucleotides*, 15(2), 94–104. <https://doi.org/10.1089/oli.2005.15.94>
- Shafer, P., Kelly, L. M., & Hoyos, V. (2022). Cancer Therapy With TCR-Engineered T Cells: Current Strategies, Challenges, and Prospects. *Frontiers in Immunology*, 13, 835762. <https://doi.org/10.3389/fimmu.2022.835762>
- Siegel, P. M., & Massagué, J. (2003). Cytostatic and apoptotic actions of TGF-β in homeostasis and cancer. *Nature Reviews Cancer*, 3(11), 807–820. <https://doi.org/10.1038/nrc1208>
- Sterner, R. C., & Sterner, R. M. (2021). CAR-T cell therapy: Current limitations and potential strategies. *Blood Cancer Journal*, 11(4), 69. <https://doi.org/10.1038/s41408-021-00459-7>

- Styczyński, J. (2020). A brief history of CAR-T cells: From laboratory to the bedside. *Acta Haematologica Polonica*, 51(1), 2–5. <https://doi.org/10.2478/ahp-2020-0002>
- Sun, S., Hao, H., Yang, G., Zhang, Y., & Fu, Y. (2018). Immunotherapy with CAR-Modified T Cells: Toxicities and Overcoming Strategies. *Journal of Immunology Research*, 2018, 1–10. <https://doi.org/10.1155/2018/2386187>
- Thomas, D. A., & Massagué, J. (2005). TGF- β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell*, 8(5), 369–380. <https://doi.org/10.1016/j.ccr.2005.10.012>
- Trapani, J. A., & Smyth, M. J. (2002). Functional significance of the perforin/granzyme cell death pathway. *Nature Reviews Immunology*, 2(10), 735–747. <https://doi.org/10.1038/nri911>
- Waldman, A. D., Fritz, J. M., & Lenardo, M. J. (2020). A guide to cancer immunotherapy: From T cell basic science to clinical practice. *Nature Reviews Immunology*, 20(11), 651–668. <https://doi.org/10.1038/s41577-020-0306-5>
- Wang, E., Wang, L.-C., Tsai, C.-Y., Bhoj, V., Gershenson, Z., Moon, E., Newick, K., Sun, J., Lo, A., Baradet, T., Feldman, M. D., Barrett, D., Puré, E., Albelda, S., & Milone, M. C. (2015). Generation of Potent T-cell Immunotherapy for Cancer Using DAP12-Based, Multichain, Chimeric Immunoreceptors. *Cancer Immunology Research*, 3(7), 815–826. <https://doi.org/10.1158/2326-6066.CIR-15-0054>
- Wang, L.-C. S., Lo, A., Scholler, J., Sun, J., Majumdar, R. S., Kapoor, V., Antzis, M., Cotner, C. E., Johnson, L. A., Durham, A. C., Solomides, C. C., June, C. H., Puré, E., & Albelda, S. M. (2014). Targeting Fibroblast Activation Protein in Tumor Stroma with Chimeric Antigen Receptor T Cells Can Inhibit Tumor Growth and Augment Host Immunity without Severe Toxicity. *Cancer Immunology Research*, 2(2), 154–166. <https://doi.org/10.1158/2326-6066.CIR-13-0027>
- Wang, Y., Jiang, H., Luo, H., Sun, Y., Shi, B., Sun, R., & Li, Z. (2019). An IL-4/21 Inverted Cytokine Receptor Improving CAR-T Cell Potency in Immunosuppressive Solid-Tumor Microenvironment. *Frontiers in Immunology*, 10, 1691. <https://doi.org/10.3389/fimmu.2019.01691>
- Weinkove, R., George, P., Dasyam, N., & McLellan, A. D. (2019). Selecting costimulatory domains for chimeric antigen receptors: Functional and clinical considerations. *Clinical & Translational Immunology*, 8(5), e1049. <https://doi.org/10.1002/cti2.1049>
- Welschof, M., Terness, P., Kipriyanov, S. M., Stanescu, D., Breitling, F., Dörsam, H., Dübel, S., Little, M., & Opelz, G. (1997). The antigen-binding domain of a human IgG-anti-F(ab')₂ autoantibody. *Proceedings of the National Academy of Sciences*, 94(5), 1902–1907. <https://doi.org/10.1073/pnas.94.5.1902>
- Yakirevich, E., Sabo, E., Lavie, O., Mazareb, S., Spagnoli, G. C., & Resnick, M. B. (2003). Expression of the MAGE-A4 and NY-ESO-1 cancer-testis antigens in serous ovarian neoplasms. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, 9(17), 6453–6460.
- Yoshimura, A., Wakabayashi, Y., & Mori, T. (2010). Cellular and molecular basis for the regulation of inflammation by TGF-. *Journal of Biochemistry*, 147(6), 781–792. <https://doi.org/10.1093/jb/mvq043>

- Zhao, Y., Deng, J., Rao, S., Guo, S., Shen, J., Du, F., Wu, X., Chen, Y., Li, M., Chen, M., Li, X., Li, W., Gu, L., Sun, Y., Zhang, Z., Wen, Q., Xiao, Z., & Li, J. (2022). Tumor Infiltrating Lymphocyte (TIL) Therapy for Solid Tumor Treatment: Progressions and Challenges. *Cancers*, 14(17), 4160. <https://doi.org/10.3390/cancers14174160>
- Zhao, Z., Chen, Y., Francisco, N. M., Zhang, Y., & Wu, M. (2018). The application of CAR-T cell therapy in hematological malignancies: Advantages and challenges. *Acta Pharmaceutica Sinica B*, 8(4), 539–551. <https://doi.org/10.1016/j.apsb.2018.03.001>
- Zhao, Z., Condomines, M., van der Stegen, S. J. C., Perna, F., Kloss, C. C., Gunset, G., Plotkin, J., & Sadelain, M. (2015). Structural Design of Engineered Costimulation Determines Tumor Rejection Kinetics and Persistence of CAR T Cells. *Cancer Cell*, 28(4), 415–428. <https://doi.org/10.1016/j.ccell.2015.09.004>
- Zheng, P.-P., Kros, J. M., & Li, J. (2018). Approved CAR T cell therapies: Ice bucket challenges on glaring safety risks and long-term impacts. *Drug Discovery Today*, 23(6), 1175–1182. <https://doi.org/10.1016/j.drudis.2018.02.012>
- Zhong, X.-S., Matsushita, M., Plotkin, J., Riviere, I., & Sadelain, M. (2010). Chimeric Antigen Receptors Combining 4-1BB and CD28 Signaling Domains Augment PI3kinase/AKT/Bcl-XL Activation and CD8+ T Cell-mediated Tumor Eradication. *Molecular Therapy*, 18(2), 413–420. <https://doi.org/10.1038/mt.2009.210>