

MASTERARBEIT | MASTER'S THESIS

Titel | Title

Development and functional evaluation of dual-chain and second-generation CAR-T cells incorporating scFv affinity variants against ErbB2-positive tumors

verfasst von | submitted by

Luca Lucia Feichter B.Sc.

angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of
Master of Science (MSc)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt |
Degree programme code as it appears on
the student record sheet:

UA 066 865

Studienrichtung lt. Studienblatt | Degree
programme as it appears on the student
record sheet:

Masterstudium Molecular Biology

Betreut von | Supervisor:

Dr. Sebastian Carotta

Sworn declaration

I hereby certify that I have prepared and written the master thesis “Development and functional evaluation of dual-chain and second-generation CAR-T cells incorporating scFv affinity variants against ErbB2-positive solid tumors” independently. Quotations, illustrations, or diagrams that do not originate from me are marked with references.

No other sources or aids have been used.

Luca Feichter

Acknowledgement

Mit dieser Danksagung möchte ich all jenen meinen Dank aussprechen, die mich während der Anfertigung dieser Masterarbeit in vielfältiger Weise unterstützt, motiviert und begleitet haben.

Mein besonderer Dank gilt PD Dr. rer. nat. Markus Chmielewski, der mir dieses spannende Thema anvertraut und mich mit seiner erstklassigen Betreuung durch die gesamte Forschungsarbeit geführt hat. Ich bin für die inspirierenden Gespräche, die wertvollen Anregungen und die großartige Unterstützung während der gesamten Arbeit zutiefst dankbar. Die Zusammenarbeit war eine große Bereicherung, und ich habe viel gelernt.

Ebenso danke ich Simon Lennartz für seine stets unkomplizierte Unterstützung und die angenehme, humorvolle Zusammenarbeit, die die Arbeit im Labor bereichert hat.

Herzlich bedanken möchte ich mich auch bei meiner gesamten Arbeitsgruppe – Dana, Tobi, Fabian, Daniel, Niklas, Sisi, Lennert, Celina und Martin – für die stets hilfsbereite, freundliche und motivierende Atmosphäre. Euer Engagement und Teamgeist haben den Arbeitsalltag bereichert und diese Zeit zu einer unvergesslichen Erfahrung gemacht.

Ein persönlicher Dank gilt meinen Eltern, die mich auf meinem gesamten Weg bedingungslos unterstützt und ermutigt haben.

Inhaltsverzeichnis

Sworn declaration	I
Acknowledgement	III
Inhaltsverzeichnis.....	IV
List of abbreviations	VII
Zusammenfassung	IX
Abstract	X
1. Introduction	1
1.1. Biological background of ErbB2	1
1.2. Chimeric antigen receptor (CAR)-T cell immunotherapy.....	3
1.3. CAR-T cell design	4
1.4. The evolution of CAR design.....	5
1.5. Dual targeting CAR-T cell	6
1.6. Limitations of CAR-T cell therapy	7
1.7. CAR-T toxicity	8
1.8. T cell subsets and function	9
2. Aim of the study.....	10
3. Material	11
3.1. Antibodies	11
3.1.1. Antibodies for flow cytometry	11
3.1.2. Antibodies for ELISA	12
3.1.3. Antibodies for T cell activation.....	12
3.2. Enzymes and buffers	12
3.2.1. Restriction enzymes	12
3.2.2. Other enzymes	13
3.2.3. Restriction enzyme buffers	13
3.3. Chemicals	13
3.4. Media and Buffer	14
3.5. Oligonucleotides for Cloning	14
3.6. Plasmids	15
3.6.1. Retroviral CAR expression plasmids	15
3.6.2. Retroviral helper plasmids	15
3.7. Human cell lines	16
3.8. Bacterial strains	16
3.9. Consumables	16
3.10. Kits.....	16
3.11. Devices	17
3.12. Programs.....	17

4. Methods	18
4.1. Microbiological Methods	18
4.1.1. Transformation of chemo-competent <i>E. coli</i> DH5 α bacteria	18
4.1.2. Storage of <i>E. coli</i> DH5 α bacteria	18
4.2. Molecular biological methods	18
4.2.1. Isolation of plasmid DNA from <i>E. coli</i> DH5 α bacteria	18
4.2.2. Determination of the DNA concentration	19
4.2.3. Cloning strategy for plasmid construct generation	19
4.2.4. Plasmid sequencing	20
4.3. Cell culture methods	20
4.3.1. Cultivation of eukaryotic cells	20
4.3.2. Passage of adherent cells	21
4.3.3. Cell counting and viability assessment	21
4.3.4. Cryopreservation and thawing of cells	21
4.3.5. Detection of mycoplasma bacteria	22
4.3.6. Isolation and activation of primary human T cells	22
4.3.7. PEI transfection of HEK 293T cells	23
4.3.8. Preparation of PDL-coated plates	24
4.3.9. Transduction of human T cells using recombinant retrovirus	24
4.3.10. CAR-T cell cytotoxicity test	25
4.3.11. Measurement of CAR-T mediated cytotoxicity by XTT assay	25
4.3.12. Repetitive CAR-T cell cytotoxicity test	25
4.3.13. Protein assay for cytokine release	26
4.4. Immunological methods	27
4.4.1. Flow cytometry	27
4.4.2. Cytokine release assay	27
5. Results	29
5.1. Dual-chain and 2 nd Gen CAR designs	29
5.2. Expression of dual-chain and 2 nd Gen CAR constructs on the surface of human T cells	31
5.3. ErbB2 expression levels on the surface of target cell lines and fibroblasts	34
5.4. Selective IFN- γ release by CAR-T cells specific for domain II and IV of ErbB2	35
5.5. T cells expressing second-generation CAR constructs 4D5 and 4D5 _{low} demonstrate superior anti-tumor efficacy in repetitive killing assays	36
5.6. T cells equipped with second-generation 4D5 CAR construct exhibit reduced cytotoxicity toward primary human fibroblasts expressing ErbB2 at low density	38
5.7. Repetitive specificity assay targeting MCF-7 ErbB2 knockout cells	39
5.8. Antigen-dependent cytokine secretion by CAR-T cells	40
5.8.1. Secretion of the pro-inflammatory cytokine IFN- γ	40
5.8.2. Secretion of the pro-inflammatory cytokine IL-2	42
5.9. Low expression of the immune checkpoint receptor PD-1 on the surface of T cells equipped with second-generation	43
5.10. CD4 ⁺ and CD8 ⁺ T cell distribution in CAR ⁺ and CAR ⁻ subsets	44
5.11. T cell phenotyping after co-culture with ErbB2 associated cell lines	48
6. Discussion	51
7. Outlook	56
8. References	57

9. Appendix.....	61
9.1. <i>IL-2 secretion after stimulation with recombinant ErbB2 domain II and IV</i>	61
9.2. <i>Retroviral expression vectors</i>	62
9.2.1. Dual-chain C6.5/4D5	62
9.2.2. Dual-chain C6.5B1D2/4D5.....	63
9.2.3. Dual-chain C6.5/4D5mut.....	64
9.2.4. Dual-chain C6.5B1D2/4D5mut	65
9.2.5. 2 nd Gen C6.5B1D2.....	66
9.2.6. 2 nd Gen C6.5	67
9.2.7. 2 nd Gen 4D5	68
9.2.8. 2 nd Gen 4D5mut	69
9.2.9. Dual-chain C6.5B1D2/B7H3.....	70
9.2.10. 1 st Gen C6.5B1D2.....	71
9.2.11. 1 st Gen C6.5	72
9.3. <i>Helper plasmids.....</i>	73
9.3.1. Helper plasmid #3077	73
9.3.2. Helper plasmid #3076	74

List of abbreviations

Abbreviation	Name
ACT	Adoptive T cell immunotherapy
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
CO ₂	Carbon dioxide
CRS	Cytokine release syndrome
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
ddH ₂ O	Double distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxiribonucleotide triphosphate
ERBB2	Erythroblastic oncogene B, member 2
ELISA	Enzyme-linked immunosorbent assay
E:T	Effector: Target
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FSC	Forward scatter
HEK	Human embryonic kidney
HER	Human epidermal growth factor
ICANS	Immune effector cell-associated neurotoxicity syndrome
IFN- γ	Interferon- γ
IL	Interleukin
ITAMs	Immune receptor tyrosine-based activation motifs
K _d	Dissociation constant
KO	Knockout
LK	Kappa leader
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

NF- κ B	Nuclear factor κ B
NK cells	Natural killer cells
OD	Optical density
Ox40	CD134
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDL	Poly-D-lysine
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed cell death protein 1
RPMI	Roswell park memorial institute medium
RT	Room temperature
scFv	Single chain variable fragment
SSC	Side scatter
TAA	Tumor associated antigen
TCR	T cell receptor
TSA	Tumor specific antigen
TM	Transmembrane
TMB	3,3',5,5'-Tetramethylbenzidine
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TRUCK	T cell redirected for universal cytokine-mediated killing
UTC	Untransduced T cell
VH	Variable heavy chain
VL	Variable light chain
v/v	Volume per volume
WT	Wild type
1G	First generation
2G	Second generation
3G	Third generation
4-1BB	CD137
XTT	2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Zusammenfassung

Die chimäre Antigenrezeptor (CAR)-T Zelltherapie hat bemerkenswerte klinische Erfolge bei der Behandlung hämatologischer Tumore erzielt, doch ihre Wirksamkeit bei soliden Tumoren ist unter anderem aufgrund der Seltenheit exklusiv exprimierter tumor-spezifischer Antigene, weiterhin begrenzt (Mitra et al., 2023). ErbB2 ist ein attraktives therapeutisches Ziel, da es häufig in verschiedenen soliden Krebsarten überexprimiert wird (Oh & Bang, 2020). Allerdings wirft die basale Expression in normalem Gewebe ein Sicherheitsrisiko auf (Morgan et al., 2010). Zur Evaluierung von Strategien zur Verbesserung der Selektivität haben wir eine Reihe von CAR-Konstrukten entwickelt, die die ErbB2-zielenden Antikörperfragmente C6.5 und 4D5 entweder in konventionellen CARs der zweiten Generation (2nd Gen) oder in Dual-Chain-Formaten integrieren. Frühere Studien zeigten, dass der von 4D5 abgeleitete Antikörper Trastuzumab zu erheblichen Hintergrundsignalen führt und Sicherheitsrisiken bergen kann (Morgan et al., 2010). Dies motivierte die Einbeziehung affinitätsveränderter Varianten in unserem Design: das hoch-affine C6.5 (C6.5B1D2) und das niedrig-affine 4D5. C6.5 ist mit der Aktivierungsdomäne CD3 ζ verbunden und kann Selektivität und Effizienz beeinflussen, während 4D5, das mit der kostimulatorischen Domäne 4-1BB verbunden ist, Parameter wie Effizienz, Persistenz oder tonisches Signal beeinflussen kann. Im Gegensatz zu früher beschriebenen Dual-Chain CARs, die zwei unterschiedliche Antigene binden, zeigte das Dual-Chain-Design, das auf zwei Epitope innerhalb von ErbB2 abzielt, eine reduzierte Aktivität und eine geringere Sicherheit im Vergleich zu konventionellen CAR Konstrukte der zweiten Generation, die ErbB2 Domäne IV erkennen. Im Gegensatz dazu demonstrierten konventionelle CARs, die entweder das native 4D5 oder seine affinitätsgereifte Variante enthielten, eine überlegene Selektivität und Antitumor-Potenz. Diese Befunde unterstreichen die Vorteile optimierter ErbB2-zielender CARs zweiter Generation gegenüber Dual-Epitop-Strategien zur Verbesserung der Sicherheit und Wirksamkeit im Kontext solider Tumoren.

Abstract

Chimeric antigen receptor (CAR)-T cell therapy has achieved remarkable clinical success in the treatment of hematological malignancies, yet its efficacy in solid tumors remains limited in part by the scarcity of exclusively expressed tumor-specific antigens (Mitra et al., 2023). ErbB2 is an attractive therapeutic target due to its frequent overexpression in several solid cancers (Oh & Bang, 2020); however, its basal expression in normal tissues raises safety concerns (Morgan et al., 2010). To evaluate strategies that enhance ErbB2 selectivity, we engineered a panel of CAR constructs incorporating the ErbB2-targeting antibody fragments C6.5 and 4D5 in either conventional second-generation (2nd Gen) or dual-chain formats. Previous studies have shown that the 4D5-derived antibody trastuzumab can induce substantial background signaling and pose safety risks (Morgan et al, 2010), motivating the inclusion of affinity-maturated variants in this design – the high-affinity C6.5 (C6.5B1D2) and the low-affinity 4D5. C6.5 which is connected to the CD3 ζ activation domain can influence selectivity and efficiency, while 4D5, connected to the co-stimulatory domain 4-1BB can influence parameters like efficiency, persistence or tonic signaling. Unlike previous described dual-chain CARs that engage two distinct antigens, the dual-chain design targeting two epitopes within ErbB2 showed reduced activity and a narrower safety margin compared with conventional 2nd Gen CAR constructs recognizing ErbB2 domain IV. In contrast, conventional CARs incorporating either the native 4D5 or its affinity-maturated variant demonstrated superior selectivity and antitumor potency. These findings highlight the advantages of optimized single-chain ErbB2-targeting CARs over dual-epitope strategies for improving safety and efficacy in solid tumor settings.

1. Introduction

1.1. Biological background of ErbB2

ErbB2, also referred to as HER2/neu, is a member of the human epidermal growth factor receptor family, comprising EGFR/ErbB1, ErbB3, and ErbB4, which collectively orchestrate signaling pathways governing cell growth, survival, and proliferation (Landgraf, 2007). Distinct to its family counterparts, ErbB2 lacks an identified ligand and is therefore unable to engage in ligand-dependent homodimerization (Bai et al., 2023). Under conditions of overexpression, however, ErbB2 can form ligand-independent homodimers, enabling downstream signaling even in the absence of ligand stimulation (Morgan et al., 2010). Under physiological conditions, its activation relies on heterodimerization with other receptors of the family following their respective ligand engagement (Bai et al., 2023). Among the possible dimerization configurations, ErbB2 most effectively heterodimerizes with EGFR and ErbB3 (Cheng, X., 2024). Formation of homo- or heterodimers results in phosphorylation of the receptor complex, thereby triggering downstream signaling pathways, including PI3K/AKT or MAPK, which stimulate proliferation, cell growth, and differentiation (Bai et al., 2023). Dysregulated ErbB2 activity plays a central role in the pathogenesis of multiple solid malignancies. The profile of ErbB2 dimerization partners is context-dependent; in breast cancer, ErbB2/ErbB3 heterodimers predominate, whereas in other tumor types ErbB2 may interact with additional receptors or become activated through mutation-driven mechanisms (Cheng, X., 2024).

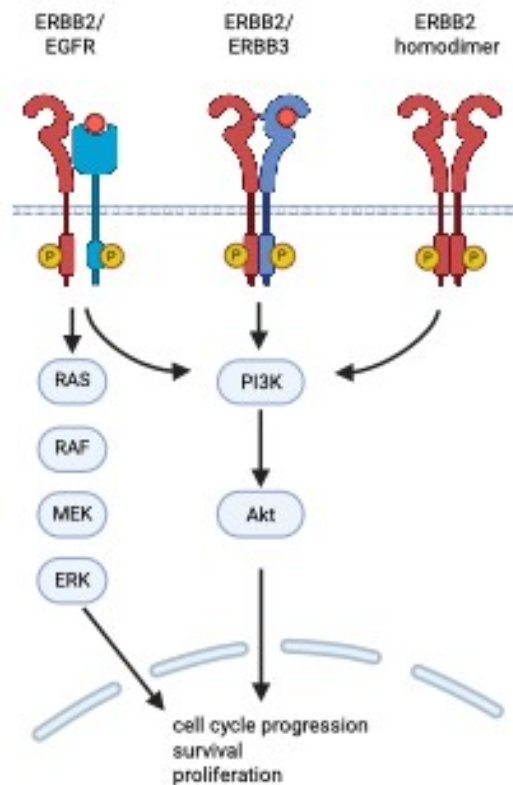


Figure 1: Schematic overview of ErbB2-mediated signaling. ErbB2 forms either ligand-independent homodimers or ligand-dependent heterodimers with other members of the EGFR/ErbB receptor family. Dimerization induces phosphorylation of the receptor complex and activates downstream signaling pathways that regulate cell cycle progression, survival, and proliferation. Created with BioRender.com and adapted from (Cheng, X., 2024).

Approximately 10 million people died from cancer in 2020. Breast cancer ranks as the leading cancer type in women and is among the top five most common cancers overall (Finn et al., 2025). In contrast, ovarian cancer is significantly less frequent, with approximately 314,000 new cases globally in 2020 – representing only around 1,6% of all cancer cases (Finn et al., 2025). What both cancer types have in common is the overexpression of ErbB2. It is overexpressed in approximately 25-30% of breast cancer cases and is associated with increased tumor aggressiveness and poor prognosis. ErbB2 overexpression is not limited to breast and ovarian cancers but is also found in a variety of other solid tumors, including gastric, esophageal, and bladder cancers, highlighting its broad relevance in oncology. Given its pivotal role in tumor biology, ErbB2 is considered a well-established oncogenic driver and a clinically relevant therapeutic target (Slamon et al., 2001). Antibody-based therapies targeting ErbB2, such as the monoclonal antibody trastuzumab (Herceptin), have achieved significant therapeutic responses in patients with ErbB2^{high} breast cancer, making

trastuzumab a promising candidate for CAR-T cell engineering (Oh & Bang, 2020). However, a major limitation is the poor tumor selectivity since ErbB2 is also expressed at lower levels in normal epithelial tissues (Morgan et al., 2010). Furthermore, efficacy is often limited by the short half-life of antibodies used and a low local concentration at the tumor site, which is often insufficient to achieve a long-lasting antitumor effect (Stern & Herrmann, 2005).

1.2. Chimeric antigen receptor (CAR)-T cell immunotherapy

CAR-T cell therapy constitutes a promising therapeutic strategy in oncology, relying on the genetic modification of patient-derived T cells to recognize and eradicate cancer cells (Dabas & Danda, 2023). CAR-T cell therapy represents an innovative form of adoptive T cell immunotherapy (ACT) that aims to redirect the antigen specificity of T cells toward malignant cells (Dabas & Danda, 2023). In this approach, autologous T cells are collected from the patient's peripheral blood through leukapheresis (Ying et al., 2019) and subsequently genetically engineered in vitro, commonly using viral vectors such as lentiviruses or retroviruses, to express recombinant CARs (Restifo et al., 2012). These synthetic immune receptors endow T cells with the capacity to recognize and eliminate tumor cells independently of the Major Histocompatibility Complex (MHC), in contrast to the conventional T cell receptor (TCR), which requires antigen presentation via MHC molecules (Labanieh et al., 2018). Following expansion in culture, the engineered CAR-T cells are reinfused into the patient (Uscanga-Palomeque et al., 2023). Upon reinfusion, CAR-T cells circulate until binding their target antigen (Benmeharek et al., 2019). Engagement of the CAR triggers CD3 ζ signaling domain, activating the T cell and inducing the release of cytolytic proteins and pro-inflammatory cytokines, such as Interferon- γ (IFN- γ) and Interleukin-2 (IL-2) (Lee et al., 2019), which mediate tumor cell lysis (Zhang et al., 2017). CAR-T cell-based immunotherapies have shown particularly promising outcomes in treating hematologic malignancies such as acute lymphoblastic leukemia and lymphomas. However, their impact on solid tumors remains limited, largely because suitable surface antigens are lacking (Mitra et al., 2023).

1.3. CAR-T cell design

Structurally, CARs consist of several key domains: an extracellular antigen-binding region, a spacer or hinge domain, a transmembrane region, and intracellular signaling domains. A CAR commonly utilizes an antibody-derived single-chain variable fragment (scFv) as its extracellular antigen-binding domain. The scFv consists of the variable domains of the heavy (VH) and light chains (VL) of an immunoglobulin, connected by a flexible peptide linker. This linker, typically 10-25 amino acids in length, is enriched in hydrophilic residues to ensure solubility and proper folding. Among the various linker designs, the tripeptide repeat (Gly₄Ser)₃ is the most common sequence (Malpiedi et al., 2013). The antigen-binding domain is linked to the transmembrane (TM) region by the hinge or spacer domain, which plays a key role in receptor flexibility, stability, and target accessibility. Commonly, the hinge is derived from IgG1, CD8a, or CD28, each influencing CAR performance differently. While IgG1 hinges provide structural stability, CD8a and CD28 hinges often enhance antigen engagement and can reduce excessive activation, leading to improved CAR persistence and function (Huang et al., 2020). The transmembrane domain forms the link between the extracellular and intracellular components and anchors it within the plasma membrane. It typically consists of a hydrophobic α -helical segment that stabilizes receptor integration and shapes the intracellular signaling profile (Huang et al., 2020). Among the most frequently used TM domains are those derived from CD8a, CD28, and 4-1BB (Huang et al., 2020) (Zhang et al., 2017). The CD8a TM domain is often favored for its association with lower activation-induced cell death, which can support longer persistence of CAR-T cells (Huang et al., 2020). The CD28 TM is considered structurally stable (Zhang et al., 2017) and helps maintain robust receptor expression (Fujiwara et al., 2020). The intracellular signaling domain of a CAR typically contains the CD3 ζ chain with three immune-receptor-tyrosine-based-activation-motifs (ITAMs) (Benmehbarek et al., 2019), which initiate T cell activation upon antigen recognition. Additional costimulatory domains such as CD28 or 4-1BB amplify these signals, promoting proliferation and prolonging CAR-T cell persistence (Huang et al., 2020).

1.4. The evolution of CAR design

CAR-T cell designs have evolved through several technological refinements, leading to five distinct generations of constructs (Uscanga-Palomeque et al., 2023). The first generation (1G) of CAR constructs represented the earliest and most minimalistic structure, composed of an extracellular antigen-recognition domain and the intracellular CD3 ζ signaling domain, without additional costimulatory input (Eshhar et al., 1993). This design resulted in poor in vivo persistence, limited proliferation, and modest antitumor activity. To improve functionality, a single costimulatory domain such as CD28, 4-1BB or OX40 was added alongside CD3 ζ in the second-generation (2nd Gen). This provided stronger activation, greater persistence, and significantly enhanced tumor-killing capacity (Pietrobon et al., 2021). CD28 as a costimulatory domain triggers faster immune response, whereas 4-1BB CARs expand more slowly but persist longer. Evidence from preclinical and clinical studies further indicates that 4-1BB costimulation favors the development of central-memory-like cells and reduces tonic signaling, thereby delaying exhaustion whereas CD28 costimulation tends to drive more effector-like phenotypes with shorter longevity. The third generation (3rd Gen) CARs combine two costimulatory domains, most commonly CD28 and 4-1BB (Huang et al., 2020). Fourth-generation CAR-T cells, known as TRUCKs (T cells redirected for universal cytokine-mediated killing), integrate antigen-specific recognition with inducible cytokine expression often controlled by NFAT-responsive promotor elements (Uscanga-Palomeque et al., 2023). These constructs can encode factors such as IL-12, IL-15, or IL-18, which are secreted locally within the tumor microenvironment upon CAR activation (Chmielewski et al., 2014) (Glienke et al., 2022). IL-12 is a potent immunostimulatory cytokine that boosts the activity of both innate and adaptive immune cells. It promotes IFN- γ production, increases granzyme B and perforin expression in T and natural killer (NK) cells, and inhibits tumor-induced regulatory T cell (Treg) expansion, thereby enhancing tumor clearance and limiting immune suppression (Tokarew et al., 2019). The fifth or 'next generation' CAR-T cells build on 2nd Gen designs but incorporate a truncated IL-2 receptor β -chain domain containing a STAT3-binding motif. Upon antigen engagement, these receptors activate CD3 ζ -mediated TCR signaling, costimulation, and JAK-STAT3/5 cytokine signaling, thereby delivering the three key signals necessary for optimal T cell activation and expansion (Tokarew et al., 2019).

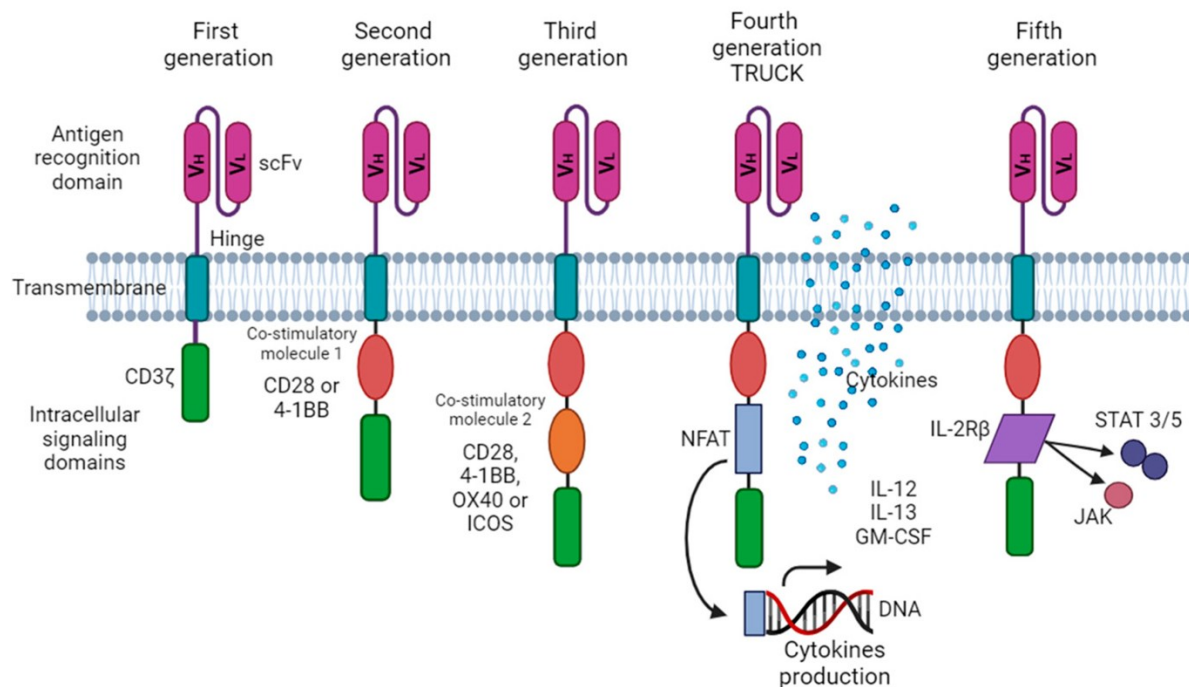


Figure 2: Overview of CAR generations. Schematic representation of the evolution of chimeric antigen receptor designs. All CARs share an antigen-recognition domain formed by an scFv, containing a V_H and V_L region, and a hinge and transmembrane segment. The first generation contains only the CD3ζ signaling domain, while the second and third generation incorporate one or two additional intracellular co-stimulatory domains. Fourth generation CARs incorporate NFAT (blue) and produce cytokines (blue dots). Fifth generation CARs integrate cytokine receptor motifs (purple). (Uscanga-Palomeque et al., 2023).

1.5. Dual targeting CAR-T cell

Several advanced CAR-T cell designs have been developed to improve specificity, efficacy, and safety beyond conventional generations. These include formats such as switchable and inhibitory CARs, that aim to increase safety and specificity with built-in safety controls. Another approach are universal CARs, which use modular adapter systems to broaden antigen recognition (Uscanga-Palomeque et al., 2023). Finally, dual-targeting, or dual-chain CARs offer an additional layer of flexibility, as they can recognize two different antigens either through separate CAR constructs co-expressed on the same T cell or within a single CAR molecule. These dual approaches can be implemented in multiple ways, including co-transduction, bivalent CARs (tandem), or split CARs (bicistronic). In co-transduction, two separate vectors are introduced into the same T cell, resulting in the parallel expression of

two distinct CARs. A bivalent or tandem CAR, encodes two scFvs within a single receptor, enabling recognition of two antigens (Marple et al., 2020). A further variation is the bicistronic CAR design, where signaling domains are distributed across two receptors, requiring engagement of both antigens for full T cell activation. One construct provides the activation signal, while the other delivers the costimulatory signaling. Full T cell activation is achieved only when both receptors are engaged simultaneously (van der Schans et al., 2020). In bicistronic dual-chain CAR designs, a 2A self-cleaving peptide is essential to enable the two receptor chains to be produced as distinct proteins (Liu et al., 2017).

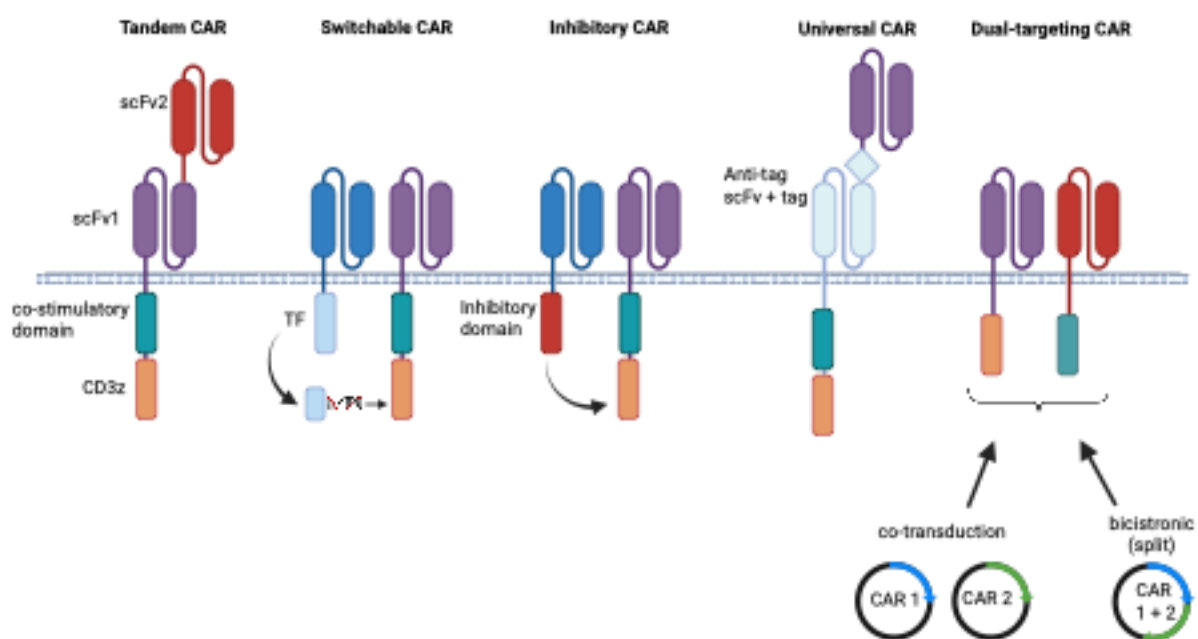


Figure 3: Overview of advanced CAR-T cell designs. Tandem CARs contain two scFvs within a single construct. Switchable CARs use adaptor molecules that can reversibly control CAR activation. Inhibitory CARs include inhibitory signaling domains that can suppress activation. Universal CARs employ an anti-tag scFv that binds to tagged adaptor molecules. Dual-targeting CARs express two distinct CAR constructs within the same T cell, either via co-transduction or bicistronic systems. Created with BioRender.com and adapted from (Uscanga-Palomeque et al., 2023).

1.6. Limitations of CAR-T cell therapy

A major limitation of CAR-T cell therapy is the emergence of antigen escape, where tumor cells downregulate or lose the targeted antigen, leading to relapse. This phenomenon has been observed across hematologic and solid malignancies. To counteract antigen escape, multi-antigen targeting strategies have been developed (Stern & Stern, 2021). Beyond

antigen loss, the immunosuppressive tumor microenvironment (TME) represents an additional barrier to durable responses. Malignant cells exploit inhibitory immune checkpoint pathways, particularly PD-1 and CTLA-4, to attenuate T cell proliferation and effector function. Under physiological conditions, immune checkpoints are essential for maintaining self-tolerance and preventing immune-mediated tissue damage during responses to infection. In the malignant setting, however, their dysregulation contributes to impaired proliferation, functional exhaustion, and diminished cytotoxicity of CAR-T cells. Mechanistically, CTLA-4 competes with the costimulatory receptor CD28 for binding to CD80 and CD86, thereby preventing full T-cell activation. CTLA-4 expression is further upregulated on activated T cells to limit prolonged TCR stimulation. PD-1 signaling dampens effector function and survival of CAR-T cells (Pardoll, 2012). Another intrinsic limitation affecting CAR-T efficacy is tonic signaling, a ligand-independent activation of the CAR that drives premature T cell exhaustion, reduces proliferation capacity, and decreases antitumor activity (Calderon et al., 2020).

1.7. CAR-T toxicity

A major barrier to the widespread clinical adoption of CAR-T cell therapy is its potential to cause severe, and in some cases life-threatening, toxicities (Morgan et al., 2010). The two most frequently observed adverse effects are cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) (Uscanga-Palomeque et al., 2023). CRS typically occurs within the first week after CAR-T cell infusion, most often beginning with fever and flu-like symptoms. Severe cases can involve hypotension, hypoxia, capillary leak, coagulopathy, and multi-organ failure. The condition is driven by excessive immune activation, with interleukin-6 (IL-6) as the primary mediator, alongside elevations in other pro-inflammatory cytokines. Management depends on grade: mild cases usually resolve with observation, while moderate to severe CRS requires IL-6 blockade (e.g. tocilizumab), sometimes with corticosteroids ("Blood and Marrow Transplant Handbook: Comprehensive Guide for Patient Care," 2011). ICANS represents the second major toxicity associated with CAR-T cell therapy and is characterized as a neurological complication. It typically develops following high-grade CRS and manifests with symptoms such as encephalopathy, aphasia, or seizures. The syndrome is driven by cytokine-mediated disruption of the blood-brain barrier

rather than direct neurotoxicity from CAR-T cells. Although most cases are reversible, management relies on corticosteroids as the primary therapeutic intervention, and anti-epileptics are used when seizures occur ("Blood and Marrow Transplant Handbook: Comprehensive Guide for Patient Care," 2011). Another major toxicity of CAR-T cell therapy is the on-target, off-tumor effect, which occurs when CAR-T cells attack normal cells that express the same antigens as tumor cells. Ideally, targeted therapy should focus on tumor-specific antigens (TSAs), which are expressed only on tumor cells. However, TSAs are rare, so most CAR-T therapies target tumor-associated antigens (TAAs), which can also be present at low levels on healthy tissues. As a result, CAR-T cells may eliminate both tumor and normal cells, sometimes causing severe or even fatal side effects (Miao et al., 2021). Optimizing CAR-T cell architecture can help reduce unintended targeting, but discovering new TSAs is complex and resource-intensive (Uscanga-Palomeque et al., 2023). Furthermore, Anaphylaxis can occur during CAR-T cell therapy, likely due to murine antibody-derived antigen-recognition domains triggering IgE-mediated reactions (Uscanga-Palomeque et al., 2023). Using fully human sequences or simplified CAR structures can reduce immunogenicity without affecting tumor-killing activity (Miao et al., 2021).

1.8. T cell subsets and function

T cells build a highly differentiated and functionally heterogeneous population that can be divided into several subsets, each with distinct roles in immunity (Golubovskaya & Wu, 2016). Naïve T cells (T_N) are antigen-inexperienced cells with high proliferation potential upon first exposure (Gattinoni et al., 2012). Memory T cells, including central memory T cells (T_{CM}) and effector memory T cells (T_{EM}), are capable of self-renewal and rapid expansion upon re-exposure to antigen (Golubovskaya & Wu, 2016). Terminally differentiated effector T cells (T_{TE}) exert strong cytotoxicity but exhibit minimal longevity. Finally, stem cell memory T cells (T_{SCM}) represent the least differentiated subset among antigen-experienced T cells. They combine the longevity and self-renewal capacity of naïve cells with the rapid responsiveness of memory T cells, making them particularly valuable for long-term immunity (Gattinoni et al., 2012). T cells can also be categorized based on surface glycoproteins: $CD8^+$ cytotoxic T cells directly kill target cells, whereas $CD4^+$ helper T cells provide supportive

signals through cytokine secretion and help maintain memory functions (Golubovskaya, Wu, 2016). Regulatory T cells (Tregs) are characterized by surface expression of CD4 and possess immunosuppressive functions (Sakaguchi et al., 2020).

Recent evidence showed that the effectiveness of CAR-T cell therapy is closely linked to the functional characteristics of these subsets (Golubovskaya, Wu, 2016). Less differentiated cells, including T_{SCM} and T_{CM} , are associated with enhanced persistence, proliferative capacity, and durability of anti-tumor responses, whereas more differentiated effector populations provide immediate cytotoxicity but limited long-term persistence (Golubovskaya, Wu, 2016) (Koh et al., 2023). $CD8^+$ CAR-T cells with phenotype resembling T_{SCM} exhibit enhanced anti-tumor activity compared to T_{CM} . High frequencies of $CD8^+$ T cells are generally linked to potent cytotoxicity, while $CD4^+$ T cells support proliferation and maintenance of memory cells, and a high proportion of Tregs may dampen anti-tumor activity (Sakaguchi et al., 2020) (Golubovskaya, Wu, 2016).

2. Aim of the study

The aim of this study was to evaluate whether dual-chain CAR constructs targeting two different epitopes of ErbB2 could enhance selectivity, efficacy, and long-term persistence of CAR-T cell responses compared to conventional CAR constructs of the second generation. Achieving high selectivity in targeting solid tumors remains a major challenge, as most surface antigens, including ErbB2, are not exclusively expressed on malignant cells. This can lead to on-target, off tumor toxicity and restrict therapeutic benefit. Furthermore, conventional second-generation CAR designs frequently exhibit tonic signaling, resulting in an earlier onset of exhaustion. The dual-chain CAR design aims to solve these problems by combining two ErbB2-specific scFvs with specificity for distinct epitopes and affinity-matured variants to improve tumor discrimination while maintaining strong effector function.

The first objective was to create and express four dual-chain CARs, each containing two distinct ErbB2-specific scFvs, alongside four CAR constructs of the second generation carrying a single scFv. After confirming successful expression of all constructs on T cells, their

functional activity was evaluated through cytotoxicity assays and cytokine secretion assays. Cytotoxicity and cytokine release were assessed in co-culture with ErbB2-positive tumor cell lines (SKOV-3, MCF-7), ErbB2 knockout MCF-7 cells as a negative control, and fibroblasts representing normal tissue with physiological expression of ErbB2. Previous studies demonstrated that the 4D5-derived scFv is associated with substantial background signaling, prompting its coupling to the 4-1BB costimulatory domain and the generation of a low-affinity variant to mitigate safety risks. Conversely, the high-affinity C6.5 variant was hypothesized to be highly effective while maintaining selectivity. Based on this rationale, we hypothesized that the C6.5_{high}/4D5_{low} dual-chain CAR construct, would exhibit the most balanced therapeutic profile among the dual-chain designs, effectively combining the high activation potential of C6.5_{high} with the safety-optimized co-stimulation of the 4D5_{low} variant to achieve an optimal balance of selectivity, efficiency, and persistence.

3. Material

3.1. Antibodies

3.1.1. Antibodies for flow cytometry

Antibody	Epitope	Isotype	Cat.nº/ Clone	Manufacturer
Goat anti-mouse IgG Fab-Biotin	Fab	IgG	1015-08	SouthernBiotech
Mouse anti-human IgG1 FITC	CD3	IgG1, κ	300440	BioLegend
Anti-human CD4 BV510	CD4	Recombinant human IgG1	REA623	Miltenyi Biotec
Anti-human CD8 PerCP-Vio700	CD8	Recombinant human IgG1	REA734	Miltenyi Biotec
Anti-human/mouse CD27 PE	CD27	Recombinant human IgG1	REA499	Miltenyi Biotec
Anti-human CD45RO-FITC	CD45RO	Mouse IgG2a, κ	304204	BioLegend
Anti-human CD45RO PE-Vio770	CD45RO	Recombinant human IgG1	REA611	Miltenyi Biotec
Anti-human CD45RA-PerCP	CD45RA	IgG1	21810455	ImmunoTools
Anti-human CD62L APC	CD62L	Recombinant human IgG1	REA615	Miltenyi Biotec
Anti-human CD95	CD95	Mouse IgG1, κ	305623	BioLegend

BV421

Rabbit anti-mouse PE	G4S	Rabbit IgG	E702V	Cell Signaling Technology
Anti-human CD279 PE	PD-1	Mouse IgG1, κ	329906	BioLegend
Anti-human CD340 APC	CD340 (ErbB2)	Mouse IgG1, κ	324408	BioLegend
Isotype APC	-	Mouse IgG1, κ	400120	BioLegend

3.1.2. Antibodies for ELISA

Antibody	Epitope	Concentration [mg/ml]	Cat.nº	Manufacturer
Purified mouse anti-human	IFN-γ	1 mg/ml	551221	BD Phamingen
Biotin mouse anti-human	IFN-γ	0,5 mg/ml	554550	BD Phamingen
Purified mouse anti-human	IL-2	0,5 mg/ml	555051	BD Phamingen
Biotin mouse anti-human	IL-2	0,5 mg/ml	555040	BD Phamingen

3.1.3. Antibodies for T cell activation

Antibody	Epitope	Cat.nº	Manufacturer
Ultra-LEAF Purified anti-human CD3 (OKT-3)	CD3	317326	BioLegend
Ultra-LEAF Purifies anti-human CD28 (15E8)	CD28	102116	BioLegend

3.2. Enzymes and buffers

3.2.1. Restriction enzymes

Enzyme	Recognition sequence	Cat.nº	Manufacturer
AarI	5'...CACCTGC(N)4↓(N)4...3' 3'...GTGGACG(N)4↑(N)4...5'	ER1582	Thermo Scientific
BamHI	5'...GGATCC...3' 3'...CCTAGG...5'	ER0051	Thermo Scientific
NcoI	5'...CCATGG...3' 3'...GGTACC...5'	R0193L	New England Bio Lab
NcoI Fast Digest	5'...CCATGG...3' 3'...GGTACC...5'	FD0575	Thermo Scientific
XcmI	5'...CCA(N)4↓(N)8TGG...3'	R0533L	New England Bio Lab

	3'...GGT(N)8↑(N)4ACC...5'		
XhoI	5'...CTCGAG...3' 3'...GAGCTC...5'	ER0695	Thermo Scientific
XhoI Fast Digest	5'...CTCGAG...3' 3'...GAGCTC...5'	FD0694	Thermo Scientific

3.2.2. Other enzymes

Enzyme	Cat.nº	Manufacturer
Taq DNA-Polymerase	EP0401	Thermo Scientific
T4 DNA Ligase	EL0011	Thermo Scientific

3.2.3. Restriction enzyme buffers

Buffer	Content buffer	Cat.nº	Manufacturer
10x Buffer AarI, Bpu10I, Scal, PstI, AclI	Contains BSA	B27	Thermo Scientific
FastDigest Green Buffer (10X)	-	B72	Thermo Scientific
Tango Buffer (10X)	33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA	BY5	Thermo Scientific

3.3. Chemicals

Chemicals	Cat.nº.	Manufacturer
Ampicillin	PHR2838	Merck
Bovine serum albumin (BSA)	250372	Sigma Aldrich
Dimethylsulfoxid (DMSO)	472301	Merk
dNTPs	N04475	NewEngland BioLabs
Glycerol	1.04095	Merk
Fetal calf serum (FCS)	P40-37500	PAN Biotech
Recombinant human IL-2		Novartis
Recombinant human IFN-γ		Boeringer Ingelheim
Lymphoprep™	1856	Serumwerk Bernburg
PEIpro	101000017	PolyPlus
Penicillin-Streptomycin	P06-07100	PAN Biotech
Poly-D-Lysine (PDL)	354210	Corning
Streptavidin-HRP	405210	BioLegend
Streptavidin-PE	405203	BioLegend
Sulfuric acid (H ₂ SO ₄)	7664-93-9	Roth
Thiazole Orange	107091-89-4	Sigma Aldrich
3,3',5,5'-Tetramethylbenzidin	79110750	Invitrogen

(TMB)		
Trypan Blue solution	T8154	Sigma Aldrich
Trypsin-EDTA (x10)	P10-024100	PAN Biotech
Tween-20	817072	Merck
Q5 x2 Master Mix	M04925	NewEngland BioLabs
5x Q solution	1005485	Qiagen
6x TriTrack DNA loading dye	R1161	Thermo Scientific

3.4. Media and Buffer

Media	Content	Manufacturer
DMEM (1X) + GlutaMAX-I XXX	Dulbecco's Modified Eagle Medium supplemented with 10%FCS, 1% penicillin- streptomycin, 1% HEPES buffer	Gibco
LB-Amp	10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl; Supplemented with 100 µg/ml Ampicillin	-
LB-media	10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaC	-
RPMI Medium 1640 (1X)- Glucose XXX	Roswell Park Memorial Institute 1640 Medium supplemented with 10%FCS, 1% penicillin-streptomycin, 1% HEPES buffer	Gibco
Coating Buffer	A+B, A: Na ₂ CO ₃ (21,2 g/l), B: NaHCO ₃ (16,8 g/l)	-
HEPES Buffer	[c]= 50 mM	Invitrogen
10x PBS	1.37 M NaCl; 27 mM KCl; 100 mM Na ₂ HPO ₄ ; 18 mM KH ₂ PO ₄	-
PBS-T	1x PBS supplemented with 0,1% Tween	-
50x TAE Buffer	50 mM EDTA disodium salt; 2 M Tris; 11 M glacial/acetic acid	-

3.5. Oligonucleotides for Cloning

Primer (Labory internal numbering)	Sequence	Manufacturer
#3166 reverse	5'ACAAATTGGACTAATCCGGATC 3'	Eurofins Genomics

#3167 reverse	5'CGAGGAGCTGGAGTTGTAGTGGTGCCTTGGGTTTTGG 3'	Eurofins Genomics
#3170 forward	5' CCCCCAAACCCAAGGACACCACTACAACCTCCAGCTCCTCG 3'	Eurofins Genomics
#3171 forward	5'GAAGGCGCGGCCCGCCTAGACCATGGATTTTCAGGTGCAGA 3'	Eurofins Genomics
#3172 reverse	5'CGAGGAGCTGGAGTTGTAGTCGCACCTAGGACGGTG 3'	Eurofins Genomics
#3173 forward	5'AGGTCACCGTCCTAGGTGCGACTACAACCTCCAGCTCCTCG 3'	Eurofins Genomics

3.6. Plasmids

3.6.1. Retroviral CAR expression plasmids

Number	Content
#714	pBullet LK_C6.5B1D2_hlgG_CD28_CD3ζ
#745	pBullet LK_C6.5_hlgG_CD28_CD3ζ
#1586	pBullet LK_4D5_hlgG_CD28_CD3ζ
#1849	pBullet LK_4D5mutant_hlgG_CD28_CD3ζ
#3165	pBullet LK_C6.5B1D2_CD8ah_CD28TM_41BB_CD3ζ
#3251	pBullet LK_C6.5_CD8ah_CD28TM_CD3ζ_T2A_LK_4D5_CD8ah_CD28TM_41BB
#3252	pBullet LK_C6.5B1D2_CD8ah_CD28TM_CD3ζ_T2A_LK_4D5_CD8ah_CD28TM_41BB
#3253	pBullet LK_C6.5_CD8ah_CD28TM_CD3ζ_T2A_LK_4D5mutant_CD8ah_CD28TM_41BB
#3254	pBullet LK_C6.5B1D2_CD8ah_CD28TM_CD3ζ_T2A_LK_4D5mutant_CD8ah_CD28TM_41BB
#3265	pBullet LK_C6.5_CD8ah_CD28TM_41BB_CD3ζ
#3267	pBullet LK_4D5_CD8ah_CD28TM_41BB_CD3ζ
#3268	pBullet LK_4D5mutant_CD8ah_CD28TM_41BB_CD3ζ

3.6.2. Retroviral helper plasmids

Number	Content
#3076	Expression of the murine Moloney leukemia virus (M-MuLV) proteins gag (group-specific antigens) and pol (reverse transcriptase polymerase)
#3077	Expression of the envelope protein env of the gibbon monkey leukemia virus (GALV); VSV-G tropism

3.7. Human cell lines

Cell line	Characteristics	Reference
Fibroblasts	Primary human fibroblasts derived from connective tissue	Ito, A., et al. 1995
HEK 293T	Derivate of renal carcinoma cell line 293; expression of SV40 large T antigen	Pear, Nolan et.al 1993
SKOV-3	Human ovarian cancer cell line, with high ErbB2 expression	Wang, S., et al. 2019
MCF-7	Human breast adenocarcinoma cells	Soule, H. D., et al. 1973
MCF-7 ErbB2 KO	Human breast adenocarcinoma cells with ErbB2 knockout	Soule, H. D., et al. 1973

3.8. Bacterial strains

Bacterial strain	Properties	Reference
E. coli DH5a	Genotyp F-, end A1, hsd R17 (rk-, mk-), sup E44, thi-1, lambda-, rec A1, gyr A96, Φ 80 d lacZ δ	(Woodcock et al., 1989)

3.9. Consumables

Consumable	Manufacturer
Cell culture plate 10 cm	Sarstedt AG
Cell culture plate 15 cm	Sarstedt AG
Cryotubes	Greiner
Microtube 1,5 ml	Sarstedt AG
Microtube 2 ml	Sarstedt AG
Falcon-tube 10 ml	Sarstedt AG
Falcon-tube 15 ml	Sarstedt AG
Falcon-tube 30 ml	Sarstedt AG
Falcon-tube 50 ml	Sarstedt AG
Maxisorb plates 96x	Thermo Scientific
Well plates 6x	Sarstedt AG
Well plates 24x	Sarstedt AG
Well plates 96x	Greiner

3.10. Kits

Kit	Manufacturer
NucleoSpin Plasmid Mini Kit	Machery-Nagel
NucleoBond Xtra Midi Kit	Machery-Nagel
NucleoSpin Gel and PCR Clean Up Kit	Machery-Nagel

3.11. Devices

Device	Manufacturer
Bacteria plate incubator	Binder
Casting System Compact	Biometra
CellDrop FL Fluorescence Cell Counter	DeNovix
Centrifuge (Maxi)	Thermo Scientific
Centrifuge (Mini)	Thermo Scientific
Elektrophoresis Power supply	Biometra
Freezer -150 °C	PCHbi
Freezer-80 °C	PHCbi
Freezer -20 °C	Liebherr
Gel Imager	Analytik Jena
MACSQuant	Miltenyi Biotech
Multicycler	Eppendorf
NanoPhotometer	Implen
Photometer Multiscan GO	Thermo Scientific
Refrigerator 4 °C	Liebherr
Shaking incubator (InnovaS44i)	Eppendorf

3.12. Programs

Benchling
Excel
FlowJo
GraphPad
Vector NTI

4. Methods

4.1. Microbiological Methods

4.1.1. Transformation of chemo-competent *E. coli* DH5 α bacteria

For transformation, chemo-competent *E. coli* DH5 α bacteria stored at -80 °C were thawed on ice. Approximately 50 μ l of bacterial suspension was combined with 100 ng of the plasmid DNA and kept on ice for 30 minutes. The cells were then exposed to heat shock at 42 °C for 90 seconds, immediately cooled on ice for 5 minutes, and subsequently supplemented with 900 μ l antibiotic-free LB medium. Following an incubation period of 1 hour at 37 °C to allow recovery and expression of antibiotic resistance, the cultures were centrifuged (2 min, room temperature, 1100 x g), resuspended in a reduced volume of LB medium, and spread on Ampicillin containing LB agar plates. Plates were incubated overnight at 37 °C to enable colony formation.

4.1.2. Storage of *E. coli* DH5 α bacteria

To preserve the transformed *E. coli*, 700 μ l bacterial culture was combined with 300 μ l of glycerol, transferred into cryovials, and stored at -80 °C.

4.2. Molecular biological methods

4.2.1. Isolation of plasmid DNA from *E. coli* DH5 α bacteria

Plasmid DNA was purified from *E. coli* DH5 α cultures for subsequent transfection of human embryonic kidney 293T (HEK293T) cells using an anion-exchange chromatography approach. DNA preparation was carried out with the NucleoBond Xtra Midi Kit (Macherey-Nagel) according to the manufacturer's protocol. For plasmid amplification, 300 ml LB medium was inoculated with bacterial colonies derived either from LB agar plates or glycerol stocks. The isolated DNA was dissolved in double-distilled water and stored at -20 °C.

4.2.2. Determination of the DNA concentration

The concentration of plasmid DNA was determined spectrophotometrically using the “NanoDrop 1000” instrument. Absorbance values were recorded at 260 and 280 nm with ddH₂O as reference blanks. DNA concentrations were adjusted to a final concentration of 1 µg/µl.

4.2.3. Cloning strategy for plasmid construct generation

For the generation of plasmid constructs, gene-specific primers were first designed to amplify DNA fragments from vectors via PCR. These primers had to be solved in nuclease free water and subsequently diluted in a 1:10 ratio. PCR reactions were prepared using 12,5 µl Q5 Mastermix, 2,5 µl of each primer, 1 µl Q solution, 1 ng DNA template, and ddH₂O to the final volume. A thermal cycler capable of applying variable annealing temperatures was used, as different primers required specific temperatures. The resulting PCR products were separated by agarose gel electrophoresis. Agarose was dissolved in 1x TAE buffer (1:100) cooled to approximately 50 °C and supplemented with Thiazole Orange (1:1000). Samples were mixed with TriTrack loading dye (1:6) and loaded alongside a DNA ladder. Electrophoresis was performed at 100 V for 25 minutes, and fragments of the expected size were excised. DNA fragments were purified from the gel using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) according to the manufacturer’s instructions and eluted in ddH₂O. In parallel, a commonly used plasmid backbone including Ampicillin resistance was excised out of another plasmid through restriction digest. For this 1 µl of each enzyme, 1 µl buffer, 1 – 3 µg Plasmid DNA were mixed and subsequently ddH₂O was added to 10 µl. Based on the fragment lengths, the required DNA amounts (in ng) were calculated using In-Fusion Ratio Calculator. These values were then divided by the DNA concentrations determined via NanoDrop (ng/µl) to calculate the exact volumes needed for each fragment in the In-Fusion reaction. For each In-Fusion reaction, 1 µl of In-Fusion Master Mix was combined with 1 µl of the backbone fragment, the calculated volumes of the respective insert fragments, and nuclease-free water to a final reaction volume of 5 µl. The reaction mixtures were incubated at 50 °C for 15 minutes and subsequently placed on ice. *E. coli* DH5α bacteria were transformed with the reaction mixture as described above. Transformed cells were plated on LB agar plates containing ampicillin and incubated overnight at 37 °C. On the following day, individual colonies were picked using pipette tips

and transferred into 10 ml falcon tubes containing 5 ml LB medium supplemented with ampicillin (1:500). Cultures were incubated overnight at 37 °C with shaking at 200 rpm. Plasmid DNA was then isolated using NucleoSpin Plasmid Mini Prep Kit (Macherey-Nagel), following the manufacturers protocol. DNA was eluted in 30 µl of nuclease-free water, and concentrations were measured spectrophotometrically. To verify the correctness of the plasmid constructs, a control digest was performed using the same conditions as described above for restriction digest. The resulting fragments were analyzed by agarose gel electrophoreses.

4.2.4. Plasmid sequencing

For plasmid sequencing, samples were prepared by combining 20 µl of plasmid DNA (50 – 100 ng/µl) with 2 µl of the corresponding sequencing primer. The prepared samples were sent to Eurofins for sequencing and were later analyzed with Benchling.

4.3. Cell culture methods

4.3.1. Cultivation of eukaryotic cells

Human MCF-7 and MCF-7 ERBB2 knockout (KO) breast cancer cell lines were cultured in DMEM GlutaMAX media supplemented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) penicillin-streptomycin, and 1 % (v/v) HEPES buffer (referred to as DMEM XXX) at 37 °C and 10% CO₂. Cells were split once or twice per week, depending on their proliferation rate. Human fibroblasts and the SKOV-3 ovarian cancer cell line were maintained in RPMI 1640 GlutaMAX media supplemented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) penicillin-streptomycin, and 1 % (v/v) HEPES buffer (referred to as RPMI XXX). These cells were cultured at 37 °C and 5% CO₂ and were split twice per week to maintain exponential growth. Primary human T cells were also cultivated in RPMI XXX at 37 °C in 5% CO₂. HEK293T cells, a retroviral packaging line originating from immortalized human embryonic kidney cells, were maintained in DMEM 1640 GlutaMAX medium supplemented with 10 % (v/v) FCS, 1 % (v/v) penicillin-streptomycin. Cultures were incubated at 37 °C and 5% CO₂.

4.3.2. Passage of adherent cells

To detach adherent cells, the culture medium was first aspirated, and the cells were rinsed once with sterile PBS to remove residual serum. After removing the PBS, approximately 3 ml of 1x Trypsin-EDTA solution was added to each T75 flask. The flasks were incubated at room temperature for about 2 minutes. If the cells remained adherent, an additional incubation step at 37 °C for 2 minutes was performed. Once the cells were fully detached, activity was neutralized by adding medium (DMEM XXX or RPMI XXX, depending on the cell line). The resulting cell suspension was collected and centrifuged at 300 x g for 5 minutes. After centrifugation, the supernatant was discarded, and the cell pellet was resuspended in fresh pre-warmed medium. A portion of the resuspended cells was then transferred to a new culture flask for further cultivation.

4.3.3. Cell counting and viability assessment

Cell counting and viability determination were performed using the “CellDrop cell counter”. Cell samples were diluted 1:2 with Trypan blue to stain non-viable cells. 10 µl of the dilution was applied to the cell counter for measurement, which automatically determined both total cell number and viability.

4.3.4. Cryopreservation and thawing of cells

For long-term preservation, cells were first harvested by centrifugation at 300 x g for 5 minutes at room temperature. A minimum of 5×10^6 cells was resuspended in 1 ml of fresh culture medium and supplemented with 10% (v/v) dimethyl sulfoxide (DMSO). After gentle mixing, the suspension was transferred into cryovials, which were immediately placed in a pre-cooled “Mr. Frosty” freezing container. The cells were then frozen at -80 °C, allowing for a controlled cooling rate of approximately -1 °C per minute. After 24 to 48 hours, the vials were transferred to a -150 °C freezer for long-term storage.

For thawing, frozen cell vials were gently warmed by adding small volumes of pre-warmed culture medium to the cell suspension during thawing. Once fully thawed, the suspension was transferred into a total volume of 10 ml warm medium. The cells were then centrifuged at 300 x g for 5 minutes, the supernatant was discarded, and the pellet was resuspended in fresh medium for further cultivation.

4.3.5. Detection of mycoplasma bacteria

All cell lines used in this study were tested for mycoplasma contamination once per week. Detection was performed using a PCR-based assay targeting a conserved region of the mycoplasma 16S rRNA gene. For this purpose, 100 µl of culture supernatant was collected from each cell line, transferred into 1,5 ml tubes, and centrifuged at 2100 x g for 5 minutes. The resulting supernatant was transferred to a new tube and subsequently heat-inactivated at 95 °C for 5 minutes. The composition for each PCR reaction mix was as detailed below (Table 1).

Table 1: Content for mycoplasma detection test

Content	Volume [µl]
Supernatant	2 µl
Forward Primer	1 µl
Reverse Primer	1 µl
25 mM MgCl ₂	2 µl
10x PCR Run buffer	2,5 µl
10 mM dNTPs	1 µl
Taq polymerase	0,2 µl
Nuclease-free water	15,3 µl

Amplification was carried out in a thermocycler using a mycoplasma program. PCR products were analyzed by agarose gel electrophoresis. A band at approximately 510 bp indicated the presence of mycoplasma contamination. Contaminated cultures were immediately discarded.

4.3.6. Isolation and activation of primary human T cells

Buffy coat samples were ordered and picked up from the blood bank of University Hospital Cologne. Four sterile 30 ml tubes were pre-filled with 10 ml of 1x PBS. Additionally, four 50 ml tubes were filled with 16 ml of Lymphoprep. The buffy coat tubing and scissors were disinfected with 70% ethanol, then the buffy coat tubing was cut near the top, and blood was distributed into the prepared 30 ml tubes. Each 30 ml tube was then filled up to 30 ml with 1x PBS. The mixture was gently homogenized by pipetting up and down once using a 25

ml pipette. The entire volume was carefully layered onto the surface of the lymphoprep in the 50 ml tubes. The tubes were tilted to allow the mixture to flow slowly onto the density gradient without disturbing it, thus avoiding any mixing of layers. The samples were centrifuged with 800 x g, at 21 °C, with no break applied, for 20 minutes. After centrifugation, the PBMC layer, appearing as a distinct ring between the plasma and lymphoprep layers, was carefully aspirated using a 10 ml pipette and transferred to a fresh 30 ml tube. The isolated PBMCs were washed three or four times with 1x PBS to remove residual platelets and contaminants. Washing was performed by centrifugation with the following settings: 1200 rpm, 5 minutes, RT. After each washing step, the supernatant was carefully discarded. The washed PBMC pellet was resuspended in 50 ml of RPMI XXX medium for further use. Cell number and viability were determined using CellDrop. For activation, 1,5 to 2 x 10⁸ PBMCs were added to 100 ml of activation medium consisting of RPMI XXX, supplemented with 5,6 µl IL-2 (10⁴ stock), 7,3 µl anti-CD3 antibody/ OKT3 (2730 µg/µl stock), and 5,6 µl anti-CD28 antibody 15E8 (890 µg/µl stock). The cell suspension was incubated lying flat in a cell culture incubator at 37 °C with 5% CO₂.

4.3.7. PEI transfection of HEK 293T cells

HEK 293T cells were seeded 1,88 – 3,75 x 10⁶ cells per 10 cm dish in 10 ml DMEM 24 hours before transfection. For transfection of HEK 293T cells, the “PEIpro” reagent was used, and plasmids were prepared at a concentration of 1 µg/µl. The helper plasmids #3076 (recombinant MLV packaging vector) and #3077 (VSV-G transfer plasmid) were included. Two separate transfection mixes were prepared in 1,5 ml tubes according to the components outlined in table 2.

Table 2: Transfection mixtures with a PEI/DNA ratio of 2:1

	Content	Volume [µl]
Mixture 1	Construct DNA	8 µl (if 1 µg/µl)
	Helper plasmid #3076	4 µl
	Helper plasmid #3077	4 µl
	RPMI	234 µl
Mixture 2	PEIpro	16 µl
	RPMI	234 µl

Each mixture was vortexed to ensure homogeneity. Subsequently mixture 2 was added entirely to mixture 1, vortexed again, and incubated at RT for 15 minutes to allow complex formation. Meanwhile the HEK 293T plates were labeled and the DMEM medium was carefully aspirated and replaced by 8,5 ml serum-free RPMI. After the incubation time, the complete 500 µl transfection mixture was added dropwise to the corresponding HEK cell culture plate. Cells were incubated at 37 °C and 5% CO₂ for 3-5 hours. Subsequently, 1 ml of FCS was added to each plate to support cell viability. The cells were then returned to the incubator and left overnight under the same conditions. The retroviral supernatant was used for transduction 24 hours later.

4.3.8. Preparation of PDL-coated plates

For transduction, poly-D-lysine (PDL)-coated plates were prepared in advanced. PDL was diluted 1:100 in ddH₂O and applied at 200 µl per well on a 24-well plate. Each construct was allocated 4 wells. Plates were incubated at 4 °C overnight. PDL coating was always prepared freshly on the day of transfection.

4.3.9. Transduction of human T cells using recombinant retrovirus

Human T cells were transduced in 24-well plates that had been pre-coated overnight at 4 °C with PDL. The next day the PDL solution was removed, and plates were allowed to dry. The 10 ml of viral supernatants per plate were collected into 15ml Falcon tubes and 10 ml fresh RPMI XXX was put on every plate. The Falcons were centrifuged (300 x g, 5 minutes) to remove HEK 293T cells and a total of 1,8 ml of clarified viral supernatant was added to each well. T cells, previously incubated for two days, were centrifuged (300 x g, 5 minutes) the supernatant was discarded, and the cells were resuspended in fresh RPMI XXX medium. Cell counts were determined using the CellDrop Cell Counter. Recombinant human IL-2 was added to a final concentration of 1000 U/ml. For each well 200 µl of cell suspension containing 5×10^5 T cells were added to the viral supernatant. The plates were centrifuged (800 x g, 90 minutes) and incubated overnight at 37 °C with 5% CO₂. The following day, 1,8 ml supernatant was replaced with 1,8 ml of fresh viral supernatant harvested from HEK 293T plates. Additionally, 100 µl of RPMI XXX supplemented with 1000 U/ml IL-2 was added per well. The 24-well plates were centrifuged again (800 x g, 90 minutes) and incubated

overnight at 37 °C with 5% CO₂. On day three, the medium was exchanged for fresh RPMI XXX supplemented with 300 U/ml IL-2. Cells were cultured under these conditions for several days before assessing transduction efficiency by flow cytometry (FACS).

4.3.10. CAR-T cell cytotoxicity test

Transduced CAR-T cells were used as effector cells and were cultured without IL-2 for at least 24 hours. CAR expression was assessed by FACS, and cell numbers of both T lymphocytes and target cells were determined. SKOV-3 cells served as cancer target cells and fibroblasts as healthy target cells. Target cells were detached using 1x trypsin-EDTA and counted using Celldrop cell counter. Per well, 100 µl cell suspension, containing 2×10^4 T cells were added. T cells were added at different effector to target (E:T) ratios (1:1, 1:3, 1:9), with an end-volume of 200 µl in every well. Co-cultures were incubated for 48 hours at 37 °C and 5% CO₂. After incubation, 150 µl of supernatant was collected from each well for ELISA analysis. The cell lysis was then measured using 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and an ELISA reader.

4.3.11. Measurement of CAR-T mediated cytotoxicity by XTT assay

The XTT assay was used to assess cell viability by measuring the ability of metabolically active cells to reduce the yellow tetrazolium salt to an orange formazan product. Following the cytotoxicity co-culture assay, wells were washed with medium to remove remaining T cells. 50 µl RPMI XXX medium and 50 µl XTT solution (50 µg/ml in RPMI 1640) supplemented with 1 µl of 1,25 mM phenazine methosulfate (PMS) was added to each well. Plates were incubated for 30-120 minutes at 37 °C, and absorbance was measured at 450-650 nm (OD₄₅₀₋₆₅₀) using an ELISA reader. The maximum turnover of XTT was determined in wells containing only tumor cells. Cell lysis was then calculated in Excel.

4.3.12. Repetitive CAR-T cell cytotoxicity test

This assay was performed similarly to the standard cytotoxicity assay, except that CAR-T cells were transferred onto fresh target cells for four rounds to assess their efficacy, selectivity, and persistency. CAR expression was first confirmed by flow cytometry, and both transduced CAR-T cells and target cells were counted. SKOV-3 ovarian cancer cells and MCF-7 breast cancer cells were used as tumor targets, while MCF-7 ErbB2 KO cells served as a negative

control and fibroblasts as healthy target cells. In flat-bottom 96-well plates, 100 μ l of medium containing 2×10^4 CAR-T cells were seeded per well, followed by 100 μ l containing 2×10^4 target cells, resulting in an E:T ratio of 1:1. For each target cell line, “target only” control wells and “CAR-T cells only” wells were included. Each construct was tested in 3-5 replicates, including untransduced T cells (UTCs). When transduction rates of the constructs differed by more than 10%, a mock adjustment was performed to normalize cell input. In this approach, UTCs were added to ensure that each well contained the same total number of T cells, while maintaining the correct proportion of CAR⁺ cells. Plates were incubated for 48 hours at 37 °C and 5% CO₂. After incubation, 120 μ l of supernatant was collected from each well, frozen for later cytokine analysis, and replaced with fresh medium. Wells were gently resuspended to detach T cells. In a new 96-well plate, 2×10^4 freshly plated target cells were prepared per well, and all T cells from the previous round were transferred to these wells to a total volume of 200 μ l. “Target only” control wells were again included. Wells from the first round were subjected to an XTT assay to evaluate target cell viability. After an additional 48 hours of incubation, this process was repeated: supernatants were collected for ELISA, 2×10^4 new target cells were seeded, and T cells from second round were transferred onto the fresh targets. Wells from the second round were analyzed with XTT. When sufficient replicates were available, T cells were also stained to assess exhaustion and phenotype (see 4.4.1.). A third and fourth round of serial stimulation were performed using the same procedure.

4.3.13. Protein assay for cytokine release

First, a 96-well plate was coated with coating buffer supplemented with various concentrations of different epitopes (ErbB2 domain II and IV). For the first condition, both epitope domains were added at 1 μ g each. For the second condition, domain II was added at 0.5 μ g and domain IV at 1 μ g to account for its lower binding affinity. Additional conditions included Domain II alone at 1 μ g and 0.5 μ g, Domain IV alone at 1 μ g and 0.5 μ g, and a control without any epitopes. Each condition was prepared in duplicates. The plate was incubated overnight at 4 °C on a shaker. On the same day, transduced T cells were deprived of IL-2 for at least 24 hours. The following day, the coated plates were emptied and 200 μ l of blocking solution (2% BSA) was added to each well, followed by incubation for 2 hours at -4 °C on a shaker and subsequent washing with PBS. Finally, 40,000 CAR-T cells

were added per well to a final volume of 200 µl and incubated for 24 hours at 37 °C in 5% CO₂. After incubation, 150 µl of supernatant was collected from each well for ELISA to determine IFN-γ and IL-2 concentration.

4.4. Immunological methods

4.4.1. Flow cytometry

From each well, 200 µl T cells were collected and centrifuged (300 x g, 5 minutes). The supernatant was discarded, and the pellet was resuspended in 50 µl PBS supplemented with each 1 µl of the respective fluorescent dye-labeled detection antibodies. Samples were incubated for 15 minutes at RT in the dark, then washed with 150 µl PBS (300 x g, 5 minutes). After removal of the supernatant, cells were resuspended in 200 µl PBS and analyzed by flow cytometry (MACSQuant Analyzer). Data analysis was subsequently performed with FlowJo software. Some constructs expressed two CARs on the T cell surface, both of which had to be detected to evaluate overall transduction efficiency. Expression of CARs in general was assessed using recombinant ERBB2 protein, which binds to both scFvs. For ERBB2 staining the protocol was slightly modified, with 100 µl of reagent added and an incubation time of 45 minutes. Individual scfvs were detected using different strategies depending on their specific tags. Scfvs containing the G4S linker, a short peptide sequence (Gly₄-Ser) used to connect VH and VL chains, were stained with CD3-FITC antibody for T cell detection and G4S-PE antibody. Other scFvs were detected either with the corresponding epitope-Biotin antibody and subsequent streptavidin staining or with mouse anti-human IgG-Biotin, followed by streptavidin-PE as a secondary reagent. For T cell exhaustion analysis, antibodies against G4S, CD3, CD4, CD8, and PD-1 were used. CD4 and CD8 were employed to distinguish T cell subpopulations, while PD-1 served as a marker of T cell exhaustion and activation. For phenotypic characterization, antibodies against CD45RO, CD45RA, CD24, CD95 and CD62L were applied to differentiate between naïve, memory, and effector T cell subsets.

4.4.2. Cytokine release assay

Cytokine concentrations in the supernatants of cytotoxicity assays were quantified by ELISA. For plate coating, maxi-sorb 96-well plates were incubated with capture antibodies: mouse

anti-human IL-2 (0.5 mg/ml) and mouse anti-human IFN- γ (1 mg/ml). The final coating concentration was adjusted to 1 μ g/ml in coating buffer, with 50 μ l applied per well. Plates were incubated overnight at 4 °C on a shaker. Plates were then blocked with 200 μ l per well of 3% BSA in PBS and incubated overnight at 4 °C. After blocking, plates were washed three times with 200 μ l PBS-T per well. Supernatants collected from the cytokine assays were then added at 50 μ l per well. The bottom row of wells was reserved for IL-2 and IFN- γ standards. The highest concentration of murine IFN- γ was 10 ng/ml, and the highest IL-2 concentration was 2 U/ml. Standards were serially diluted 1:2 in PBS across the row, with PBS alone used as a negative control in the final well. Plates were incubated overnight at 4 °C on a shaker and subsequently washed four times with PBS-T. Cytokines were detected using mouse anti-human biotin-conjugated antibodies (both 0.5 mg/ml), diluted 1:500 in PBS, and added at 50 μ l/well. Plates were incubated for 1 hour at RT on a shaker and washed four times with PBS-T. Streptavidin-HRP was diluted 1:3000 and added at 50 μ l/well, followed by 30 minutes incubation at RT on a shaker. Plates were washed again four times with PBS-T and detection was performed using 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution, with 100 μ l added per well. The reaction was stopped once wells developed a medium-to-dark blue color by adding 50 μ l of 0,5 M H₂SO₄ resulting in a color change to yellow. Optical density (OD) was measured at 450-570 nm using an ELISA plate reader. OD values were first corrected by subtracting the OD value of the PBS control from each measurement. A linear standard curve was then generated in Excel, and the linear regression coefficient was used to convert each OD value into cytokine concentration.

5. Results

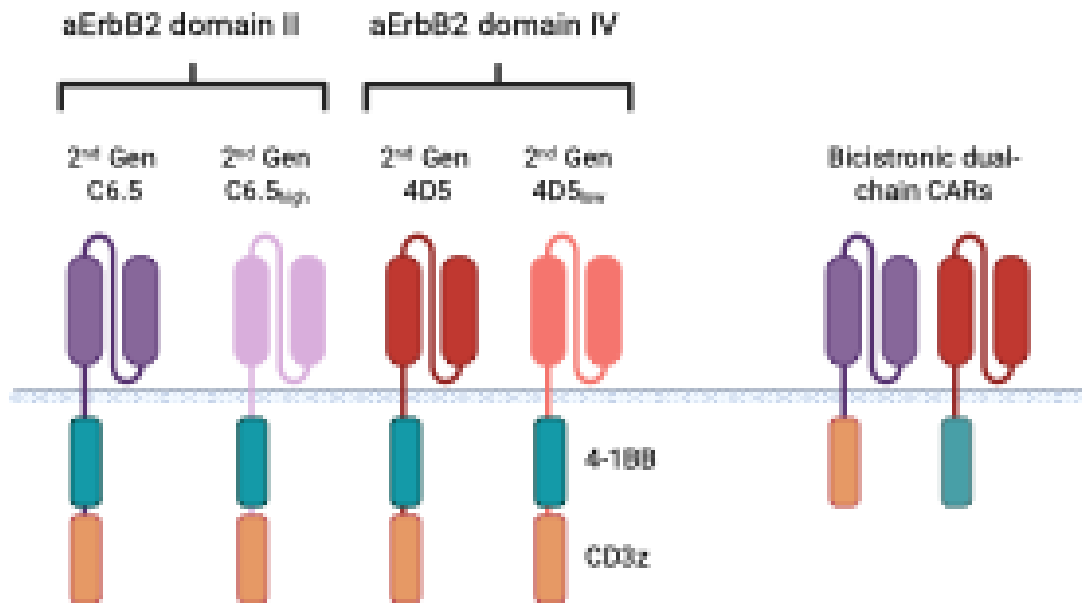
5.1. Dual-chain and 2nd Gen CAR designs

In this study we designed and cloned eight different anti-ErbB2 CARs, comprising four bicistronic dual-chain CARs and four conventional second-generation CAR constructs. All plasmids share the same backbone fragment. This backbone contains, among other elements, UTRs, promoters, and ampicillin resistance. All domains and scFvs inserted into the linearized plasmid backbone were obtained from pre-existing plasmids and assembled using in-fusion cloning. All scFvs used in this study are specific for ErbB2, with the C6.5 variants recognizing domain II and the 4D5 variants binding domain IV of ErbB2 (Figure 4A).

The CAR constructs of the second generation consist of a CD8 hinge and TM domain, the 4-1BB costimulatory domain, and the CD3 ζ activation domain. Each construct incorporates only one ErbB2-specific binding domain, either C6.5, its affinity-maturated variant C6.5B1D2 (hereafter C6.5_{high}), 4D5, or its low-affinity variant 4D5_{low} (Figure 4).

The dual-chain CAR constructs were designed to co-express two distinct receptor chains from a single transcript by incorporating a T2A self-cleaving peptide. This design enables simultaneous recognition of two distinct ErbB2 epitopes, with the C6.5-derived CAR providing the primary activation signal through CD3 ζ upon antigen binding, while the 4D5-derived CAR delivers complementary co-stimulation via 4-1BB. Four dual-chain configurations were generated: one pairing the wild-type (WT) versions of both domains, one combining the high-affinity C6.5_{high} variant with 4D5 WT, the third one integrating C6.5 WT with the low-affinity 4D5_{low} domain, and a final construct combining the two affinity-maturated domains C6.5_{high} and 4D5_{low} (Figure 4B).

A



B



Figure 4: Schematic overview of the 2nd Gen CAR and dual-chain CAR constructs

A: Structural representation of the CAR constructs used in this study. The left side shows 2nd Gen CAR constructs carrying different scFVs: purple indicates the C6.5 variants, and red indicates the 4D5 variants. Both are equipped with CD3ζ and 4-1BB signaling domains. The right panel illustrates the cis dual-chain CAR design, displaying both scFVs expressed on the surface of a T cell. **B:** Expression cassette of four cis dual-chain CARs and four 2nd Gen CAR constructs. The 2nd Gen CARs each contain a 4-1BB (turquoise) and CD3ζ (orange) domain and differ in their scFv. The dual-chain CARs comprise a scFv fused to CD8aTM (dark blue) and CD3ζ and a second scFv fused to CD28TM (light blue) and 4-1BB. The two CAR chains are separated by a T2A cleaving sequence. Created with BioRender.com

5.2. Expression of dual-chain and 2nd Gen CAR constructs on the surface of human T cells

To assess CAR expression in human T cells, they were first isolated from human peripheral blood and activated for 48 hours. The cells were then separately transduced retrovirally with seven different CAR plasmids. CAR surface expression was analyzed three days later by flow cytometry. Because the CAR constructs contain scFvs with varying binding affinities, multiple staining approaches were required to detect surface expression of both CAR chains. All staining approaches followed the same initial gating strategy: first, SSC-A versus FSC-A, excluding debris; second, FSC-A versus FSC-H selecting single cells; third, SSC-A versus CD3 to identify T cells; and finally, SSC-A versus the relevant fluorophore to detect CAR expression (Figure 5). A distinct and nearly complete CD3⁺ T cell population was detected.

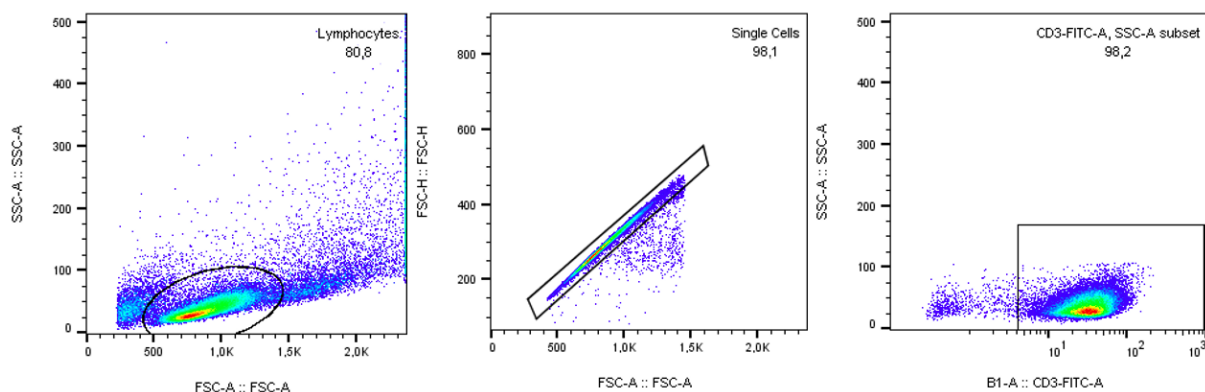


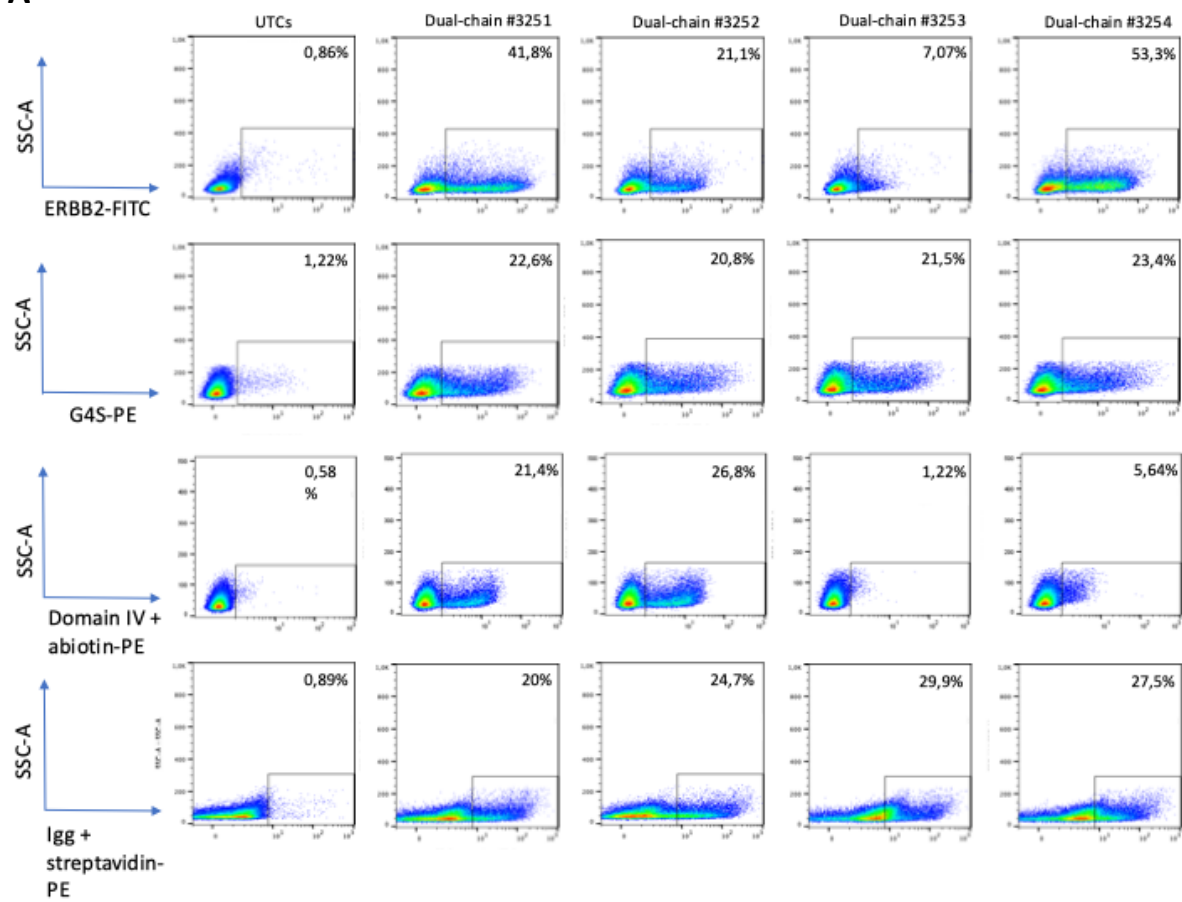
Figure 5: Gating strategy applied for flow cytometric analysis. Live lymphocytes were first selected, followed by single cells and subsequent gating of CD3-positive T cells. This gating strategy was applied to all samples analyzed.

First, ErbB2-GFP recombinant protein was used to stain all constructs, as all CAR constructs target ErbB2. Untransduced T cells (UTCs) were used as a negative control and showed no staining. All dual-chain CAR constructs, except the C6.5/4D5_{low} variant showed transduction rates of at least 23%, whereas the C6.5/4D5_{low} CAR showed a much lower rate of ~6% (Figure 5A). Next, a G4S linker specific antibody, present only in C6.5-containing CAR designs, confirmed expression of this chain, with transduction rates of approximately 22% for all dual-chain constructs. Staining with biotinylated ErbB2 domain IV recombinant protein, followed by detection with anti-biotin PE antibody, was used to verify expression of the 4D5 CAR chain in dual-chain CARs. Constructs C6.5/4D5 and C6.5_{high}/4D5 displayed transduction rates of ~25%. Detecting the 4D5_{low} domain within the dual-chain CARs was restricted by its

markedly reduced binding affinity. As a result, the C6.5/4D5_{low} CAR exhibited no detectable staining, whereas the C6.5_{high}/4D5_{low} CAR showed around 6% positivity. Since the only distinction between these two dual-chain constructs lies in the C6.5 scFv variant, all 2nd Gen CAR constructs were similarly analyzed to provide a consistent comparison (Figure 6B).

Analysis of domain IV staining in the 2nd Gen CAR constructs revealed unintended cross-reactivity of the C6.5_{high} scFv to ErbB2 domain 4, which showed 16% positivity despite being designed to exclusively recognize domain II of ErbB2 (Figure 6B). Among the 4D5-derived constructs, the 4D5 2nd Gen showed a transduction rate of ~33%, whereas the 4D5_{low} 2nd Gen showed no detectable staining. CAR expression of C6.5 2nd Gen and C6.5_{high} 2nd Gen was successfully confirmed using G4S staining, with rates of around 22%. Since CAR expression of the 4D5_{low} 2nd Gen had not been demonstrated at this point, additional staining was performed using anti-mouse IgG and streptavidin-PE. Mouse IgG is present only in the 4D5-based CAR designs. However, positive staining was also observed for the C6.5-based 2nd Gen CAR constructs, indicating non-specific binding of the antibody. Notably, as the 2nd Gen CAR constructs express only a single CAR chain, the detection of any staining signal with anti-IgG in the 4D5_{low} 2nd Gen nevertheless confirms that this construct is expressed. In summary, all constructs demonstrated stable transgene expression levels above 20%.

A



B

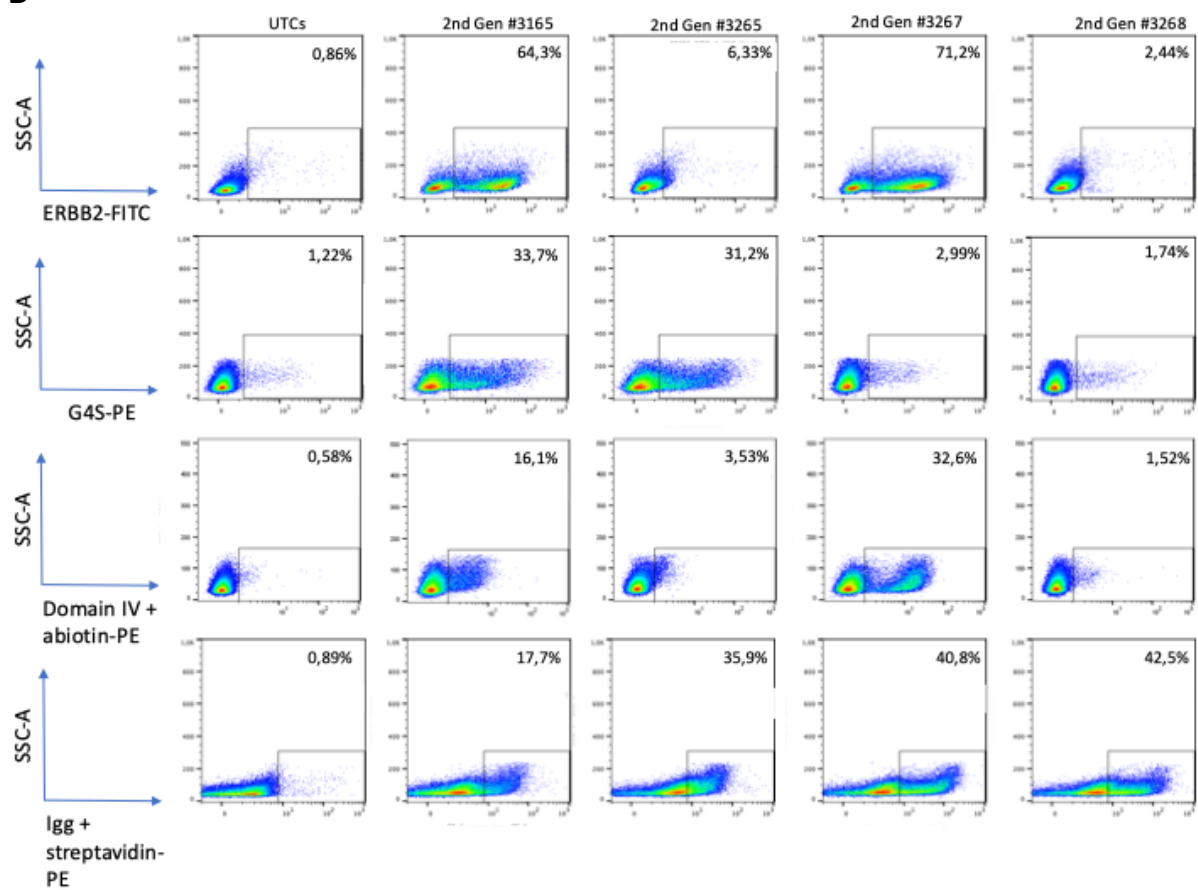


Figure 6: Expression of dual-chain and second-generation CAR constructs on the surface of T cells. Flow cytometric analysis of transduction efficiency and detection of both CARs on the cell surface using ErbB2-GFP, G4S-PE, and biotinylated ErbB2 domain IV protein as primary reagent, followed by anti-biotin PE as the secondary antibody. UTCs served as negative controls, and the percentage of CAR-positive cells among viable T cells is indicated. Staining is shown on the x-axis, and SSC-A is shown on the y-axis. **A:** dual-chain CAR transduction efficiencies **B:** Second-generation CAR constructs transduction efficiencies.

5.3. ErbB2 expression levels on the surface of target cell lines and fibroblasts

ErbB2 surface expression was quantified across the target cell panel and additionally assessed in fibroblasts to validate antigen availability for subsequent cytotoxicity assays. As expected, cells of the cell line SKOV-3 showed the highest ErbB2 expression, consistent with their established classification as ErbB2_{high} tumor model. The breast cancer cell line MCF-7 displayed moderate antigen levels, fibroblasts low levels, and the MCF-7 ErbB2 knockout cell line did not show any expression (Figure 7). These profiles established SKOV-3 and MCF-7 as ErbB2-positive targets of differing antigen density, while fibroblasts and ErbB2-KO cells served as appropriate ErbB2-low and ErbB2-negative controls.

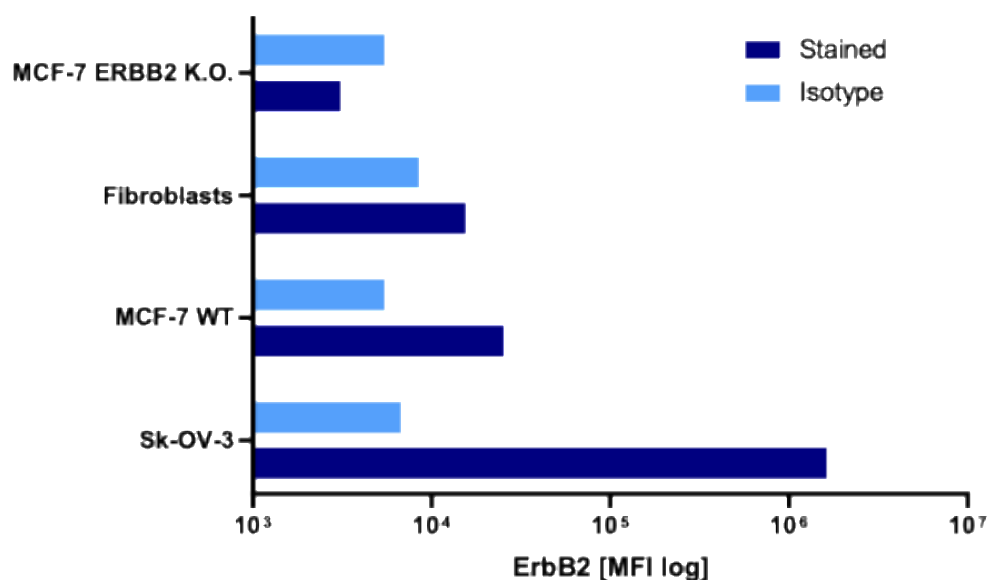


Figure 7: Quantification of ErbB2 surface expression on target cell lines and fibroblasts. ErbB2 expression levels on the indicated cell lines were measured by flow cytometry using an ErbB2 specific antibody. Fluorescence intensity is depicted in comparison to the respective Isotype control. SKOV-3 cells displayed the highest ErbB2 expression, followed by MCF-7, then Fibroblasts and ErbB2-knockout MCF-7 cells exhibited no detectable signal. The x-axis shows the mean fluorescence intensity.

5.4. Selective IFN- γ release by CAR-T cells specific for domain II and IV of ErbB2

To evaluate antigen-specific activation of CAR-T cells, a protein-based assay was performed using recombinant ErbB2 domains as target antigens, with domain II being the C6.5 epitope and domain IV the 4D5 epitope. CAR-T cells were incubated on plates coated with the following conditions: both domains at 1 μ g each, only domain II at 1 μ g, only domain IV at 1 μ g, and a control without antigen coating. The domains were coated both in combination and individually to determine their relative contribution to CAR activity and to exclude non-specific binding. After 24 hours, supernatants were collected and analyzed by ELISA for IFN- γ and IL-2 secretion.

Analysis of IFN- γ secretion revealed only domain-specific activation and a clear hierarchy of CAR activity driven by scFv affinity and antigen domain specificity. The C6.5 2nd Gen CAR responded exclusively to domain II, while the 4D5 2nd Gen CAR was activated only by domain IV, confirming that each binder recognizes its intended epitope without detectable cross-reactivity. Notably, the analysis lacks the C6.5_{high} 2nd Gen CAR, which showed low-level non-specific binding in the expression analysis (Figure 6). Next, dual-chain constructs were evaluated by comparing stimulation with domain II alone versus simultaneous stimulation with both domains. For dual-chain CARs incorporating 4D5 WT, the presence of both domains led to a modest increase in IFN- γ secretion relative to domain II alone, indicating that the 4D5 chain contributes measurable, but smaller, signaling input in dual-chain context. In contrast, dual-chain CARs containing the low-affinity 4D5 scFv showed no increase in IFN- γ secretion after incubation with both domains compared to domain II alone. Stimulation with domain IV alone did not induce substantial activation in any of the dual-chain constructs, consistent with the fact that 4D5 binds this region but is linked only to the 4-1BB costimulatory module and not to CD3 ζ . This also confirms the absence of cross-reactivity of the C6.5 scFv towards domain IV. Across all stimulation conditions, activation in dual-chain CARs was driven primarily by the C6.5 chain. CARs incorporating the affinity-matured C6.5_{high} variant produced substantially higher IFN- γ levels than constructs harboring C6.5 WT, regardless of whether stimulation occurred with domain II alone or with both domains. In summary, the C6.5-based scFvs generally induced higher IFN- γ secretion than the 4D5-based fragments. Among the different variants, a further distinction is observed: the C6.5_{high} domain elicited the strongest cytokine response, whereas the 4D5_{low}

domain showed the lowest activity. Activity in dual-chain CARs appeared to be predominantly driven by the C6.5_{high} variant, as construct C6.5_{high}/4D5_{low} showed high IFN- γ secretion despite carrying the 4D5_{low} domain. The negative control, lacking both target epitopes, did not induce a significant increase in IFN- γ secretion in any of the constructs (Figure 8).

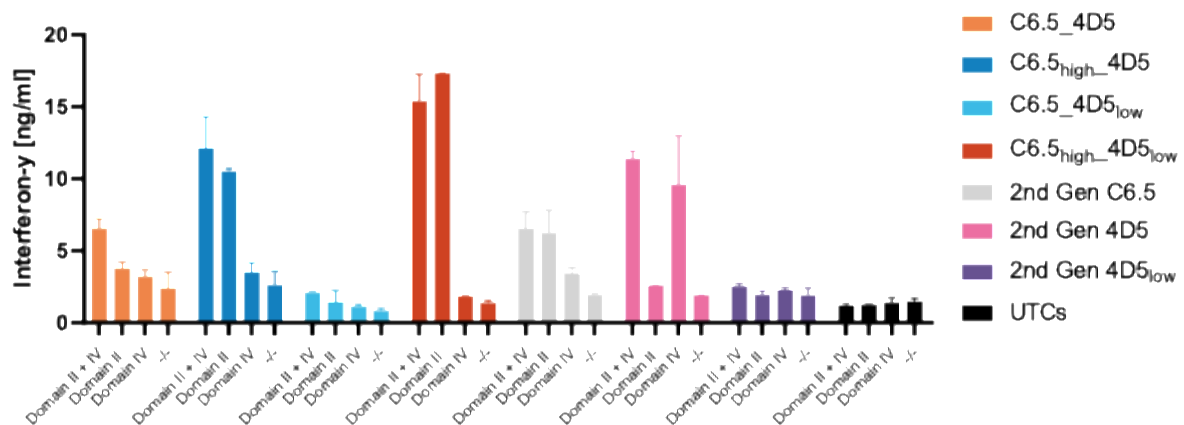


Figure 8: Specific activation of CAR-T cells by their respective epitopes. IFN- γ secretion [ng/ml] of CAR-T cells after 24-hour stimulation with ErbB2 domains II and IV. C6.5-based 2nd Gen CAR constructs responded exclusively to domain II, whereas 4D5-based CARs were activated only by domain IV. Dual-chain CARs showed varying degrees of activation depending on scFv affinity. Bar plots represent mean $\pm$ SD, n=2.

The analysis of IL-2 showed that the UTCs exhibited the highest levels across all conditions (Figure A1). Accordingly, the CAR constructs did not secrete a relevant amount of IL-2 in this experiment, preventing further meaningful analysis.

5.5. T cells expressing second-generation CAR constructs 4D5 and 4D5_{low} demonstrate superior anti-tumor efficacy in repetitive killing assays

Because the protein-based stimulation assay provided only limited insights into overall CAR activation dynamics, a repetitive cytotoxicity assay was performed to further characterize functional CAR-T cell behavior. This approach allows assessment of how CAR-T cells respond in co-culture with different target cell lines and to what extent varying levels of ErbB2 expression influence CAR activation and tumor cell lysis. It was designed to assess whether combining two different scFvs, with distinct binding affinities against different epitopes of the same target protein, results in improved efficacy, selectivity, and persistency compared

to conventional 2nd Gen CAR constructs. 2×10^4 target cells were co-cultured with CAR-T cells 1:1 for 48 hours. A UTC adjustment was performed because transduction rates differed by more than 10%. CAR-T cells were repeatedly exposed to freshly seeded target cells every two days for four times. Four cell lines were tested in total, including two tumor cell lines for evaluation of cytotoxic efficacy. The first tumor cell line, SKOV-3 ovarian cancer, exhibits a high ErbB2 expression. All CAR constructs achieved an average lysis rate of more than 80% during the first three rounds of co-culture. However, by round four, only the two 4D5-derived 2nd Gen CAR constructs maintained high cytotoxic activity of over 80%, whereas all remaining constructs showed marked decline, reaching average lysis levels of approximately 50% (Figure 9).

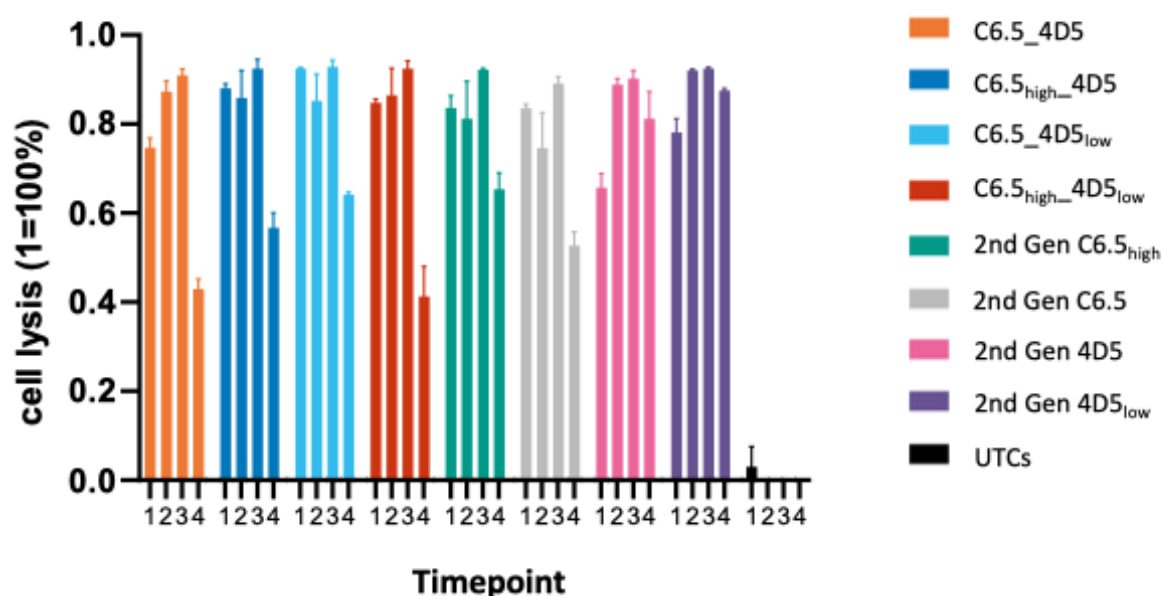


Figure 9: Cell lysis after a repetitive co-culture of CAR-T cells with ErbB2_{high} SKOV-3 ovarian cancer cells, measured by XTT. All eight constructs as well as UTCs were included in the analysis. The x-axis indicates the repetitive rounds 1-4, and the y-axis represents the extent of cell lysis, with 1 corresponding to 100% lysis. Both 4D5-derived 2nd Gen CAR designs show a superior efficacy and persistence through all four rounds.

MCF-7, an ErbB2_{dim} breast cancer cell line, was included to assess whether reduced antigen density affects CAR-T cell efficacy and functional persistence, and to determine whether CAR activation remains stable under conditions of lower ErbB2 expression. Despite the reduced antigen density, MCF-7 co-cultures yielded the same overall outcome as seen with SKOV-3 cells. The two 4D5-derived 2nd Gen CAR constructs maintained consistently high cytotoxicity of approximately 90% across all rounds. In contrast, all other constructs showed strong

initial activity between 80 and 90% during the first two rounds, followed by a pronounced decline to an average of ~40% in later timepoints (Figure 10). These findings indicate that the relative performance differences between the CAR constructs were maintained across ErbB2_{high} and ErbB2_{dim} targets.

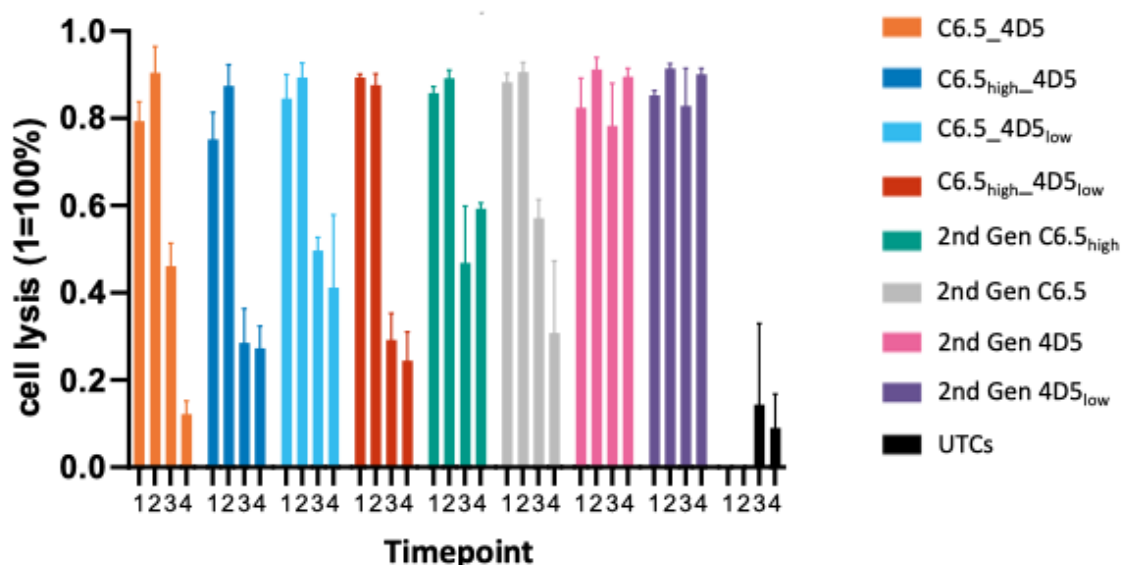


Figure 10: Cell lysis after a repetitive co-culture of CAR-T cells with ErbB2_{dim} MCF-7 breast cancer cells, measured by XTT. All eight constructs as well as UTCs were included in the analysis. The x-axis indicates the repetitive rounds 1-4, and the y-axis represents the extent of cell lysis, with 1 corresponding to 100% lysis. Both 4D5-derived 2nd Gen CAR designs show a superior efficacy and persistence through all four rounds.

5.6. T cells equipped with second-generation 4D5 CAR construct exhibit reduced cytotoxicity toward primary human fibroblasts expressing ErbB2 at low density

To assess CAR-T cell on-target, off-tumor activity, fibroblasts were included in this assay. Fibroblasts are healthy human cells that express basal levels of ErbB2, and thus represent a model for potential off-tumor recognition. As shown in Figure 11 all constructs showed some cell lysis, but by round three, most CAR-T cell constructs showed fibroblast lysis rates lower than 20%. The dual-chain CAR, incorporating C6.5 and 4D5_{low} consistently mediated the significantly highest lysis, still reaching ~60% by the third round. In contrast, the two 4D5-derived 2nd Gen CAR designs emerged as the most selective constructs within the panel, displaying cytotoxicity values above 20% only in the second round and markedly lower activity in all other rounds. Notably, the 4D5 WT 2nd Gen CAR showed even weaker

cytotoxicity than its affinity-maturated counterpart. Among the dual-chain CARs, the C6.5/4D5_{low} construct performed comparatively well and showed the greatest degree of selectivity (Figure 11).

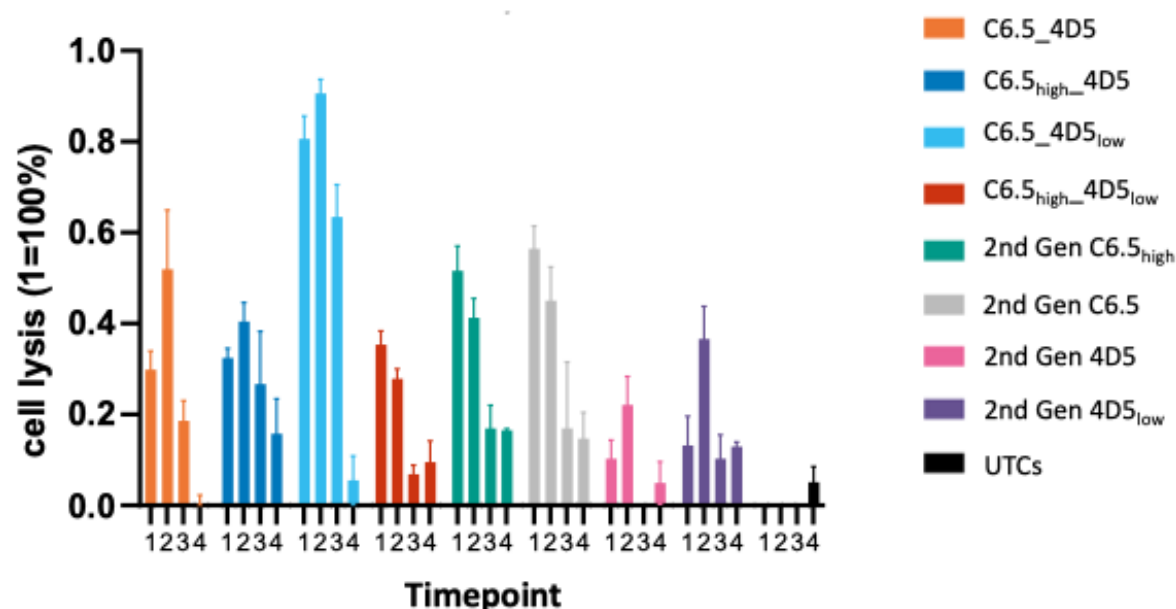


Figure 11: Cell lysis after a repetitive co-culture of CAR-T cells with ErbB2_{low} fibroblasts measured by XTT. All eight constructs as well as UTCs were included in the analysis. The x-axis indicates the repetitive rounds 1-4, and the y-axis represents the extent of cell lysis, with 1 corresponding to 100% lysis. The 4D5-derived 2nd Gen CAR designs showed a superior selectivity through all four rounds.

5.7. Repetitive specificity assay targeting MCF-7 ErbB2 knockout cells

The MCF-7 ErbB2 KO line was shown to be completely ErbB2-negative (Figure 7) and was included as a specificity control. As expected, the overall cytotoxicity was markedly lower than in any ErbB2-expressing cell line; however, a low but detectable level of background activity remained. Notably, the 2nd Gen C6.5-based CARs showed the highest residual activity. The C6.5 WT 2nd Gen CAR reached approximately 50% lysis in the first round, and both C6.5-derived 2nd Gen CAR constructs still retained 30-40% activity in round three. Among the dual-chain designs, the C6.5/4D5_{low} construct exhibited the greatest residual activation, reaching around 30% lysis in round three, although with considerable variability, and approximately 20% in round four (Figure 12).

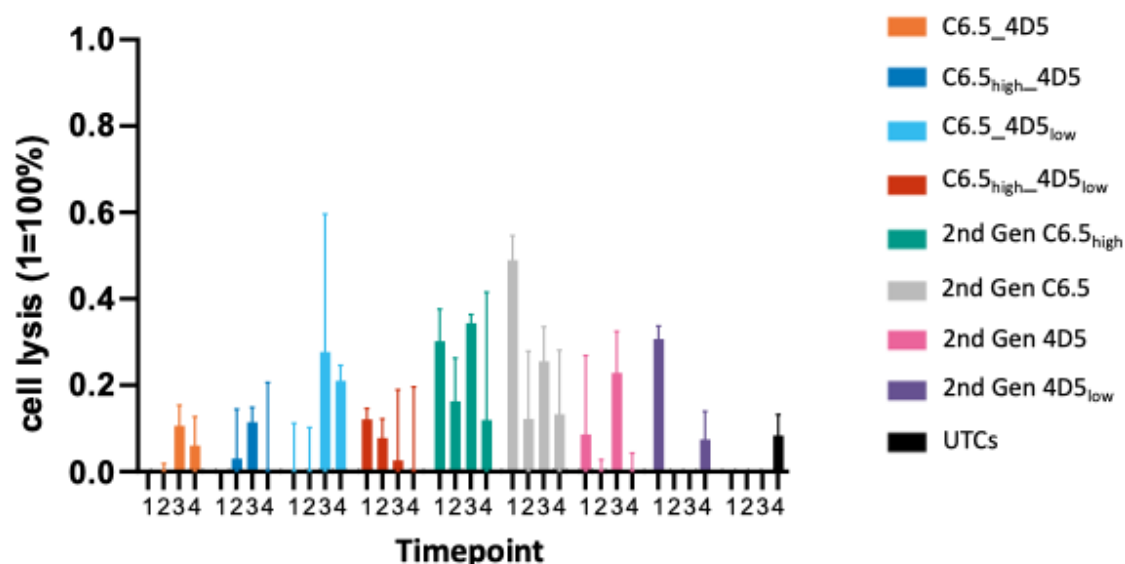


Figure 12: Cell lysis after a repetitive co-culture of CAR-T cells with MCF-7 breast cancer cells, with ErbB2 knocked out. All eight constructs as well as UTCs were included in the analysis. Cell lysis was measured by XTT. The x-axis indicates the repetitive rounds 1-4, and the y-axis represents the extent of cell lysis, with 1 corresponding to 100% lysis. The C6.5-derived 2nd Gen CAR constructs show higher cell lysis than all other constructs.

5.8. Antigen-dependent cytokine secretion by CAR-T cells

5.8.1. Secretion of the pro-inflammatory cytokine IFN- γ

To assess whether target cell lysis correlates with cytokine secretion, IFN- γ secretion was quantified in supernatants from cytotoxicity assays including eight distinct CAR constructs and UTCs as a negative control. IFN- γ levels were determined by ELISA following co-culture with ErbB2-associated cell lines, including SKOV-3, MCF-7, fibroblasts, and MCF-7 ErbB2 KO cells. In addition to the four cell lines, a CAR-T only condition was included to evaluate potential tonic signaling in the absence of antigen. Across all constructs, IFN- γ secretion was antigen-dependent. In the case of SKOV-3 cancer cells, all CAR constructs secreted significantly more IFN- γ than UTCs throughout all rounds in both assays, this was evaluated by two-way ANOVA analysis. During the first round, all CAR designs produced similar cytokine levels, however the later timepoints showed that several dual-chain constructs exhibited slightly higher IFN- γ production than their 2nd Gen counterparts (Figure 13). This pattern does not fully align with the XTT-based cytotoxicity data, where 2nd Gen designs demonstrated stronger and more durable cell lysis (Figure 9). A different pattern was observed with ErbB2_{dim} MCF-7 cells. Although MCF-7 induced high levels of tumor cell lysis

comparable to SKOV-3 in the XTT assay, IFN- γ secretion remained minimal and did not exceed the levels measured with fibroblasts. The 4D5-derived 2nd Gen CAR constructs showed a modest increase in round three, and only the 4D5 WT 2nd Gen CAR showed some IFN- γ secretion in the last round. These findings correlate with the XTT results (Figure 10). Co-culture with fibroblasts induced only a transient cytokine signal in round one. In line with the XTT-based selectivity assay (Figure 11), the dual-chain construct C6.5/4D5_{low} and both 4D5-derived 2nd Gen CAR constructs exhibited the lowest IFN- γ secretion under these conditions, further supporting their comparatively high selectivity toward ErbB2-expressing targets. Co-culture with ErbB2 KO MCF-7 cells, as well as the CAR-T only condition resulted in no detectable IFN- γ secretion (Figure 13).

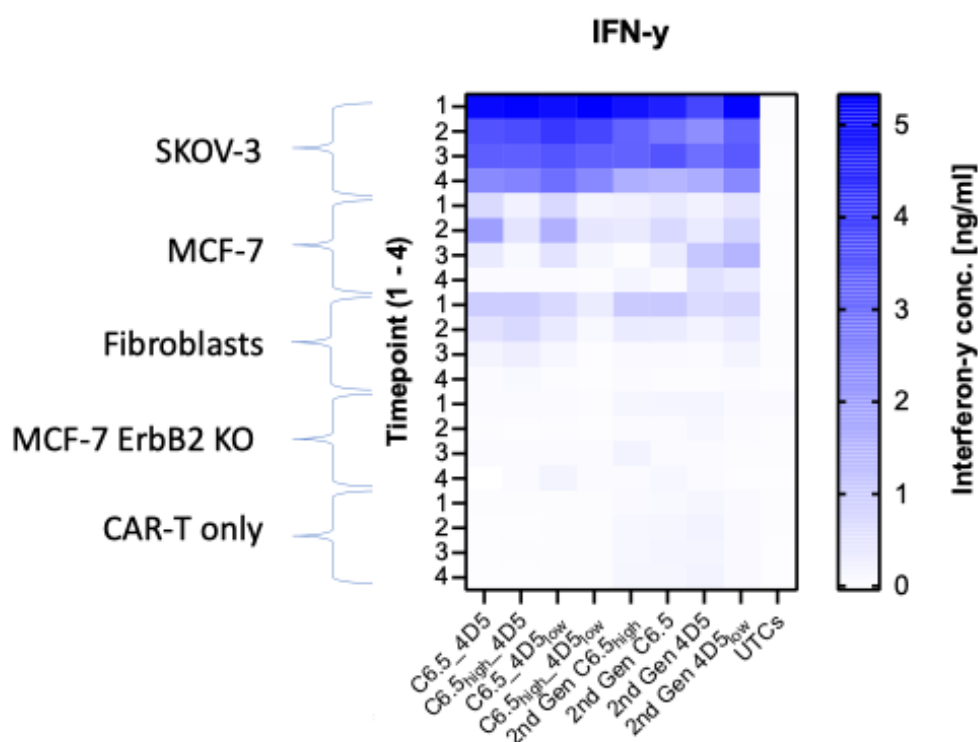


Figure 13: IFN- γ levels triggered by the dual-chain CAR and 2nd Gen CAR constructs co-cultured with ErbB2 associated cell lines. The functional activity of eight CAR constructs was assessed by quantifying IFN- γ release in supernatants from co-cultures with human ErbB2 associates cell lines, including fibroblasts, SKOV-3 ovarian cancer cells, MCF-7 breast cancer cells, and MCF-7 ErbB2 KO cells. Data are shown as heatmaps representing IFN- γ concentrations [ng/ml] determined by ELISA at four consecutive time points. Co-cultures were performed at an E:T ratio of 1:1, with each condition tested in triplicate and displayed as mean values. The x-axis shows the constructs. The y-axis shows the cell lines.

5.8.2. Secretion of the pro-inflammatory cytokine IL-2

Although IL-2 production is generally low in CAR designs containing only 4-1BB costimulatory domain, IL-2 secretion was nevertheless quantified to evaluate potential differences in activation strength across target cell lines (Figure 14). Co-culture with SKOV-3 cells elicited the strongest IL-2 secretion across all constructs. Most CAR designs showed pronounced IL-2 increase in round two, except the 4D5-derived 2nd Gen CAR constructs, which instead showed a higher IL-2 secretion in round three. In contrast, co-culture with ErbB2_{dim} MCF-7 cells did not induce a meaningful IL-2 response. Despite the high levels of tumor cell lysis observed in the XTT assay, IL-2 secretion remained minimal for all constructs. There is only shown a minimal secretion of all 2nd Gen CAR constructs in round one. Fibroblasts also induced only small, transient IL-2 signal in round one, followed by complete loss of activity in subsequent rounds. The ErbB2 KO line as well as the CAR-T only condition demonstrated minimal to no IL-2 release in any dual-chain CAR, whereas the 2nd Gen CAR constructs exhibit a minimal but detectable IL-2 signal, especially in the first round (Figure 14). Overall, IL-2 secretion levels were substantially lower than IFN- γ production.

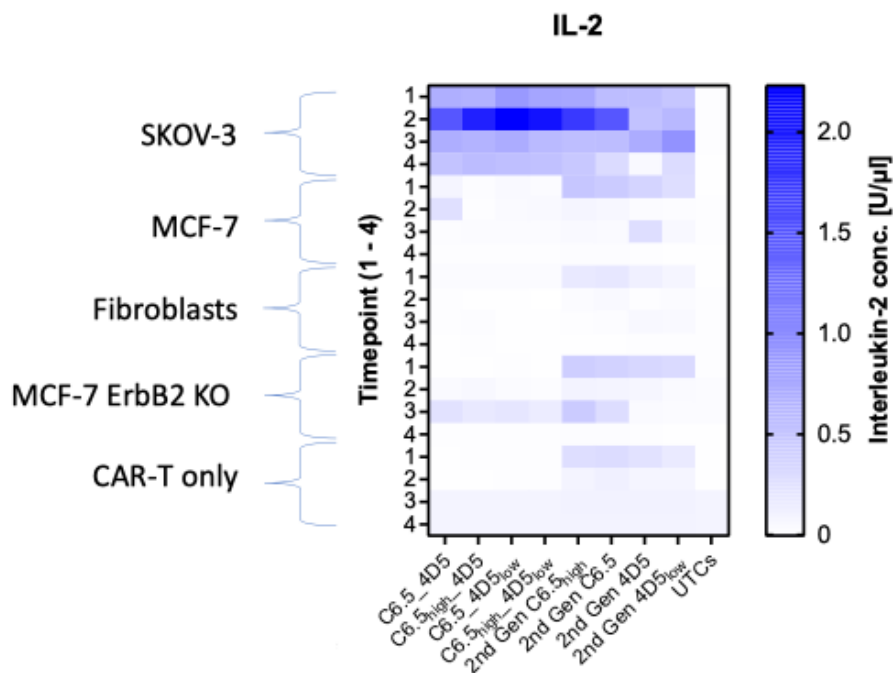


Figure 14: IL-2 levels triggered by the dual-chain CAR and 2nd Gen CAR constructs cocultured with ErbB2 associated cell lines. The functional activity of eight CAR constructs was assessed by quantifying IL-2 release in supernatants from co-cultures with human ErbB2 associates cell lines, including fibroblasts, SKOV-3 ovarian cancer cells, MCF-7 breast cancer cells, and MCF-7 ErbB2 KO cells. Data are shown as heatmaps representing IL-2 concentrations [U/μl] determined by ELISA at four consecutive time points. Co-cultures were performed at an E:T ratio of 1:1, with each condition tested in triplicate and displayed as mean values. The x-axis shows the constructs. The y-axis shows the cell lines.

5.9. Low expression of the immune checkpoint receptor PD-1 on the surface of T cells equipped with second-generation

PD-1 (Programmed Cell Death Protein 1) is an inhibitory receptor upregulated upon T-cell activation and is widely used as a marker of activation-induced exhaustion. Therefore, PD-1 expression was analyzed to determine which CAR design may exhibit the slowest onset of exhaustion and, consequently, the greatest persistence potential. For flow cytometric analysis, living cells were first gated, followed by single cell gating to exclude aggregates. Subsequently, CD3⁺ T cells were identified and separated into CAR⁺ and CAR⁻ populations (Figure 16). PD-1 expression was assessed at two timepoints: prior to assay initiation (baseline) and after round 2 (T2), following co-culture with the respective target cell lines. For the CAR⁺ population, the mean fluorescence intensity (MFI) of PD-1 expression was calculated using FlowJo. The results showed that PD-1 levels after co-culture with MCF-7, fibroblasts and MCF-7 ErbB2 KO cells, remained largely unchanged compared to timepoint 0. In contrast, SKOV-3 represented the only target cell line showing a pronounced increase in PD-1 expression, particularly in the dual-chain CAR constructs and C6.5 2nd Gen CAR, whereas the 4D5-derived 2nd Gen constructs did not display this elevation (Figure 15).

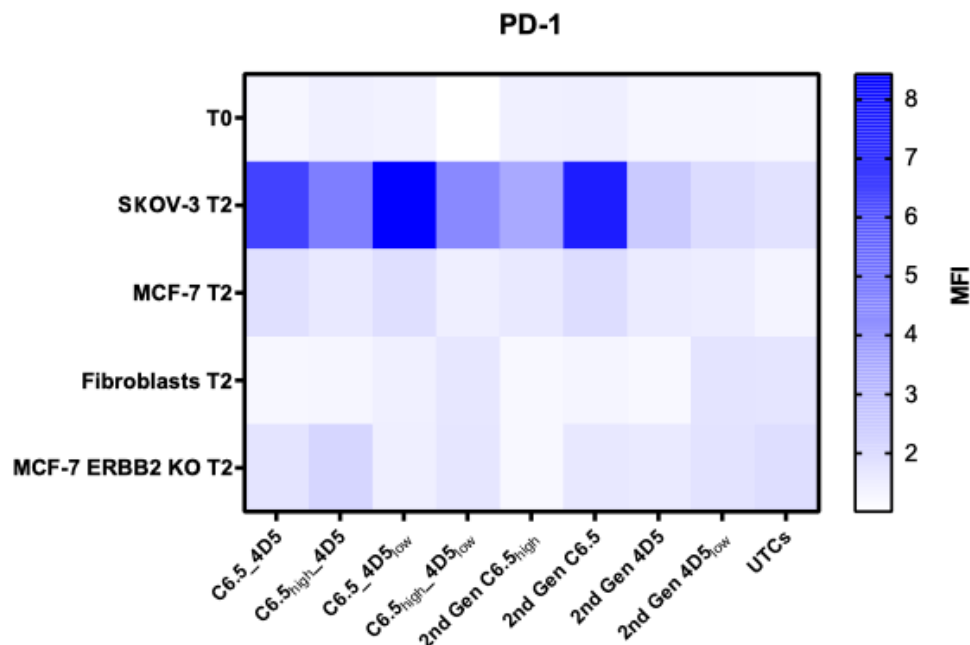


Figure 15: PD-1 expression on CAR-T cells before and after co-culture with ErbB2 associated cell lines. CAR-T cells were FACS-analyzed for PD-1 expression prior to the assay to ensure comparable baseline activation levels (T0). A repetitive cytotoxicity assay was performed using ErbB2 associated target cell lines. In this heatmap the cell lines are ordered according to their ErbB2 expression levels. At timepoint 2 (T2), following incubation with target cells, CAR⁺ T cells were analyzed by flow cytometry, and the MFI of PD-1 was quantified and depicted in the heatmaps.

5.10. CD4⁺ and CD8⁺ T cell distribution in CAR⁺ and CAR⁻ subsets

To further characterize the functional state of CAR-T cells following repetitive antigen stimulation, a subtype analysis was performed by flow cytometry. The expression of CD4 and CD8 was assessed to distinguish helper and cytotoxic T cell subsets and to determine potential shifts in subset composition resulting from repeated target cell engagement. Following standard gating of live, single, CD3⁺ T cells, CAR⁺ and CAR⁻ populations were analyzed for CD4⁺ and CD8⁺ expression at round two of the repetitive cytotoxicity assay. Figure 16 shows a clear differentiation between CD4⁺ and CD8⁺ subpopulations.

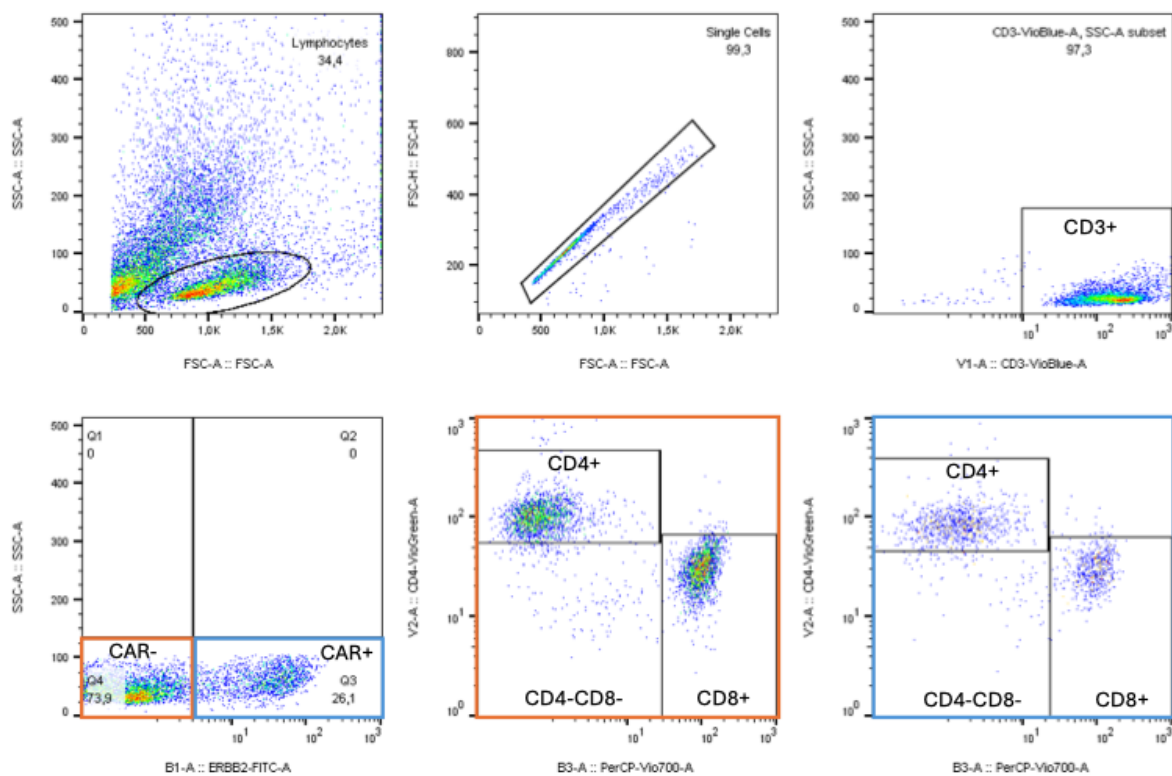


Figure 16: Gating strategy applied for flow cytometric analysis of PD-1, CD4, and CD8 expression.

Live lymphocytes were first selected, followed by single cells and subsequent gating of CD3-positive T cells. CD3⁺ T cells were then separated into CAR⁺ and CAR⁻ populations. For each population, the mean fluorescence intensity (MFI) of PD-1 expression was determined, while the frequencies of CD4⁺ and CD8⁺ T cells were calculated as percentages within both the CAR⁺ and CAR⁻ subsets.

After co-culture with fibroblasts (T2), both CAR⁺ and CAR⁻ T cell populations displayed distinct CD4/CD8 subset distributions. Within the CAR⁺ population, the proportion of CD8⁺ T cells varied between constructs, while CD4⁺ T cells were generally less abundant. Dual-chain CAR C6.5/4D5_{low} and C6.5 2nd Gen CAR showed a notably higher proportion of CD4⁺CD8⁻ cells, whereas the remaining constructs were less distinct and contained fewer CD4⁺CD8⁻ cells, leading to a predominance of CD8⁺ cells (Figure 17). In the CAR⁻ population, a slightly

higher proportion of CD4⁺ T cells was observed compared to the CAR⁺ population. All constructs showed a similar CD4/CD8 distribution without noticeable differences.

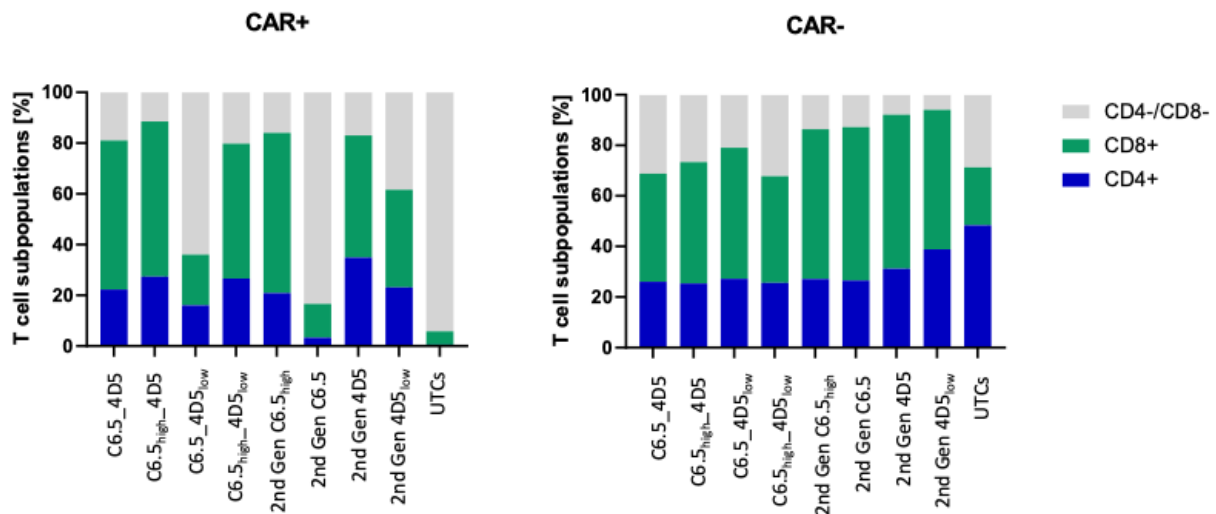


Figure 17: CD4 and CD8 expression [%] on CAR⁺ and CAR⁻ T cell subsets after co-culture with fibroblasts. Stacked bar graphs depict distribution of CD4⁺, CD8⁺, and CD4⁻CD8⁻ T-cell subsets within each CAR construct and UTCs. Data are shown separately for CAR⁺ and CAR⁻ populations. The flow cytometric analysis was performed at timepoint 2 (T2), following co-culture of CAR-T cells with fibroblasts.

After co-culture with SKOV-3 cancer cells, the CAR⁺ population showed an increase in CD4⁺ T cells and a reduction in CD8⁺ T cells across all constructs. Based on the cytotoxicity results, particular attention was given to 4D5_{low} 2nd Gen CAR, where a noticeable lower proportion of CD8⁺ cells was observed compared to the other CARs. This observation contrasts with prior XTT assay results that had indicated the highest activity against SKOV-3 cells for this construct. In contrast, the CAR⁻ population maintained a relatively stable proportion of CD4⁺ cells (~50%), while the CD8⁺ fraction was higher than in the CAR⁺ population (around 45%). No major differences were observed between the constructs, as their distribution appeared similar (Figure 18).

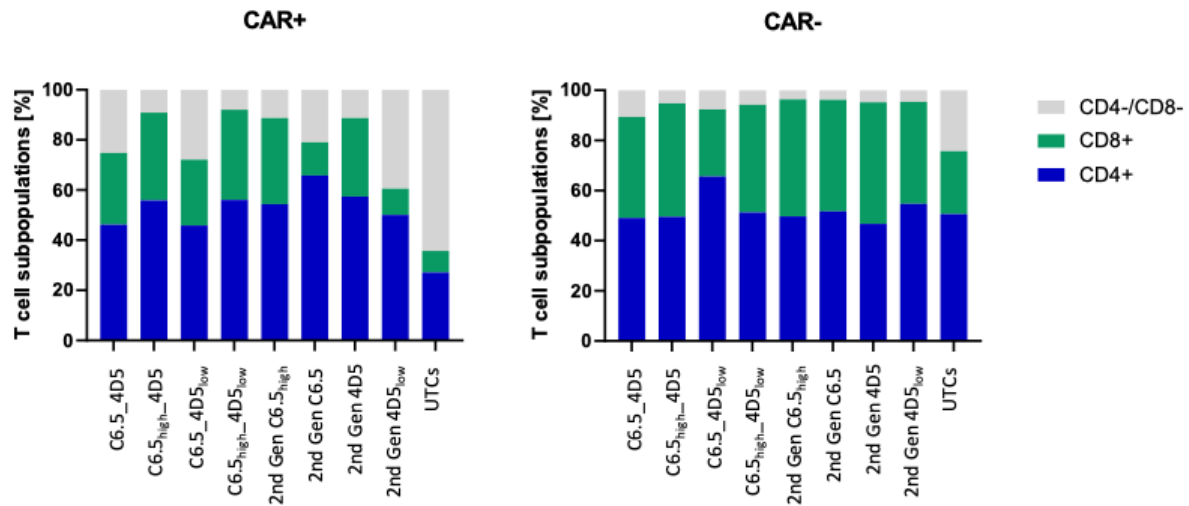


Figure 18: CD4 and CD8 expression [%] on CAR⁺ and CAR⁻ T cell subsets after co-culture with SKOV-3 ovarian cancer cells. Stacked bar graphs depict distribution of CD4⁺, CD8⁺, and CD4⁻CD8⁻ T-cell subsets within each CAR construct and UTCs. Data are shown separately for CAR⁺ and CAR⁻ populations. The flow cytometric analysis was performed at timepoint 2 (T2), following co-culture of CAR-T cells with SKOV-3 cancer cells.

Following co-culture with MCF-7 cancer cells, the CAR⁺ population showed a minimal increase in the relative proportion of CD8⁺ T cells across all constructs, with dual-chain CAR C6.5/4D5_{low} displaying the lowest CD8⁺ fraction despite exhibiting a high cytotoxicity in the XTT assay. In the other constructs, CD4⁺ and CD8⁺ T cells were approximately equally distributed. In the CAR⁻ population, the distribution pattern was similar to that observed after co-culture with SKOV-3 cells, with CD4⁺ and CD8⁺ cells being nearly equally represented across all constructs, including the UTCs, with approximately 45% each (Figure 19).

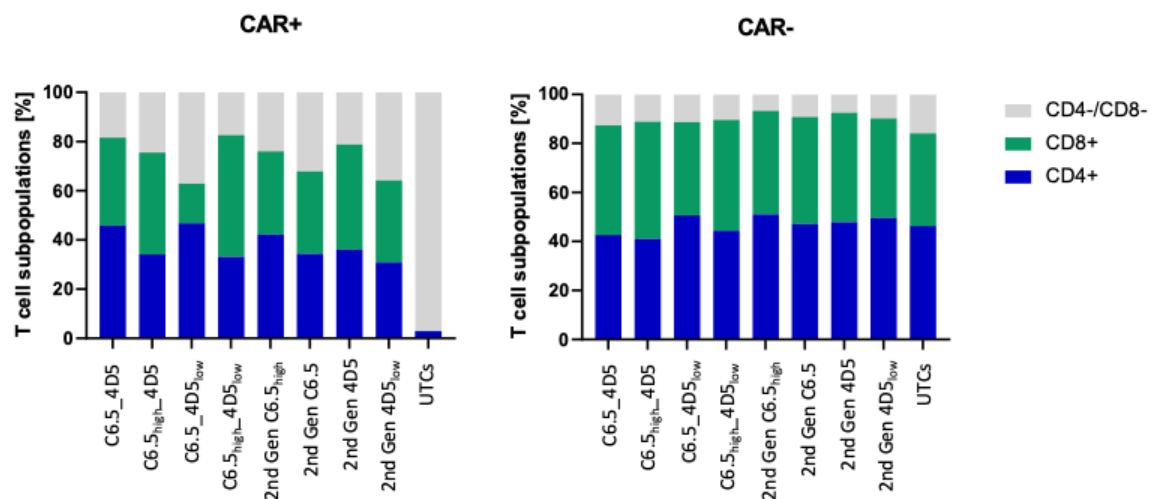


Figure 19: CD4 and CD8 expression [%] on CAR⁺ and CAR⁻ T cell subsets after co-culture with MCF-7 breast cancer cells. Stacked bar graphs depict distribution of CD4⁺, CD8⁺, and CD4⁻CD8⁻ T-cell subsets within each CAR construct and UTCs. Data are shown separately for CAR⁺ and CAR⁻ populations. The flow cytometric analysis was performed at timepoint 2 (T2), following co-culture of CAR-T cells with MCF-7 cancer cells.

Finally, cells were analyzed after co-culture with MCF-7 ErbB2 KO cells to assess CD4 and CD8 distribution. A striking observation was the high proportion of CD4⁻CD8⁻ cells within the CAR⁺ population. Moreover, most cells were CD4⁺, with only a small fraction of CD8⁺ T cells. The CD4/CD8 distribution varied a lot among the different constructs, with almost every construct showing a distinct pattern. The dual-chain CARs C6.5/4D5 and C6.5_{high}/4D5_{low} consisted predominantly of CD4⁻CD8⁻ cells, and dual-chain C6.5_{high}/4D5_{low} contained mainly CD4⁺ cells. The remaining constructs displayed an average amount of 20% CD8⁺ T cells. In the CAR⁻ population, all constructs, including the UTCs, showed a comparable distribution. The proportion of CD8⁺ cells was slightly higher than in the CAR⁺ population, averaging 30%. In contrast to the other cell lines, these samples displayed a predominance of CD4⁺ over CD8⁺ T cells, with an average of about 50% CD4⁺ cells (Figure 20).

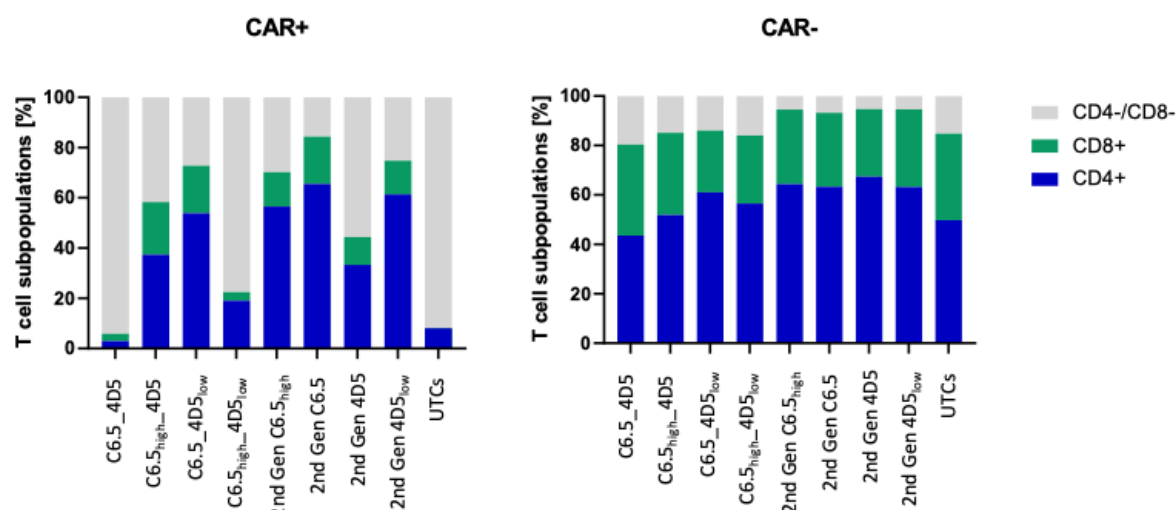


Figure 20: CD4 and CD8 expression [%] on CAR⁺ and CAR⁻ T cell subsets after co-culture with MCF-7 ErbB2 KO cells. Stacked bar graphs depict distribution of CD4⁺, CD8⁺, and CD4⁻CD8⁻ T-cell subsets within each CAR construct and UTCs. Data are shown separately for CAR⁺ and CAR⁻ populations. The flow cytometric analysis was performed at timepoint 2 (T2), following co-culture of CAR-T cells with MCF-7 ErbB2 KO cells.

5.11. T cell phenotyping after co-culture with ErbB2 associated cell lines

Beyond this lineage profiling, additional markers including CD27, CD45RO, CD45RA, CD95, and CD62L were analyzed to evaluate the differentiation and memory status of the surviving T cell populations. These markers enable the discrimination between naïve (CD45RA⁺CD27⁺CD62L⁺), central memory (CD45RO⁺CD27⁺CD62L⁺), effector memory (CD45RO⁺CD27⁻CD62L⁻), terminal effector (CD45RA⁺CD62L⁻), and stem cell memory (CD45RA⁺CD27⁺CD95⁺) subsets. Assessing this phenotypic profile after repetitive killing provides insights into the persistence potential, activation history, and differentiation trajectories of CAR-T cells, thereby complementing functional readouts such as cytotoxicity and PD-1 expression. For the analysis of T cell subpopulations, cells were first gated for CD45RO⁺ and CD45RA⁺ populations. As shown in figure 21, CD45RA staining was not optimal and resulted in weak signal intensity, while CD45RO staining produced a clear positive population (Figure 21). Within each of these populations, additional gating was performed using CD27 and CD62L expression. In the CD45RO⁺ population, this allowed the identification of T_{TM}, T_{CM}, and T_{EM} subsets. In the CD45RA⁺ population, T_{TE} cells were identified, and further staining with CD95 enabled the distinction between T_N and T_{SCM} subsets.

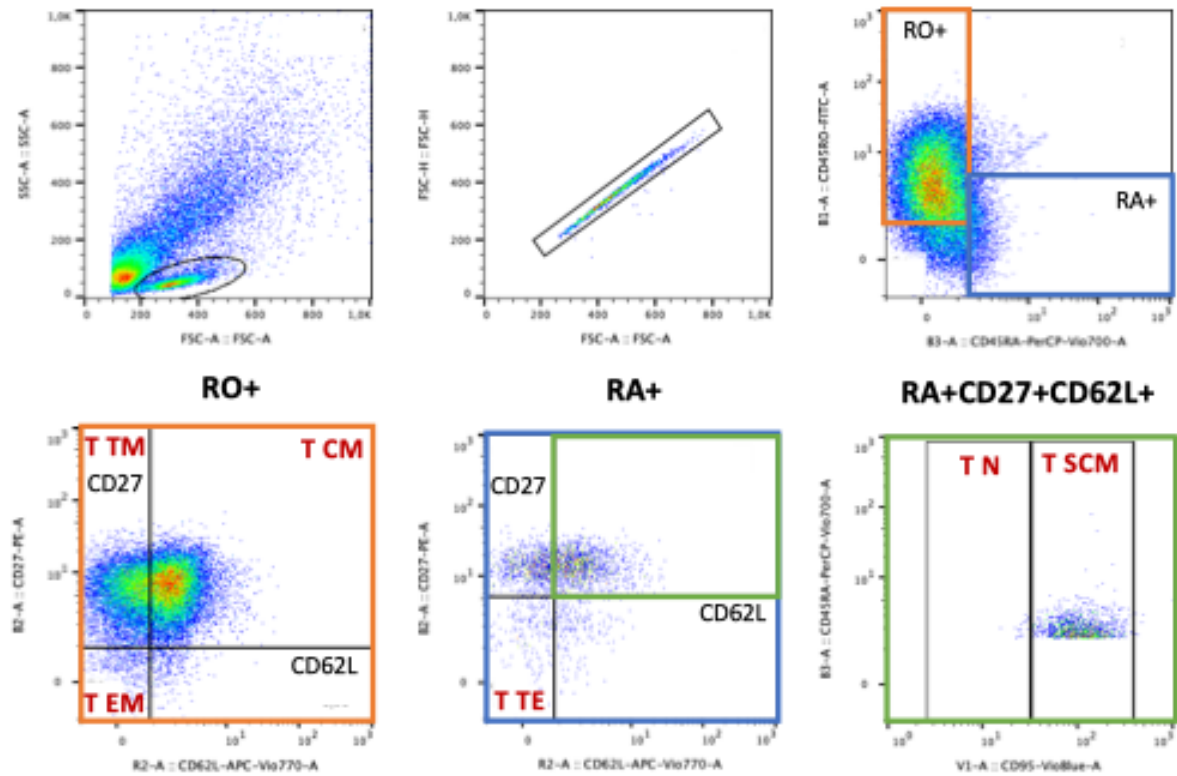


Figure 21: Gating strategy applied for flow cytometric analysis of T cell subpopulations. Live lymphocytes were first selected based on FSC and SSC properties, followed by singlet discrimination. Subsequently, CD45RO⁺ and CD45RA⁺ populations were gated. Within the CD45RO⁺ fraction, staining with CD27-PE and CD62L-APC allowed the identification of central memory (T CM), transitional memory (T TM), and effector memory (T EM) T cell subsets. In the CD45RA⁺ population, CD27 and CD62L staining enabled the detection of terminal effector (T TE) cells. Further analysis of the CD45RA⁺CD27⁺CD62L⁺ subset using CD95-VioBlue staining distinguished naïve (T N) from stem cell memory (T SCM) T cells.

Across all co-cultures with target cells, a clear difference between CAR-transduced and UTCs was observed. The UTCs consistently displayed the highest proportion of naïve T cells across all cell lines. After co-culture with Fibroblasts, some naïve T cells were retained in the dual-chain CAR constructs, whereas the 2nd Gen CARs showed lower T_N fraction. In SKOV-3 co-cultures, naïve T cells were largely restricted to the UTCs, while CAR-expressing cells showed a shift toward more differentiated subsets. In MCF-7 co-cultures with the two 4D5-derived 2nd Gen CARs, there are no naïve T cells. Terminal effector cells (T_{TE}), indicative for strong stimulation, were rare across all cell lines, except for C6.5 2nd Gen CAR construct after co-culture with MCF-7. Notably, in MCF-7 and MCF-7 ErbB2 KO co-cultures, the proportion of T_{SCM} was higher compared to fibroblasts and SKOV-3. In SKOV-3 co-cultures, dual-chain CARs retained slightly more T_{SCM} cells than the 2nd Gen CARs. Across all constructs and cell lines, T_{CM} cells, representing persistence potential, and T_{TM}, indicative for early activation,

consistently contributed a significant fraction in the T cell distribution. In SKOV-3 co-cultures, only dual-chain construct C6.5_{high}/4D5_{low} showed a modest reduction in T_{CM}, while dual-chain CAR C6.5/4D5_{low} exhibited the highest relative abundance of T_{CM}. In MCF-7 co-cultures, T_{CM} levels were largely similar across all constructs. In SKOV-3, and MCF-7 co-cocultures, the T_{TM} fraction was slightly higher with 2nd Gen CARs compared to dual-chain CARs (Figure 22).

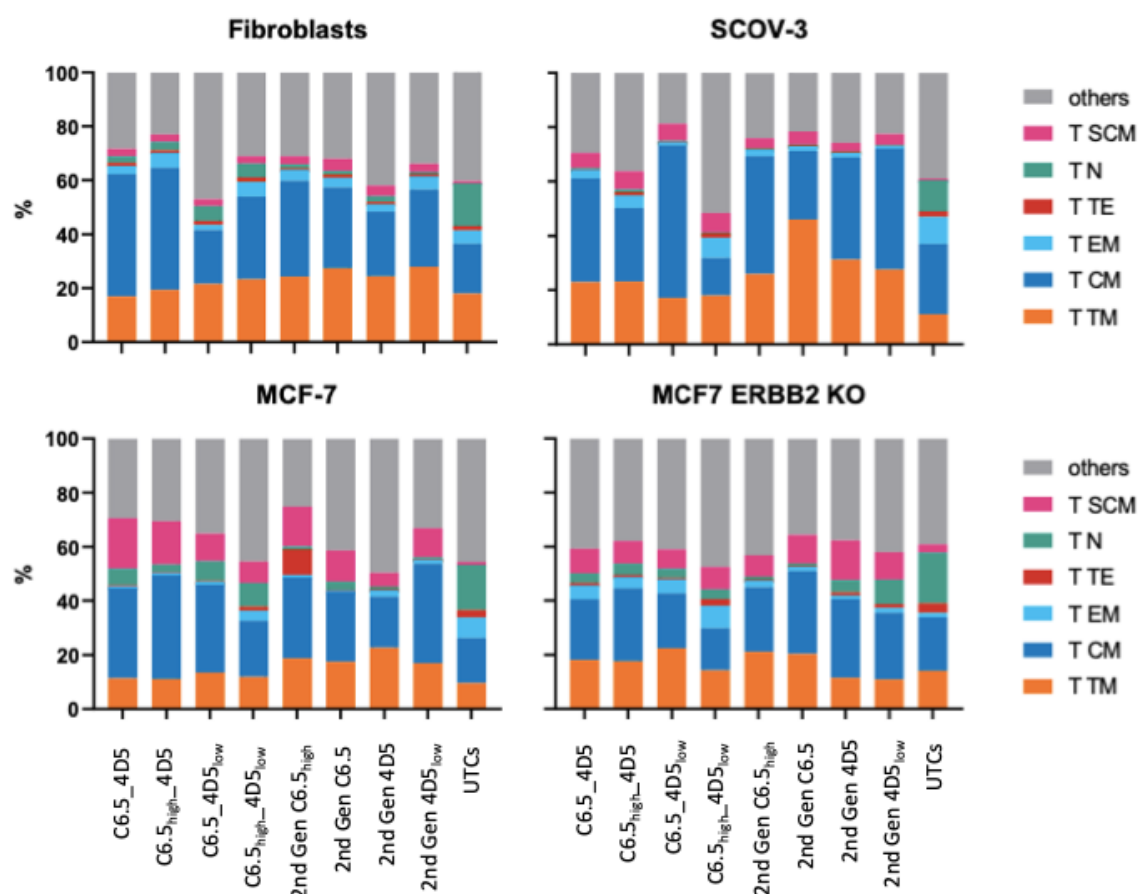


Figure 22: Phenotypic characterization of CAR-T cells after repetitive cytotoxicity assay. T cells transduced with dual-chain or 2nd Gen CAR constructs were analyzed by flow cytometry following the third round of repetitive co-culture with fibroblasts, SKOV-3, MCF-7, and MCF-7 ErbB2 KO cells. The stacked bar graphs show the distribution of T cell subpopulations (y-axis, %) for each CAR construct and UTCs (x-axis). Subpopulations were defined based on surface marker expression as follows: T SCM (stem cell memory), T N (naïve), T EM (effector memory), T CM (central memory), T TM (transitional memory).

6. Discussion

CAR-T cell therapy has achieved remarkable clinical success in the treatment of hematological malignancies. However, its application to solid tumors remains highly challenging due to significant biological and technical hurdles. Major limitations include on-target, off-tumor toxicity, where healthy tissues expressing low levels of the target antigen are unintentionally attacked (Mitra et al., 2023), and insufficient CAR-T cell persistence, which can lead to suboptimal therapeutic efficacy. To address these issues, a panel of CAR constructs was designed. The rational approach involved combining scFvs with different binding affinities for ErbB2 and incorporating the co-stimulatory domain 4-1BB. The aim was to improve target selectivity through affinity tuning, while enhancing CAR-T cell persistence and survival via 4-1BB signaling. The primary focus of this study was to investigate whether dual-chain CARs containing two ErbB2-specific scFvs outperform conventional 2nd Gen CARs, and, if so, to determine which combination of scFv affinities yields the most favorable functional performance.

The first aim was the successful cloning of four distinct dual-chain CARs and four 2G CARs, the latter of which represent clinically established CAR designs already used in therapy (Hamieh et al., 2023), and to express them in primary T cells for functional characterization. Retroviral vectors were used for T cell transduction in this study. These vectors integrate their genetic material only into dividing cells (Watanabe & McKenna, 2023), which is not a limitation in this context since T cells are pre-activated prior to transduction and therefore proliferative. Retroviral systems are widely used in clinical CAR-T cell production and are considered both safe and stable (Watanabe & McKenna, 2023). The transduction and subsequent validation of CAR expression presented challenges, and the staining strategies employed provided only limited information. The detection of both scFvs on the dual-chain CARs represents a fundamental prerequisite, as all subsequent analyses rely on successful expression. The G4S-specific antibody could only detect C6.5-based CARs, whereas 4D5 variants lack the G4S linker, and instead contain an IgG domain. Staining with an anti-mouse IgG antibody resulted in nonspecific binding and could not provide a reliable detection of the 4D5 scFv in dual-chain constructs. Consequently, staining was performed using domain IV, which was intended to bind specifically to the 4D5 scFv. However, this approach also produced nonspecific signals, as seen when comparing dual-chain CARs C6.5/4D5_{low} and

C6.5_{high}/4D5_{low} (Figure 6A). These constructs differ only in the C6.5 variant, yet C6.5_{high}/4D5_{low} exhibited stronger staining, supporting the notion that C6.5_{high} scFv can also bind domain IV and thus confound interpretation. ErbB2-based staining was also problematic: low-affinity variants bound poorly (Figure 6), making it difficult to assess true expression. These limitations lead to a critical issue: the expression of both scFvs in the dual-chain CARs could not be definitively confirmed, particularly for 4D5 variants. While some detection was achieved, these results must be interpreted with caution.

To assess the contribution of each scFv to CAR-T cell activity, a protein-based assay was performed. It showed that both binding domains recognized only their respective epitopes, implementing the absence of non-specific binding, and induced activation in an affinity-dependent manner. Among all four domains, C6.5_{high} contributed most strongly to activation, whereas 4D5_{low} showed the lowest impact on activation. The expectation that the C6.5 variants would elicit higher activity than the 4D5 variants was based on their respective binding affinities, with C6.5 WT exhibiting a K_D value of 10 nM (Suksanpaisan et al., 2014) and 4D5 WT showing a K_D value of ~0,3 nM (Liu et al., 2016).

Furthermore, this study aimed to investigate whether dual-chain CARs outperform conventional 2nd Gen CARs in terms of selectivity, efficiency, and persistence, as well as to determine which combination of scFv affinities provides the most optimal performance. Based on their design principles, the C6.5_{high}/4D5_{low} dual-chain CAR was initially expected to perform best. This construct combines several theoretically advantageous features, including the dual-chain architecture, which was anticipated to reduce tonic signaling, due to the separated intracellular domains, which are then less prone to form clusters (Calderon et al., 2020). Furthermore, the C6.5_{high} scFv was predicted to provide strong but selective cytotoxic efficacy, and the 4D5_{low} domain was expected to minimize background activation while still offering sustained but moderate co-stimulation, thereby supporting prolonged persistence. Indeed, the C6.5_{high}/4D5_{low} CAR emerged as the most selective out of all dual-chain constructs; however, its overall cytotoxic performance remained limited. When comparing all constructs, two clear top performers emerged: the two 4D5-derived 2nd Gen CARs, which showed the most favorable balance of cytotoxic potency and selectivity across assays. This raises the question of why the dual-chain approach did not yield superior outcomes in this setting. One possible explanation relates to the structural organization of

ErbB2. Unlike previously described dual-chain CARs, which target two distinct antigens, the constructs in this study engaged two epitopes of the same target protein. It is conceivable that the special arrangement or structural dynamics of ErbB2 do not readily permit simultaneous engagement by both binding domains. The dual-chain CAR C6.5/4D5_{low}, composed of the two lower-affinity scFvs, performed unexpectedly poorly, exhibiting high on-target, off-tumor activity. One possible explanation for this observation is the incomplete verification of transduction efficiency. Because accurate staining of both CAR chains could not be achieved, the actual number of CAR-positive cells may have been underestimated. As a result, an excessive number of CAR-T cells could have been added to the assay compared to other constructs, leading to artificially elevated cytotoxicity.

Moreover, analysis of MCF-7 ErbB2 knockout line, revealed low but detectable cytotoxic activity, indicating the presence of tonic signaling. This effect was more pronounced in the C6.5-derived 2nd Gen CARs, a difference potentially rooted in their signaling architecture: While dual-chain CARs separate domains like CD3 ζ and 4-1BB across two chains, which might mitigate tonic signaling, 2nd Gen CARs integrate both domains within a single receptor. This closer spatial proximity and clustering in single-chain designs is hypothesized to promote enhanced basal activation (Zhou et al., 2024). This conclusion is further supported by the elevated IL-2 secretion levels evidence of enhanced tonic signaling in this design.

Cytokine secretion patterns showed only partial correlation with results derived from XTT-based tumor cell viability assays. While both the ErbB2_{high} and ErbB2_{dim} target cell lines exhibited strong cytolytic activity in the cytotoxicity assays, a key difference emerged in cytokine release: only the ErbB2_{high} cell line showed elevated cytokine secretion, whereas the ErbB2_{dim} failed to induce a corresponding release. This distinct response pattern strongly suggests that cytokine secretion is highly dependent on the ErbB2 expression level. Further analysis of cytokine secretion, showed less IL-2 release in comparison to IFN- γ . The reason might be, that all the constructs incorporate 4-1BB as the co-stimulation domain, which is generally associated with sustained T-cell persistence but often drives signaling pathways less conducive to maximum IL-2 secretion compared to CD28. Despite this, the results from the “CAR-T only” condition provided evidence for differences in basal receptor activation. Dual-chain CARs showed no detectable IL-2 secretion, suggesting minimal tonic signaling. Conversely, the 2nd Gen CARs exhibited low but detectable IL-2 secretion in the absence of

target cells. This observation confirms the presence of enhanced tonic signaling in the single-chain 2nd Gen architecture, consistent with the hypothesis that the close spatial proximity and clustering of the integrated CD3ζ and 4-1BB domains promote basal receptor activation. Additionally, a significant increase in IL-2 secretion was successfully induced upon SKOV-3 cancer cell. While all CARs showed a peak secretion at round two, only the 4D5 2nd Gen CARs displayed a delay in activation profile, indicating a later activation but better persistence.

Further analysis of T cell exhaustion and phenotypic markers was performed to evaluate more precisely which construct exhibits the greatest persistence. This analysis supported the indication of 4D5 2nd Gen CARs having a beneficial persistence and slower exhaustion. Both 4D5-derived 2nd Gen CARs, previously identified as a strong candidate based on cytotoxicity assays, showed favorable characteristics in PD-1 expression. PD-1 is an activation-induced inhibitory receptor that plays a central role in regulating T-cell responses. Upon T-cell activation, PD-1 is upregulated and contributes to the induction and maintenance of T cell tolerance (Keir et al, 2008). Through interaction with its ligand PDL-1, expressed on tumor cells, PD-1 delivers inhibitory signals inducing T cell inactivation (Ameli et al, 2022). Consequently, PD-1 serves as both an activation marker and an indicator of exhaustion when its expression remains elevated under chronic antigen exposure. Consistent with this regulatory role, the cytotoxic assays demonstrated that the two 4D5 2nd Gen CARs displayed the greatest functional persistence across multiple rounds. Their comparatively low PD-1 expression after round two in ErbB2_{high} conditions therefore suggests a reduced propensity toward activation-induced exhaustion, supporting the notion that these constructs may sustain effector function more effectively than the other CAR designs examined.

In contrast, the CD4⁺ and CD8⁺ T cell distribution deviated from initial assumptions. It was anticipated that SKOV-3 and MCF-7 co-cultures, representing higher antigenic activity, would yield higher proportions of cytotoxic CD8⁺ T cells (Golubovskaya, Wu, 2016). However, 4D5 WT 2nd Gen displayed the lowest CD8⁺ fraction in the SKOV-3 condition, indicating diminished cytotoxic activity, in contrast to strong activity seen in the cytotoxic assays. This discrepancy may be attributed to impaired CAR detection after such a killing assay, which could have led to biased CD4/CD8 analyses when gating on CAR⁺ cells. The phenotypic characterization further demonstrated that the SKOV-3 co-culture contained the fewest naïve T cells among all tested conditions. This observation is favorable, as it indicates

enhanced differentiation and activation of CAR-T cells upon exposure to target antigen. Nevertheless, certain technical limitations must be acknowledged. The CD45RA staining did not perform optimally, likely due to an insufficient antibody concentration, which hindered reliable discrimination between T_{TE}, T_N, and T_{SCM} subsets.

Several limitations of this study must be acknowledged. Most importantly, the reliability of the flow cytometry staining proved challenging, making it difficult to draw definitive conclusions from marker-based analysis. Additionally, certain aspects of the CAR designs themselves limited the comparability and interpretability of the results. Specifically, the dual-chain architecture was built utilizing a C6.5 variant paired with CD3 ζ signaling domain, instead of 4D5. This selective pairing created an incomplete comparative framework. To conclusively establish the superiority of the 2nd Gen 4D5 CARs, it is essential to directly benchmark them against dual-chain constructs that incorporate 4D5 coupled to the CD3 ζ domain. Based on the current dataset, the 4D5-derived 2nd Gen CAR designs, incorporating either the WT scFv or the low-affinity variant show superior results for selectivity, efficiency and persistence and outperform all tested dual-chain designs.

Overall, the 4D5-derived 2nd Gen CAR constructs exhibited strong and selective cytotoxic activity in the XTT assays. However, detailed phenotypic analysis revealed a key divergence between the 4D5 low- and wildtype variant. The 4D5_{low} 2nd Gen CAR displayed signs of pronounced activation and differentiation, evidenced by an almost complete loss of naïve T cells after co-culture with primary human fibroblasts, and subsequently showed a low CD8⁺ T cell frequency after co-culture with cells of the SKOV-3 cell line, suggesting potential limitations in cytotoxic efficacy or survival of the effector subset. In contrast, the 4D5 WT 2nd Gen CAR maintained a small population of naïve T cells following fibroblast co-culture and exhibited higher relative CD8⁺ T cell frequencies after exposure to cells of the tumor cell lines SKOV-3 and MCF-7. Crucially, and consistent with their overall strong cytotoxic and selective performance, both constructs displayed very low PD-1 expression after SKOV-3 co-culture, indicating minimal T cell exhaustion. This data suggests that especially the 4D5 WT variant achieves a superior balance between potent killing and the maintenance of a favorable, non-exhausted phenotype essential for long-term clinical efficacy. Both 4D5-derived CARs are highly promising and warrant further investigation and clinical development.

7. Outlook

A critical prerequisite for robust conclusion is reliable CAR expression conformation. Future CAR designs should consider alternative hinge domains, such as CD34, which would in combination with G4S staining allow for reliable detection of both CAR chains on the T cell surface. Additionally, to fully characterize the dose-response relationship and to also see binding of the low affinity variants, the target concentration ranges in protein titration assays should be expanded. To further investigate the hypothesis of tonic signaling, future studies should include single-chain control constructs expressing only 4-1BB co-stimulatory domain to verify that it alone cannot induce basal activation.

Another important consideration is the arrangement of the signaling domains. In the current dual-chain CARs, CD3 ζ was linked to the C6.5 scFv and 4-1BB to the 4D5 scFv. Given that the 4D5 variant demonstrated beneficial selectivity and stronger activation, it would have been more logical to pair CD3 ζ with 4D5. Moreover, for comprehensive risk assessment, a functional comparison of all constructs against an ErbB2-specific 3rd Gen CAR is necessary because these designs have previously shown severe safety problems (Morgan et al., 2010).

To bridge the gap between in vitro findings and clinical potential, a rigorous in vivo assessment is required. This must include autologous in vitro assays with primary tumor and healthy tissues and matched CAR-T cells to prove realistic selectivity and safety against ErbB2_{low} expressing healthy tissues. Ultimately, preclinical testing in mouse models is necessary to better approximate potential effects in humans. Specifically, a double tumor xenograft model utilizing ErbB2_{high} SKOV-3 and a cell line expressing physiological levels of ErbB2 should be established to test selectivity and the safety of the CAR design in a complex in vivo environment.

In summary, the results suggest that further optimization efforts should focus on the 4D5-based 2nd Gen CAR variants, whereas the dual-chain CAR strategy, which targets two different epitopes within ErbB2, did not show any functional benefit under the conditions tested and should therefore not be prioritized for future dual chain CAR strategies.

8. References

- Ameli, F., Shajareh, E., Mokhtari, M. *et al.* (2022). Expression of PD1 and PDL1 as immune-checkpoint inhibitors in mantle cell lymphoma. *BMC Cancer* **22**, 848. <https://doi.org/10.1186/s12885-022-09803-x>
- Birke, C., Peter, H., Langenberg, U., Müller-Hermes, W., Peters, J., Heitmann, J., Leibold, W., Dallügge, H., Krapf, E., & Kirchner, H. (1981). *J Immunol.* 127(1):94-8. <https://pubmed.ncbi.nlm.nih.gov/7195412/>
- Bai, X., Sun, P., Wang, X. *et al.* (2023). Structure and dynamics of the EGFR/HER2 heterodimer. *Cell Discov* **9**, 18. <https://doi.org/10.1038/s41421-023-00523-5>
- Blood and Marrow Transplant Handbook: Comprehensive Guide for Patient Care. (2011). *Blood and Marrow Transplant Handbook: Comprehensive Guide for Patient Care*, 1-324. <https://doi.org/10.1007/978-1-4419-7506-5>
- Brudno, J. N., & Kochenderfer, J. N. (2019). Recent advances in CAR T-cell toxicity: Mechanisms, manifestations and management. *Blood Rev*, 34, 45-55. <https://doi.org/10.1016/j.blre.2018.11.002>
- Calderon H, Mamonkin M, Guedan S. (2020). Analysis of CAR-Mediated Tonic Signaling. *Methods Mol Biol.*;2086:223-236. https://doi.org/10.1007/978-1-0716-0146-4_17
- Cheng X. (2024). A Comprehensive Review of HER2 in Cancer Biology and Therapeutics. *Genes (Basel)*. 15(7):903. <https://doi.org/10.3390/genes15070903>
- Chmielewski, M., Hombach, A. A., & Abken, H. (2014). Of CARs and TRUCKs: chimeric antigen receptor (CAR) T cells engineered with an inducible cytokine to modulate the tumor stroma. *Immunological Reviews*, 257(1), 83-90. <https://doi.org/10.1111/imr.12125>
- Dabas, P., & Danda, A. (2023). Revolutionizing cancer treatment: a comprehensive review of CAR-T cell therapy. *Med Oncol*, 40(9), 275. <https://doi.org/10.1007/s12032-023-02146-y>
- 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. *Proc Natl Acad Sci U S A*, 90(2), 720-724. <https://doi.org/10.1073/pnas.90.2.720>
- Finn, R. S., Gu, K. S., Chen, X., Merle, P., Lee, K. H., Bouattour, M., Cao, P. G., Wang, W., Cheng, A. L., Zhu, L. J., Lim, H. Y., Kudo, M., Pan, Y. Y., Chang, T. T., Edeline, J., Li, W., Yang, P., Li, C., Li, J. F., . . . Qin, S. K. (2025). [1] Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021;71:209-249. *Jhep Reports*, 7(6). https://doi.org/ARTN_101350_10.1016/j.jhepr.2025.101350
- Fujiwara, K., Tsunei, 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://www.mdpi.com/2073-4409/9/5/1182>
- Gattinoni, L., Klebanoff, C., & Restifo, N. (2012) Paths to stemness: building the ultimate antitumor T cell. *Nat Rev Cancer* 12(10):671-684. <https://pmc.ncbi.nlm.nih.gov/articles/PMC6352980/pdf/nihms-1004443.pdf>
- Glienke, W., Dragon, A. C., Zimmermann, K., Martyniszyn-Eiben, A., Mertens, M., Abken, H., Rossig, C., Altvater, B., Aleksandrova, K., Arseniev, L., Kloth, C., Stamopoulou, A., Moritz, T., Lode, H. N., Siebert, N., Blasczyk, R., Goudeva, L., Schambach, A., Köhl, U., . . . Esser, R. (2022). GMP-Compliant Manufacturing of TRUCKs: CAR T Cells targeting

- GD and Releasing Inducible IL-18. *Frontiers in Immunology*, 13. <https://doi.org/ARTN839783> 10.3389/fimmu.2022.839783
- Golubovskaya, V., & Wu, L. (2016). Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy. *Cancers*, 8(3), 36. <https://doi.org/10.3390/cancers8030036>
- Hamieh, M., Mansilla-Soto, J., Riviere, I., et al. (2023). Programming Car T Cell Tumor Recognition: Tuned Antigen Sensing and Logic Gating. *Cancer Discovery*, 13(4), 829-843. <https://doi.org/10.1158/2159-8290.CD-23-0101>
- Hoff, F., Hu, C., Qutub, A., Qui, Y., Graver, E., Hoang, G., Chauhan, M., Bont, E., & Kornblau, S. (2018). Mycoplasma contamination of leukemic cell lines alters protein expression determined by reverse phase protein arrays. *Cytotechnology* 70(6): 1529-1535. <https://doi.org/10.1007/s10616-018-0244-2>
- Huang, R., Li, X., He, Y., Zhu, W., Gao, L., Liu, Y., Gao, L., Wen, Q., Zhong, J. F., Zhang, C., & Zhang, X. (2020). Recent advances in CAR-T cell engineering. *J Hematol Oncol*, 13(1), 86. <https://doi.org/10.1186/s13045-020-00910-5>
- Ito, A., Nakajima, S., Sasaguri, Y., Nagase, H., & Mori, Y. (1995) "Co-culture of human breast adenocarcinoma MCF-7 cells and human dermal fibroblasts enhances the production of matrix metalloproteinases ..." *Br. J. Cancer*, 71(5):1039-45. <https://pubmed.ncbi.nlm.nih.gov/7734296/>
- Keir ME, Butte MJ, Freeman GJ, Sharpe AH (2008). PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 26:677-704. <https://doi.org/10.1146/annurev.immunol.26.021607.090331>
- Koh, C., Lee, S., Kwak, M., Kim, B., & Chung, Y. (2023). CD8 T-cell subsets: heterogeneity, functions, and therapeutic potential. *Exp Mol Med*. 55(11):2287-2299 <https://doi.org/10.1038/s12276-023-01105-x>
- Landgraf, R. (2007). HER2 therapy. HER2 (ERBB2): functional diversity from structurally conserved building blocks. *Breast Cancer Res* 9, 202 <https://doi.org/10.1186/bcr1633>
- Lee YG, Chu H, Lu Y, Leamon CP, Srinivasarao M, Putt KS, Low PS. (2019). Regulation of CAR T cell-mediated cytokine release syndrome-like toxicity using low molecular weight adapters. *Nat Commun*, 10(1):2681. <https://doi.org/10.1038/s41467-019-10565-7>
- Liu, Z., Chen, O., Wall, J.B.J. et al. (2017). Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector. *Sci Rep* 7, 2193. <https://doi.org/10.1038/s41598-017-02460-2>
- Liu, X., Jiang, S., Fang, C., ...& Zhao, Y. (2016). Affinity-tuned ErbB2 or EGFR chimeric antigen receptor T cells exhibit an increased therapeutic index against tumors in mice. *Cancer Res*. 1:75(17):3596-3607. <https://doi.org/10.1158/0008-5472.CAN-15-0159>
- Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, Ingaramo M, Smith JP, Walker AJ, Kohler ME, Venkateshwara VR, Kaplan RN, Patterson GH, Fry TJ, Orentas RJ, Mackall CL. (2015). 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. *Nat Med*. Jun;21(6):581-90.
- Malpiedi, L. P., Díaz, C. A., Nerli, B. B., & Pessoa, A. (2013). Single-chain antibody fragments: Purification methodologies. *Process Biochemistry*, 48(8), 1242-1251. <https://doi.org/10.1016/j.procbio.2013.06.008>
- Marple, A. H., Bonifant, C. L., & Shah, N. N. (2020). Improving CAR T-cells: The next generation. *Semin Hematol*, 57(3), 115-121. <https://doi.org/10.1053/j.seminhematol.2020.07.002>
- Miao, L., Zhang, Z., Ren, Z., & Li, Y. (2021). Reactions Related to CAR-T Cell Therapy. *Front Immunol*, 12, 663201. <https://doi.org/10.3389/fimmu.2021.663201>

- Mitra, A., Barua, A., Huang, L., Ganguly, S., Feng, Q., & He, B. (2023). From bench to bedside: the history and progress of CAR T cell therapy. *Front Immunol*, 14, 1188049. <https://doi.org/10.3389/fimmu.2023.1188049>
- Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. (2010). Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther.*, 18(4):843-51. <https://doi.org/10.1038/mt.2010.24>
- Oh, D. Y., & Bang, Y. J. (2020). HER2-targeted therapies - a role beyond breast cancer. *Nat Rev Clin Oncol*, 17(1), 33-48. <https://doi.org/10.1038/s41571-019-0268-3>
- Pardoll, D. M. (2012). The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*, 12(4), 252-264. <https://doi.org/10.1038/nrc3239>
- Pear, W. S., Nolan, G. P., Scott, M. L., & Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. *PNAS*, 90 (18) 8392-8396 <https://www.pnas.org/doi/abs/10.1073/pnas.90.18.8392>
- Pietrobon, V., Todd, L. A., Goswami, A., Stefanson, O., Yang, Z., & Marincola, F. (2021). Improving CAR T-Cell Persistence. *Int J Mol Sci*, 22(19). <https://doi.org/10.3390/ijms221910828>
- Restifo, N. P., Dudley, M. E., & Rosenberg, S. A. (2012). Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat Rev Immunol*, 12(4), 269-281. <https://doi.org/10.1038/nri3191>
- Sakaguchi, S., Mikami, N., Wing, J., Tanaka, A., Ichiyama, K., & Ohkura, N. (2020). Regulatory T Cells and Human Disease. *Annu Rev Immunol*.26:38:541-566. <https://doi.org/10.1146/annurev-immunol-042718-041717>
- Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., & Norton, L. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *New England Journal of Medicine*, 344(11), 783-792. <https://doi.org/Doi 10.1056/Nejm200103153441101>
- Soule, H. D., Vazquez, J., Long, A., Albert, S., & Brennan, M. (1973). "A human cell line from a pleural effusion derived from a breast carcinoma." *J. Natl. Cancer Inst.* 51(5):1409-16 <https://pubmed.ncbi.nlm.nih.gov/4357757/>
- Stern, M., Herrmann, R., (2005). Overview of monoclonal antibodies in cancer therapy: present and promise. *Clinical Reviews in Oncology/Hematology*, 54(1):11-29 <https://doi.org/10.1016/j.critrevonc.2004.10.011>
- Sterner, R. C., & Sterner, R. M. (2021). CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J*, 11(4), 69. <https://doi.org/10.1038/s41408-021-00459-7>
- Suksanpaisan, L., Russell, S., & Peng, K. (2014). High scFv-receptor affinity does not enhance the antitumor activity of HER2 retargeted measles virus. *Cancer Gene Ther.* 30;21(6):256-260. <https://doi.org/10.1038/cgt.2014.25>
- Tokarew, N., Ogonek, J., Endres, S., von Bergwelt-Baildon, M., & Kobold, S. (2019). Teaching an old dog new tricks: next-generation CAR T cells. *Br J Cancer*, 120(1), 26-37. <https://doi.org/10.1038/s41416-018-0325-1>
- Uscanga-Palomeque, A. C., Chavez-Escamilla, A. K., Alvizo-Baez, C. A., Saavedra-Alonso, S., Terrazas-Armendariz, L. D., Tamez-Guerra, R. S., Rodriguez-Padilla, C., & Alcocer-Gonzalez, J. M. (2023). CAR-T Cell Therapy: From the Shop to Cancer Therapy. *Int J Mol Sci*, 24(21). <https://doi.org/10.3390/ijms242115688>

- van der Schans, J. J., van de Donk, N., & Mutis, T. (2020). Dual Targeting to Overcome Current Challenges in Multiple Myeloma CAR T-Cell Treatment. *Front Oncol*, 10, 1362. <https://doi.org/10.3389/fonc.2020.01362>
- Wang, S., Zhang, J., Wang, T., Ren, F., Liu, X., Lu, Y., Xu, L., Zhang, Y., Wang, D., Xu, L., Wu, Y., Liu, F., Li, Q., Zaky, M., Liu, F., Zou, K., & Zhang, Y. (2019). Endocytic degradation of ErbB2 mediates the effectiveness of neratinib in the suppression of ErbB2-positive ovarian cancer. *Int J Biochem Cell Biol*. 117:105640 <https://pubmed.ncbi.nlm.nih.gov/31689531/>
- Watanabe, N., & McKenna, M. (2023). Generation of CAR-T cells using γ -retroviral vector. *Methods Cell Biology*. 30;167_171-183 <https://doi.org/10.1016/bs.mcb.2021.06.014>
- Woodcock, D. M., Crowther, P. J., Doherty, J., Jefferson, S., DeCruz, E., Noyer-Weidner, M., Smith, S. S., Michael, M. Z., & Graham, M. W. (1989). Quantitative evaluation of Escherichia coli host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res*, 17(9), 3469-3478. <https://doi.org/10.1093/nar/17.9.3469>
- Ying, Z., Huang, X. F., Xiang, X., Liu, Y., Kang, X., Song, Y., Guo, X., Liu, H., Ding, N., Zhang, T., Duan, P., Lin, Y., Zheng, W., Wang, X., Lin, N., Tu, M., Xie, Y., Zhang, C., Liu, W., . . . Chen, S. Y. (2019). A safe and potent anti-CD19 CAR T cell therapy. *Nat Med*, 25(6), 947-953. <https://doi.org/10.1038/s41591-019-0421-7>
- Zhang, C., Liu, J., Zhong, J. F., & Zhang, X. (2017). Engineering CAR-T cells. *Biomark Res*, 5, 22. <https://doi.org/10.1186/s40364-017-0102-y>
- Zhou, J., Shi, F., Luo, X., Lei, B., Shi, Z, Huang, C., Zhang, Y., Li, X., Wang, H., Li, X., He, X. (2024). The persistence and antitumor efficacy of CAR-T cells are modulated by tonic signaling within the CDR. *International Immunopharmacology*, Volume 126. <https://www.sciencedirect.com/science/article/pii/S1567576923015667>

9.2. Retroviral expression vectors

9.2.1. Dual-chain C6.5/4D5

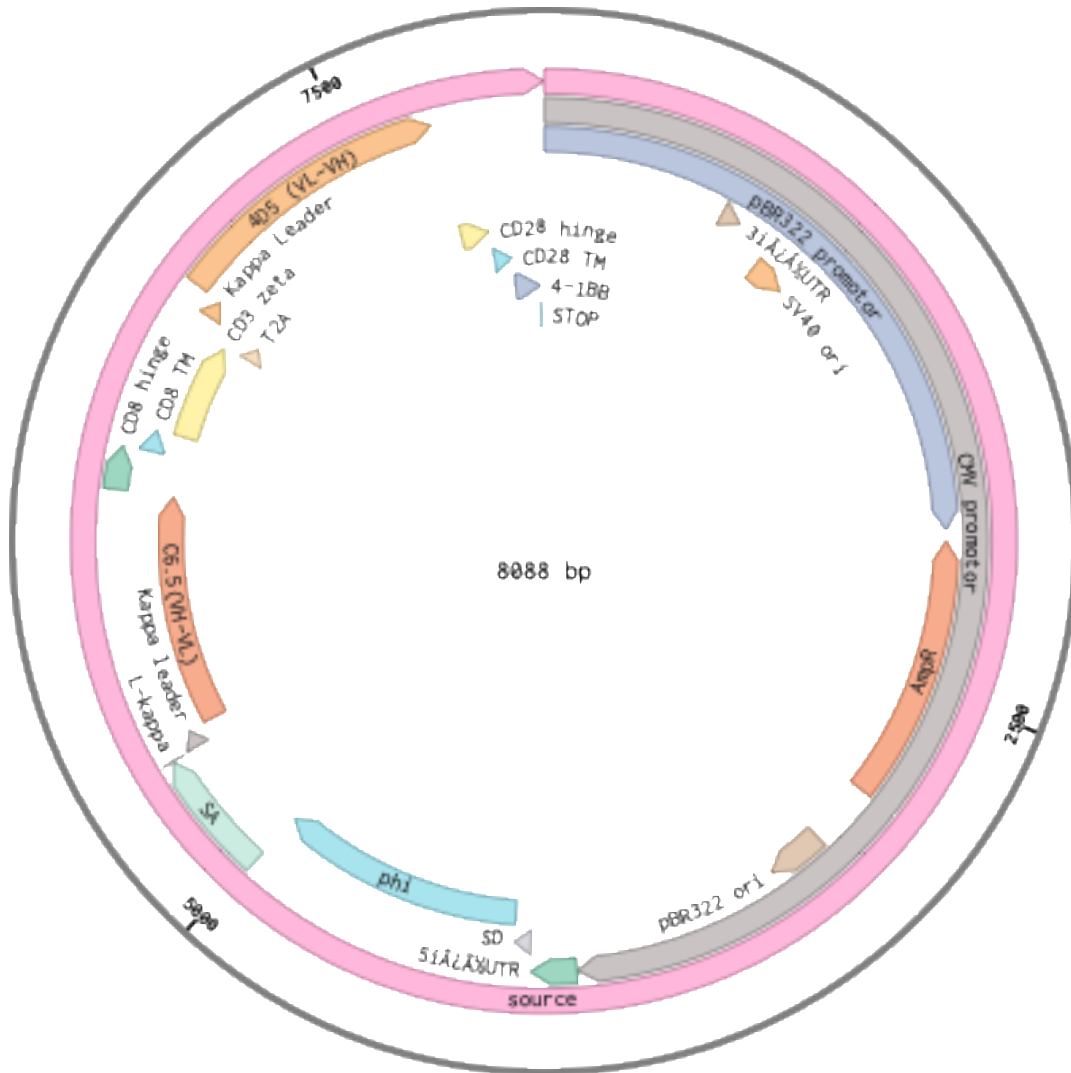


Figure A2: Plasmid map of pBullet_LK_C6.5_CD8ah_CD28TM_CD3ζ_T2A_LK_4D5_CD8ah_CD28TM_41BB. The plasmid contains the expression domains for a dual-chain CAR, incorporating the C6.5 WT scFv bound to CD3ζ, and 4D5 WT scFv bound to 4-1BB.

9.2.2. Dual-chain C6.5B1D2/4D5

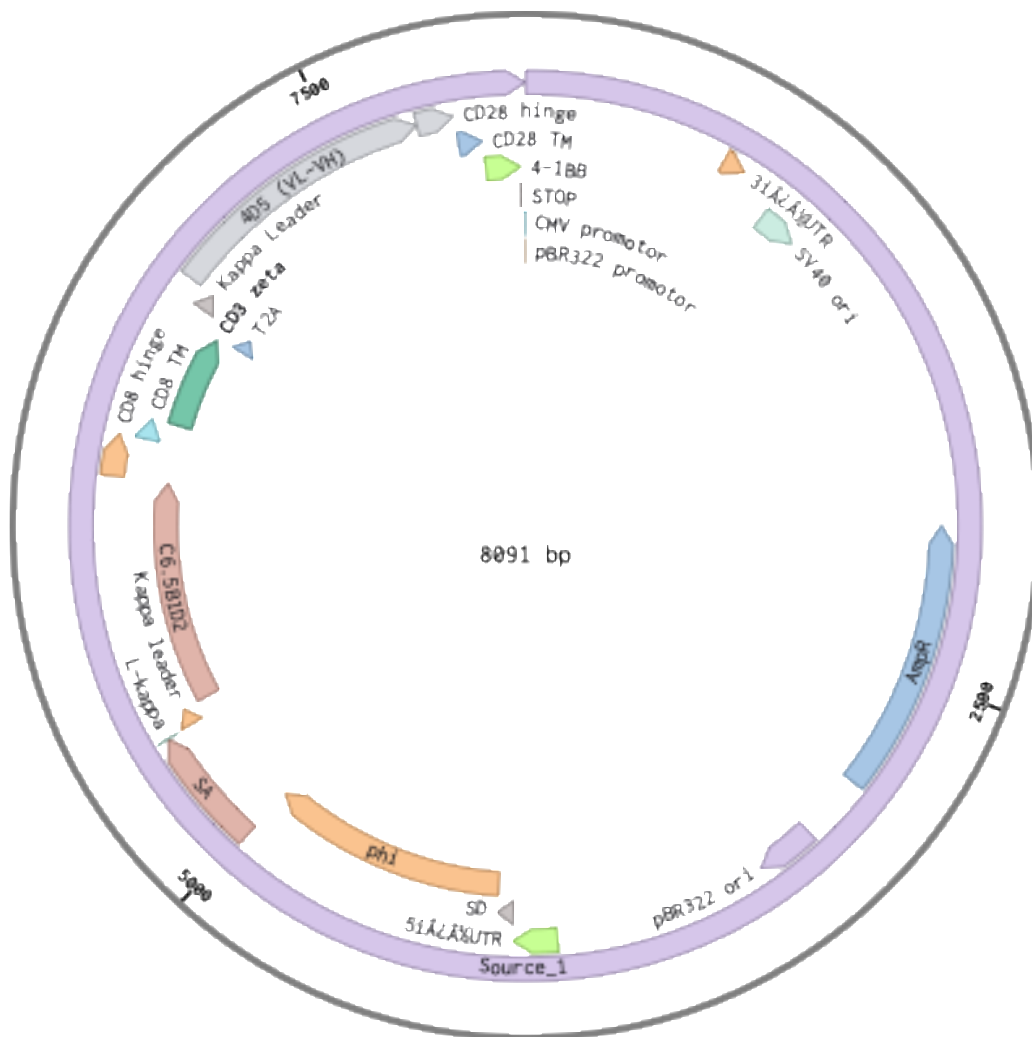


Figure A3: Plasmid map of pBullet_LK_C6.5B1D2_CD8ah_CD28TM_CD3ζ_T2A_LK_4D5_CD8ah_CD28TM_41BB. The plasmid contains the expression domains for a dual-chain CAR, incorporating the C6.5B1D2 scFv bound to CD3ζ, and 4D5 WT scFv bound to 4-1BB.

9.2.3. Dual-chain C6.5/4D5mut

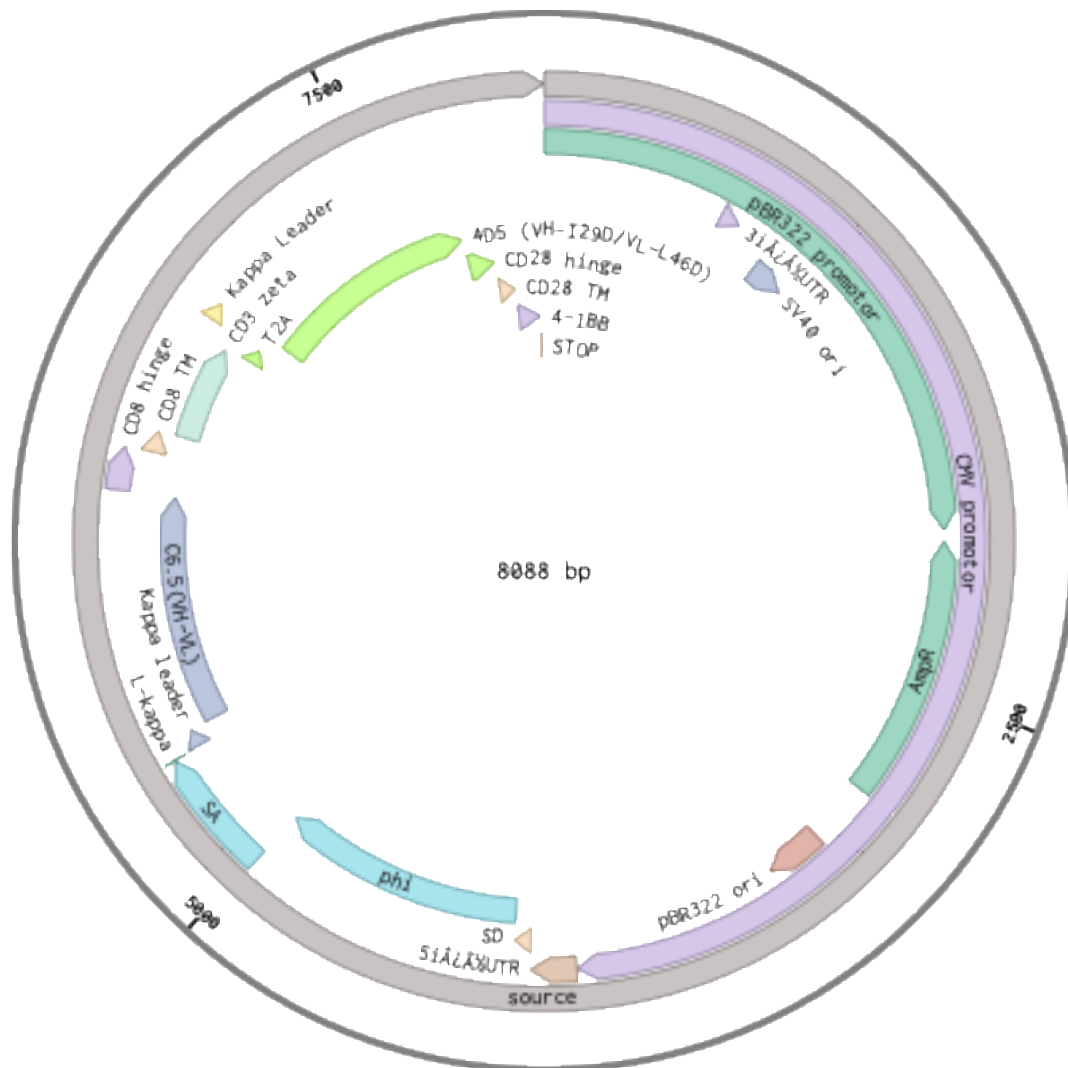


Figure A4: Plasmid map of #3253 pBullet_LK_C6.5_CD8ah_CD28TM_CD3ζ_T2A_LK_4D5mut_CD8ah_CD28TM_41BB. The plasmid contains the expression domains for a dual-chain CAR, incorporating the C6.5 scFv bound to CD3ζ, and 4D5mut scFv bound to 4-1BB.

9.2.4. Dual-chain C6.5B1D2/4D5mut

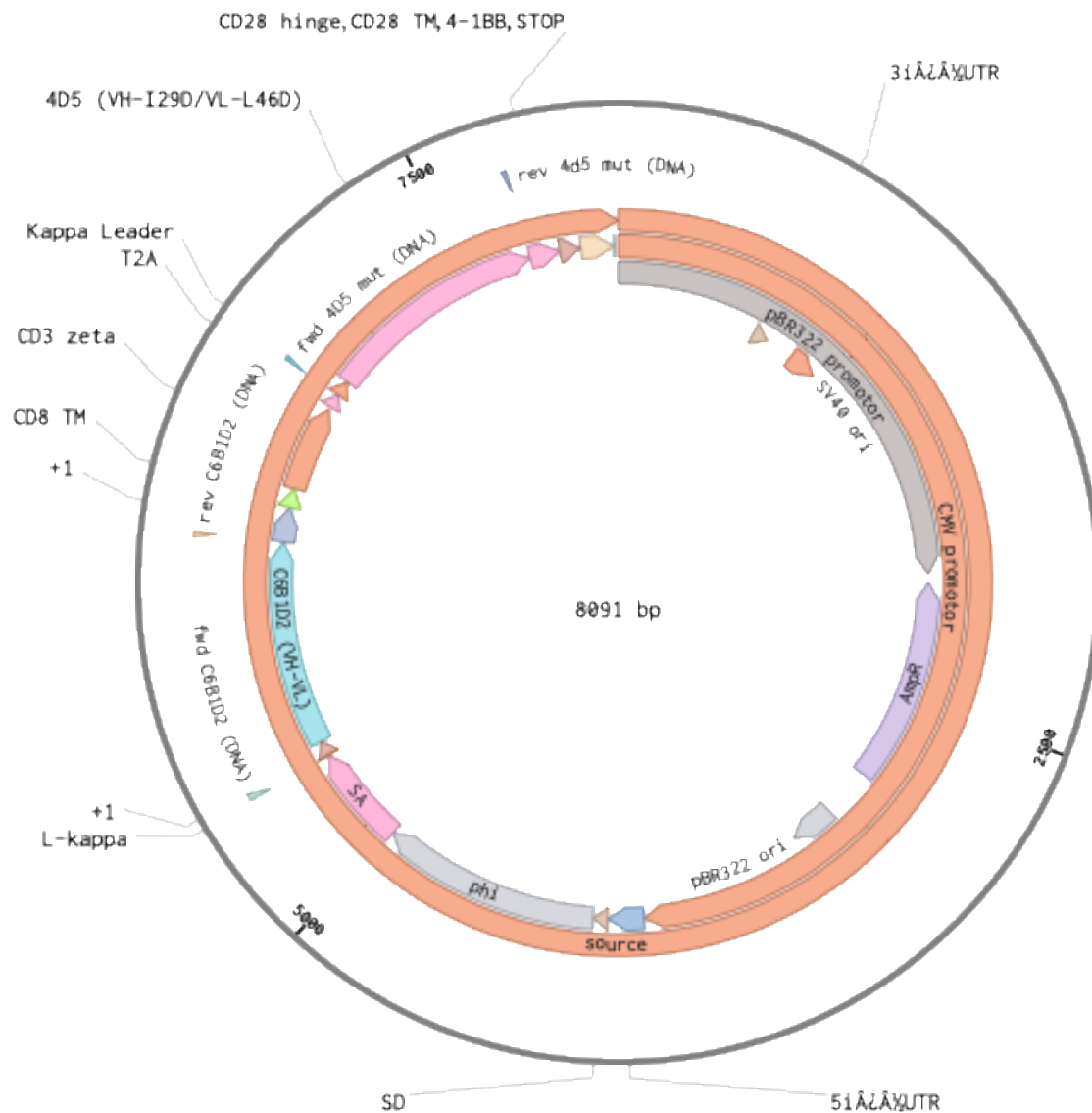


Figure A5: Plasmid map of pBullet_LK_C6.5B1D2_CD8ah_CD28TM_CD3ζ_T2A_LK_4D5mut_CD8ah_CD28TM_41BB. The plasmid contains the expression domains for a dual-chain CAR, incorporating the C6.5B1D2 scFv bound to CD3ζ, and 4D5mut scFv bound to 4-1BB.

9.2.5. 2nd Gen C6.5B1D2

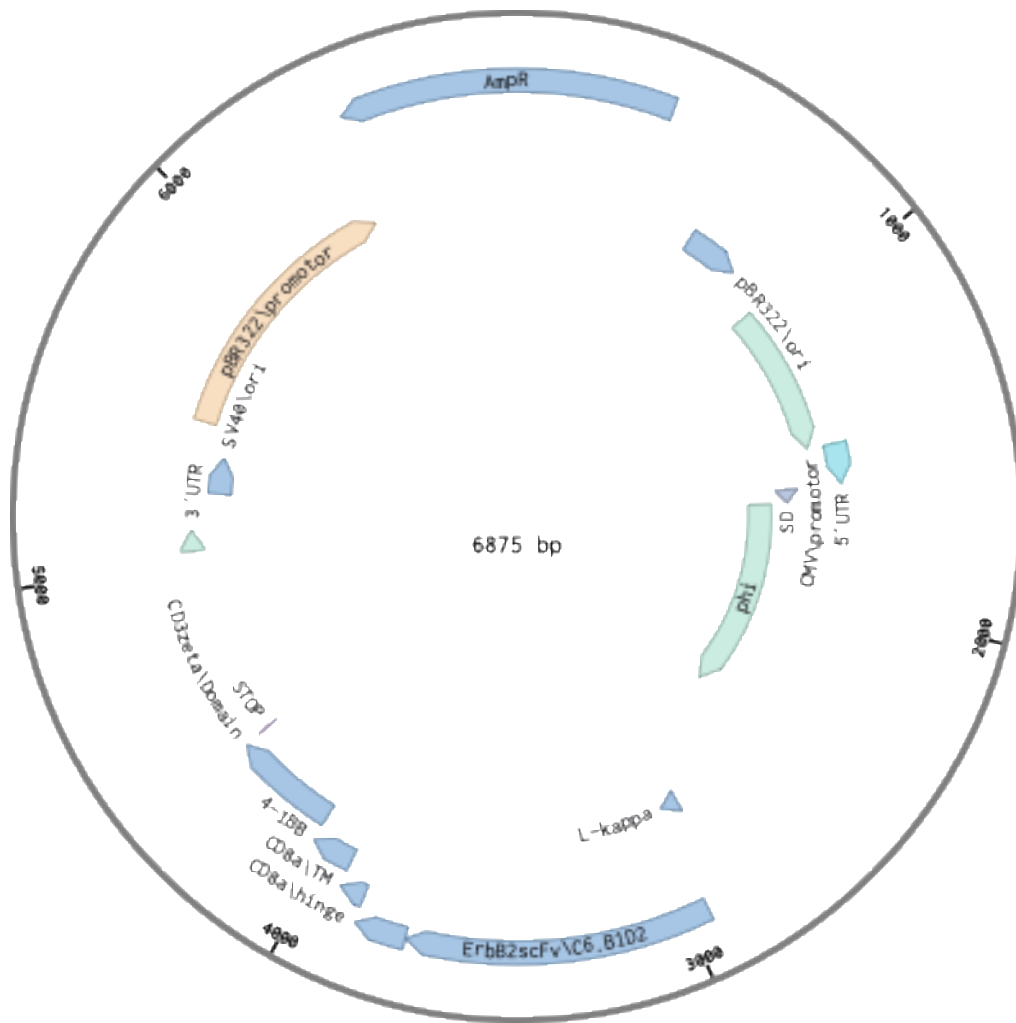


Figure A6: Plasmid map of pBullet_LK_C6.5B1D2_CD8ah_CD8aTM_4-1BB_CD3 ζ . The plasmid contains the expression domains for a 2G CAR, incorporating the C6.5B1D2 scFv bound to 4-1BB and CD3 ζ .

9.2.6. 2nd Gen C6.5

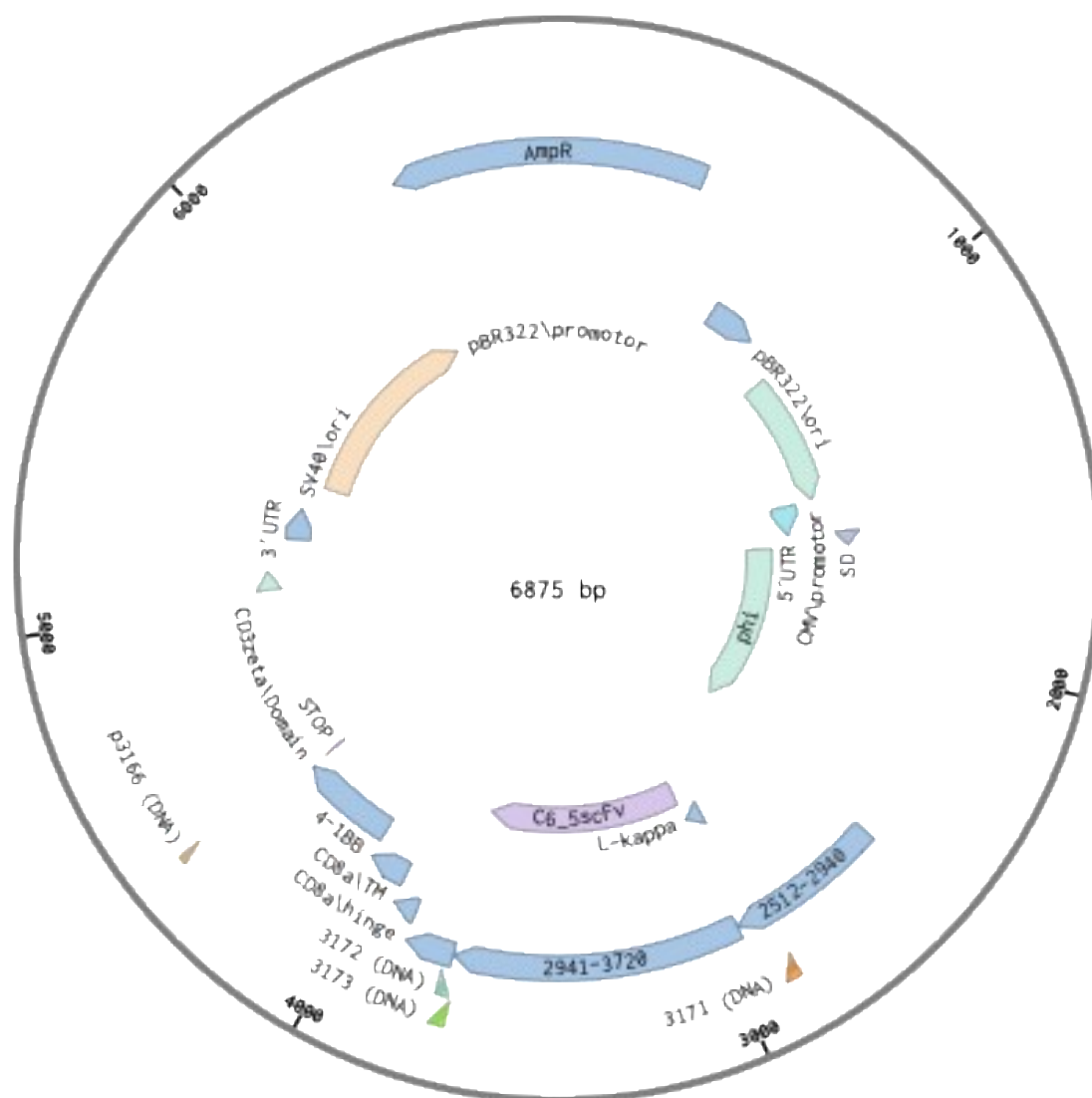


Figure A7: Plasmid map of pBullet_LK_C6.5_CD8aH_CD8aTM_4-1BB_CD3ζ. The plasmid contains the expression domains for a 2G CAR, incorporating the C6.5 scFv bound to 4-1BB and CD3ζ.

9.2.7. 2nd Gen 4D5

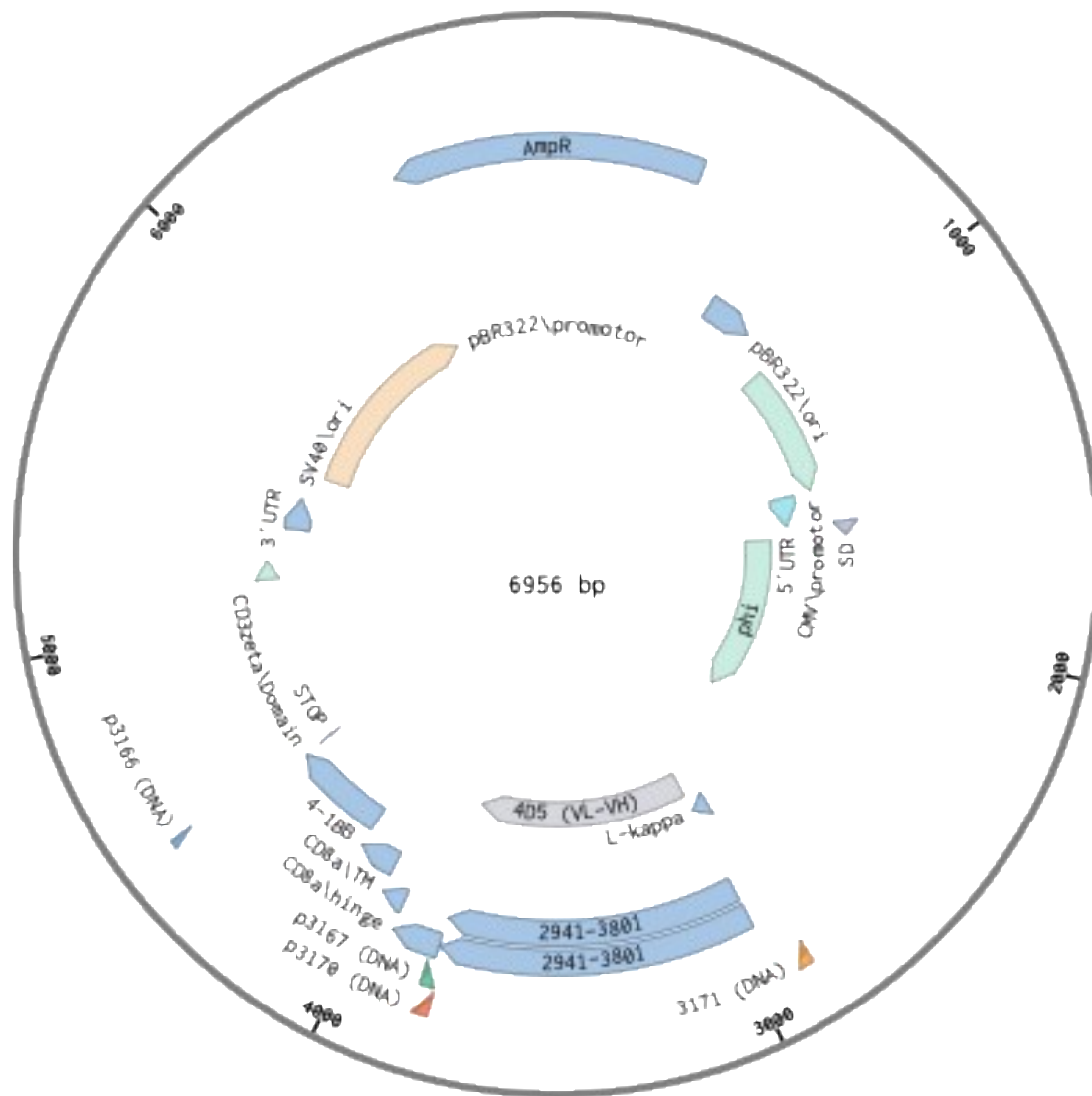


Figure A8: Plasmid map of #3267 pBullet_LK_4D5_CD8ah_CD8aTM_4-1BB_CD3ζ. The plasmid contains the expression domains for a 2G CAR, incorporating the 4D5 scFv bound to 4-1BB and CD3ζ.

9.2.8. 2nd Gen 4D5mut

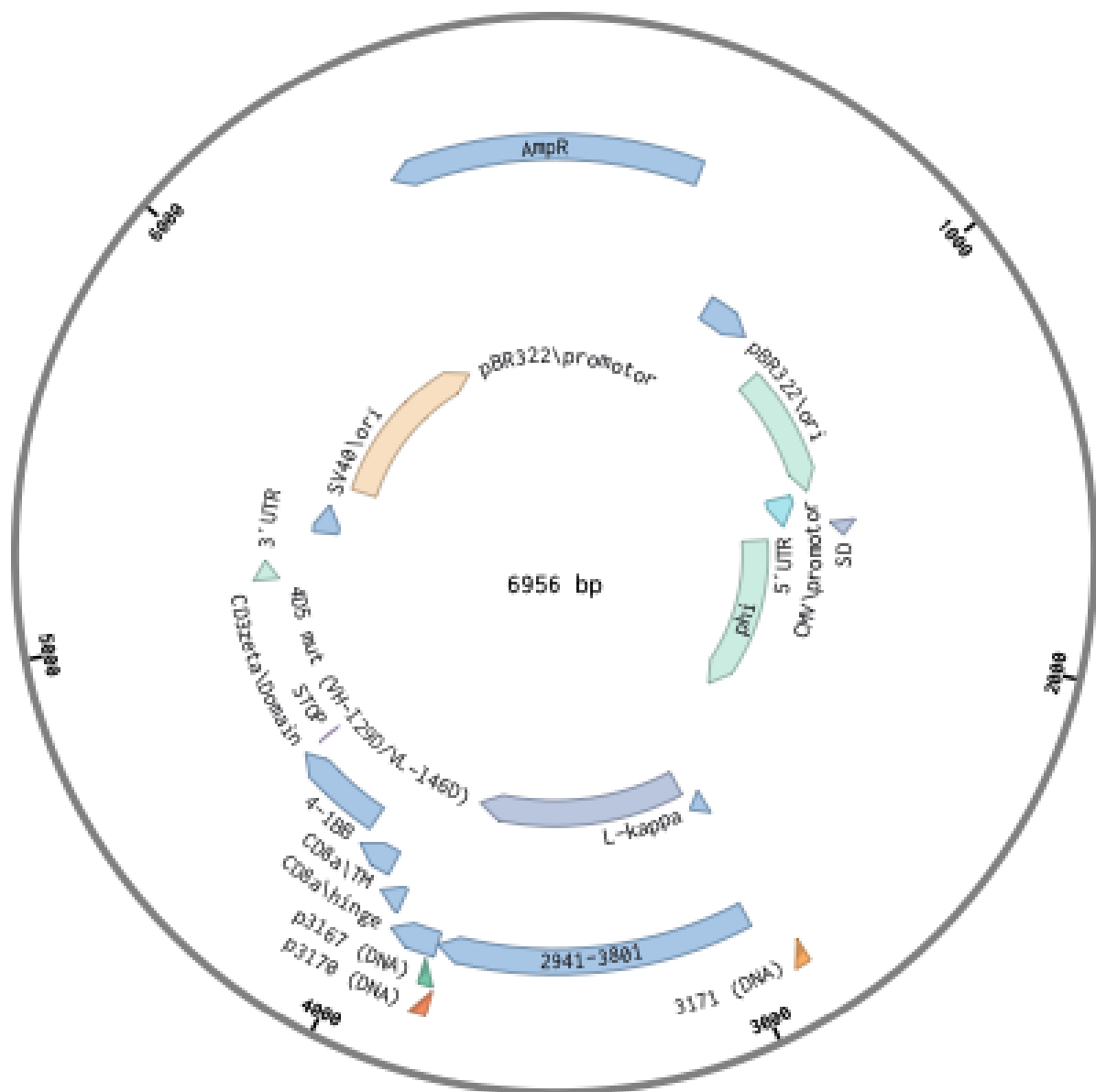


Figure A9: Plasmid map of pBullet_LK_4D5mut_CD8ah_CD8aTM_4-1BB_CD3ζ. The plasmid contains the expression domains for a 2G CAR, incorporating the 4D5mut scFv bound to 4-1BB and CD3ζ.

9.2.9. Dual-chain C6.5B1D2/B7H3

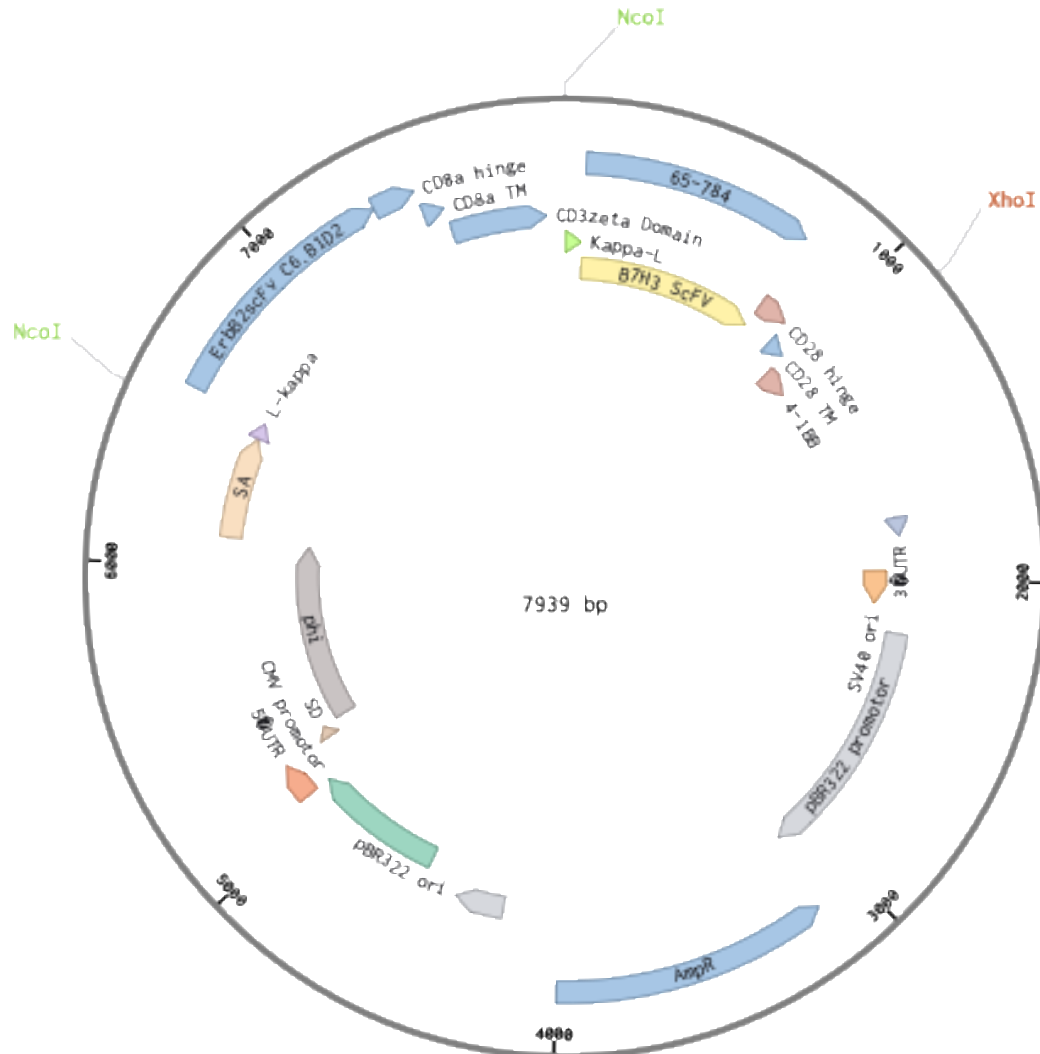


Figure A10: Plasmid map of pBullet_LK_C6.5B1D2_CD8ah_CD28TM_CD3ζ_LK_B7H3_CD28h_CD28TM_41BB. The plasmid contains the expression domains for a dual-chain CAR, incorporating the C6.5B1D2 scFv bound to CD3ζ, and B7H3 scFv bound to 4-1BB. This plasmid was digested with NcoI, and XhoI and used as the backbone fragment.

9.2.10. 1st Gen C6.5B1D2

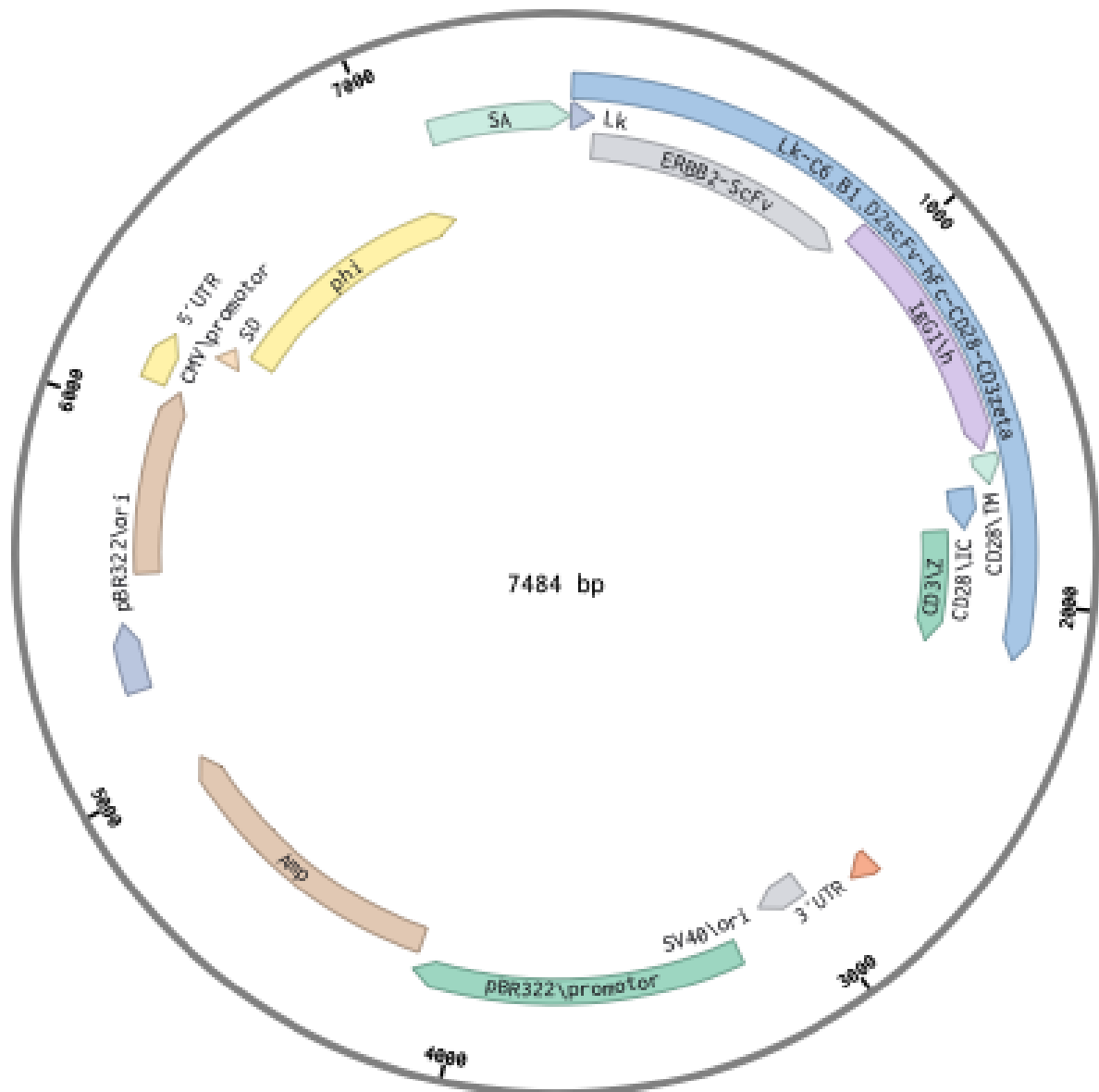


Figure A11: Plasmid map of pBullet_LK_C6.5B1D2_IgG1h_CD28TM_CD3ζ_CD28h_CD28TM_41BB. The plasmid contains the expression domains for 1st Gen CAR, incorporating the C6.5B1D2 scFv bound to CD3ζ.

9.2.11. 1st Gen C6.5

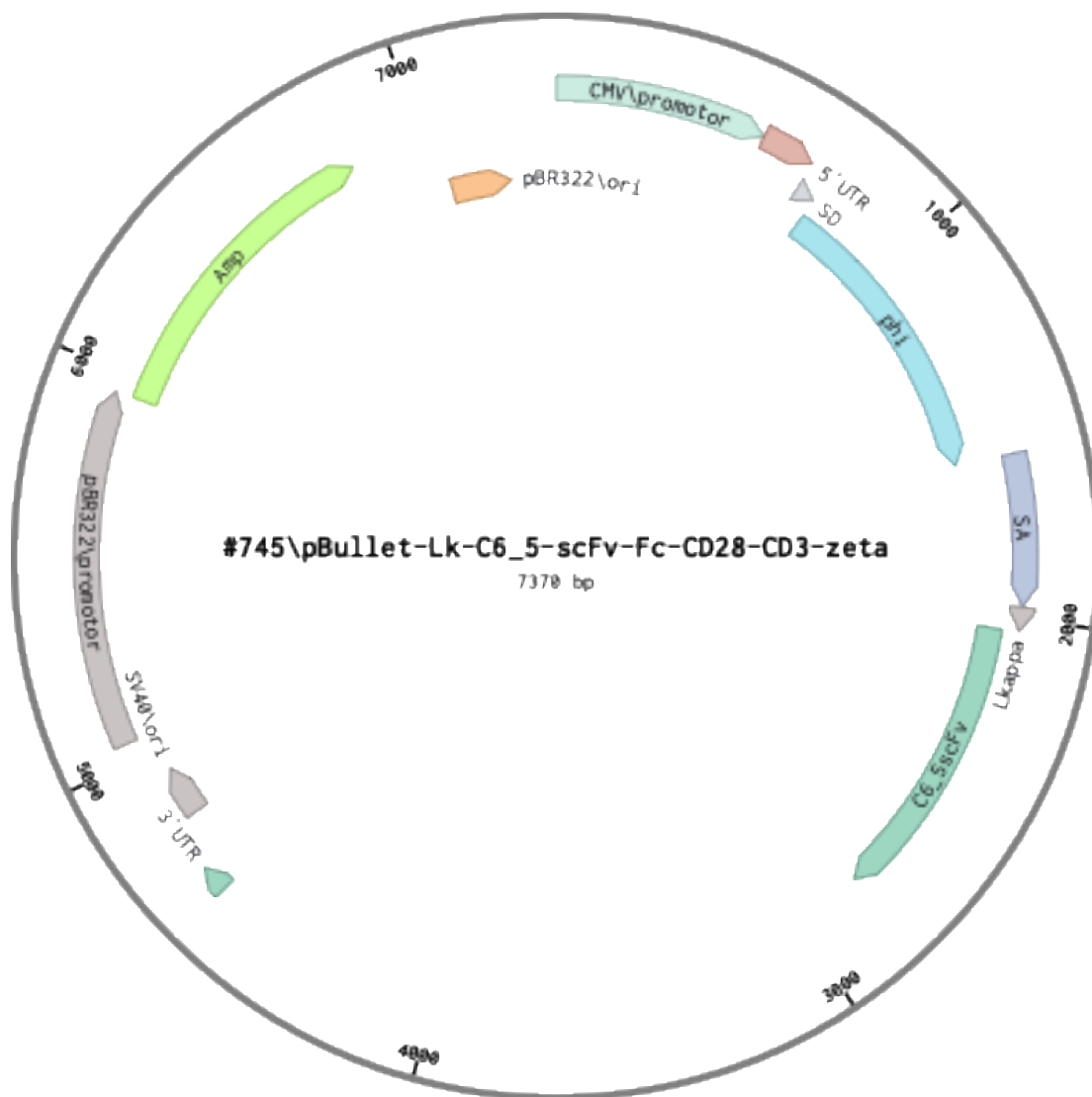


Figure A12: Plasmid map of pBullet_LK_C6.5_IgG1h_CD28TM_CD3 ζ _CD28h_CD28TM_41BB. The plasmid contains the expression domains for 1st Gen CAR, incorporating the C6.5B1D2 scFv bound to CD3 ζ .

9.3. Helper plasmids

9.3.1. Helper plasmid #3077

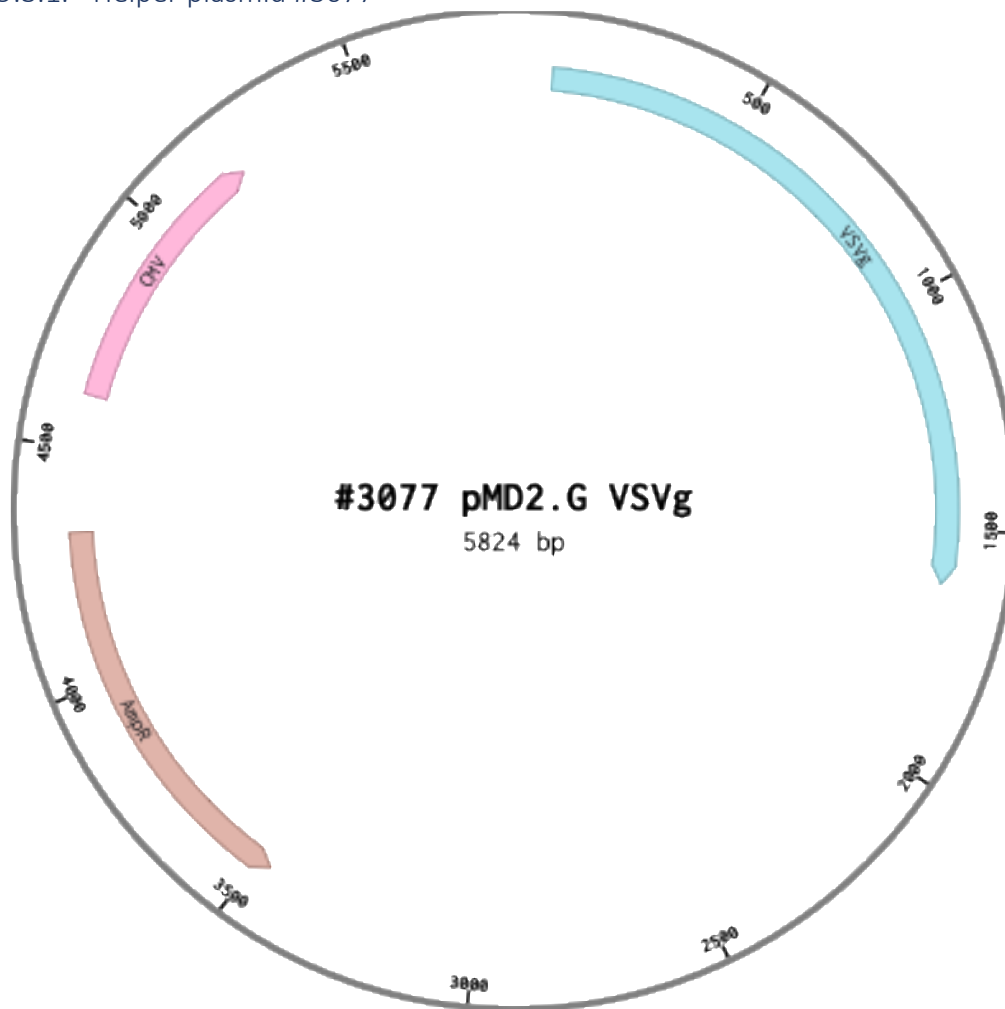


Figure A13: Plasmid map helper plasmid #3077 pMD.G VSVg. Expression of the murine Moloney leukemia virus (M-MuLV) proteins gag (group-specific antigens) and pol (reverse transcriptase polymerase)

9.3.2. Helper plasmid #3076

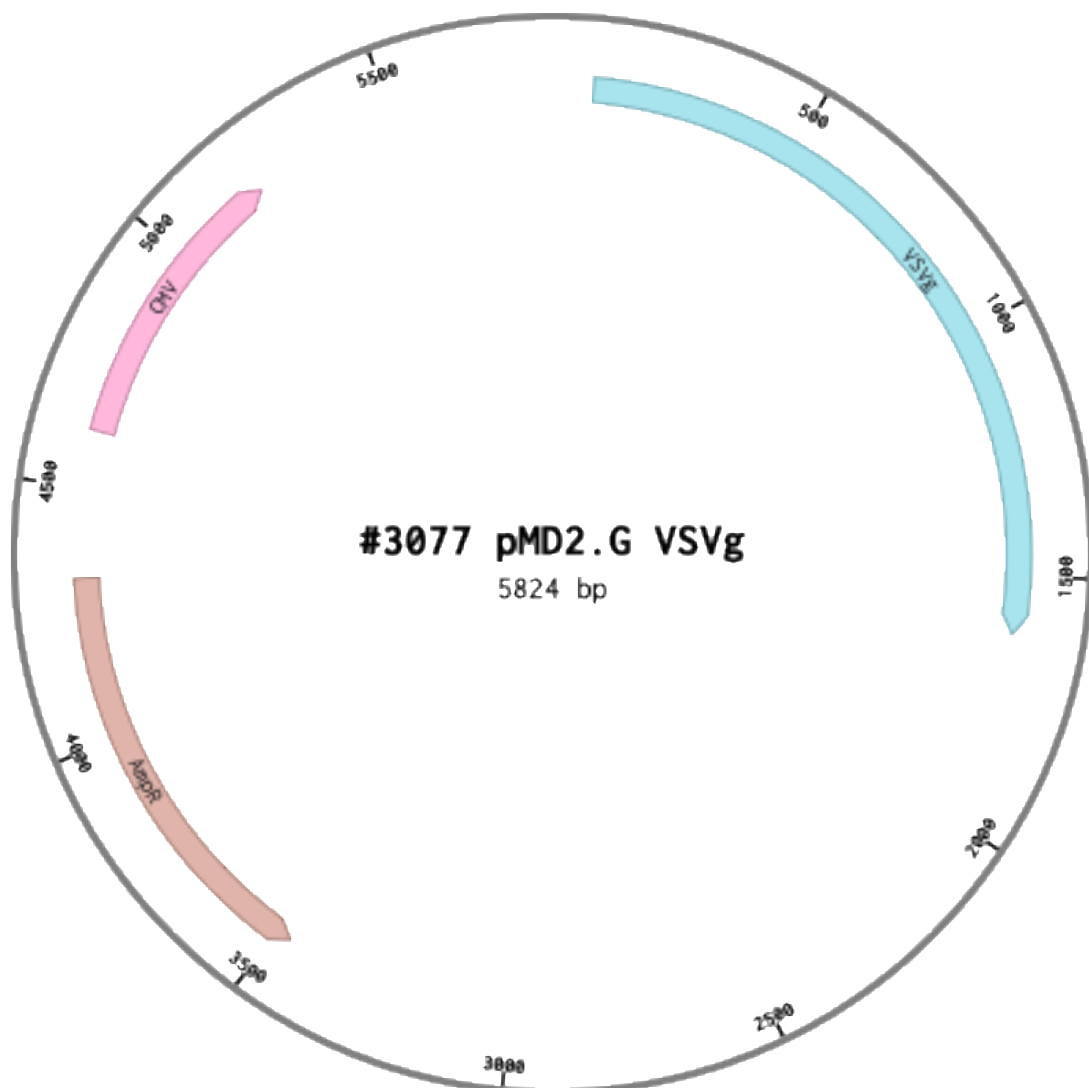


Figure A14: Plasmid map of helperplasmid #3077 pMD2.G VSVg. Expression of the envelope protein env of the gibbon monkey leukemia virus (GALV); VSV-G tropism