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Mgr. Martin Štach

Enhancing Anti-tumor Efficacy and Improving Manufacturing of Chimeric Antigenic Receptor T Cells

Zvyšování protinádorové účinnosti a zlepšování výrobních postupů T-lymfocytů nesoucích chimerické antigenní receptory

Doctoral Thesis

Supervisor: doc. MUDr. Pavel Otáhal, Ph.D.

Institute of Hematology and Blood Transfusion

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Prohlášení:

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ABSTRAKT

T-lymfocyty exprimující chimérické antigenní receptory (CAR) představují nový léčebný postup, který je obzvláště účinný proti B-lymfocytárním malignitám, a mění dosavadní paradigma léčby rakoviny. V současné době existuje několik schválených a komerčně dostupných CAR T-lymfocytárních produktů. Nicméně tato léčba je stále neúnosně finančně nákladná. Proto existuje poptávka po nahrazení obzvláště nákladných retrovirových/lenvirových vektorů levnějšími, nevirovými alternativami. Navíc se současný výzkum nadále zaměřuje na další vylepšení CAR T-lymfocytů posílením jejich protinádorových funkcí, expanze a perzistence *in vivo*. Kromě toho je usilováno o rozšíření spektra antigenů a nemocí proti kterým by byly CAR T lymfocyty použitelné. Tato disertační práce se zabývá výrobou CD19 specifických CAR T-lymfocytů z hlediska použitých cytokinů při kultivaci, vektoru pro genetickou modifikaci, testováním nového konstruktu CARu sekretujícího interleukin (IL)-21 a analýzou CAR T-lymfocytů od pacientů léčených produktem tisagenlecleucel.

V rámci této práce byl vyvinut alternativní protokol kultivace CAR T-lymfocytů, nahrazující tradičně používaný IL-2, který způsobuje efektorovou diferenciaci T-lymfocytů. Je založen na cytokinech IL-4 a IL-7, které výrazně podporují přežívání T-lymfocytů, přidání IL-21 pak podporuje zachování časně paměťového fenotypu T-lymfocytů a nízkou expresi inhibičních receptorů. CAR T-lymfocyty byly navíc úspěšně produkovány pomocí transpozonového vektoru piggyBac.

Experimenty s přidáváním exogenního IL-21 navíc potvrdily jeho zesilující účinek na proliferaci CAR T-lymfocytů, snížení apoptózy a zamezení terminální diferenciaci. Proto byly připraveny CAR T-lymfocyty inducibilně sekretující IL-21, které vykazovaly zvýšenou míru infiltrace nádorů a inhibovaly jejich růst *in vivo* v myším modelu. IL-21 také snižoval imunosupresivní účinky buněk chronické lymfocytární leukemie (CLL) během společné kultivace. To naznačuje, že by CAR T-lymfocyty vyzbrojené IL-21 mohly zlepšit výsledky terapie malignit rezistentních vůči klasickým CAR T-lymfocytům.

V rámci výzkumné práce byla rovněž testována nová metoda produkce CAR T-lymfocytů pomocí transpozonového vektoru piggyBac. Namísto běžně používaných plazmidů byla použita enzymaticky produkovaná lineární DNA kódující CAR a mRNA kódující enzym transpozázu. Tímto přístupem – bez využití bakterií – bylo dosaženo účinné produkce CAR T-lymfocytů s kontrolovaným počtem kopií integrovaného vektoru. To jej činí kompatibilním se současnými standardy správné výrobní praxe (SVP) a usnadňuje schválení regulačním úřadem. Takto vyrobené CAR T lymfocyty se funkčně nelišily od buněk vyrobených standardní a v současnosti schválenou metodou.

V neposlední řadě byla provedena studie reálného použití CAR T-lymfocytů u pacientů s relabovaným/refrakterním difuzním velkobuněčným B-lymfomem (DLBCL) a B-buněčnou akutní lymfoblastickou leukemií (B-ALL) léčených produktem tisagenlecleucel. Imunofenotyp T-lymfocytů aferetického materiálu, CAR T-lymfocytů produktu a vzorků periferní krve byl stanoven pomocí

multiparametrické průtokové cytometrie. Účinnost léčby korelovala spíše s expanzí CAR T-lymfocytů *in vivo* než s imunofenotypem produktu. Dále bylo zjištěno, že pacienti s vyšším procentem časně paměťových T-lymfocytů ve vzorcích aferézy reagovali na léčbu lépe. Nejhorší výsledky měli pacienti s primárně refrakterním onemocněním a velkou nádorovou zátěží.

V souhrnu tato práce představuje několik metod zlepšení výroby CAR T-lymfocytů a posílení jejich funkcí. Na základě získaných výsledků byl pro potřeby klinické studie fáze I (NCT05054257) zaveden výrobní protokol pro přípravu CAR T-lymfocytů. Dizertační práce také poskytuje vhled do reálného použití CAR T-lymfocytů a parametrů ovlivňujících s výsledky léčby.

Klíčová slova: CAR T-lymfocyty, paměťový fenotyp, IL-21, piggyBac, tisagenlecleucel

ABSTRACT

Chimeric antigenic receptor (CAR) T cells are a paradigm-shifting cancer treatment option that is particularly effective against B-cell malignancies. Currently, several approved, commercially available CAR T-cell products exist. Nonetheless, this therapy is still prohibitively expensive. Thereby, there is an incentive to substitute the particularly costly retroviral/lentiviral vectors with cheaper non-viral alternatives. Moreover, ongoing research strives to further improve CAR T cells by enhancing their anti-tumor functions, expansion, and *in vivo* persistence. Additionally, broadening the spectrum of antigens and diseases of CAR T-cell applicability is highly sought after. This thesis addresses CD19-specific CAR T-cell manufacturing regarding cytokines used for the cultivation and vector for genetic alteration, new interleukin (IL)-21 secreting CAR construct testing, and analysis of CAR T cells from patients treated with tisagenlecleucel.

As part of this thesis, an alternative CAR T-cell cultivation protocol was developed. Replacing the traditionally used IL-2, which drives the effector differentiation of T cells, the protocol is based on the strongly pro-survival cytokines IL-4 and IL-7, with IL-21 supporting the stem cell memory-like T-cell phenotype retention and low inhibitory receptor expression. Moreover, the CAR T cells were successfully produced using a piggyBac transposon vector.

Furthermore, experiments with the addition of exogenous IL-21 confirmed its enhancing effect on CAR T-cell proliferation, reduction of apoptosis, and prevention of terminal differentiation. Therefore, CAR T cells inducibly secreting IL-21 were prepared. They exhibited increased tumor infiltration and inhibited tumor growth *in vivo* in a mouse model. Additionally, IL-21 decreased the immunosuppressive effects of chronic lymphocytic leukemia (CLL) cells during a co-culture. This suggests that IL-21-armed CAR T cells could improve therapy outcomes of CAR-resistant malignancies.

In this thesis, a novel production method using the piggyBac transposon vector was tested. Instead of the regularly used plasmids, enzymatically produced linear DNA encoding CAR and transposase coded by mRNA were used. This bacteria-free approach achieved efficient CAR T-cell production with controlled vector copy numbers, which makes it compatible with current good manufacturing practice (cGMP) standards and facilitates easier approval by a regulatory agency. The CAR T cells were functionally indistinguishable from those produced by standard and currently approved methods.

Lastly, a real-world study was conducted on patients with relapsed/refractory diffuse large B-cell lymphoma (DLBCL) and B-cell acute lymphoblastic leukemia (B-ALL) treated with tisagenlecleucel. The immunophenotype of T cells in the apheretic material and CAR T cells in the product and peripheral blood samples was assessed by multiparametric flow cytometry. The treatment efficacy correlated with *in vivo* CAR T-cell expansion rather than the product immunophenotype. In addition, patients with

higher early-memory T-cell percentages in apheresis samples responded better to therapy. The worst outcomes were observed in patients with primary refractory disease and a large tumor load.

In conclusion, this thesis presents several methods for improving CAR T-cell manufacturing and enhancing CAR T-cell functions. These results were used to establish a manufacturing protocol for a phase I clinical trial (NCT05054257). It also provides insight into real-world CAR T cell usage and parameters affecting clinical outcomes.

Keywords: CAR T cells, memory phenotype, IL-21, piggyBac, tisagenlecleucel

LIST OF ABBREVIATIONS

aAPCs	artificial antigen-presenting cells
Ab	antibody
ACT	adoptive cell therapy
ALL	acute lymphoid leukemia
APCs	antigen-presenting cells
B2M	β2-microglobulin
BCMA	B-cell maturation antigen
BiTE	bispecific T-cell engager
bp	base pairs
CAR	chimeric antigenic receptor
CCR7	C-C chemokine receptor 7
CD	cluster of differentiation
CID	chemical inducer of dimerization
CML	chronic lymphocytic leukemia
CRISPR/Cas	clustered regularly interspaced short palindromic repeat/CRISPR-associated
CRS	cytokine release syndrome
CSR	cytokine switch receptor
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
DC	dendritic cell
DKO, TKO	double, triple knock-out
DLBCL	diffuse large B-cell lymphoma
DNR	dominant negative receptor
ECD	extracellular domain
EGFR	epidermal growth factor receptor
FDA	Food and Drug Administration
GMP	good manufacturing practice
GvHD	graft-versus-host disease

HCC	hepatocellular carcinoma
HER2	human epidermal growth factor receptor 2
HSCT	hematopoietic stem cell transplantation
ICD	intracellular domain
ICR	inverted cytokine receptor
IFN-γ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
kbp	kilobase pairs
KO	knock-out
LAG-3	lymphocyte-activation gene 3
LTR	long terminal repeats
LV	lentivirus
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MDSCs	myeloid-derived suppressor cells
MHC	major histocompatibility complex
NFκB	nuclear factor kappa B
NFAT	nuclear factor of activated T cells
NK	natural killer
PB	piggyBac
PBMCs	peripheral blood mononuclear cells
PD-1	programmed cell death 1
PDX	patient-derived xenograft
r/r	relapsed/refractory
RV	retrovirus

SB	*Sleeping Beauty*
scFv	single chain variable fragment
STAT	signal transducerand activator of transcription
synNotch	synthetic Notch
TALEN	transcription activator-like effector nucleases
T_{CM}	central memory T cell
TCR	T-cell receptor
T_{EM}	effector memory T cell
T_{EMRA}	terminally differentiated T cell
TGF-β	transforming growth factor beta
Th cell	T helper cell
TILs	tumor-infiltrating lymphocytes
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TME	tumor microenvironment
TNF	tumor necrosis factor
TRAC, TRBC	T-cell receptor alpha constant, T-cell receptor beta constant
Tregs	regulatory T cells
TRUCKs	T cells redirected for antigen-unrestricted cytokine-mediated killing
T_{SCM}	stem cell memory T cell
TSSs	transcriptional start sites
VCN	vector copy number

1. INTRODUCTION

1.1. Adoptive T-cell therapy

Adoptive T-cell transfer (ACT) is a type of cellular immunotherapy that harnesses the patient's own T cells to combat malignant diseases. The ACT is mostly autologous; the T cells are isolated, expanded, either genetically modified or activated *ex vivo* to enhance their anti-tumor activity, and then reinfused. ACT includes methods based on tumor-infiltrating lymphocytes (TILs), chimeric antigen receptors expressing T cells (CAR T cells), and transgenic tumor-specific T-cell receptor-expressing T cells (TCR T cells). When reinfused, the enhanced T cells target a tumor-associated antigen (TAA), leading to precise tumor cell eradication. Clinical trials have demonstrated outstanding potential in treating hematological malignancies, while this success has not yet been replicated for solid tumors. This thesis will solely focus on CAR T cells.

1.2. Principles of CAR T cells

A chimeric antigenic receptor (CAR) is an artificial receptor formed by a combination of domains from different receptors by genetic engineering. It consists of an extracellular antigen-recognition domain derived from an antibody (Ab), a transmembrane domain, intracellular signaling (CD3ζ), and costimulatory domains (Figure 1). The extracellular domain (ECD) recognizes surface antigens in a non-MHC-restricted manner. This feature of CAR T cells is a great advantage in comparison to other ACTs, as many tumors downregulate their MHC class I molecules on the cell surface, resulting in immune escape (Garrido et al., 2016). CAR-modified T cells are activated by engaging with their target, which triggers the proliferation of CAR T cells, the production of cytokines, and the killing of target cells. Through this mechanism of action, CAR T cells directly destroy target cancer cells.

1.2.1. Structure

The extracellular part of a CAR is a ligand-recognizing domain that is responsible for high-affinity antigen binding. It is generally derived from a monoclonal antibody (mAb) as a single-chain variable fragment (scFv). The scFv comprises heavy and light variable (V_H, V_L) immunoglobulin (Ig) chains connected with a flexible linker. Another option is the so-called ligand-based CAR, which uses a natural ligand of its target molecule, e.g., the cytokine GM-CSF, for targeting cells that carry its receptor (Ramírez-Chacón et al., 2022). The ECD also contains a signaling peptide on its N-terminal end that directs the protein into the endoplasmic reticulum during proteosynthesis to be transported to the plasmatic membrane (C. Zhang et al., 2017).

A short hinge domain connects the antigen binding and transmembrane domains. It indirectly influences the CAR expression and its antigen-binding function by adding flexibility. The length of the spacer affects the proper formation of an immunological synapse; a shorter hinge is more optimal for targeting membrane-distal epitopes and vice versa (Guest et al., 2005; Hudecek et al., 2015). The hinge domain

sequence is derived from a hinge region of Ig-family molecules such as IgG, or CD8 and CD28 (Guedan et al., 2019).

The transmembrane domain anchors the CAR in the plasma membrane. Structurally, it is a hydrophobic α-helix spanning the membrane. The sequence is usually derived from CD4, CD8, or CD28 (Guedan et al., 2019). There is limited evidence that the origin of the transmembrane sequence can affect the CAR's dimerization with membrane-bound molecules, which might affect the signaling (Morin et al., 2015). For example, a CD3-derived transmembrane domain is not optimal, as it can cause CAR dimerization with the endogenous TCR, which could hinder proper CAR signaling.

The intracellular domain (ICD) consists of TCR-signaling CD3ζ and costimulatory domains. The two most often used costimulatory domains are derived from CD28 and 4-1BB (CD137). Others include ICOS (CD278), OX40 (CD134), and CD27-derived domains. The CD3 ζ domain contains three immunoreceptor tyrosine-based activation motifs (ITAMs), which, after phosphorylation, are responsible for recruiting kinases and starting the TCR signaling cascade. The specific characteristics of these various costimulatory domains influence T-cell functions like activation, cytotoxic functions, proliferation, persistence, metabolic activity, and cytokine secretion (Guedan et al., 2019).
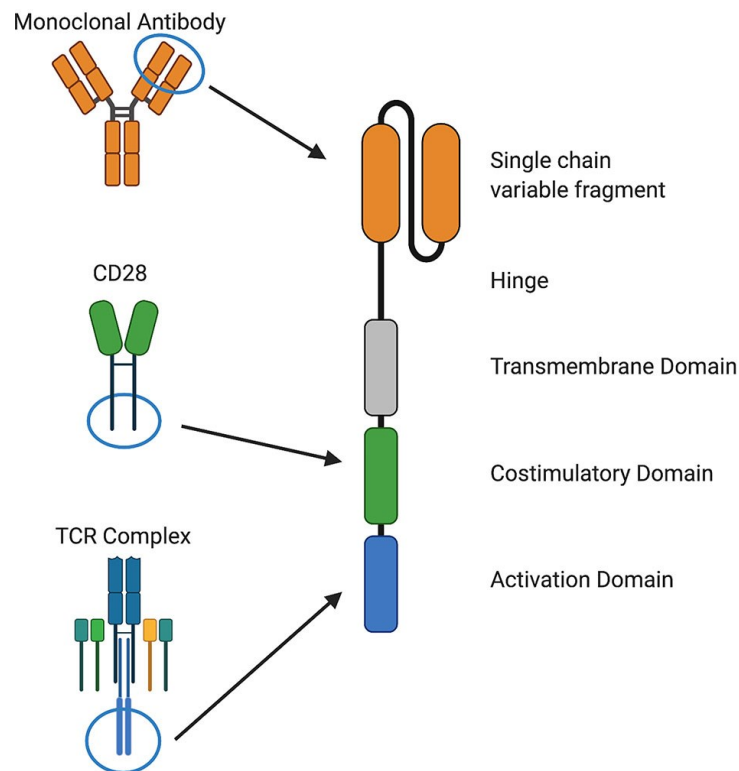
Figure 1. The composition of a chimeric antigenic receptor. Figure by Bell & Gottschalk, 2021.

1.2.2. CAR signaling

Functionally, CAR combines the ligand-recognition ability of an antibody with TCR and costimulatory signaling (Figure 2). Upon antigen recognition, CAR multimerizes and mimics TCR signaling. The three CD3ζ ITAMs are phosphorylated by a Src family kinase LCK. In physiological TCR signaling, this creates a positive feedback loop, with more and more LCK engagement. Phosphorylated ITAMs create Src homology 2 (SH2) and SH3 binding sites for the recruitment of Syk family kinase ZAP70. ZAP70 further phosphorylates the linker of activated T cells (LAT), SLP-76, and phospholipase C–γ (PLC-γ). This consequently leads to the activation of transcription factors nuclear factor kappa B (NFκB), activator protein 1 (AP-1, Fos/Jun heterodimer), and nuclear factor of activated T cells (NFAT) (Gaud et al., 2018). Constructs bearing the CD28 costimulatory domain have higher levels of LCK activation than the 4-1BB ones, suggesting that a positive feedback loop mechanism occurs. In 4-1BB constructs, there is only a minimal LCK association (Salter et al., 2018). Despite the differences in early activation signaling in both types of CAR constructs, 24 hours post-activation, the levels of phosphorylated ZAP70 seem to be equal (Drent et al., 2019).

The first signal alone, mediated by CD3ζ, is insufficient for T-cell activation and leads to T-cell anergy (Otten & Germain, 1991). The second signal by a costimulatory molecule is required for the activation. Physiologically, it is spatially and timewise separated from TCR signaling, which is not the case in CAR T cells, where both signaling domains are part of one receptor. Costimulatory domains from the CD28 family (CD28, ICOS) contribute through phosphoinositide 3-kinase (PI3K)/Akt signaling pathway (Boomer & Green, 2010), while the tumor necrosis factor receptor (TNFR) family (4-1BB, OX40, CD27) signals via TNF receptor-associated factor (TRAF) signaling (Watts, 2005).

The difference in CD28 and 4-1BB signaling have functional implications. CD28-incorporating CARs demonstrate rapid and enhanced T-cell activation, leading to effector-like phenotype with high cytotoxic capability, IL-2 secretion, and glycolytic metabolism (Kawalekar et al., 2016; Porter et al., 2015; Van Der Stegen et al., 2015; Z. Zhao et al., 2015). Nevertheless, this is connected to limited *in vivo* persistence and higher susceptibility to activation-induced cell death (AICD) (Brentjens et al., 2013; D. W. Lee et al., 2015). On the contrary, 4-1BB constructs exhibit a delayed effector activity, preference for a memory-like phenotype, and increased oxidative phosphorylation metabolism and mitochondrial biogenesis (Kawalekar et al., 2016; Porter et al., 2015; Van Der Stegen et al., 2015; Z. Zhao et al., 2015). Additionally, their persistence is augmented through elevated anti-apoptotic protein expression (G. Li et al., 2018).
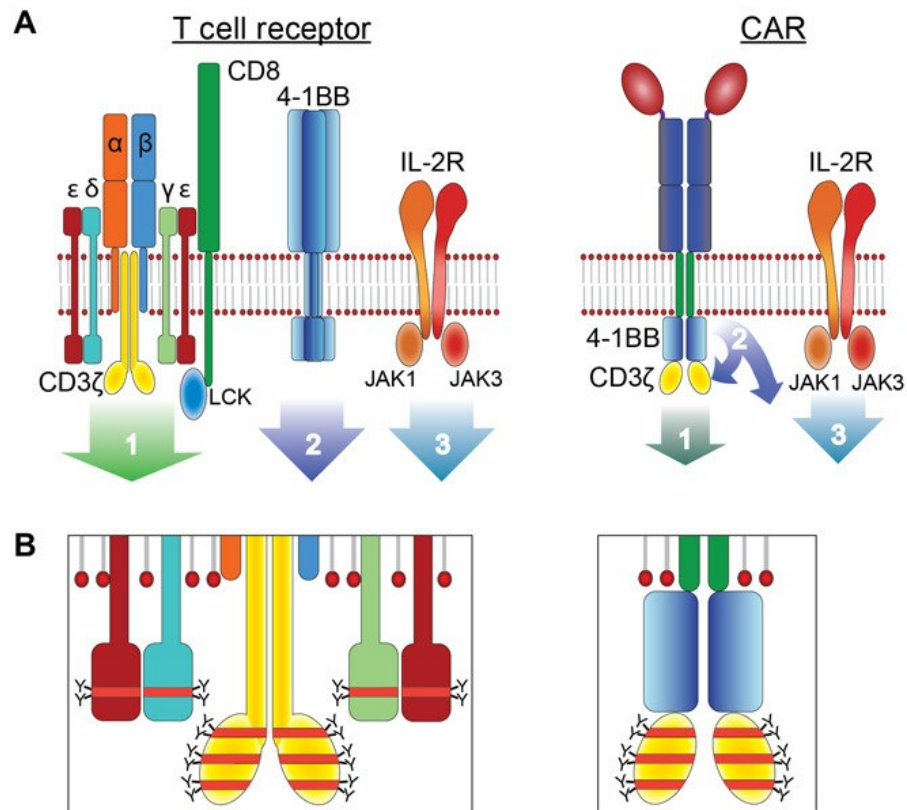
Figure 2. The signaling components of TCR and CAR. (A) TCR mediates signal 1, and costimulatory receptors (4-1BB) contribute to signal 2. The CAR molecule alone provides signals 1 and 2; it contains TCR and costimulatory signaling domains. In both cases, signal 3 is facilitated via cytokine receptors. (B) The immunoreceptor tyrosine-based activation motifs (ITAMs) on TCR chains and CAR. Each ITAM contains two tyrosine phosphorylation sites. Figure by Lindner et al., 2020.

1.2.3. Mechanism of action

CAR T cells recognize and eliminate cells expressing the target molecule. Once the CAR's antigen-binding domain binds the target, it triggers signaling pathways as described above. Consequently, this manifests in T-cell activation, proliferation, differentiation, contact antigen-specific cytotoxicity, and cytokine secretion (Figure 3).

Cytolysis of tumor target cells is caused by exocytosis of granules containing perforin/granzyme (degranulation) and expression of Fas Ligand (FasL), which interacts with the Fas receptor (Meiraz et al., 2009). Degranulation is a fast-acting mechanism. Perforin facilitates the formation of pores in the cell membrane, and granzyme protease cleaves pro-caspases into their active state, thus starting the cascade leading to apoptosis (Cullen & Martin, 2007). FasL binding to the Fas receptor on target cells causes its trimerization and the formation of a death-inducing signaling complex (DISC) with pro-caspase 8 (Fu et al., 2016). The caspase is activated and similarly induces apoptosis by activating downstream caspases. Traditionally, the main focus was on cytotoxic CD8^{+} CAR T cells. However, CD4^{+} CAR T cells can be cytotoxic as well (Oh & Fong, 2021), although the killing might be delayed, as they contain lower amounts of perforin/granzyme levels than CD8^{+} T cells (Hombach et al., 2006; Liadi et al., 2015).

Besides contact cytolysis, the secretion of pro-inflammatory cytokines (tumor necrosis factor α (TNFα), interferon- γ (IFN-γ), IL-2, IL-6) is a secondary, antigen-independent mechanism of tumor cell killing. The cytokines sensitize the tumor stroma and activate and recruit other immune cells, such as natural killer (NK) cells, B cells, and macrophages (Benmebarek et al., 2019; Chekol Abebe et al., 2022). In this context, CD4^{+} CAR T cells invigorate the overall anti-tumor effect by producing large amounts of IFN-γ. IFN-γ activates macrophages to secrete IL-12, enhancing CD8^{+} CAR T-cell, endogenous T-cell, and NK-cell killing functions (Boulch et al., 2021). Additionally, IFN-γ upregulates the expression of major histocompatibility complex (MHC) molecules and thus might sensitize tumor cells with previously downregulated MHC (Toffalori et al., 2019). By this indirect mechanism, even perforin-deficient CD4^{+} CAR T cells maintain the tumor-controlling ability (Boulch et al., 2023).

However, potent cytokine secretion by CAR T cells during peak tumor lysis can lead to a systemic inflammatory response – cytokine release syndrome (CRS) with potentially life-threatening toxicities, particularly neurotoxicity called "immune effector cell-associated neurotoxicity syndrome (ICANS)." (Xiao et al., 2021).
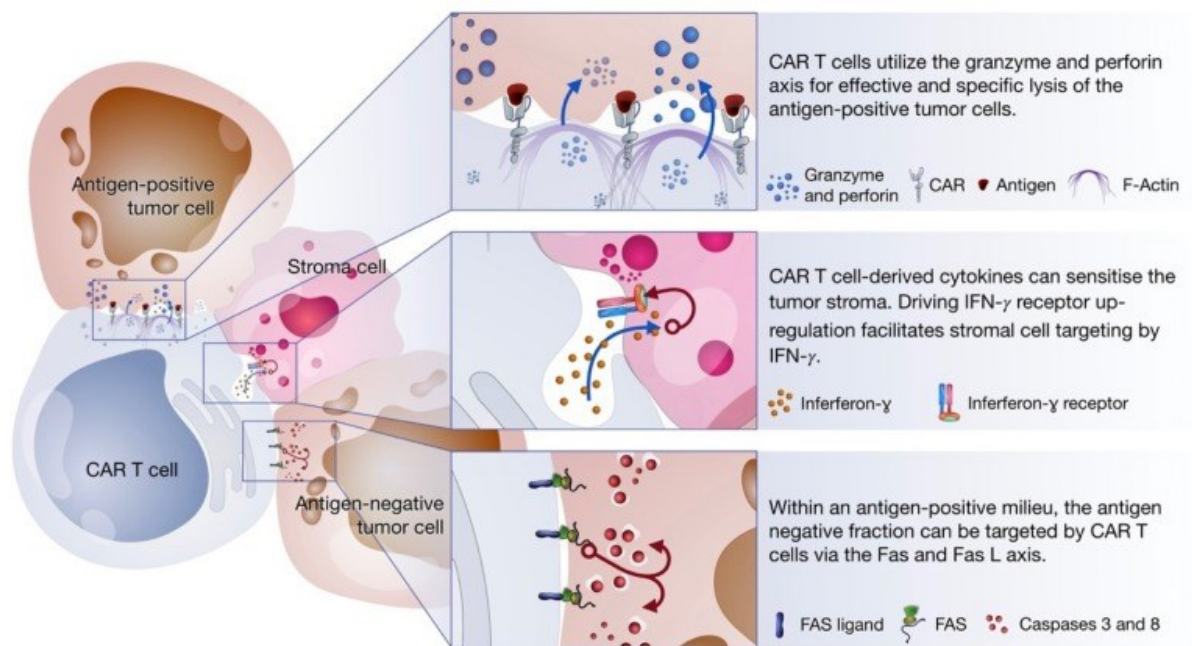
Figure 3. CAR T-cell mechanism of action. The CAR T's cytotoxicity is mediated by the degranulation of perforin and granzyme, by contact via the Fas-FasL axis, and indirectly by cytokine secretion (e.g. IFN-γ). Figure by Benmebarek et al., 2019.

1.3. Evolution of CAR T cells

1.3.1. First generation

The first mention of chimeric receptors engineered from an antibody binding domain and TCR-derived constant domain is from Japan (Kuwana et al., 1987). This Kurosawa-led group produced constructs with Ig variable domains ($V_{H, L}$) attached to the TCR constant α and β chains. The Ig variable domains conferred specificity towards *S. pneumoniae* cell-wall constituent phosphorylcholine. Transfected T cells specifically reacted with intracellular calcium influx after stimulation with phosphorylcholine-positive bacteria. Nevertheless, the birthplace of CAR T cells is considered the Weizmann Institute in Israel, where the concept of combining antibodies with the TCR signaling domain was independently postulated as "T-bodies" by immunologists Zelig Eshhar and Gideon Gross (Gross et al., 1989). It was first tested as a "proof-of-concept" to study TCR activation. Still, the exploitability of this approach to combat tumors or virally infected cells was evident and rapidly caught on. Their first construct was an engineered hybrid TCR with variable regions (V α, β) swapped for Ig V_H and V_L, the so-called Ig-TCR CAR (Gross et al., 1989). Combinations of V_H and V_L of 2,4,6-trinitrophenol (TNP)-specific mAb with either TCR α or β chains were tested (Figure 4). The procedure required a double transfection of two vectors to contain both Ig and TCR chains. Transduced T cells in contact with TNP-coated cells started secreting IL-2 and could induce specific lysis of target cells in a non-MHC hapten-specific manner (Gross et al., 1989). To simplify this method, the first CAR with a single chain variable fragment (scFv) was developed (Eshhar et al., 1993). Ig V_H and V_L domains were joined with a linker to form scFv connected to the intracellular TCR constant domain (CD3ζ). This design was later named the 1st-generation CAR (Hwu et al., 1993).

In primary research on TCR signaling, Irving and Weiss utilized a chimeric receptor comprised of a CD8 molecule fused with CD3ζ. This tool provided insight into the fundamental role of Lck and ZAP70 kinases (A. C. Chan et al., 1991; Irving & Weiss, 1991).

The first clinical trial with 1st generation CARs was conducted to treat HIV infection by the company Cell Genesys Inc. (Mitsuyasu et al., 2000; Walker et al., 2000). They used ligand-based CD4-CD3ζ CAR, which targeted viral gp120 on the surface of HIV-infected cells (Romeo & Seed, 1991). Later, Cell Genesys led the first anti-cancer trial with a CAR against colorectal cancer (Hege et al., 2017). Nonetheless, the 1st-generation CARs showed minimal efficacy in clinical trials due to low cytotoxicity, expansion, and persistence (Tokarew et al., 2018).
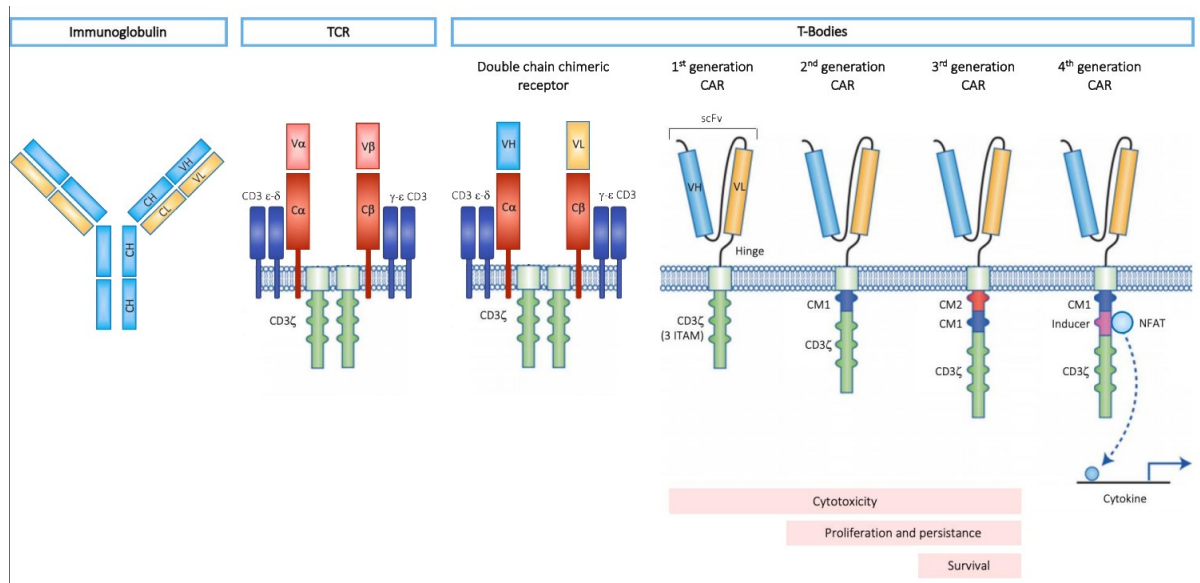
Figure 4. The evolution of CAR T cells. In the 1st-generation CAR, the intracellular domain comprised only CD3ζ; the 2nd generation added a costimulatory domain (CM1), and the 3rd generation incorporated two costimulatory domains. In the 4th generation, the CAR is engineered to induce the secretion of a cytokine. Figure by Bourbon et al., 2021.

1.3.2. Second generation

As previously mentioned, physiological T-cell activation is dependent on two signals. The TCR mediates the first signal by recognizing antigens as a peptide/MHC complex. This signal alone is insufficient for T-cell activation and may lead to T-cell anergy. A second, costimulatory signal is needed for survival, proliferation, and cytokine secretion. A costimulatory molecule on T cells binds ligands on activated antigen-presenting cells (APCs) – for example, pair CD28 and CD80 or CD86.

The 1st-generation CARs had limited cytotoxicity, proliferation, and persistence. Therefore, the 2nd-generation CAR incorporates a costimulatory domain in addition to the CD3ζ (Figure 4). Including CD28 costimulatory domain in the construct increased proliferation, cytokine secretion, and survival and led to an overall more potent anti-tumor activity (H. M. Finney et al., 1998, 2004; Maher et al., 2002). Similarly, the 4-1BB (CD137) signaling domain in anti-CD19 CAR led to robust, specific cytotoxicity against B-ALL cells, a high rate of expansion, and IL-2 secretion (Imai et al., 2004).

Comparing 4-1BB and CD28 costimulatory domains revealed that their signaling leads to the activation of distinct metabolic pathways (Kawalekar et al., 2016), as mentioned in chapter 1.2.2. Additionally, it has been shown that CD28ζ CAR T cells have higher early cytotoxicity and initial expansion (Kawalekar et al., 2016). CAR T cells with 4-1BB can be detected in peripheral blood for up to several years post-administration (Fraietta et al., 2018; Porter et al., 2015), while CD28 constructs are typically detectable solely within a couple of months (Brentjens et al., 2013; D. W. Lee et al., 2015). In theory, highly potent and rapid cytotoxic CD28-based CARs could be a better option for transient tumor elimination in instances such as bridging therapy before hematopoietic stem cell transplant (HSCT). The lasting persistence of 4-1BB constructs could offer a permanent solution in disease management.

However, real-world data indicate that the CD28-based CAR T cells have better clinical outcomes at the cost of higher toxicities than the 4-1BB-based ones (Bachy et al., 2022; Gagelmann et al., 2024).

Other costimulatory domains derived from OX40, ICOS, and CD27 have been tested as viable alternatives (Guedan et al., 2014; Pulè et al., 2005; Song et al., 2012). The "28ζ" and "BBζ" constructs are today's established standard in clinical practice, as they are present in all the CAR T-cell products approved by the Food and Drug Administration (FDA) so far (chapter 1.7).

1.3.3. Third generation

CAR constructs combining multiple intracellular costimulatory domains are labeled as 3rd-generation CAR (Figure 4). For example, CAR ICDs composed of CD28-4-1BB-CD3ζ (28BBζ) in anti-CD19 CAR (Tammana et al., 2010) or CD28-OX40-CD3ζ targeting either CD33 (Pulè et al., 2005) or GD2 (Marin et al., 2010) have been tested. Results of *in vitro* experiments suggest that including an additional costimulatory domain may augment CAR T-cell activation, proliferation, and survival. Clinical trials with 28BBζ constructs targeting HER-2/neu for colon cancer (Morgan et al., 2010a) and CD20 for B-cell lymphoma (Till et al., 2012) reported similar efficiency and safety as 2nd-generation CARs. Another phase I clinical trial simultaneously compared 2nd-generation (28ζ) and 3rd-gen (28BBζ) anti-CD19 CAR T cells for relapsed/refractory (r/r)-non-Hodgkin lymphomas (Ramos et al., 2018). Both cellular products were administered to each patient at the same time. In this study, 3rd-generation CAR T cells were superior in expansion and persistence *in vivo*. However, these data are always for small groups of patients, and therefore, more studies are needed to determine the optimal composition of costimulatory domains.

1.3.4. Fourth generation

The next evolutionary stage of CAR design (4th generation) is also referred to as T cells redirected for antigen-unrestricted cytokine-mediated killing (TRUCKs), armored, or armed CARs (Chmielewski & Abken, 2015). These constructs are derived from the 2nd generation (one costimulatory domain and CD3ζ) with incorporated transgenic protein production, most commonly a cytokine, especially inducible upon CAR T-cell activation (Figure 4). This further stimulates CAR T cells and possibly recruits bystander non-transduced T cells to fight tumor cells and modulate the tumor microenvironment (TME). This could be a solution to overcome immunosuppression in solid tumors, for which CAR T cells are ineffective. Moreover, targeted cytokine production in the tumor site could present a significantly lower risk of side effects than systemic administration (Chmielewski & Abken, 2015). The most widely used inducible promoter is based on six NFAT binding motifs together with minimal IL-2 promoter (Northrop et al., 1993). Novel promoters, such as NR4A, could represent an alternative offering higher expression than the standard NFAT (T. Guo et al., 2022).

IL-12 is a cytokine that remarkably enhances inflammatory response. It is produced by antigen-presenting cells and phagocytes – dendritic cells (DCs), monocytes, and macrophages as a response to infections. IL-12 stimulates T cells and NK cells, inducing IFN-γ secretion, and increases cytotoxicity by elevating perforin and granzyme levels (Ferlazzo et al., 2004; Kubin et al., 1994). It drives T cells toward a Th1 phenotype (Manetti et al., 1993) and also blocks the expansion of T-regulatory (Treg) cells (X. Cao et al., 2009). Consequently, as a positive feedback loop, IFN-γ activates monocytes and macrophages, further augmenting IL-12 production and aiding pathogen elimination (X. Ma et al., 1996). However, IL-12 bears safety issues, especially when administered systemically due to the strong induction of IFN-γ production. In this case, hyper-activated immune cells eventually cause liver, lung, gastrointestinal, and hematopoietic toxicities (Car et al., 1999). Thus, CAR T-cell direct secretion at the tumor site might effectively boost the anti-tumor response without the abovementioned risks. Delivering IL-12 by CAR T cells to the tumor could recruit other immune cells—macrophages, NK, and T cells – to fight cancer in a Th1-driven response. A broad immune response could mitigate the risk of immune escape of tumor cells by antigen loss (Chmielewski et al., 2011).

Several pre-clinical studies have confirmed a superior effect of IL-12 secreting CAR T cells for B-cell malignancies (Kueberuwa et al., 2018; Pegram et al., 2012), and especially against large established solid tumors, which are not sensitive to CAR T cell without added cytokines such as for melanoma (L. Zhang et al., 2012), ovarian carcinoma (Koneru et al., 2015; Yeku et al., 2017), hepatocellular carcinoma (Y. Liu et al., 2019), and glioblastoma (Agliardi et al., 2021). Increased cytotoxicity, proliferation, persistence, and resistance to Tregs were among the observed effects on CAR T cells. In the first human clinical trial, TILs with inducible IL-12 secretion were used to treat melanoma. IL-12 boosted TIL anti-tumor activity; nevertheless, the dose escalation had to be stopped due to toxicities connected with high levels of IL-12 and IFN-γ (L. Zhang et al., 2015). To increase safety by bypassing peripheral IL-12 secretion, membrane-bound IL-12 (and membrane-bound tumor-targeted IL-12) CAR T cells were shown to be highly effective for treating large solid tumors on patient-derived xenograft (PDX) osteosarcoma and melanoma models (J. Hu et al., 2022), and ovarian cancer model (E. H. J. Lee et al., 2023). In another non-secretory approach, a single chain IL-12 was inserted in the extracellular domain of CAR, while both IL-12 and CAR remained functional. IL-12 enhancement was evident, together with increased antigen-dependent cytotoxicity. Furthermore, IL12-CAR T cells gained NK-like antigen-independent killing ability. Specifically, CD8+CD56+CD62L^{high} population with upregulated genes linked with NK cell cytotoxicity – CD94/NKG2D. This hybrid killing mechanism could be suitable against heterogeneous tumors containing tumor cells without target antigens (Hombach et al., 2022).

 An alternative method for antigen-specific, tumor-targeted, non-leaky, and reversible inducible cytokine expression was developed by Yang and colleagues (Yang et al., 2023). It is a non-gene editing

CRISPR-based system of inducible IL-12 expression utilizing co-transduction of two constructs. The first encodes human epidermal growth factor receptor 2 (HER2)-specific 2nd-generation CAR with an added tobacco etch virus (TEV) protease at the end of its ICD. It also codes two single guide RNAs (sgRNA) targeting IL-12A and IL-12B transcription start sites. The second construct contains a LAT molecule connected to a nuclease-deactivated Cas9 (dCas9) with a nuclear localization signal by a TEV-cleavage site. Upon CAR activation, LAT molecules are brought to its proximity, and then TEV protease releases dCas9 (with bound sgRNAs), which translocates to the nucleus, inducing endogenous IL-12 expression (Yang et al., 2023).

A cytokine delivery system called "IL-12 nanostimulant-engineered CAR T cells biohybrids" was developed to increase CAR T cell effectivity and safety. In this method, CAR T cells are externally conjugated by click chemistry with IL-12-packed nano chaperones made of chemically modified human serum albumin (HSA). IL-12 release from nano chaperones is triggered only after contact with free thiol groups that are increased on the cell surface after T cell activation, in this case by CAR recognizing target tumor cells (Luo et al., 2022).

In a phase I clinical trial for the treatment of MUC16^{ecto+} ovarian cancer using CAR T cells that secrete IL-12, all patients experienced CRS. However, no dose-limiting toxicities were observed in patients who did not receive additional chemotherapy, only in those who received post-lymphodepletion chemotherapy before the treatment (O'Cearbhaill et al., 2020).

1.3.4.2. IL-18 TRUCKs

IL-18 is often used instead of IL-12 as a safer substitute. In a clinical trial, systemic administration of IL-18 to cancer patients did not cause any significant toxicities (Robertson et al., 2008). IL-18 is a pro-inflammatory cytokine with pleiotropic function. It stimulates proliferation and IFN-γ secretion in T and NK cells. Furthermore, it affects monocytes, DCs, natural killer T (NKT) cells, B cells, mast cells, and basophils (Yasuda et al., 2019). CAR T cells releasing IL-18 displayed superior anti-tumor efficacy against CD19$^+$ B-cell malignancies, melanoma (B. Hu et al., 2017), and pancreatic and metastatic lung cancer in mouse models (Chmielewski & Abken, 2017). Again, adding IL-18 was crucial in eradicating large solid tumors, as they were refractory to unenhanced CAR T cells. IL-18 drove T cells into T-bet^{high} FoxO1^{low} effector phenotype together with Th1 polarization. Moreover, IL-18 production altered tumor-associated immune cell composition – a rise in M1 macrophages and NK cells was observed, while the numbers of suppressive M2 macrophages, DCs, and Tregs decreased (Chmielewski & Abken, 2017). Another pre-clinical study of lung cancer treated with CAR T cells with IL-18 similarly reported a durable response and activation of TILs and APCs. IL-18 also prevented the exhaustion of CAR T cells, which retained a memory phenotype (Jaspers et al., 2023). The first human clinical trial with anti-CD19 CAR T cells co-expressing IL-18 is currently being

conducted at the University of Pennsylvania with promising efficacy and controllable adverse toxicities (Svoboda et al., 2022).

1.3.4.3. IL-15 TRUCKs

Another cytokine used to augment CAR constructs is IL-15. It enhances T-cell proliferation and cytotoxic function by elevating granzyme B production (Tamang et al., 2006). IL-15, like all other γ-chain cytokines, further promotes T-cell survival by regulating B-cell lymphoma-2 (Bcl-2) family proteins (Shenoy et al., 2014). In a preclinical mouse model, anti-CD19 CAR T cells expressing IL-15 had enhanced anti-tumor activity, significantly enhanced expansion, improved survival, and diminished expression of the exhaustion marker programmed cell death 1 (PD-1) (Hoyos et al., 2010). In another approach, anti-CD19 CAR T cells improved by membrane-bound IL-15 had an elevated percentage of a long-lived stem cell memory (T_{SCM})- like phenotype and enhanced cytotoxicity. (Hurton et al., 2016). To combat unwanted toxicities of secreted cytokines in CAR T-cell therapy, co-expressing IL-15 receptor α subunit, in addition to IL-15, showed reduced systemic adverse effects of IL-15 in a mouse xenograft model (Y. Zhang et al., 2022). In a xenograft glioblastoma model, IL-15 secreting GD2-specific CAR T cells effectively infiltrated the tumors and exhibited remarking tumor control, with a complete response in half of the treated mice (Gargett et al., 2022). Another glioblastoma study tested anti-IL13 receptor α2 (anti-IL13Rα2) CAR T cells secreting IL-15 or with IL-15 fused to the CAR's scFv. These CAR T cells simultaneously targeted glioblastoma tumor cells and myeloid-derived suppressor cells (MDSCs) in the TME, as they express IL13 and IL-15 receptors. Both CAR T cells were effective against the MDSCs, while the IL-15-fused CAR was more potent (Zannikou et al., 2023).

In a case study, a B-ALL patient who relapsed twice after treatment with anti-CD19 and anti-CD22 CAR T cells received CD19-specific CAR T cells with membrane-bound IL-15 and achieved complete remission with high CAR T expansion and no severe side effects. However, the patient relapsed again (CD19-negative), and further treatment with another dose of anti-CD22 CAR T cells did not have a curative effect (Sun et al., 2021).

1.3.4.4. IL-7 TRUCKs

IL-7 is widely used in (CAR) T cell expansion protocols, as it promotes T-cell proliferation and has anti-apoptotic properties by upregulating protein Bcl-2. It further promotes a low degree of differentiation of T cells (MacKall et al., 2011; Schluns et al., 2000). Therefore, it might be suitable for enhancing CAR T cell survival and persistence *in vivo*. Studies with CAR T cells producing IL-7 have confirmed this effect in preclinical models with NKG2D-specific CAR against prostate cancer (He et al., 2020) and CD19-specific CAR (L. Li et al., 2022). IL-7 promoted less differentiated CAR T cells, prevented exhaustion, and led to better metabolic fitness, persistence, and overall anti-tumor function. In addition to increased proliferative capacity by IL-7, the subsequent studies further combined IL-7 with co-expression of another cytokine. For example, secretion of chemokines would improve migration

to the tumor site and infiltration into the tumor. To utilize this, Adachi et al. combined CD20 CAR T cells with IL-7 and CCL19, and then similarly, Luo et al. tested IL-7 plus CCL21 for solid tumor models (Adachi et al., 2018; H. Luo et al., 2020). CCL19 and CCL21 act as chemoattractants for T cells and DCs via interaction with C-C chemokine receptor 7 (CCR7) (Luther et al., 2002). Moreover, CCL21 has additional anti-tumor properties due to its angiostatic function by binding to CXCR3 (Vicari et al., 2000). In both cases, IL-7 improved the proliferation of CAR T cells, and co-expressed chemokines caused higher T-cell/DCs tumor infiltration than conventional CAR T cells (Adachi et al., 2018; H. Luo et al., 2020). A clinical trial with anti-CD19 CAR T cells expressing IL-7 and CCL19 reported manageable adverse toxicities and superior anti-tumor effects than conventional CAR T cells (Lei et al., 2024). Another tested co-expressed protein together with CAR and IL-7 is hyaluronidase PH20. It further enhanced the penetration of CAR T cells through dense extracellular matrix into solid tumors in the hepatocellular carcinoma model (Xiong et al., 2021).

A CAR T cell approach toward heterogenous solid tumors containing target-antigen-negative tumor cells is challenging. Swan et al. designed a CAR construct that produces IL-7 and Fms-like tyrosine kinase receptor 3 ligand (Flt3L) against 50% epidermal growth factor receptor (EGFR)vIII+ glioblastoma that would rely on recruiting endogenous immune cells and CAR T cells for tumor eradication (Swan et al., 2023). Flt3L is an essential growth factor for DC development (Cueto & Sancho, 2021). Increasing intra-tumoral DC numbers could lead to new tumor-specific cytotoxic T-cell priming. Tumors treated with CAR T cells with IL-7 and Flt3L had elevated populations of conventional DCs and CD103+XCR1+ migratory and cross-presenting DC populations, and these tumor-bearing mice had higher survival rates than with non-enhanced CAR T cells (Swan et al., 2023).

A modification of the IL-7 itself represents another direction for further improvements. The short half-life of a cytokine might be a limiting factor for its *in vivo* function. Kim et al. constructed a long-acting recombinant human IL-7 by fusing it with hybrid Fc (rhIL-7-hyFc) (M. Y. Kim et al., 2022). It promoted CAR T-cell proliferation, cytotoxicity, and memory phenotype in CD19+ lymphoma and CD33+ leukemia models. Although they did not present a direct comparison with CAR T cells secreting unmodified IL-7, they presume that rhIL-7-hyFc could surpass it in future human trials.

To avoid unwanted bystander lymphocyte activation and following potential side effects such as neurotoxicity and CRS, a constitutive active IL-7 receptor (C7R) was developed (Shum et al., 2017). CAR T cells with C7R showed superior anti-tumor activity, proliferation, and survival in neuroblastoma and glioblastoma models (Shum et al., 2017) and breast cancer models, but not *in vivo* (Z. Zhao et al., 2020).

1.3.4.5. IL-21 TRUCKs

IL-21 is another interesting cytokine in the context of CAR T-cell therapy. IL-21 bears pleiotropic functions (Tian & Zajac, 2016), stimulating T-cell expansion and maintaining early memory phenotypes

desired for CAR T cells. In our study (Štach et al., 2020), anti-CD19 CAR T cells producing IL-21 had increased expansion, survival, retainment of T_{SCM}-like phenotype, and tumor infiltration *in vitro* and in a mouse model. Moreover, IL-21 might make CAR T cells more effective for CAR T-resistant diseases like chronic lymphocytic leukemia (CLL) (Štach et al., 2020). Another group working on solid tumors engineered CAR T cells against HCC targeting glypican-3 with co-expression of IL-21 and IL-15. Likewise, enhanced CAR T cells had an elevated percentage of early differentiated T_{SCM} and T_{CM} populations and increased expansion and persistence (Batra et al., 2020).

1.3.4.6.   *IL-10 TRUCKs*

IL-10 is a pleiotropic cytokine that used to be generally connected with inhibition, although it has been described that it has anti-tumoral properties as well (Fujii et al., 2001; Mumm et al., 2011; Tanikawa et al., 2012). Zhao et al. showed that CAR T cells producing IL-10 had improved metabolical fitness, proliferation, and effector functions even against established and metastatic tumor models. Moreover, IL-10 promoted the formation of the early-differentiated T_{SCM} phenotype and thus established a durable response (Y. Zhao et al., 2024).

1.3.4.7.   *Combating the tumor microenvironment*

Besides cytokines, various other transgenic proteins could be expressed by CARs. Strategies discussed so far mostly improved CAR T-cell cytotoxicity, expansion, persistence, and recruiting bystander T cells. Immune escape is one of the characteristics of "successful" cancer. The immune escape mechanisms include immune-suppressive cytokines (transforming growth factor β (TGF β), IL-4, IL-10), checkpoint molecules (PD-1, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)), and in solid tumors, dense extracellular matrix, hypoxia, and suppressor cells (Tregs, MDSCs). Hence, neutralizing immunosuppression by tumor microenvironment (TME), especially in solid tumors, might be equally crucial as enhancing CAR T cells (Xia et al., 2019).

1.3.4.7.1.   Inverted cytokine receptor (chimeric switch receptor)

The inverted cytokine receptor (ICR), also known as chimeric switch receptor (CSR), was developed to reverse an inhibitory signal into a stimulatory one (Figure 5A). One of the suppressive cytokines associated with TME is IL-4. It was shown that IL-4 promotes tumor growth and protects tumor cells from cytotoxic T cells by promoting Th2 polarization (Gocheva et al., 2010; Wynn, 2015). To combat this effect, Leen et al. fused the cytokine-binding exodomain of the IL-4 receptor with the intracellular signaling domain of the IL-7 receptor into the IL-4R/IL-7R (4/7) ICR. They reported that adding IL-4 improved proliferation, cytotoxic function, and Th1 phenotype sustainment of the transduced T cells (Leen et al., 2014). Similarly, prostate stem cell antigen (PSCA)-specific CAR T cells bearing the 4/7 ICR thrived in the IL-4-rich TME and exhibited boosted anti-tumor functions in a pancreatic cancer preclinical model (Mohammed et al., 2017). Next, a novel IL-4R/IL-21R ICR provided CAR T cells

with increased tumor-lytic ability and a Th17-like polarization against IL-4^{+} tumors, both *in vitro* and *in vivo* HCC model (Y. Wang et al., 2019).

TGF-β is another textbook example of immunosuppressive cytokine. It dampens T-cell activation and proliferation while it supports tumor invasion and metastasis (M. O. Li et al., 2006). The TGF-β/IL-7R CSR developed by Noh et al. enhanced target-specific CAR T cell cytotoxicity and overall survival on a CD19^{+} B-cell lymphoma mouse model (Noh et al., 2021).

Physiological signaling via inhibitory receptors PD-1 and CTLA-4 causes T-cell exhaustion and dysfunction. Expression of their ligands on tumor cells is one of the resistance mechanisms (Schnell et al., 2020). Engineering PD-1 exodomain with CD28 ICD into PD-1/CD28 CSR (Figure 5A) boosted anti-tumor functions, proliferation, and cytokine secretion of tumor-specific T cells (Ankri et al., 2013; Kobold et al., 2015; Prosser et al., 2012) and CAR T cells (C. Chen et al., 2021). Similar results were observed with CTLA-4/CD28 chimera in T cells against melanoma (Shin et al., 2012).

1.3.4.7.2. Dominant negative receptors

Another strategy for counteracting the immunosuppressive molecules is the expression of dominant negative receptors (DNRs). They represent the receptor's truncated, non-signaling form, which acts as a decoy for its ligand (Figure 5B). The TGF-β DNR and PD-1 DNR are the most promising in CAR T-cell engineering. The TGF-β DNR was tested in various contexts such as Eppstein-Barr virus (EBV)-specific T cells (Foster et al., 2008), mesothelin-specific CAR T cells in ovarian cancer PDX model (K. Li et al., 2023), anti-HER2 CAR T cells co-expressing CCR8 (Strzalkowski et al., 2022), anti-B-cell maturation antigen (BCMA) CAR T cells against multiple myeloma (Alabanza et al., 2022), and PSMA-targeting CAR T cells in prostate cancer preclinical model (Kloss et al., 2018) and a phase I clinical trial (Narayan et al., 2022). The TGF-β DNR enhanced tumor infiltration, proliferation, and facilitation of anti-tumor responses of CAR T cells in the presence of TGF-β.

Likewise, the PD-1 DNR protects T cells from inhibition mediated by PD-L1-positive tumors (Figure 5B). The PD-1 DNR on mesothelin-specific CAR T cells bolstered their anti-tumor functions, cytokine secretion, and expansion in preclinical models (N. Chen et al., 2017; Cherkassky et al., 2016). Moreover, the first human clinical trial with PD-1 DNR anti-CD19 CAR T cells showed promising efficacy against follicular lymphoma without causing significant toxicity (X. Liu et al., 2021).

Next, disruption of the apoptotic Fas/FasL axis by Fas DNR in CAR T cells improved their survivability and the durability of the anti-tumor response against solid tumor models (Yamamoto et al., 2019).
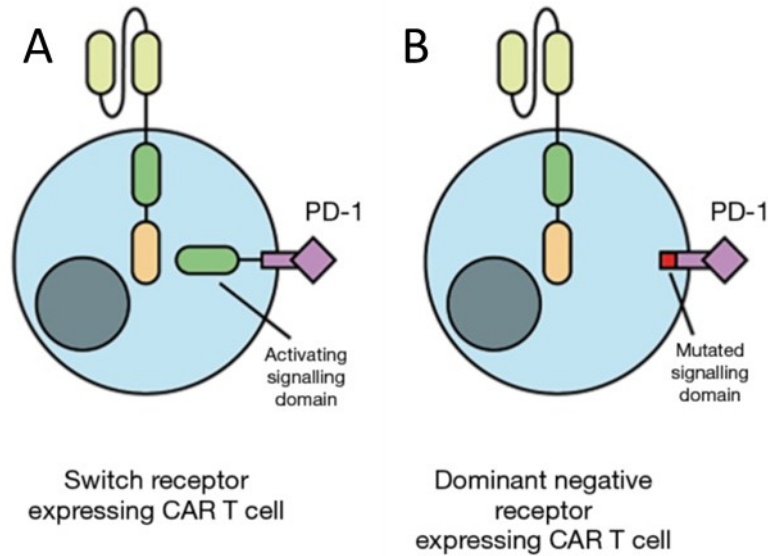
Figure 5. Strategies of CAR T cells to overcome immunosuppression. (A) Chimeric switch receptor, (B) Dominant negative receptor. Figure by J. D. Chan et al., 2020.

1.3.4.7.3.   Secretory antibodies

With the boom of checkpoint inhibitor therapy, i.e. mAbs against inhibitory receptors or their ligands, can be harnessed in CAR T-cell engineering to tackle immunosuppressive TME. In addition, secreting the mAbs by CAR T cells directly at the tumor site maximizes their concentration at the site of action while minimizing systemic exposure and potential off-target effects. Moreover, continuous production and sustained release of the mAbs can enhance the duration of their therapeutic effect and help overcome the limitations of passive mAb administration, such as short half-life and inadequate tumor penetration.

In several studies, blocking PD-1 on CAR T cells by secretory anti-PD-1 scFv was tested on different models. First, anti-CD19 CAR T cells secreting anti-PD-1 scFv exhibited enhanced proliferation and effector functions in a xenograft model of solid tumors artificially expressing CD19 (S. Li et al., 2017). Rafiq et al. showed a similar effect on CD19- or Muc-16-specific CAR T cells against PD-L1-positive leukemia and ovarian cancer models. Furthermore, the produced anti-PD-1 scFv bolstered the anti-tumor response in an autocrine and paracrine manner for CAR T cells and bystander endogenous tumor-specific T cells (Rafiq et al., 2018). In other studies, the anti-PD-1 scFv enhanced tumor-killing and tumor infiltration of EGFR-specific CAR T cells in gastric cancer xenograft and PDX mouse models (J. T. Zhou et al., 2020), and anti-mesothelin CAR T cells in lung cancer xenograft model (Ping et al., 2020).

As an example of blocking programmed death ligand 1 (PD-L1) on tumor cells, anti-PD-L1 mAb-producing CAR T cells outperformed conventional CAR T cells in a mouse xenograft model of clear cell renal cell carcinoma. Additionally, the anti-PD-L1 IgG1 mAb mediated antibody-dependent cellular cytotoxicity (ADCC) by NK cells on PD-L1^{+} cancer cells, therefore employing an additional mechanism of tumor lysis (Suarez et al., 2016).

The inhibitory receptor CTLA-4 suppresses T-cell functions upon binding to CD80/CD86 either on APCs or tumor tissue. In a study by Yin et al., a sub-therapeutic dose of anti-CTLA-4 minibody-secreting CAR T cells displayed a prolonged anti-tumor effect compared to conventional CAR T cells in a mouse xenograft glioma model (Yin et al., 2018).

Another molecule, CD47, acts as a "don't eat me" signal via the signal regulatory protein-α (SIRPα) receptor on macrophages and DCs. CD47 is widely expressed and usually overexpressed on tumor cells (Willingham et al., 2012). Systemic blocking of CD47 would pose a risk of toxicity, while localized delivery could enhance tumor sensitivity to phagocytosis. Xie et al. demonstrated that CAR T cells secreting an anti-CD47 VHH fragment fused to Fc stimulated macrophage engulfment of cancer cells (Xie et al., 2020). Another group engineered CD19 CAR T cells with CV1, a secretory variant of SIRPα. They named this system "Orexi" CAR T cells due to facilitating orexigenic activity, meaning enhanced phagocytosis (Dacek et al., 2023).

An opposite strategy to checkpoint blockade is secreting agonist Abs to activate stimulatory pathways. One example is the production of CD40 agonist Ab by meso3-specific CAR T cells, which enhanced the anti-tumor efficacy, increased cytokine release, and cytotoxicity in an ovarian cancer xenograft model. Moreover, a comparatively larger ratio of the CD40 Ab-secreting CAR T cells retained central memory phenotype (Y. Zhang, Wang, et al., 2021).

To prevent antigen escape or to target multiple antigens in heterogenous tumors, coupling CAR T cells with the production of bispecific T cell engagers (BiTE) might empower the T cells to mount a more robust and effective anti-cancer response. The BiTE consists of two scFv domains, one targeting CD3 and the other a tumor antigen. The BiTE bridges a T cell with tumor cells upon binding both antigens, allowing their lysis. Glioblastoma, an example of a heterogeneous tumor, can express wild-type (wt) EGFR and its mutated variant EGFRvIII. However, targeting EGFRvIII by CAR T cells led to antigen-negative outgrowth, and systemic anti-EGFR therapy poses a high risk of on-target off-tumor toxicity (Choi et al., 2019). The localized secretion of CD3-EGFR BiTE by EGFRvIII-specific CAR T cells mediated the complete eradication of heterogeneous tumors in a glioma mouse model. According to expectations, the BiTE redirected and recruited CAR T and bystander T cells against wt EGFR (Choi et al., 2019).

1.3.5. Fifth-generation, next-generation

The 4th-generation CAR engineering incorporated a cytokine or a receptor into the CAR construct. The next-generation CARs rely on more innovative approaches that utilize insights from synthetic biology and logic-gating (AND, OR, NOT, IF). They address topics like targeting multiple tumor antigens simultaneously, alleviating toxicities, and safety switches.
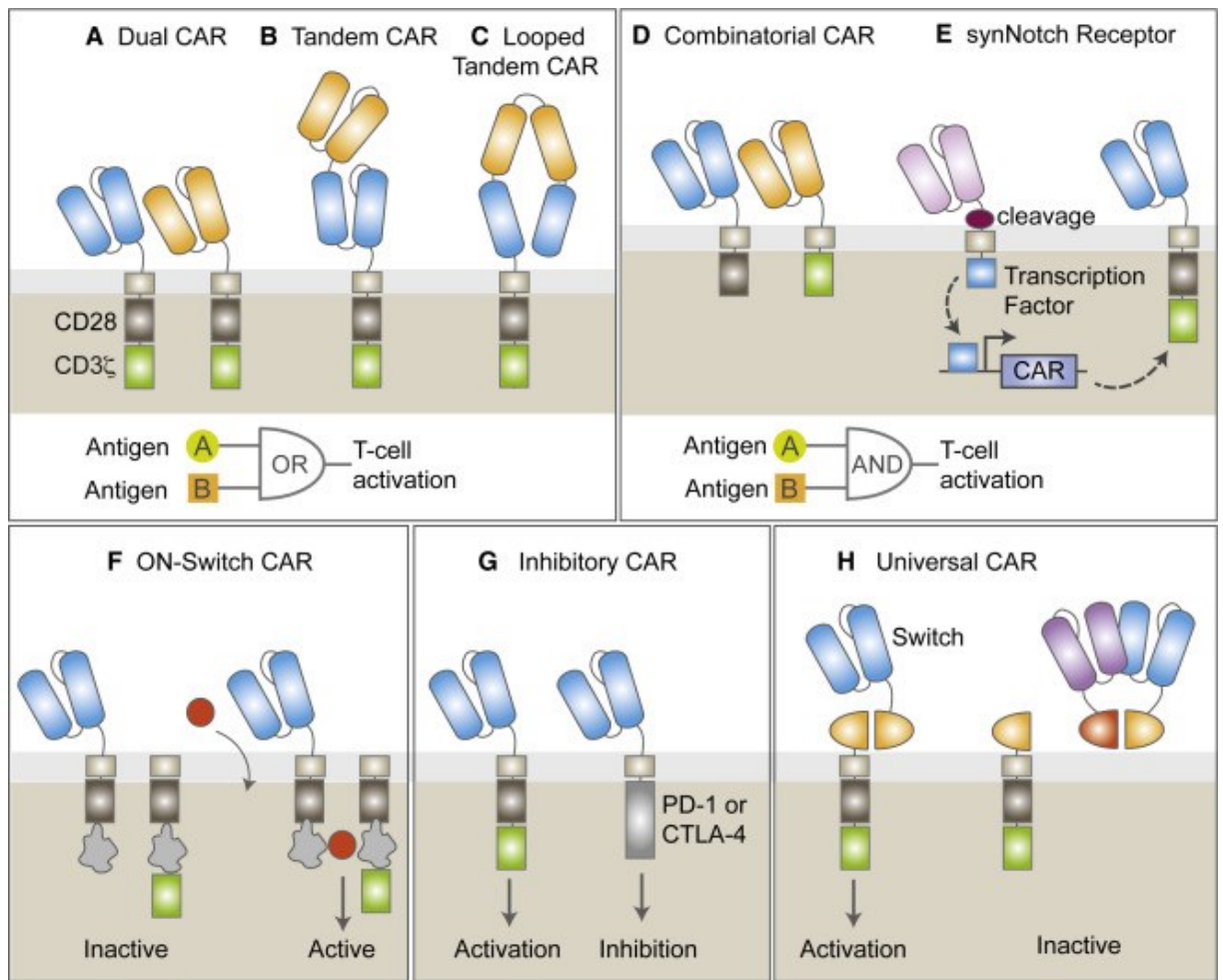
Figure 6. The next-generation CAR designs. Figure by Guedan et al., 2019.

1.3.5.1. "OR-gate"

1.3.5.1.1. Dual targeting

Targeting two antigens simultaneously can prevent the escape of tumor cells by antigen loss or its downregulation. Under evolutionary pressure by the CAR T cells, the selection of an antigen-negative clone and its following outgrowth can occur and is responsible for resistance to the treatment (Brown et al., 2015; Fry et al., 2018; Orlando et al., 2018; O'Rourke et al., 2017; Sotillo et al., 2015). Dual targeting applies when either antigen is expressed on tumor cells, corresponding to the logical "OR-gate" (Figure 6A, B, C).

One way of dual targeting with the traditional 2nd-generation CAR constructs is co-administrating two CAR T products (Ruella et al., 2016; T. Wang et al., 2023). A step-up would be the expression of two different CAR molecules on one T cell – dual CAR T cells, also called dual-signaling CAR T cells (Figure 6A). It requires either co-transfection with different constructs or one large bicistronic vector (K. H. Chen et al., 2018; Kokalaki et al., 2023; Ruella et al., 2016; H. Wang et al., 2022). In the case of the single vector, additional changes like codon optimization may be necessary

to prevent recombination between duplicate sequences. These two CARs can bear different costimulatory domains to combine their respective signaling advantages.

Another option is a single-chain bi-specific construct with a single intracellular domain, in which scFvs can be assembled in series – tandem CAR (TanCAR) (Grada et al., 2013) or in a looped structure – looped tandem CAR (Figure 6B, C) (Z. Chen et al., 2023; Qin et al., 2018). Regarding genetic engineering, tandem CARs require optimization of the spacer and linker domain lengths and the scFv orientations for optimal dual-antigen recognition (Zah et al., 2016). Computational tools can aid in the design of such constructs by predicting the structure and functionality of the double scFv complex (Grada et al., 2013). These tandem CARs can react against either antigen alone. Notably, binding both antigens at the same time causes a synergetic effect by strengthening the signal transduction, which makes the TanCAR T cells functionally superior to two mixed monovalent CAR T cell populations (Grada et al., 2013; Hegde et al., 2013, 2016; Ruella et al., 2016; Schneider et al., 2017). At present, numerous clinical trials are being conducted with dual and tandem CARs against B-cell malignancies with a combination of antigens CD19, CD20, and CD22 and against multiple myeloma targeting BMCA, CD38, CS1, and PD-1 combinations (Stock et al., 2023).

1.3.5.1.2. Triple targeting

Targeting multiple antigens is especially important with heterogeneous tumors like glioblastoma, where antigen escape is impending. A triple-CAR strategy by Bielamowicz et al. employs a tricistronic construct with three CAR molecules divided by viral 2A self-cleaving peptide sequences. It targeted three glioma tumor-associated antigens (TAAs) – HER2, interleukin-13 receptor subunit α-2 (IL13Rα2), and ephrin-A2 (phA2) (Bielamowicz et al., 2018). Similarly, B-cell targeted triple-CAR T cells engineered with tricistronic CD19-CD20-CD22 CAR construct could be an improved option to regular CD19-specific CAR T cells that prevent CD19-negative relapses (Fousek et al., 2020). Another group developed a bicistronic vector with CD19-CD20-specific TanCAR and CD22 CAR as "tri-specific dual CAR T cells" and employed the 2A sequence (Schneider et al., 2021). In their dual-CAR architecture, they also reported that ICOS, OX40, and CD27 CAR costimulatory domains were more potent than the CD28 or 4-1BB. An alternative design of the antigen-binding domain leveraged ankyrin repeat proteins (DARPins) rather than scFvs (Balakrishnan et al., 2019). This tri-specific TanCAR contained three high-affinity binding domains specific for EGFR, EpCAM, and HER2 in one CAR molecule. A Chinese company, Legend Biotech, presented additional non-scFv tri-TanCAR with three heavy-chain variable domains (VHH) fragments specific for CD19-CD20-CD22 in tandem (Z. (Joy) Zhou et al., 2021). The triple-targeted CAR T cells displayed cytotoxicity against heterogeneous tumor cell mixtures. The single-antigen recognitions were comparable to monospecific CARs, while binding multiple antigens on the same tumor cells manifested the synergetic enhanced activity in triple-CAR T cells.

The production of more complex constructs might be challenging due to the low capacity of LV vectors, but alternatives like transposons might be better suited for next-generation designs (Wagner et al., 2022).

Combinatorics between mono-, bi-, or even tri-specific TanCARs, some of them even 4th-generation design, brings up a nearly infinite number of possibilities, which makes this approach effective against antigen-loss in tumor target cells. However, multiple-antigen targeting with "OR-gate" increases the likelihood of on-target off-tumor toxicities.

1.3.5.2. "AND-gate"

Unfortunately, most tumor cells do not have a distinct antigen that is not expressed on any other cell type, as in the case of B-cell malignancies. Even a minor expression on healthy tissue could pose a risk of life-threatening on-target off-tumor toxicity in the CAR T-cell treatment (Lamers et al., 2006; Morgan et al., 2010; Parkhurst et al., 2011). Therefore, approaches that offer specific killing only when a combination of target antigens is recognized on tumor cells and not independently are highly sought after. This corresponds to the logical "AND-gate".

What is being referred to as combinatorial or conditional CAR are two CAR molecules against different antigens with split signaling (Figure 6D, F). One CAR contributes with signal one by CD3ζ, and the other with costimulatory signal 2 (Kloss et al., 2013; Lanitis et al., 2013). Signal 1 alone leads to suboptimal T-cell activation, thereby diminishing the degree of on-target off-tumor toxicities, yet it might still occur in some form.

1.3.5.2.1. "IF-THEN-gate"

The "IF-THEN-gate" is represented by a synthetic Notch receptor (synNotch) circuit developed to improve the precision of on-target cytotoxicity by dual-targeting. It is an "AND-gated" dual CAR circuit system where recognition of the first antigen induces the expression of a second CAR, which is specific for the second antigen (Roybal et al., 2016). The binding of the first CAR to its ligand does not activate T-cell signaling. Instead, similarly to the wild-type Notch receptor, the synNotch receptor is cleaved, releasing its intracellular part, which serves as a transcription factor that triggers the expression of the second CAR (Figure 6E) (Yuan et al., 2015). This approach was tested with CARs on various models, for example, glioblastoma (Choe et al., 2021; Moghimi et al., 2021) and ROR1+ tumors (Srivastava et al., 2019), and was deemed safe and accurate. In addition, the synNotch system can be used for target-induced cytokine secretion (Cho, Okuma, et al., 2018; H. Luo et al., 2019). However, the possibility of a tumor escaping by losing the first antigen is still a constraint of this approach.

A more sophisticated use of a synNotch-based circuit was developed by Williams et al. as a 3-input "AND-gate" CAR (Figure 7) with either in-series (cascade) or in-parallel design (Williams et al., 2020). In the in-series circuit, synNotch1 against antigen A is constitutively expressed. After activation, it induces the expression of synNotch2 against antigen B, which consequently causes the expression

of CAR against antigen C. In a parallel circuit, the two synNotch receptors against antigens A and B each trigger the expression of a part of a split-CAR, a scFv against antigen C and intracellular CAR domain, both with dimerization motives. In a mouse model, CAR T cells with the in-series circuit reported superior and accurate cytotoxicity against triple-positive tumor cells. On the contrary, their in-parallel system suffered from a leaky expression and exhibited partial cytotoxicity against single-positive target cells (Williams et al., 2020).
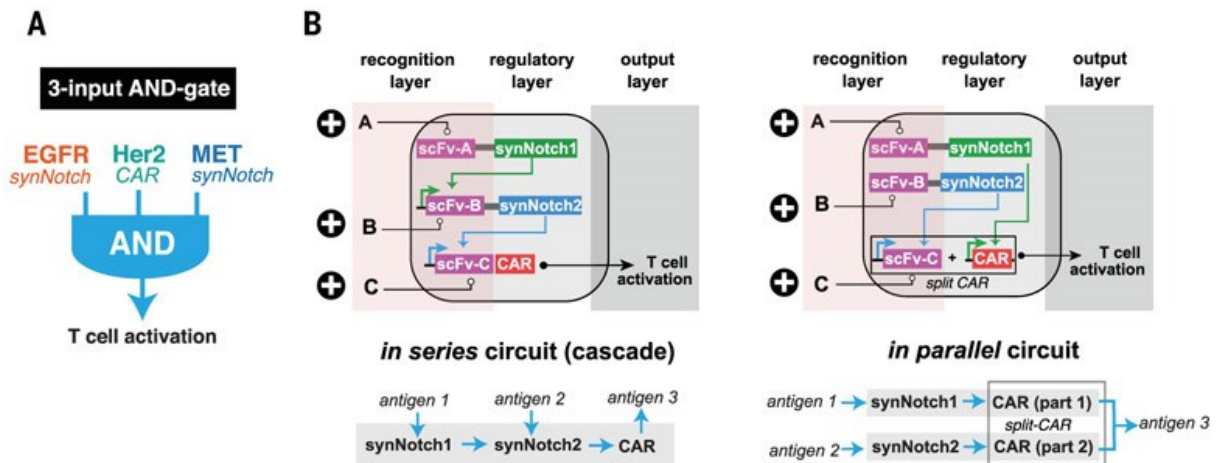
Figure 7. The 3-input "AND-gate" CAR system. (A) All three antigens must be on the target cell's surface to activate the CAR T cell. (B) This system can be engineered as an "in series" or "in parallel" circuit. Figure by Williams et al., 2020.

1.3.5.2.2.   "ON/OFF-Switch"

A strategy for controlled switchable CAR T-cell activation by a small molecule is called split-CAR (C. Y. Wu et al., 2015). In this approach, the CAR is split into two parts (Figure 6F). The first part contains the extracellular antigen binding domain (mostly scFv), the transmembrane part, and the intracellular part only with the costimulatory domain. The second part lacks an extracellular part and has the membrane-anchoring and CD3ζ signaling domains. In addition, both parts have heterodimerization domains that interact only in the presence of a small molecule called chemical inducer of dimerization (CID), which makes the CAR functional (C. Y. Wu et al., 2015). This allows stringent in-situ control of CAR potency, and titrable CID dosage can regulate the degree of activation and its duration. Other designs may include the costimulatory domain with the signaling CD3ζ, leaving the antigen-binding subunit only with the heterodimerization domain (Leung et al., 2019). One example of a heterodimerization module is the FK506-binding protein (FKBP) and FKBP-rapamycin binding domain (FRB) that heterodimerize after the addition of rapamycin or its analog (Juillerat et al., 2016; Leung et al., 2019; C. Y. Wu et al., 2015). Although rapamycin is immunosuppressive and toxic to some extent, the dosage used for CAR activation is sub-immunosuppressive (Leung et al., 2019). Leung et al. named this system dimerizing agent-regulated immunoreceptor complex (DARIC). They also developed a DARIC plug-in for targeting a second antigen. The plug-in is a soluble scFv with the FKBP domain

that, in the presence of rapamycin, competes with the membrane-bound antigen-recognition module for interaction with the signaling module (Leung et al., 2019).

In another study on small molecule-regulated CARs, Jan and colleagues utilized a lenalidomide-induced interaction between CRL4^{CRBN} E3 ubiquitin ligase and zinc finger degron C2H2 motif to design an "ON-switch" split-CAR and "OFF-switch" degradable CAR systems (Jan et al., 2021). In the "OFF-switch" system, the CAR bears the zinc finger degron tag in its ICD. After the addition of lenalidomide, the CRL4^{CRBN} E3 ubiquitin ligase is recruited, and the CAR is polyubiquitinylated and directed to proteasome degradation (Fink & Ebert, 2015). In the "ON-switch" split-CAR, the CRBN domain is uncoupled from the ubiquitin ligase and incorporated in the split-CAR signaling subunit. Again, lenalidomide induces dimerization with the zinc-finger tag in the antigen-recognition split-CAR subunit (Jan et al., 2021).

Boosting the co-stimulation of CAR T cells by a small molecule could be a viable strategy to magnify their effector functions and expansion. Narayanan et al. designed a composite activation receptor that incorporates the toll-like receptor (TLR) adaptor protein MyD88 and the cytosolic part of CD40 (Narayanan et al., 2011). The MyD88/CD40 (iMC) dimerization is induced via the aforementioned FKBP domain by a synthetic dimerizing ligand ricmiducid. Although primarily developed for DCs, the conserved TLR-signaling pathways activate NFκB, Akt, mitogen-activated protein kinase (MAPK), and Janus kinase (JAK), leading to a pro-survival program in T cells (Narayanan et al., 2011). Utilizing the iMC in CAR T cells improved their efficacy (Foster et al., 2017; Mata et al., 2017).

Similarly, leveraging secretory cytokines in enhancing CAR T cells is a well-established approach, which has already been discussed in chapter 1.3.4, 4th-generation CAR T cells. However, safety concerns of systemic and secretory cytokines led to the development of strategies like membrane-bound cytokines, constitutively active cytokine receptors, and inverted cytokine receptors, as mentioned earlier. In a novel approach, engineering CAR T cells with an inducible cytokine signaling "Turbo" domain (iTurbo) provides desired synergetic cytokine signaling without any bystander or systemic effect. The iTurbo domain is activated by a small molecule-induced homodimerization. Furthermore, different types of iTurbo domains can drive the CAR T cells to a desired immunophenotype (R. J. Lin et al., 2022).

For regulating CAR expression on the transcriptional level, the inducible tetracycline (TET)-ON system is commonly used (Drent et al., 2018; Gu et al., 2018; Sakemura et al., 2016; R. Y. Zhang et al., 2019). This system utilizes a recombinant fusion protein called reverse Tet transactivator (rTA) comprising bacterial doxycycline-binding Tet-repressor and the C-terminal activator domain of VP16 protein from herpes simplex virus. After adding the TET analog doxycycline (DOX), rTA binds promoters with Tet-operator sequences and activates their expression (Gossen & Bujard, 1992; Loew et al., 2010). The DOX dosage can titrate the induction level. Moreover, by discontinuing DOX supplementation,

the expression diminishes, although it is not an instantaneous process. A further limitation of the TET-ON system is that the bacterial and viral-derived elements might be immunogenic.

Quite unconventional methods of the "ON-switch" CARs are light-inducible systems (Allen et al., 2019; Z. Huang et al., 2020; Nguyen et al., 2021) or ultrasound-activated system (Y. Wu et al., 2021). Although they present interesting cellular mechanisms and T-cell engineering, their contribution is more theoretical and a proof-of-concept rather than having an immediate clinical impact.

Exploiting characteristic traits of tumors for specific targeting CAR T cells would be an elegant way to reduce adverse toxicities. Particularly, hypoxia of the TME is one of the textbook cancer hallmarks. In a study by Juillerat et al., they incorporated the oxygen-sensitive subdomain of hypoxia-induced factor 1α (HIF1α) into the CAR scaffold (Juillerat et al., 2017). Under average oxygen concentrations, the CAR is driven into degradation due to the HIF1α domain. Contrarily, the HIF1α domain stabilizes the CAR under hypoxic conditions, thus enabling its killing ability. Unfortunately, they reported residual on-target cytotoxicity under normoxia (Juillerat et al., 2017). Kosti et al. upgraded this system with an additional control element for a stringent hypoxia-restricted activity (Kosti et al., 2021). Besides the oxygen-sensing domain of HIF1α, hypoxia-responsive elements (HREs) were added to the CAR promoter for control at the transcriptional level. This double safety switch allowed CAR T cells to be used against a widely expressed ErB2 antigen, for which conventional CARs would cause lethal toxicities, as shown in a xenograft mouse model (Kosti et al., 2021).

Another trait of the TME is the presence of tumor-associated proteases. In so-called masked CARs (Figure 8), the antigen-binding domain is blocked by a masking peptide with a cleavable linker by a TME-specific protease, making them inert in healthy tissue (X. Han et al., 2017). This concept was first developed as a prodrug antibody – "probody", and it displayed a remarkable safety index, which was then translated to an example of anti-EGFR CAR T cells (Desnoyers et al., 2013; X. Han et al., 2017).
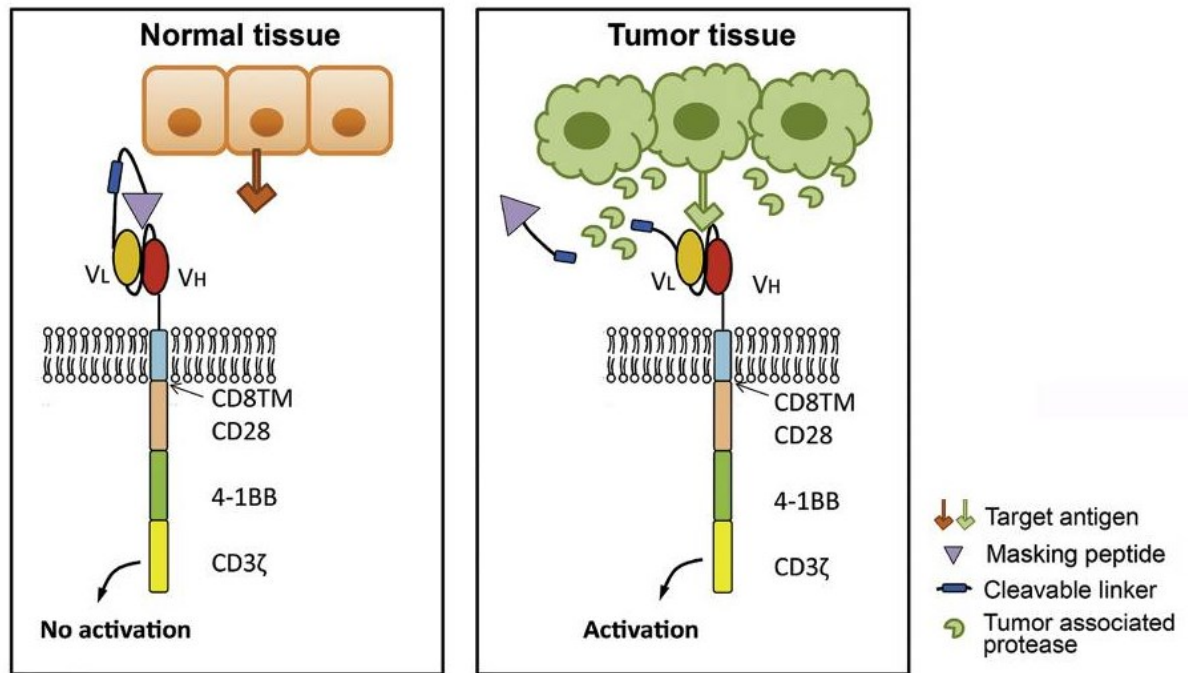
Figure 8. Masked CAR. A masking peptide with a cleavable linker blocks the CAR's antigen binding domain. The CAR is only activated in tumor tissue in the presence of a tumor-associated protease. Figure by X. Han et al., 2017.

Avidity-controlled CAR (AvidCAR) is a platform based on combining controllable CAR dimerization and two low-affinity antigen-binding domains (Salzer et al., 2020). The substantial decrease in the antigen-binding affinities of each antigen-binding domain allows for necessitated reliance on multivalent interaction, known as avidity. This system can be functionally applied as "ON-switch" or "AND-gated" AvidCARs. In the "ON-switch" case, both antigen-binding domains need to recognize the target antigen, and the dimerization inducer needs to be present. The "AND-gated" AvidCAR can be engineered in different ways. First, as a CAR specific to a single surface antigen A that is homodimerized with a soluble antigen B. Second, as two CARs specific towards two distinct surface markers in a non-switchable or switchable format. The non-switchable version employs constitutive CAR heterodimerization, while the switchable one is further inducible by a small molecule (Salzer et al., 2020).

1.3.5.3. *"NOT-gate"*

1.3.5.3.1. Inhibitory CAR

The inhibitory CAR (iCAR) is a type of CAR whose ICD was swapped for that of an inhibitory receptor (Figure 6G), such as PD-1 or CTLA-4 (Fedorov et al., 2013) or LIR-1 (Tokatlian et al., 2022). The iCAR T cells are provided with specific inhibitory signals against non-tumor antigens, which should prevent adverse toxicities in healthy tissue. In practice, the iCAR is co-expressed with classical CAR against a tumor antigen. The iCAR dampens CAR signaling, preventing cytotoxicity, cytokine secretion, and proliferation. However, iCAR has an inherent design flaw: it works only when

the recognized antigen is missing or downregulated on tumor cells, while it is abundantly expressed on normal cells (Fedorov et al., 2013). Next, Aoyama et al. developed an iCAR variant named "Scissor-CAR" with a protease as its ICD. After the binding of the healthy-cell antigen, it cleaves a specific cleavage site on the coupled classical signal-CAR (Aoyama et al., 2022).

1.3.5.3.2.   "AND-NOT"

Another way of designing the "NOT-gate" system is by the OFF-Notch receptor (Williams et al., 2020). With a similar mechanism as the synNotch, the OFF-Notch receptor, engineered by Williams et al., induces the expression of a pro-apoptotic factor truncated BH3-interacting domain death agonist (tBID). Thereby, the inhibition takes place downstream of CAR signaling, on the level of effector functions where inducing apoptosis overrides the signal for proliferation. Concurrently, the same group combined the OFF-Notch receptor into a 3-input "AND-NOT" circuit (Figure 9). First, a synNotch receptor specific to antigen A induces the expression of CAR against antigen B, and in the case of recognition of antigen C by the OFF-Notch receptor, the CAR T cell is driven into apoptosis (Williams et al., 2020).
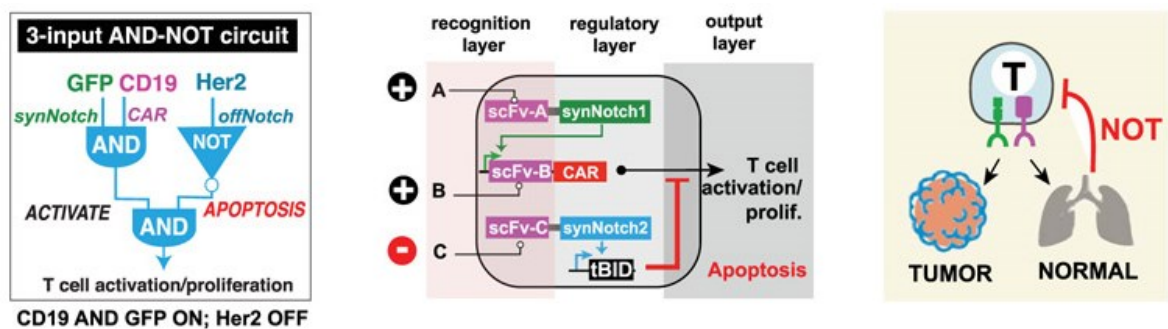
Figure 9. The 3-input "AND-NOT" circuit. The CAR is activated only by recognizing two antigens (A, B; GFP and CD19) on the tumor's surface. However, recognizing a healthy-tissue antigen (C, Her2) drives the CAR T cell into apoptosis. Figure by Williams et al., 2020.

1.3.5.4.   Universal CARs

Unlike traditional CAR T cells, which are typically designed to recognize a single antigen expressed on cancer cells, universal (also adaptor or modular) CAR T cells are engineered as a two-component system, CAR T-cell product specific for a tumor-targeting adaptor molecule and the adaptor itself (Figure 6G). The universal CAR T cells provide adaptability against antigen escape. They may widen the scope of treatable diseases, increasing effectiveness in patients with heterogeneous tumors or relapses in the case of antigen loss or its downregulation. Instead of engineering separate CAR T cells for each antigen, universal CAR T cells streamline the process, potentially reducing costs associated with development and manufacturing. In addition, administering the adaptor molecule and its concentration can control the degree and length of CAR T activation. This corresponds to the "AND-gate" and, in the case of multiple adaptors, the "OR-gate" strategy. Depending on the adaptor molecule, there are several types of universal CARs – Tag-specific, Fc-binding, and bispecific antibody-binding CARs (Arndt et al., 2020).
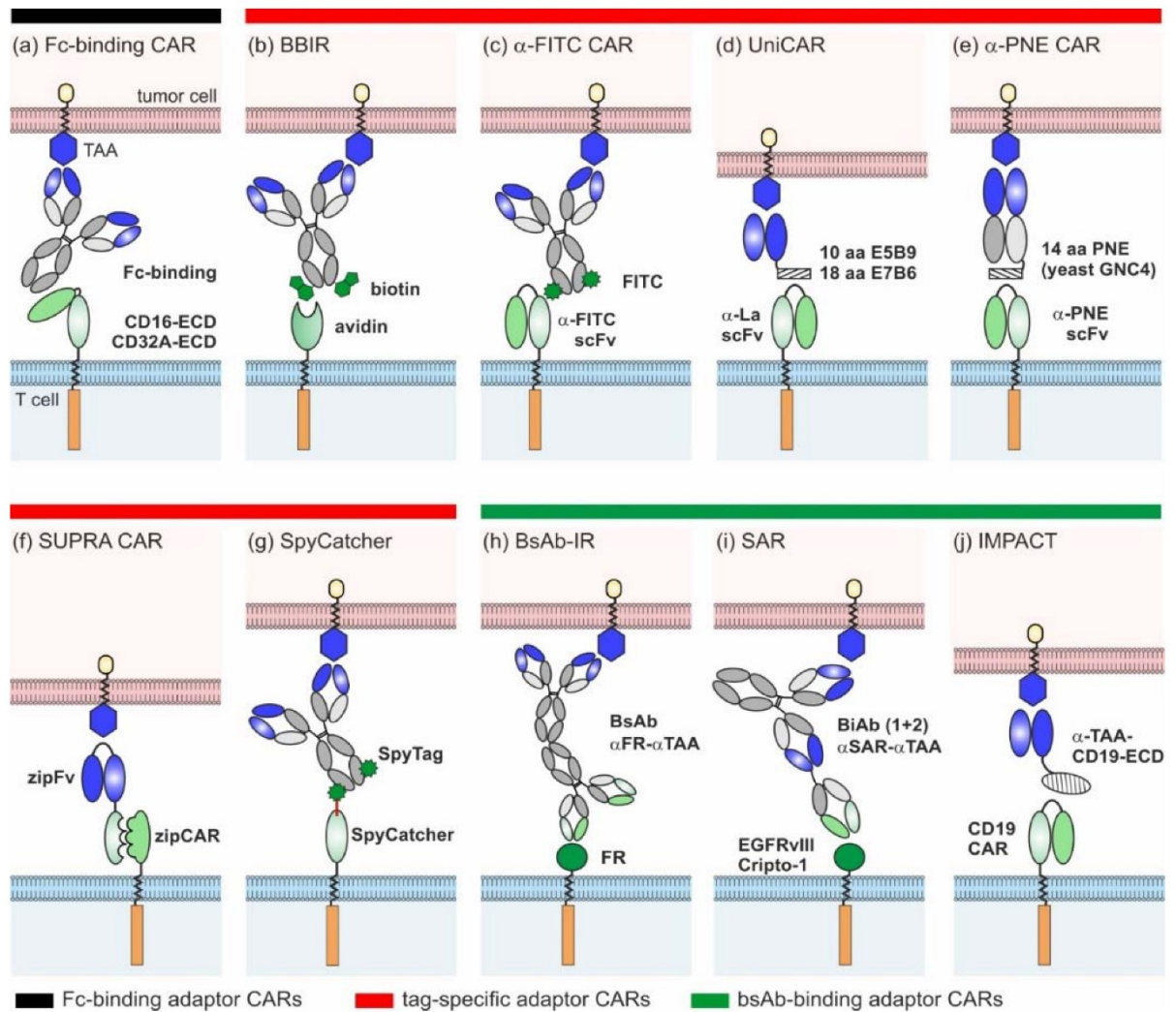
Figure 10. Universal CAR designs. Figure by Arndt et al., 2020.

1.3.5.4.1. Fc-binding adaptor CARs

The Fc-binding CARs are based on the extracellular binding domain of the Fc-receptors CD16 (Kudo et al., 2014; Rataj et al., 2018) or CD32 (Caratelli et al., 2020). A tumor-specific antibody acts as the adaptor for CAR T cells to trigger the tumor lysis (Figure 10a). The main upside of this approach is the wide variety of clinically approved mAbs like rituximab, cetuximab, and trastuzumab. Nevertheless, the binding domains of CD16 and CD32 do not differentiate between therapeutic mAbs and endogenous IgG Abs, which might lead to adverse toxicities (Rataj et al., 2018; Stock et al., 2022).

1.3.5.4.2. Tag-specific adaptor CARs

Tag-specific CARs are engineered to recognize tagged anti-tumor adaptor molecules. First, biotin-binding immune receptors (BBIR) are CARs with avidin or streptavidin as the extracellular domain (ECD), which naturally binds to biotin (Figure 10b). Dimeric avidin and monomeric streptavidin CARs were effective with biotinylated mAbs or scFvs (Lohmueller et al., 2018; Urbanska et al., 2012).

Second, the classical scFv CAR technology can be adopted as the adaptor system by using CARs specific to the synthetic fluorescent dye FITC (fluorescein isothiocyanate; Figure 10c). Many tumor-targeting molecules have been tested as the FITC-conjugated adaptor molecule, for example, clinical-grade mAbs, Fab fragments, and small molecules like folate (Y. Cao et al., 2016; Y. G. Lee et al., 2019; J. S. Y. Ma et al., 2016; Tamada et al., 2012). Additionally, the performance of the anti-FITC CAR T cells depends on the design of stoichiometry and position of the FITC conjugation, and it is target-antigen-specific, which diminishes the simplicity of the "universal" approach (Y. Cao et al., 2016; J. S. Y. Ma et al., 2016).

Third, peptide tags represent a short amino acid (aa) sequence of an epitope not physiologically expressed in any human cell type (Figure 10e). The anti-peptide neo-epitope (PNE) CARs target the PNE tag, a 14 aa peptide from yeast transcription factor GNC4 (Rodgers et al., 2016). The preferred PNE-tagged adaptors were Fab fragment-based instead of IgG-based due to their shorter half-life *in vivo*, thus allowing better control of the CAR activation (Rodgers et al., 2016). Furthermore, similar to the FITC tag, the design of the adaptor molecule in terms of PNE-valency and conjugation sites, as well as the CAR hinge region, affects the geometry and formation of the immunological synapse. The optimal adaptor and CAR design also depended on the choice of target antigen (Y. Cao et al., 2016; Rodgers et al., 2016).

Another design is represented by the UniCAR platform (Figure 10c), developed by the group of Michael P. Bachmann (Cartellieri et al., 2016). It utilizes an scFv CAR against the E59B 10-aa epitope obtained from the human nuclear protein La/SS-B (Koristka et al., 2013), alternatively the E7B6 18-aa epitope (Koristka et al., 2019). Moreover, they developed a broad spectrum of adaptor molecules called target modules (TM) ranging from scFv-based, nanobody-based, TCR-based, and even small molecule-based adaptors against numerous target antigens (Bachmann, 2019). Concerning autoimmunity, the La-derived epitome is a cryptic antigen, and no patients' La-specific autoantibodies have been reported to react against it (Bachmann, 2019).

An adaptor CAR technology based on leucine zippers interaction is called the split, universal, and programmable (SUPRA) CAR (Figure 10f) (Cho, Collins, et al., 2018). This includes a zipCAR that has the leucine zipper (ZIP) domain instead of classical scFv, which binds to the cognate ZIP domain on a tumor-specific soluble scFv – the zipFv (Cho, Collins, et al., 2018).

The SpyCatcher/SpyTag (Figure 10g) is a novel system utilizing covalent attachment of the adaptor molecule in universal CARs (Minutolo et al., 2020). This system is derived from a bacterial adhesin protein containing an internal isopeptide bond. The adhesin was modified and split into the SpyCatcher and a 13 aa SpyTag. After the association of the two fragments, the isopeptide bond is formed again by an autocatalytic mechanism (Zakeri et al., 2012). The CARs with SpyCather as their ECD effectively function with SpyTag-containing IgG mAbs and DARPins adaptors (Minutolo et al., 2020).

Another adaptor CAR platform is the convertibleCAR™ system (Landgraf et al., 2020). The CAR ECD consists of the inert form of the human NKG2D (iNKG2D) ectodomain. The endogenous NKG2D is an NK-cell activation receptor binding to the MIC antigens (MIC-A, MIC-B, ULBP1-6), while the iNKG2D exclusively engages with a specific ULBP2 variant and not the endogenous one. The so-called MicAbody™, an engineered mAb fused with the ULBP2 domain, serves as an adaptor molecule to convertibleCAR (Landgraf et al., 2020).

The barstar CAR (BsCAR) system is based on a high-affinity toxin-antitoxin interaction, such as the RNAse toxin barnase and its inhibitor barstar (Stepanov et al., 2022). The BsCAR ectodomain is a barstar-modified IgG4 Fc spacer, and the corresponding adaptor molecule represents a barnase-DARPin fusion protein (Stepanov et al., 2022).

1.3.5.4.3. Bispecific molecule-binding adaptor CARs

Immunotherapy based on bispecific antibodies (bsAb) or BiTEs relies on bridging an activating receptor of effector cytotoxic cells with a specific antigen on tumor cells (Stamova et al., 2012). The first combinatory approach using bsAb with CAR together was termed the bsAb-binding immune receptor (bsAb-IR; Figure 10h). The bsAb-IR consisted of the extracellular portion of human folate receptor α (FRα) and conventional CAR ICDs. The tested adaptor was an anti-FRα + anti-CD20 bsAb, which unfortunately did not meet the functional expectations against B-cell lines (Urbanska et al., 2014). Another group developed a similar system, synthetic agonistic receptor (SAR; Figure 10i) (Karches et al., 2019). The SAR employs ECDs of EGFRvIII and Cripto-1 proteins. As the tumor-specific adaptors, tetravalent and trivalent bsAbs were studied. Due to SAR cross-linking, the tetravalent bsAb activated the SAR-T cells without tumor cells and caused off-target killing. The trivalent bsAbs were shown to be effective; the CAR T-cell activation and the killing of tumor target cells were both antigen and bsAb-dependent (Karches et al., 2019).

Next, a modular technology named IMPACT™ (Integrated Modules oPtimize Adoptive Cell Therapy) repurposes the conventional and well-established anti-CD19 CAR T cells to target new antigens via an adaptor (Figure 10j). The novel adaptor molecule is a fusion protein of CD19 ECD and an anti-tumor scFv. It was initially developed to treat CD19-negative relapses; however, this system was effective even against solid tumor antigens (Klesmith et al., 2019; Rennert et al., 2019).

The RevCAR system represents a similar design to the UniCAR; however, it is in reverse orientation (Feldmann et al., 2020). The RevCAR lacks an antigen-binding domain and has either one of the UniCAR Tag epitopes (E5B9, E7B6) instead. Interaction with a tumor antigen is mediated by a bispecific targeting molecule named RevTM, comprised of two fused scFvs – one tumor-specific and the other tag-specific. This design reduces the CAR gene size, allowing for easier multicistronic vector engineering and transduction (Feldmann et al., 2020).

1.3.5.5.   CAR with JAK/STAT signaling

Cytokines provide the essential signal 3 for T cells to proliferate, survive, and differentiate. The TRUCK strategies relied on the transgenic expression of these cytokines. Kagoya et al. developed a 28ζ CAR construct that incorporated the signaling domains of cytokine receptors directly into the CAR ICD (Kagoya et al., 2018). Thereby, these CAR T cells would require no additional cytokine stimulation. One signaling domain was derived from the IL-2Rβ; the other was the tyrosine-X-X-glutamine (YXXQ) motif. These domains bind and activate signal transducer and activator of transcription 5 (STAT5) and STAT3, respectively. STAT5 is physiologically activated in IL-2, IL-7, and IL-15 signaling, STAT 3 by IL-21 (J. X. Lin & Leonard, 2018). This construct performed according to expectations with sustained proliferation, anti-tumor effect, and prevention of terminal CAR T-cell differentiation (Kagoya et al., 2018).

1.3.5.6.   Suicide switches

While potentially life-saving, CAR T-cell therapy can act as a double-edged sword. Adverse reactions like on-target off-tumor toxicity, CRS, and graft-versus-host disease (GvHD) can lead to life-threatening conditions. Therefore, the option of selective and inducible CAR T ablation increases the therapy's safety. Introducing a suicide gene that initiates apoptosis after the administration of the inductor acts as a permanent "OFF-switch". The most common mechanisms of suicide genes are metabolic switches, agent dimerization, and markers for elimination by mAbs (Figure 11).
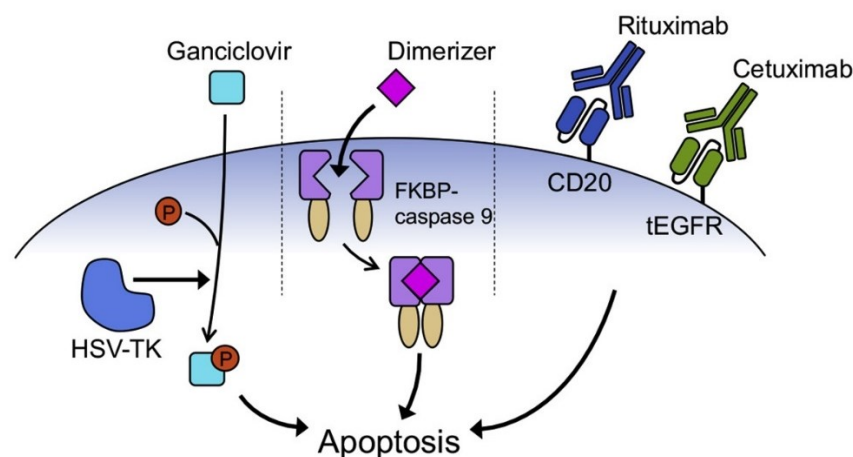
Figure 11. Suicide gene systems. Figure by Hong et al., 2020.

The metabolic switches are enzymes that convert a nontoxic prodrug into a toxic substance. Herpes simplex virus-thymidine kinase (HSV-TK) phosphorylates certain nucleoside analogs, such as ganciclovir (GCV), into GCV-triphosphate. Incorporating the GCV-triphosphate into a DNA strand by DNA polymerase causes chain termination, which eventually induces apoptosis (Bonini et al., 1997; Elion et al., 1977). The limitation of HSV-TK is its immunogenicity due to its viral origin (Berger et al., 2006) and its slow mode of action – it takes up to three days of GCV exposure to cause cell death (Marin

et al., 2012). Furthermore, by implementing the HSV-TK system, it is not possible to use GCV to treat cytomegalovirus (CMV) infection, which is common in post-HSCT and immunosuppressed patients, as the treatment would eradicate the engineered cells (Bonini et al., 2007; Emery et al., 2013). An alternative to the HSV-TK is mutant human thymidylate kinase (mTMPK) (Sato et al., 2007). Although it is advantageous in posing no risk of immunogenicity, it was shown to be less effective than the HSV-TK (Marin et al., 2012). An additional example of a metabolic suicide switch is cytosine deaminase. This enzyme converts 5-fluorocytosine into 5-fluorouracil, a cytotoxic compound that inhibits pyrimidine synthesis (Tiraby et al., 1998).

Safety switches based on induced dimerization utilize components of apoptotic pathways. They are fused with dimerization domains whose interaction is conditioned by adding a synthetic metabolically inert drug. Namely, inducible Caspase9 (iCasp9) (Stasi et al., 2011; Straathof et al., 2005) and inducible FAS (iFAS) (Clackson et al., 1998; Thomis et al., 2001) coupled with the FKBP dimerizing domains are widely utilized. The dimerizing agents employed are drugs like rimiducid (AP1903), AP20187 (Minagawa et al., 2019), and rapamycin (Stavrou et al., 2018). The iCasp9 system has a low immunogenic profile and is rapid-acting; the apoptosis occurs within 30 minutes of stimulation (Marin et al., 2012; Stasi et al., 2011).

Surface antigens typically not expressed on T cells allow for targeted elimination of CAR T cells via administration of mAbs. The mAb-labeled CAR T cells are susceptible to eradication via ADCC or complement-dependent cytotoxicity (CDC). The availability of approved, pharmaceutical-grade mAbs makes them an ideal tool. Transgenic expression of CD20 or a truncated form of EGFR (EGFRt) allows targeting by rituximab (Griffioen et al., 2009; Philip et al., 2014; Vogler et al., 2010) or cetuximab (Paszkiewicz et al., 2016; X. Wang et al., 2011), respectively. Rituximab causes B-cell aplasia and would interfere if the patient were already undergoing treatment with rituximab. Targeting CD52 with alemtuzumab can act as a natural safety switch, as CD52 is expressed on mature T cells and not on hematopoietic stem cells (G. Ma et al., 2019; Tasian et al., 2015, 2017). Other examples of target epitopes are the 10-aa tag derived from the human c-Myc protein (Kieback et al., 2008) and a cryptic EGFR epitope engineered into folate receptor 1 (FOLR1), termed FR806 (X. Wu et al., 2017). Nonetheless, the efficacy of this mAb-based approach might be undermined due to the constrained capacity for ADCC/CDC in patients subjected to chemotherapy before CAR T-cell administration (Ericson et al., 1995). Additionally, the mAbs may exhibit restricted biodistribution and tissue penetration. Therefore, CAR T-cell-based approaches were developed to target, for example, CD19 CAR by an anti-idiotype scFv (Ruella et al., 2020) or an E-tag in the primary CAR's ECD (Koristka et al., 2019).

1.4. Production of CAR T cells

A vital requirement for producing CAR T cells is the introduction of the CAR transgene into the T cells. The CAR expression should be stable in dividing T cells, which is achieved by integration into cell genomes. From the arsenal of integrative vectors, the most common method of choice is transduction with retro/lentiviral (RV/LV) vectors, followed by the emergence of non-viral approaches, including transposons and CRISPR- or transcription activator-like effector nucleases (TALEN)-based methods. These non-viral approaches require either electroporation of naked DNA or coating the DNA in a liposome or similar polymer particle. The advantages and disadvantages of genetic modification methods are reviewed in Table 1. In the next step, the cultivation of adequate numbers of viable and functional CAR T cells is needed. Last but not least, for practical reasons, the procedure should be economical and easily approved by regulatory agencies.

1.4.1. T-cell sources

The first step in the CAR T-cell manufacturing procedure (Figure 12) is isolating the starting material. As a mainly autologous therapy, the patient's peripheral blood mononuclear cells (PBMCs) containing T cells are isolated. This is achieved mostly by leukapheresis, a specific type of apheresis when leukocytes are isolated, and the rest of the blood components are returned to circulation. Alternatively, PBMCs can be separated from whole blood by centrifugation over the Ficoll gradient. The PBMCs may be a subject for further cell fractionation to enrich the T cells or a particular T-cell population, like $CD4^+$, $CD8^+$, or a distinct memory subset.

Although CAR T cells are regularly manufactured from the general $CD3^+$ T-cell population, choosing a specific T-cell memory subset as the starting material may increase the product's functional benefits. Several studies have reported that CAR T cells produced from naïve (Hinrichs et al., 2011), stem cell memory (Gattinoni et al., 2011), and central memory (Berger et al., 2008) T cells showed enhanced proliferation and persistence, which was linked to increased treatment efficacy.

Additionally, after T-cell separation, the cells can be cryopreserved for future use or transportation to the manufacturing facility.

CAR T-cell Therapy

Remove blood from patient to get T cells

T cell

Make CAR T cells in the lab

Insert gene for CAR

T cell

Chimeric antigen receptor (CAR)

CAR T cell

CAR T cells bind to cancer cells and kill them

Cancer cell

Antigens

CAR T cell

Cancer cell

Grow millions of CAR T cells

Infuse CAR T cells into patient
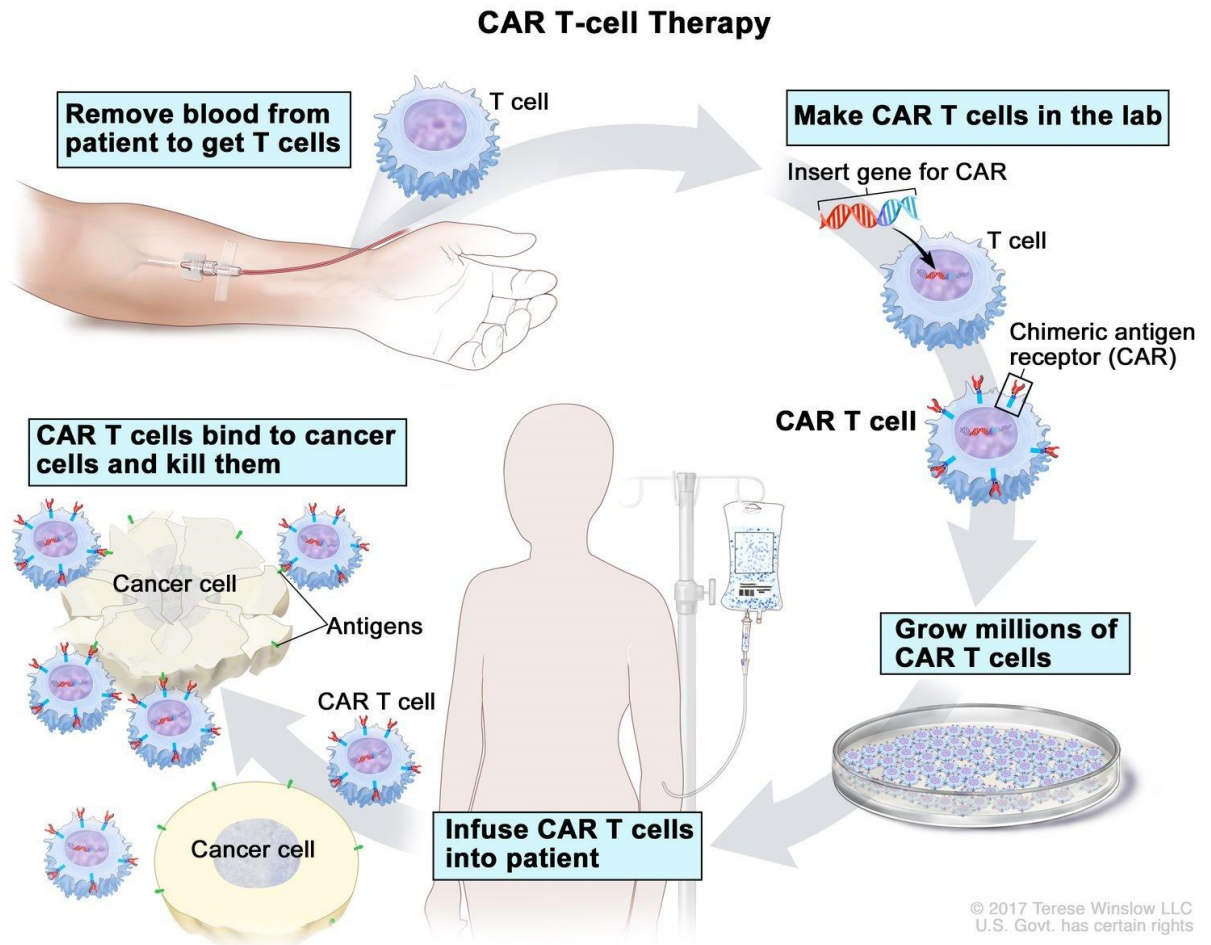
Figure 12. CAR T-cell therapy overview. Figure by https://www.cancer.gov/about-cancer/treatment/types/immunotherapy/t-cell-transfer-therapy.

1.4.2. Genetic modification

1.4.2.1. *Viral transduction*

The biological ability of retroviruses to infect host cells and integrate their DNA into the cell's genome makes them natural vectors. Retroviruses (RV) and lentiviruses (LV) contain viral diploid (+) ssRNA, which is reversely transcribed to DNA and then integrated into the host cell genome. In gene engineering, replication-deficient RV/LV with removed virulent factors and a sequence for a gene of interest (e.g., CAR) instead are used. Most RV vectors are based on the Moloney murine leukemia virus (MMLV) from the γ-*retroviridae* family. LV vectors, which are derived chiefly from modified human immunodeficiency virus (HIV), have the advantage of transducing nondividing cells, unlike RVs (Lewis & Emerman, 1994). The first human experiment using a γ-RV vector was introducing an antibiotic-resistance gene to TILs for treating melanoma. This was to study TIL trafficking and persistence *in vivo*, and it demonstrated the safety and efficacy of retroviral vectors, albeit in a small patient group (Rosenberg et al., 1990). From there, the use of RV/LV vectors became widespread. Their main advantages are reliability, high transfection efficiency, the introduction of a low copy number of inserts, relatively low genotoxicity, and low immunogenicity (Morgan & Boyerinas, 2016).

On the negative side, the RV capacity is limited to 3-4 kbp long transgene, as the whole viral DNA from 5' LTR to 3' LTR is approximately 7-8 kbp. This usually permits transduction merely of a single gene. The total LV capacity is up to 10 kbp, although, with increasing insert lengths, the produced virus titer is diminished (Kumar et al., 2001). The RV/LV vector production in a GMP regime is exceptionally costly and requires a facility with biosafety level 2. Consequently, the regulatory requirements are usually an insurmountable obstacle for hospital in-house production (Poorebrahim et al., 2019).

The RV/LV vectors are produced by co-transfecting a packaging cell line with a transfer vector and helper plasmids (Figure 13). Transfer vector contains the gene of interest and *cis*-acting elements - packaging signal (Ψ), viral LTRs (long terminal repeats), and LVs additionally central polypurine tract (cPPT)/central termination signal (CTS). Helper plasmids contain RV/LV genes required for viral replication and packaging (*trans*-acting elements) – *gag* ("group-antigen specific," capsid proteins), pol ("polymerase," replication enzymes), *env* ("envelope," envelope glycoproteins). LVs require additional accessory genes – *rev* (binds to RRE (rev-responsive element) and stimulates nuclear export and expression of *gag/pol* genes), and in 2nd generation, *tat* ("trans-activator of transcription"). The *tat* protein was required due to the weak promoter in 5'LTR, which has been modified in the 3rd generation by fusion with a heterologous promoter, making the transcription tat-independent. The 2nd generation of LV vectors used two helper plasmids – *gag/pol* and *env/rev/tat*. The 3rd generation uses three helper plasmids – *gag/pol, env*, and *rev* separate, with *tat* absent (Labbé et al., 2021). The division to a transfer plasmid and more helper plasmids is vital for safety to minimize the risk of recombination into a replication-competent virus. Alternatively, an engineered cell line (e.g. Phoenix) modified with stable expression of *gag/pol* and *env* genes can be used instead of helper plasmids. In LV vectors, the envelope glycoprotein is mainly changed to vesicular stomatitis virus G protein (VSV-G) to broaden its tropism to virtually all human cells (Cronin et al., 2005). However, the expression of the VSV-G receptor, low-density lipoprotein receptor (LDL-R), is low on resting T cells, B cells, and HSCs. Therefore, activation of these cells, which stimulates LDL-R expression, is needed for their efficient transduction (Amirache et al., 2014).

The integration of RV/LV vectors, carried out by reverse transcriptase and integrase (*pol*), is not random; both exhibit distinct preferences. The γ-RV integration is biased towards transcriptional start sites (TSSs) and CpG islands, corresponding with promoters. However, LV integration targets active transcription units without preference between introns or exons and not in upstream TSSs (Ciuffi, 2008; X. Wu et al., 2003). Hence, RVs pose a higher risk of insertional mutagenesis, leading to malignant transformation of transduced cells, a relevant safety concern. The insert could disrupt a tumor-suppressor gene or activate a proto-oncogene by proximity with viral LTR containing an enhancer and a promoter. This was the case in a clinical trial for treating SCID-X1 (X-linked severe combined immunodeficiency) with γ-RV-modified hematopoietic stem cells (HSCs) for expression of γ-chain IL-2 receptor subunit, where several patients developed leukemia from the transduced cells (Cavazzana-

Calvo et al., 2000; Hacein-Bey-Abina et al., 2003). To additionally increase the safety of RV/LV integration, approaches using insulator sequences or self-inactivating (SIN) vectors are being developed (Cesana et al., 2014; Ellis, 2005). Despite the integration patterns, CAR T cells transduced by RV/LVs were shown as safe with observations over a decade (Cappell & Kochenderfer, 2023; Scholler et al., 2012). Notably, mature T cells resist oncogenic transformation due to pro-apoptotic and epigenetic mechanisms (Newrzela et al., 2008).

RV/LV vectors are routinely used for CAR T manufacturing, including all currently approved commercial products. They are further discussed in chapter 1.7.
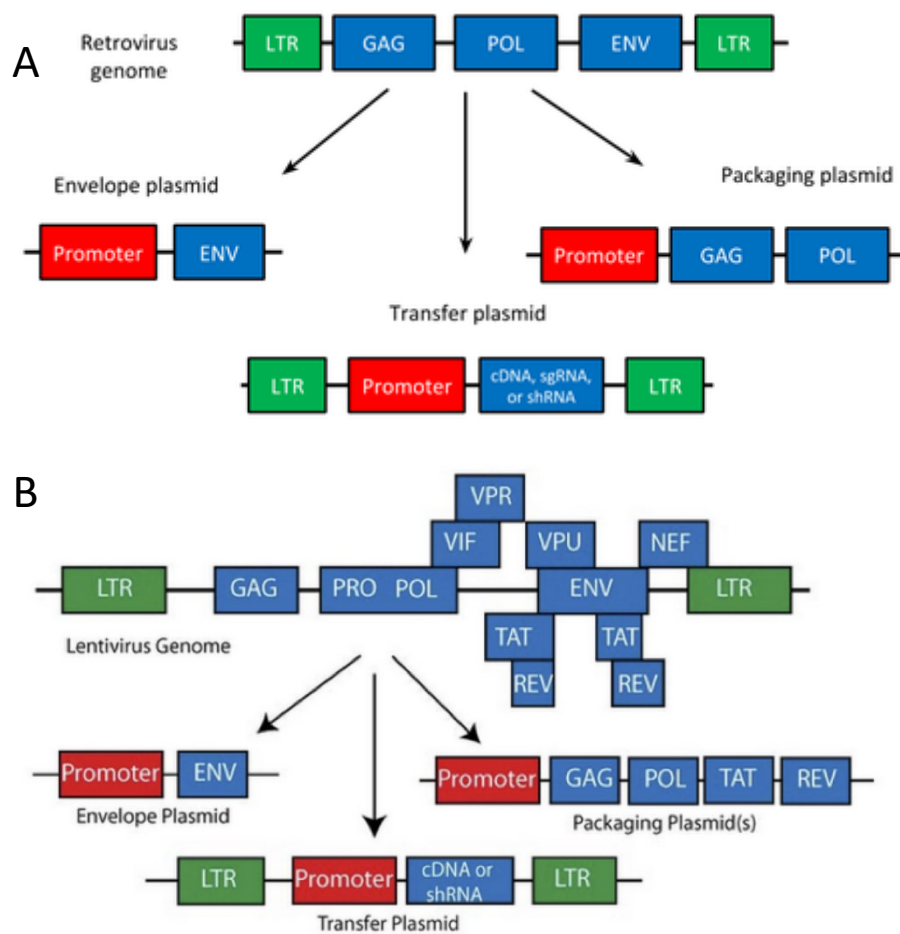
Figure 13. Organization of the RV (A) and LV (B) vector genome. To produce RV/LV vectors, simultaneous transfection by the transfer plasmid and envelope and packaging helper plasmids. The long terminal repeats (LTRs) on plasmids are modified to prevent recombinations. Figure by https://www.addgene.org/guides/retrovirus/ and https://www.addgene.org/guides/lentivirus/.

1.4.2.2.1.   Transposons

Transposons are naturally occurring integrative mobile genetic elements. They consist of a sequence usually flanked with inverted repeats (IRs). A transposase enzyme recognizes IRs and can integrate or move the transposon to a different locus through a "cut-and-paste" mechanism (Figure 14). The transposase can be either encoded by the transposon itself (autonomous elements, *cis*) or not in the case of a mutation defect (non-autonomous elements), which could still be transposed by exogenous transposase (*trans*). These attributes make transposons useful in genetic engineering as vectors for transgenesis or insertional mutagenesis. For vector purposes, the gene for transposase is usually present on a separate non-integrative plasmid or even as synthetic mRNA so that its expression remains transient. One reason why transposon vectors are particularly enticing is their cost effectiveness compared to RV/LV vectors, which could dramatically reduce overall therapy costs. Furthermore, they pose a low immunogenicity risk, albeit they often exhibit lower transfection efficacy than RV/LVs (Wagner et al., 2022).
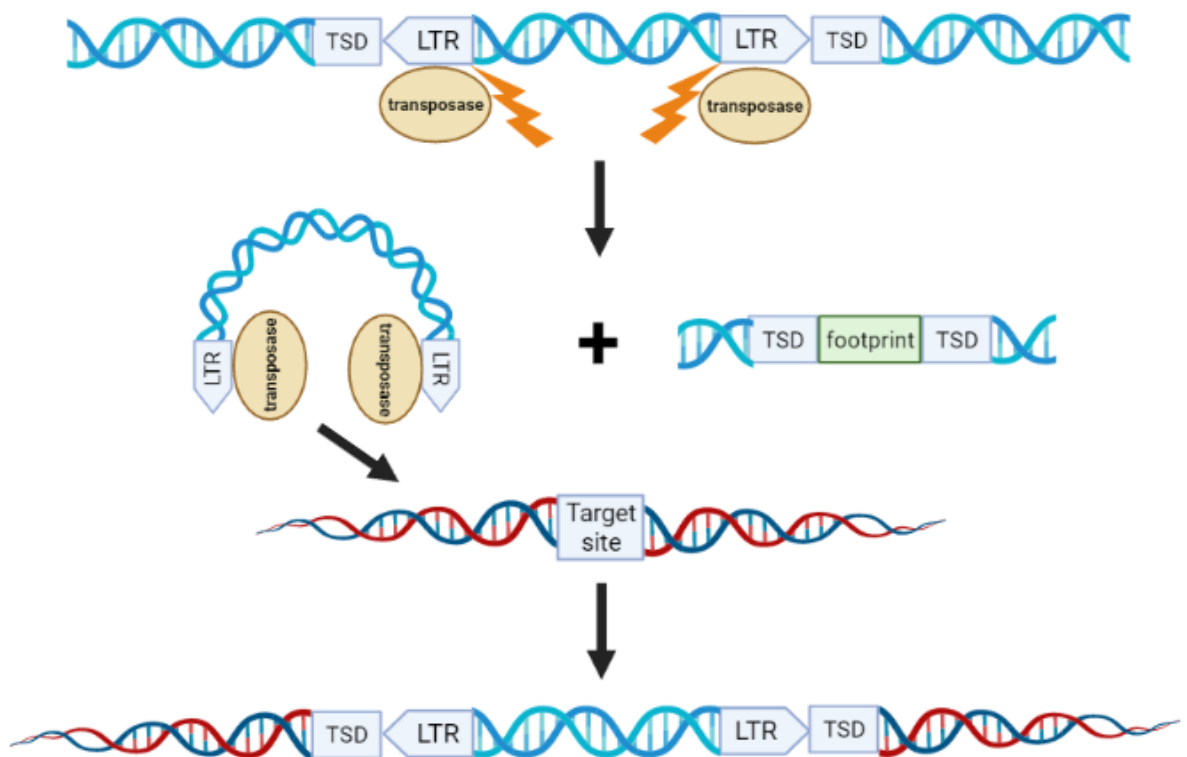
Figure 14. Cut-and-paste mechanism of transposon vector. TSD – target site duplications, LTR – long terminal repeat. Figure by https://blog.addgene.org/plasmids-101-using-transposons-in-the-lab.

1.4.2.2.1.1. Sleeping Beauty

Sleeping Beauty (SB), a Tc1/*mariner* family member, is one of the most frequently used transposon vectors for vertebrates, including human cells. It was "awakened from its slumber" by a comparative analysis of dysfunctional transposon sequences from genomes of eight teleost fish species to predict a functional one (Ivics et al., 1997). Its total size with only the transposase gene is about 1.6 kbp, including two approximately 230 bp terminal inverted/direct repeats.

SB exhibits a widespread, close-to-random integration profile, with small preferences toward genes and their upstream regulatory sequences. Structurally, a minor bias for microsatellite repeats in non-coding and bent DNA was found (Yant et al., 2005), and the integration site targets mostly TA dinucleotides (Vigdal et al., 2002). When integrating, SB creates a 3-bp footprint from either side, flanked with 2-bp TA duplication (G. Luo et al., 1998). It is deemed a somewhat safer integration profile than RV/LV vectors (Field et al., 2013). Also, as SB transposase is an exogenous enzyme, no other factor can move the transposon in human cells. Expressing SB transposase from a mRNA (Dupuy et al., 2002) or delivering it as a protein (Querques et al., 2019) would exclude even an unlikely random integration of transposase DNA (X. Huang, Haley, et al., 2010).

A significant disadvantage of SB used to be the limited capacity of SB transposon. Increasing insert size dramatically reduces transposition efficiency by about 50% at approx. 5.5 kbp, with an upper limit of around 7-10 kbp (Geurts et al., 2003; Izsvák et al., 2000; Karsi et al., 2001). Another downside is that SB can be prone to "overexpression inhibition". Transposons of *mariner* and Tn5 families decrease transposition efficacy when high levels of transposase are present (Izsvák & Ivics, 2004). There have been many attempts to increase the transposition efficacy by mutagenesis of the transposase. This has resulted in 17-fold (Baus et al., 2005) to a 100-fold increase in transposition by some variants (SB100X, Mátés et al., 2009). A transposase version SB100xco (human codon-optimized SB100x) was able to transpose a 100-kb bacterial artificial chromosome (BAC) (Rostovskaya et al., 2012). Furthermore, rational *in silico* design yielded a transposase with another 30% increase in efficacy compared to SB100X (Voigt et al., 2016) or increased enzyme solubility and stability for more effective delivery in protein form (Querques et al., 2019). Another way to improve the SB system is to replace conventional plasmids, which promote higher cytotoxicity and have lower transposition rates, with minicircle DNA (mcDNA). Briefly, mcDNA is a minimal-sized DNA circle lacking the bacterial plasmid backbone; they are further discussed in chapter 1.4.2.2.3 (Prommersberger et al., 2022).

Currently, SB is used in various applications, including induced pluripotent stem cells, insertional mutagenesis screens, transgenic animals, and both *in vitro* and *ex vivo* gene therapy (Sandoval-Villegas et al., 2021). Notably, CAR T cells generated with SB are used in several clinical trials (Magnani et al., 2020; Prommersberger et al., 2021; Singh et al., 2022).

The PiggyBac (PB) transposon was discovered while studying mutant baculoviruses that were changing phenotype during passaging in cabbage looper moth (*Trichoplusia ni*)-derived cell lines (Potter et al., 1976). It was found that sequences responsible for these insertional mutations originated from repetitive sequences in the *T. ni* genome (Fraser et al., 1983). It was later confirmed that it was, in fact, a transposable element, then named IFP2 (Cary et al., 1989). Even later, the transposase that could excise and integrate sequences bordered with IFP2 terminal repeats (TR) was identified and named piggyBac (Elick et al., 1996). The structure of the original PB is one open reading frame (ORF) coding transposase (2374 bp) between asymmetric TRs, with a total length of transposon of 2472 bp (Cary et al., 1989). Both TRs include 13-bp terminal inverted repeats and 19-bp internal repeats divided by a spacer sequence. In 5'TR, the spacer is 3 bp long, and in the 3'TR, it is 31 bp long (X. Li et al., 2005). Even though PB is derived from insects, it is efficient for use in vertebrate cells, including human cells (Wilson et al., 2007).

Similarly to SB, PB transposition is mediated by a "cut-and-paste" mechanism. However, unlike many other transposons, the PB integration and re-excision are "footprint-free" without leaving any target site duplications (Elick et al., 1996). PB integration is targeted predominantly into TTAA sites (Fraser et al., 1995). Mapping of PB integration revealed a less random pattern than SB, which was similar to γ-retroviral vectors, with enrichment at TSSs of genes (Gogol-Doring et al., 2016) and long terminal repeats elements (Wilson et al., 2007). For the CAR T-cell application, PB integration assessment showed a lower preference for TSSs than RVs and a higher bias towards transcriptionally silent chromatin ("safe harbors") than LV vectors (Hamada et al., 2018).

A substantial advantage of the PB system is its cargo capacity. Unlike SB, inserts around 10 kb did not reduce transposition frequency (Ding et al., 2005). Moreover, large 100 kb inserts (M. A. Li et al., 2011), a whole bacterial artificial chromosome (BAC) of size 150 kb (Rostovskaya et al., 2012), and even a 200 kb fragment (R. Li et al., 2013) have been successfully transposed. Conversely to SB, overproduction inhibition seems not to be a limitation of the PB system (Wilson et al., 2007). Through mutational screens, a range of hyperactive PB transposases (hyPBase) has been developed (Meir et al., 2013; Wen et al., 2020; Yusa et al., 2011), with activity up to an order of magnitude higher than the wild-type (Yusa et al., 2011).

An alternative to the plasmid template, similar to mcDNA with SB, a linear, covalently closed DNA called doggybone DNA (dbDNA) can be used for PB transposition (Bishop et al., 2020). To further reduce costs and bypass production in bacteria, dbDNA can be produced enzymatically *in vitro*, even at a large scale. After vector optimization by adding flanking regions at the dbDNA ends, it was successfully used to generate CAR T cells (Bishop et al., 2020).

The PiggyBac offers similar usage as SB in preclinical applications. It has been successful in CAR T-cell production (Manuri et al., 2010; Morita et al., 2018; Nakazawa et al., 2016). The PB system's evident advantages have made it an established platform for CAR T-cell production in clinical settings. Numerous clinical trials have been successfully conducted so far, for example, with anti-CD19 CAR (Nishio et al., 2021), anti-BCMA CAR (Costello et al., 2021), anti-EGFR for non-small cell lung carcinoma (Y. Zhang, Zhang et al., 2021), and anti-PSMA for prostate cancer (Slovin et al., 2022).

1.4.2.2.1.3. Other transposon systems

Another fish-derived transposon system is *Tol2* (Koga et al., 1996). Comparably to PB, *Tol2* can effectively transpose large 100-200 kb inserts in mammalian cells (Suster et al., 2009; Urasaki et al., 2006). *Tol2* integration is considered close to random, with small preferences for a palindromic-like octanucleotide sequence, AT-rich regions, DNaseI hypersensitive sites, CpG islands, and in proximity to TSSs. Upon integration, *Tol2* creates 8-bp target site duplication (Grabundzija et al., 2010; X. Huang, Guo, et al., 2010; Ni et al., 2016). *Tol2* has been used to manufacture functional anti-CD19 CAR T cells (Tsukahara et al., 2015).

Tc buster™ is a transposon system discovered in insects (*Tribolium castaneum*) of the same hAT superfamily as *Tol2* (Arensburger et al., 2011). A hyperactive mutant was developed by Bio-Techne (Minneapolis, MN, USA) and is commercially available (Patrinostro et al., 2022). The integration profile of *Tc buster* is near random and potentially safer than LVs, with fewer integrations near TSSs in exons and introns (Patrinostro et al., 2022). In application, it can transfer cargo over 10-12 kb and introduce low transgene copy numbers (3-5 for T cells, 2-3 for NK cells). *Tc buster* efficiently and stably introduced CAR Transgene to CAR T and CAR NK cells (Pomeroy et al., 2021).

Several other transposon systems exist, such as Frog Prince (FP) and Leap-In® (ATUM, Newark, CA, USA); however, they have not yet been used for CAR T-cell manufacturing. With new emerging transposon systems, more direct comparison studies are needed to determine the most efficient and safe for each application and cell type.

1.4.2.2.2.   Programmable nucleases (CRISPR/Cas, TALEN)

In recent times, there has been a boom in the development of programmable nucleases, namely zinc finger nucleases (ZFNs), TALENs, and mainly the CRISPR/Cas system. These enzymes can be designed to cleave DNA at specific sequences, potentially targeting any genome locus. Creating a double-strand break (DSB) leads to reparation through either nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) (O'Driscoll & Jeggo, 2006). Homologous recombination in mammal cells is relatively ineffective (Kaniecki et al., 2017). However, it is known to be vastly enhanced by the occurrence of DSBs (Rouet et al., 1994). Given that, HDR at the DSB site is conditioned by the presence of an exogenous donor DNA with homologous sequences at its ends. In the case of NHEJ, the re-joining of DNA ends is accompanied by insertions or deletions at the DSB site, which

usually results in a gene disruption (Chang et al., 2017). In conclusion, programmable nucleases facilitate target gene inactivation or targeted transgene insertion (Figure 15).
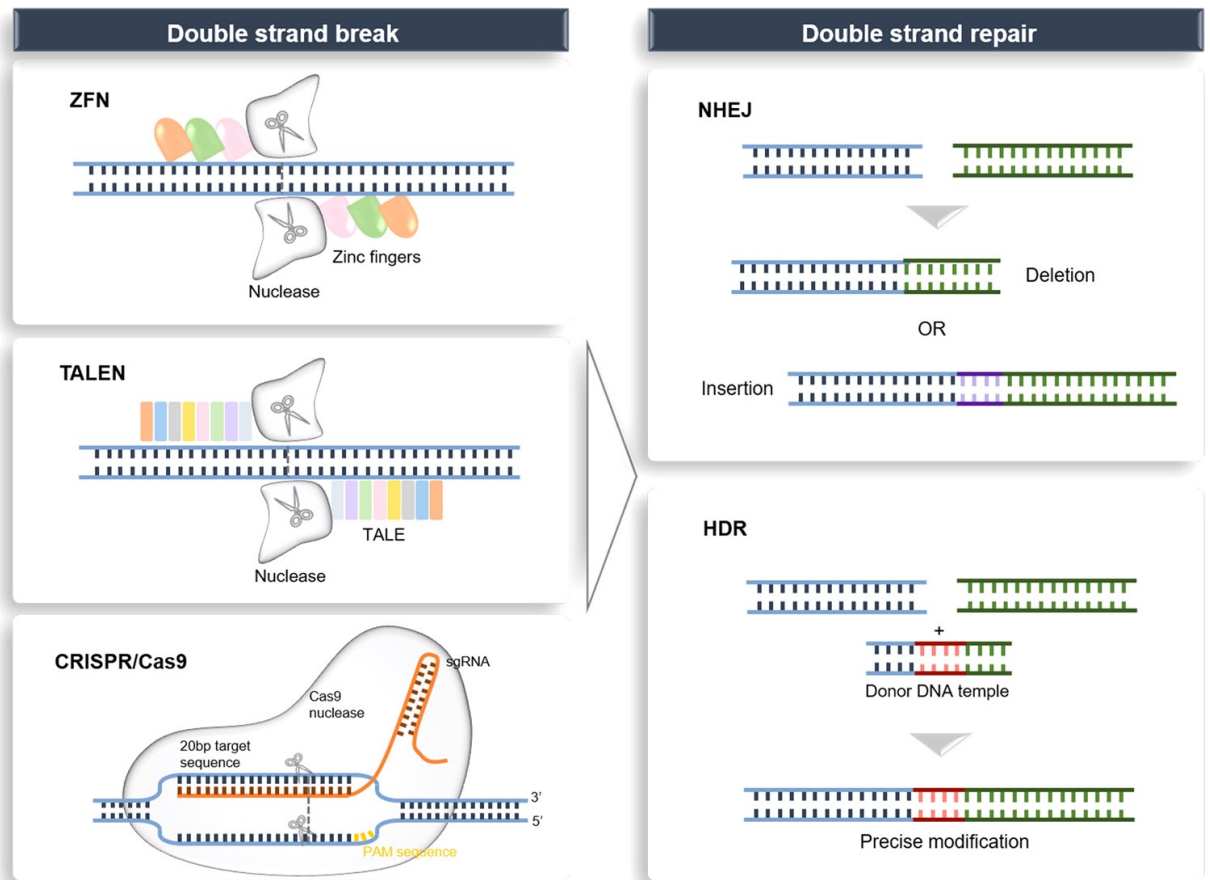
Figure 15. Mechanism of function of programmable nucleases. NHEJ – non-homology end joining, HDR – homology-directed repair. Figure by H. Li et al., 2020.

Concerning CAR T cells, this approach allows the introduction of CAR transgene into a specific locus while disrupting a particular gene. Removing endogenous TCR and human leukocyte antigen (HLA) might open doors to universal "of-the-shelf" allogenic CAR T cells. Disruption of TCR genes eliminates graft-versus-host reaction while knocking out HLA or β-2microglobulin (B2M) genes prevents host-versus-graft rejection (C. Li et al., 2020). Moreover, the ablation of inhibitory molecules such as PD-1 could make CAR T cells more potent, and the knock-in of additional genes, e.g., cytokines, could be used to make armored CAR T cells, as described in chapter 1.3.4 (C. Li et al., 2020).

ZFNs were the first programmable nucleases developed. They are based on DNA-binding zinc finger (ZF) domains, and each ZF recognizes a three-bp sequence. Combining different ZF domains into a multimeric protein enables recognition of a specific sequence. They are fused with FokI endonuclease, which cleaves DNA, creating a DSB (Y. G. Kim et al., 1996). There has been an effort to develop universal allogeneic CAR T cells by ZFNs via disrupting TCR α constant (TRAC), TCR β constant (TRBC), and HLA-A genes. TCR⁻ CAR T cells were anergic to TCR signaling while being CAR-

dependent functional. HLA-A⁻ CAR T cells evaded HLA-A-restricted cytotoxic T-cell lysis (Torikai et al., 2012, 2013).

TALENs are engineered nucleases with a design similar to ZFNs. A combination of DNA binding domains derived from a transcription factor is fused to FokI nuclease. Unlike triplets in ZFNs, each TAL domain corresponds to one nucleotide (Moscou & Bogdanove, 2009). In contrast to ZFNs, TALENs have simpler designs and have further improved specificity and efficiency (H. Li et al., 2020). In a preclinical study, anti-CD19 CAR T cells were engineered by TALENs to knock-out (KO) both TCR α and β chains and CD52, a target of the therapeutic mAb alemtuzumab. The alloreactivity of these CAR T cells was dampened by TCR deficiency, and the potential residue of TCR-expressing CAR T cells can be ablated by alemtuzumab. The KO-CAR T-cell lytic function was not disrupted, even in the presence of alemtuzumab (Poirot et al., 2015). Others used TALENs to generate universal CAR T cells by disrupting TRAC and B2M while introducing CAR (Das et al., 2023; Jo et al., 2022), and in one case, disrupting additionally HLA-E (Jo et al., 2022). However, one of the downsides of this approach is that the HLA-I negative CAR T cells can be eliminated by NK cells. Expression of HLA-E inhibits NK-mediated killing of HLA-deficient cells, thus further supporting CAR T engraftment (Jo et al., 2022).

However, both ZFNs and TALENs need tedious optimization during development, and every change in the recognized sequence requires re-engineering a whole enzyme pair.

CRISPR/Cas system serves its natural purpose as an adaptive immune system in bacteria and archaea. It is based on an RNA-guided endonuclease which cleaves foreign DNA (Jinek et al., 2012). In microbes, the system requires two RNAs, crRNA – CRISPR RNA and tracrRNA – trans-activating crRNA. To simplify the system, genetic engineering was used to create a chimeric single guide RNA (sgRNA). The sgRNA includes a 20-nt targeting sequence complementary to the specific locus. However, there must be a particular oligonucleotide "protospacer adjacent motif" (PAM) on the genomic target that serves as a binding signal for a specific Cas enzyme (Sternberg et al., 2014). The most widely used Cas nuclease is the Cas9 from *Streptococcus pyogenes* (SpCas9), with favorable 3'NGG PAM (Jiang et al., 2013). Upon successful binding, CRISPR/Cas9 ribonucleoprotein creates a DSB (Gasiunas et al., 2012) that is repaired by NHEJ or HDR (Ran et al., 2013). The main advantage of a CRISPR-based system is that altering the target sequence requires only re-designing the sgRNA, not a whole enzyme.

The application of the CRISPR/Cas system in CAR T-cell engineering is vast. First, similar to the previous systems, it can integrate the CAR insert while knocking-out endogenous TCR and HLA-I to make universal allogenic CAR T cells. Knocking-in the CAR gene into the TRAC locus reduced the risk of insertion-induced oncogenesis and TCR-prompted GvHD. Also, it resulted in superior T-cell potency and postponed T-cell exhaustion compared to regular CAR T cells (Eyquem et al., 2017). Moreover, CRISPR/Cas9 conveniently allows for multiplex editing. To produce CAR T cells that are

both universal and more potent, CRISPR/Cas9-mediated double-knock-outs (DKO) of TRAC and B2M and triple-knock-outs (TKO) with the addition of PD-1 were generated and showed that the gene editing did not hamper CAR T functionality (X. Liu et al., 2017). Subsequently, a one-shot CRISPR protocol was developed by including multiple gRNAs in the CAR-bearing LV vector (Ren, Zhang, et al., 2017). It was tested to make TRAC and HLA-I DKO universal CAR T cells and further Fas-deficient universal TKO CAR T cells to resist AICD. To take this to the next level, quadruple KO of universal CAR T cells with disrupted inhibitory receptors PD-1 and CTLA-4 was also feasible to generate (Ren, Zhang, et al., 2017). In another study, the researchers combined CAR delivery by LV vector with electroporation of Cas9 mRNA and multiple gRNAs to create TRAC/TRBC, B2M, and PD-1 TKO CAR T cells (Ren, Liu et al., 2017). More examples of mitigating CAR T-cell dysfunction by CRISPR-mediated PD-1 KO were tested with anti-CD19 CAR (Rupp et al., 2017), mesothelin-specific CAR for breast cancer (W. Hu et al., 2019), anti-glypican-3 CAR in HCC model (X. Guo et al., 2018), and anti-CD133 CAR on a glioma model (B. Hu et al., 2019). The KO of another inhibitory receptor, lymphocyte-activation gene 3 (LAG-3), in anti-CD19 CAR T cells was functionally comparable to regular CAR T cells (Y. Zhang et al., 2017). The disruption of TGF-β receptor II (TGFBR2) makes CAR T cells inert to its immunosuppressive effect, as was demonstrated for mesothelin-specific CAR T cells in pancreatic carcinoma xenograft and PDX models (Tang et al., 2020) and in mesothelin- and claudin-specific CAR T cells *in vitro* (Alishah et al., 2021). In another study, the KO of diacylglycerol kinase (DGK), an enzyme that participates in immunosuppressive signaling, made CAR T cells resistant to immunosuppressive mediators like TGFβ and prostaglandin E2 (Jung et al., 2018). CRISPR can also reduce CAR T-cell-related toxicities by knocking out CRS driver cytokines such as GM-CSF (Sterner et al., 2019).

A base-editing variant of the Cas9 enables precise, single-base transition changes without introducing DNA breaks. It is achieved by the fusion of catalytically impaired Cas9 protein (dCas9) with a DNA-modifying enzyme, such as cytidine deaminase or adenine deaminase (Komor et al., 2016). This approach allows gene disruption by introducing a premature STOP codon or interference with the splicing sites. This editing approach presents a significantly reduced risk of genotoxicity. It was reported that DSB generation and translocation frequency were dramatically reduced compared to Cas9 nuclease in an example of TRAC, B2M, and PD-1 TKO anti-CD19 CAR T cells (Webber et al., 2019). The following example of the base editor use in CAR T cells was to solve a problem of "T vs T" fratricide with CAR T cells against T-cell malignancies. These CAR T cells are engineered to target either CD3 or CD7 molecules. Hence, TCR/CD3 and CD7 TKO fratricide-resistant CD3- or CD7-specific CAR T cells were made by introducing premature STOP codons to circumvent this problem (Georgiadis et al., 2021).

Another employment of the dCas9 is CRISPR interference (CRISPRi), transcription repression via fusion with a Krüppel associated box (KRAB) domain transcriptional repressor. In CAR T-cell

design, this system was applied as a two-part "IF-THEN-gate" (Yang et al., 2021). The first vector contains HER2-specific CAR with fused TEV protease on its ICD, and it additionally codes sgRNA specific for PD-1 TSS. The second part represents LAT protein tethering a dCas9-KRAB via a TEV-cleavable site. Once the CAR binds HER2, the TEV protease releases the dCas9-KRAB to repress the transcription of PD-1. These CAR T cells showed increased cytokine secretion, proliferation, and *in vivo* persistence (Yang et al., 2021). Madison et al. developed a modified dCas9 fused with Clo051 nuclease domain, named Cas-CLOVER (Madison et al., 2022). In CAR T-cell manufacturing, it was successfully used to KO TRBC and B2M. Compared to conventional Cas9, the Cas-CLOVER exhibited high fidelity while maintaining reduced off-target activity and maintaining a higher percentage of the favorable stem cell memory T-cell phenotype (Madison et al., 2022).

For now, from the regulatory agency point of view, the CRISP/Cas9 system presents a quite high risk of off-target activity, deletions, and chromosomal translocations, which makes it difficult to approve in GMP-certified conditions (Kosicki et al., 2018). However, the novel-engineered Cas9 variants could solve this problem (Dimitri et al., 2022). Nonetheless, this technology was quickly adopted for testing in CAR T-cell clinical trials in the USA and China (Wei et al., 2023).

1.4.2.2.3.   Episomal vectors

Minicircle DNA (mcDNA) is a covalently closed circular DNA. Unlike conventional plasmids, it lacks any bacterial elements like replication origin and antibiotic resistance (Almeida et al., 2020). It is a non-integrating episomal vector, which allows long (up to 20 days) yet not persistent expression. The lack of bacterial immunogenic sequences makes mcDNA more likely to persist over time, although it is not replicated in transfected cells. mcDNA is associated with chromosomes and is evenly distributed into dividing cells (Kobelt et al., 2013). Minicircles are produced from a parental plasmid (PP), which contains an origin of replication, a selection marker, and a gene of interest flanked by specific recombination sites (att, loxP, MRS, attP/attB) (Figure 16). A specific recombinase mediates recombination between the two particular sites, and two circular DNA molecules are produced – mcDNA containing the gene of interest and a miniplasmid (mP) with the bacterial backbone. Subsequently, mcDNA has to be isolated from a mixture of mcDNA, mP, and residual PP by a chromatographic technique (Almeida et al., 2020). For CAR T-cell production, mcDNA can either be an episomal vector for transient expression or used for transposon-mediated integration. In preclinical studies, prostate stem cell antigen (PSCA)-targeted (J. Han et al., 2020) and CD44-targeted CAR T cells, made with electroporated episomal mcDNA, showed tumor infiltration and positive anti-tumor effect in a mouse model. The PSCA CAR T cells persisted for up to 28 days in mouse peripheral blood (J. Han et al., 2020). Another group developed a 48-hour CD19 CAR T-cell manufacturing protocol with episomal mcDNA. They emphasized the cost-effectiveness and safety of this method (Ye et al., 2023). A non-integrative and transient CAR expression might be superior to integration concerning safety. Nevertheless, long-term CAR T-cell persistence, in terms of months or even years, might be necessary

for disease control and long-term patient survival. On the other hand, using mcDNA for SB transposition in CAR T cells improves transposition rates and cell viability post-electroporation in CAR T-cell manufacturing (Hudecek et al., 2016; Monjezi et al., 2016; Prommersberger et al., 2022). Together, mcDNA as a complete virus- and bacteria-free system represents a substantial advantage for clinical regulatory requirements over LV and conventional plasmid vectors.
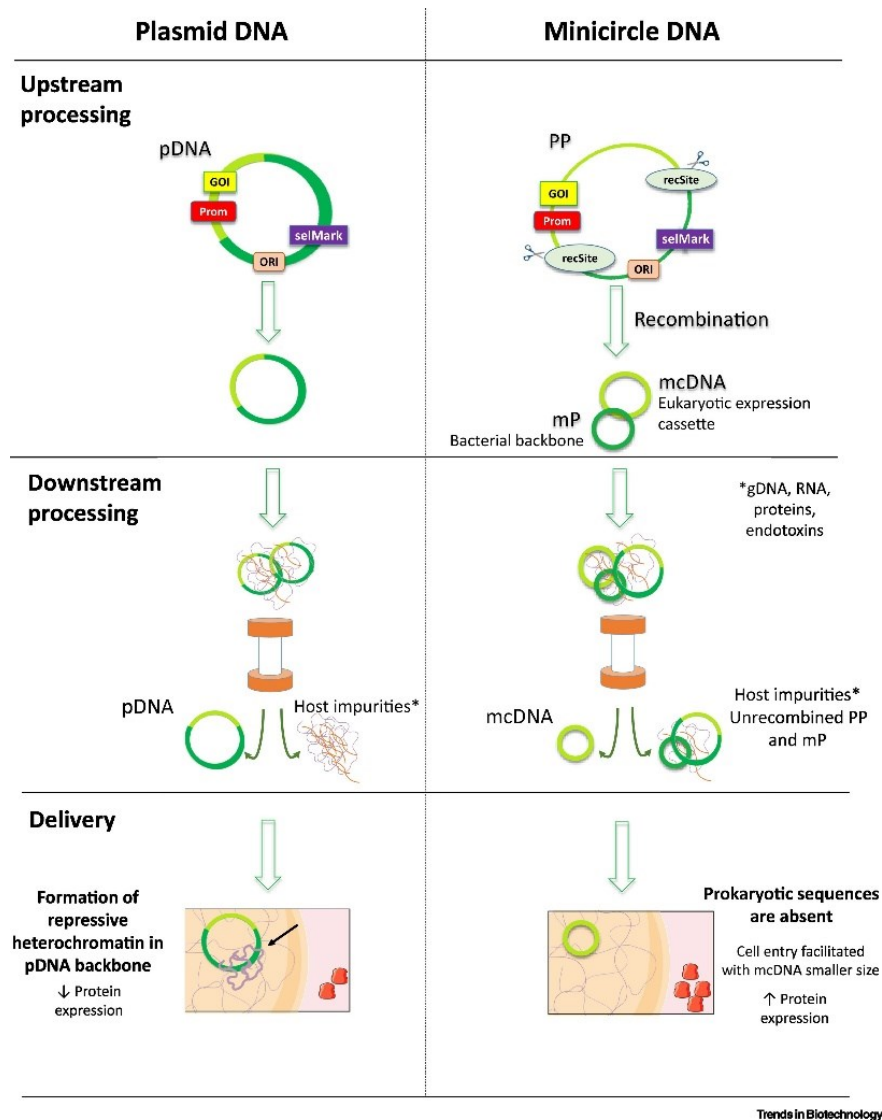
Figure 16. The production methods of plasmid and minicircle DNA vectors. Figure by Almeida et al., 2020.

Variations of plasmid vectors based on scaffold/matrix attachment region (S/MAR) motifs are unique tools that allow non-integrating yet stable transgene expression (Figure 17). Therefore, the possibility of integration-related genotoxicity is eliminated. S/MAR sequences are responsible for interaction with chromatin via nuclear matrix proteins in so-called looped domains. This enables a transcriptionally active state by association with transcription activators, extra-chromosomal maintenance and replication, and segregation during cell division (Argyros et al., 2011). These vectors have been improved in 2nd generation by decreasing immunogenic CpG motifs (Haase et al., 2010) or removing bacterial elements, as in mcDNA (Nehlsen et al., 2006). S/MAR plasmid vectors can have further

enhanced replication by human replication-initiation region (derived from β-globin locus) and were used to stably transfect T cells to make CAR T cells (Gomes et al., 2021). An alternative to mcDNA, minimally sized plasmids without antibiotic resistance gene called nano-S/MAR DNA vectors were shown to have increased transgene expression and reduced cytotoxicity in transfected cells (Bozza et al., 2020; Luke et al., 2014). This platform has been successfully used to manufacture CAR T cells by electroporation in a clinical-approved closed system. In contrast to classical LV transduction, this approach reduced costs, increased safety, and produced a clinically relevant yield of CAR T cells within just five days (Bozza et al., 2021). Another method of T-cell engineering using S/MAR elements is non-integrating lentiviral (NILV) vectors. They combine the high efficacy of the LV delivery system and circumvent integration-related risks. Tested applications ranged from long-term transgene expression and gene downregulation by RNA interference using small hairpin RNA (shRNA) to anti-CD19 CAR T-cell manufacturing, functionally indistinguishable from CAR T cells made with integrative LV vector (Jin et al., 2016).
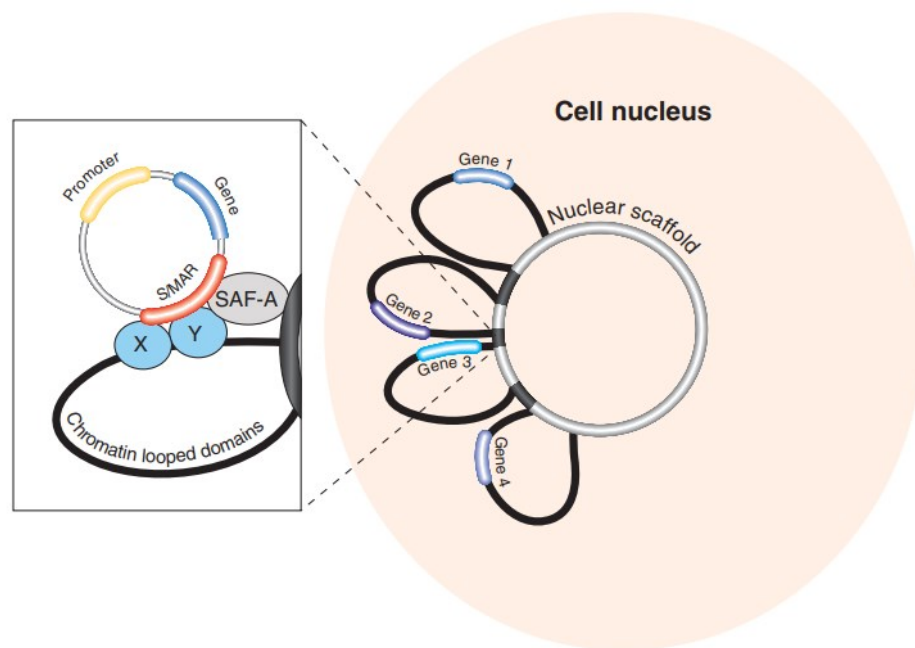
Figure 17. Non-integrative S/MAR plasmid vector. SAF-A – scaffold attachment factor A. Figure by Argyros et al., 2011.

1.4.2.2.4. mRNA

Using mRNA for genetic modifications is a promising and versatile approach with skyrocketing popularity in recent years thanks to the COVID-19 vaccines (Dolgin, 2021). After transfection, mRNA localizes in the cytoplasm and does not enter the cell nucleus. This allows transfection of non- or slow-dividing cells. Again, mRNA is not integrative, which mitigates any integration-related safety concerns. By its nature, mRNA's half-time is limited and expressed only in the short term. One of the biggest challenges with mRNA is ensuring its stability; hence, various modifications have been developed to increase its half-time and translation efficacy. They involve 5'cap analogs, 5' and 3' untranslated

regions (UTR) optimizations, codon optimization, nucleotide modifications, and 3' poly-A tail elongation (Figure 18). mRNA is delivered into cells either as naked mRNA by electroporation or a gene gun or by endocytosis in a complex with cationic polymer vehicles – lipoplexes and polyplexes (Moretti et al., 2022; Tavernier et al., 2011).
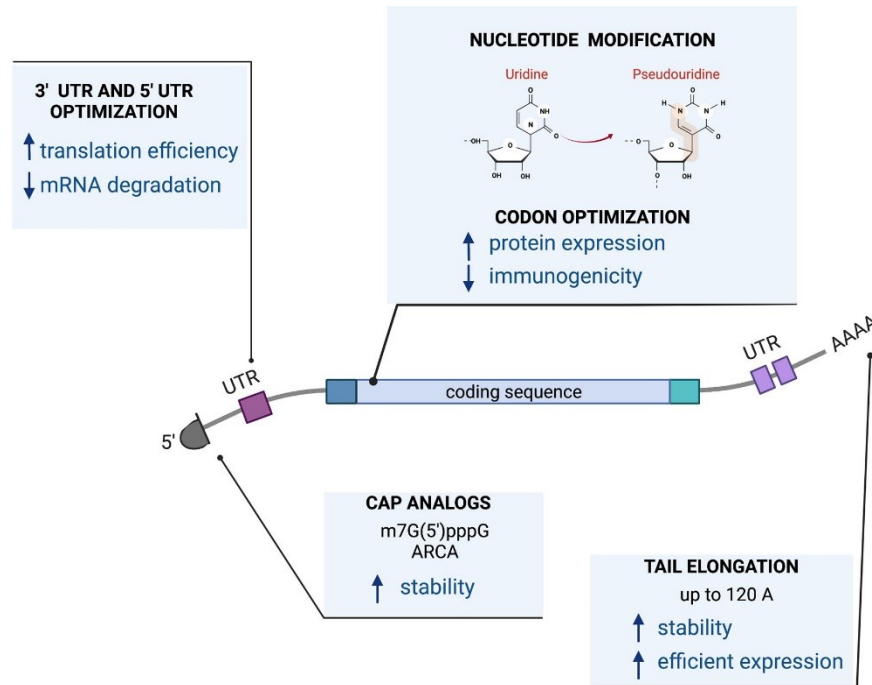
Figure 18. Optimization options of mRNA vector for transient protein expression. Figure by Moretti et al., 2022.

Transient CAR expression could improve safety when the target molecule is partially expressed on healthy tissue or with cross-reactive and highly cytotoxic CAR T cells. Conventional constitutive CAR T cells could represent a significant safety hazard in these cases. Moreover, from a regulatory point of view, mRNA transfection might be the safest and least burdensome method to approve for clinical use. Due to the transiency, multiple-dose administration protocols would likely be necessary for optimal clinical effect. For instance, in a mouse model, CAR T cells were detected only for seven days, and multiple CAR T doses were reported as beneficial (Y. Zhao et al., 2010). In preclinical models, mRNA-electroporated CAR T cells have been tested against hematological malignancies like B-ALL, B-CLL, and AML, and solid tumor models in mesothelioma and colon cancer, ovarian and breast cancer, neuroblastoma, glioblastoma, and melanoma. Generally, mRNA-CAR T cells were on-target cytotoxic, reduced tumor size, and showed lower unwanted on-target off-tumor toxicities (Rajan et al., 2020). In clinics, studies targeting antigens CD19 and CD123 for hematological malignancies and mesothelin and c-Met for solid tumors have been performed. The studies used substantial amounts of CAR T cells with 3-6 repeated administrations. (Rajan et al., 2020). No severe side effects were observed, except anaphylaxis in one study due to the IgE-mediated reaction against mouse antibody-derived scFv in CAR (Maus et al., 2013). Another critical point is that multiple dosing schemes require a tremendous amount of material from patients to manufacture the necessary number

of CAR T cells. This might not be easily feasible, especially in leukopenic, heavily pretreated patients after several lines of chemotherapy.

Another use of mRNA in CAR engineering is transposase-encoding mRNA for transposon systems such as piggyBac and *Sleeping Beauty*. Short-term transposase expression efficiently prevents random plasmid integration (Bire et al., 2013; Wilber et al., 2006).

1.4.2.2.5.   *In vivo*-generated CAR T cells

Classical CAR T-cell therapy is an *ex vivo* approach, meaning that the patient's cells are isolated, modified, and expanded *in vitro* and then reintroduced to the patient. This approach requires substantial resources, a clean room facility, GMP-certified conditions, quality control, and qualified personnel. Logistics represent another issue because the manufacturing facility is usually not a part of the hospital. After the patient's cells are collected, they need to be cryopreserved and sent for manufacturing, and then the expanded product is cryopreserved again and shipped back.   Unfortunately, due to the autologous nature, no "off-the-shelf" option is yet available. Such a complicated and costly process is often prohibitive for it to be used among the first lines of treatment. Changing the process to *in vivo* transfection would solve many of these limitations. However, new safety concerns arise, such as the transfection of non-T cells.

Transfecting circulating T cells *in vivo* is achieved by delivering nucleic acids enveloped in liposomal or polymer nanoparticles modified to target T cells. Initially, Smith et al. created DNA-carrying nanoparticles from a biodegradable polymer (poly β-amino ester) and polyglutamic acid with anchored anti-CD3e F(ab′)2 antibody fragments for T-cell specificity. These nanoparticles were loaded with two plasmids, one encoding CAR in PB transposon vector and the second PB transposase. In a preclinical mouse model, the nanoparticles effectively delivered plasmids to T-cell nuclei, prompting stable transfection. The *in vivo*-generated anti-CD19 CAR T cells expanded after contact with antigen and were successful in leukemia eradication. As for the safety measures, they argued that toxicity of CAR expressed in non-T-cell types would likely be minimal, and the short half-life of nanoparticles further secured it. In addition, they cloned CAR under a T-cell-specific promoter (Smith et al., 2017). In the following study, the same group used *in vitro*-transcribed mRNA for CAR and TCR as a vector for transient expression. The same nanoparticles were, this time, coupled with an anti-CD8 antibody instead of CD3. When compared to the use of plasmid DNA a higher transfection rate was observed, although multiple doses of nanoparticles were necessary for optimal anti-tumor effect. The approach was tested on various mouse models, with CD19-specific CAR for leukemia, receptor tyrosine kinase-like orphan receptor 1 (ROR1)-specific CAR for prostate cancer, and hepatitis B virus (HBV)-specific TCR for HBV-induced HCC, with similar outcomes to conventional *ex vivo*-generated CAR T cells (Parayath et al., 2020). A compelling study used *in vivo* generated, mRNA-transfected, non-tumor-specific CAR T cells to treat cardiac injury (Rurik et al., 2022). The CAR-coding mRNA was

encapsulated in CD5-targeted lipid nanoparticles. The CAR was targeted against fibroblast activation protein (FAP), a marker of activated fibroblasts causing fibrosis by excessive secretion of extracellular matrix. This method successfully reduced fibrosis and restored cardiac functions in a mouse model. In this context, the short-term CAR expression was an upside because, in contrast to malignancies, no long-term immune surveillance is needed. Moreover, persisting CAR T cells would interfere with physiological fibroblast activation in wound healing (Rurik et al., 2022).

As an alternative to nanoparticles, viral vectors could be used for *in vivo* CAR T-cell transduction. Two studies have been carried out with CD8-targeted LV vector for *in vivo* anti-CD19 CAR T-cell production in a mouse model with xenografted human PBMCs and lymphoma cell lines (Agarwal et al., 2019; Pfeiffer et al., 2018). Tumor cell clearance was observed in CAR-LV-injected mice. However, some mice experienced a CRS, which might have been caused by CAR-positive NK and NKT cells. A different group developed a bispecific binder targeting LV to T cells (Huckaby et al., 2021). They observed a superior anti-tumor effect in a mice xenograft lymphoma model treated with the CAR-LV vector plus the bispecific binder compared to the CAR-LV vector alone. However, not a very large number of CAR T cells were generated. Alternatively, adeno-associated virus (AAV) vectors are a non-integrative option for viral transduction. As a proof-of-concept, a CAR-carrying AAV vector was tested in a mouse xenograft model for *in vivo* T-cell transduction, leading to tumor regression (Nawaz et al., 2021). However, the issue with non-specific delivery is a concern.

The *in vivo* CAR T approach could offer significant overall cost reduction, a simplified manufacturing process, and a long-term stable "off-the-shelf" therapeutic product, which could facilitate the widespread use of CAR T therapy. Another prospect could be combining *in vivo* CRISPR-based technology with CAR T cells, although it has not yet been reported. However, from the regulatory point of view, the *in vivo* transduction must prevent off-target transduction, as it might pose risks of unexpected adverse effects.

	Transgene Insertion	Transgene Expression	Transgene Lifespan	Transgene Delivery	Types	Features and Observations
Retroviral vectors	non-targeted integration	exogenous promoter	long	*ex vivo* transduction	gamma retrovirus	safe, optimized, and FDA-approved protocols; the production of the therapeutic cells is expressive
					lentivirus	transgene size limitation
Transposase enzymes	non-targeted integration	exogenous promoter	long	*ex vivo* electroporation	Sleeping Beauty	FDA-approved protocols, more economical than viral vectors, but less developed technology; transposase have to be electroporated along with the donor DNA (plasmid or minicircles)
					piggyBac	
mRNA	non- integrative	N/A	short	*ex vivo* electroporation	N/A	fast and economical method to produce CAR T cells; the transgene expression is rapidly diluted over the expansion of the T cells; ideal when first introducing a novel CAR into patients
Non-integrative lentivirus	non-integrative, episomal	exogenous promoter	mid-short	*ex vivo* transduction	NILV-S/MAR	the transient expression can be extended up to 30 days; the production of the non-integrative lentivirus is expensive and will still require constant re-dosing
Endonuclease enzymes	targeted integration	endogenous promoters	long	*ex vivo* electroporation	zinc-finger	directed transgene insertion into the host cell genome; ability to ablate specific host cell genes; endonucleases have to be electroporated along with donor DNA (AAV or linear dsDNA); further protocol optimization is required
					TALENs	
					CRISPR/Cas9	

Table 1. Summary of genetic modification methods used for CAR T cell manufacturing. NILV-S/MAR, non-integrating lentiviral vector containing a scaffold/matrix attachment region. Table by Guedan et al., 2019.

1.4.3. Activation of CAR T cells

For (CAR) T cells to start proliferating, they must be activated. This step varies, depending on the protocol. Usually, RV/LV transduction requires T-cell activation before transduction. Electroporated cells and cells transfected with non-viral vectors can be activated before or after transfection.

Engagement of both TCR (signal 1) and a costimulatory molecule, e.g. CD28 (signal 2), is pivotal, as only signal 1 alone leads to T-cell anergy or apoptosis (Schwartz, 1990). Anti-CD3 plus anti-CD28 antibodies are the golden standard for *in vitro* T-cell activation (Levine et al., 1997; Vormittag et al., 2018). They can be either immobilized by coating on the bottom of the cultivation well or by using beads – magnetic (Dynabeads[1] by Gibco, Thermo Fisher Scientific, Waltham, MA, USA), polymer beads (TransAct[2] by Miltenyi Biotec, Bergisch Gladbach, Germany), or dissolvable beads (Cloudz[3], by Bio-Techne, Minneapolis, MN, USA). Magnetic beads have to be removed after manufacturing.

Physiological T-cell activation is mediated by APCs. Autologous APCs would be ideal. However, their isolation from patients in sufficient numbers and further logistics for CAR T-cell manufacturing would be troublesome (Levine, 2015). Artificial APCs (aAPC) and antigenic stimulation by feeder cells expressing CAR target molecules and their combination could represent a feasible and efficient way for stimulating CAR T-cell expansion. However, APCs or feeder cells need to be irradiated to prevent their overgrowth. A way to engineer aAPCs is by transduction of cell line K562 with genes

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[1] https://www.thermofisher.com/order/catalog/product/11132D
[2] https://www.miltenyibiotec.com/US-en/products/t-cell-transact-human.html#130-111-160
[3] https://www.bio-techne.com/p/cell-culture/cloudz-human-t-cell-activation-kit_cld001

of costimulatory molecules such as CD80, CD86, CD83, and 4-1BB ligand (4-1BBL, CD137L) (Suhoski et al., 2007). These K562-based aAPCs were compared with similarly transduced autologous PBMC-based aAPCs for stimulating anti-CD19 CAR T cells. Both types of cells potently stimulated CAR T-cell growth. It has to be pointed out that K562s have an advantage in reproducibility over PBMCs due to their interpersonal variability (Nakazawa et al., 2009). Another group successfully tested K562 aAPCs expressing 4-1BBL and major histocompatibility complex class I chain-related molecule A (MICA) in their anti-CD19 CAR T-cell expansion protocol (Numbenjapon et al., 2007). A similar study confirmed the positive effect of K562-based aAPCs expressing CD64, CD86, and 4-1BBL on CAR T cells, and they further showed that X-ray irradiation was a viable alternative to γ-irradiation (Bui et al., 2023). In a study by Nakamura et al., they emphasized the importance of (artificial) immunological synapses for optimal T-cell activation. To generate aAPCs, they transfected autologous PBMCs with truncated versions of the CAR target molecule (HER2) and costimulatory molecules (CD80, 4-1BBL). The anti-HER2 CAR T cells showed substantial expansion and were of an early memory phenotype with minimal expression of exhaustion marker PD-1 (Nakamura et al., 2021). Two studies by a Japanese group tested irradiated autologous activated T cells as feeder cells for CD19-specific CAR T cells (Morita et al., 2018; Saito et al., 2014). The generated CAR T cells exhibited an early-memory phenotype, and in the second study, they observed enhanced CAR expression when compared to CAR T cells without the feeder (Morita et al., 2018).

1.4.4. Cultivation vessels

The cultivation step is crucial for expanding cells to reach the desired number of cells for clinical use. Large-scale cultivation of clinical-grade CAR T cells in GMP-compliant conditions requires additional safety measures to avoid contamination and human error. Rather than conventional cultivation flasks, unique vessels with closed or semi-closed systems and possible automation are more favorable in this context.

The G-Rex (Wilson Wolf Manufacturing, St. Paul, MN, USA) platform offers cultivation flasks or bioreactors with a gas-permeable membrane at the flask's bottom, allowing oxygen and carbon dioxide to be exchanged (Figure 19A). This leads to increased proliferation of cultivated cells in much higher concentrations than in regular flasks. The bioreactors are fully closed systems with tubes connected to a pump, allowing media exchange and cell collection (Ludwig & Hirschel, 2020).

WAVE bioreactor (Cytiva, Marlborough, MA, USA) is a cell bag on an agitation platform with an automatic system that controls the concentration of oxygen and pH (Figure 19B). Exchanging cultivation media and collecting cells is facilitated by tubes with sterile filters and a pump (Meng et al., 2018).
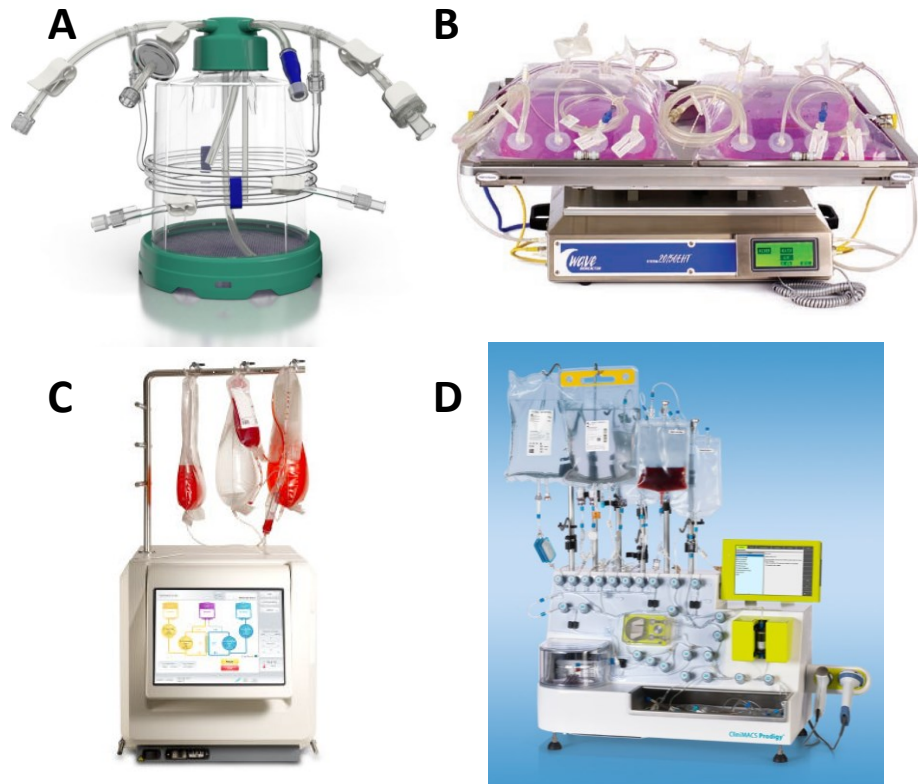
Figure 19. Cultivation vessels for CAR T cells.
(A) Fully closed G-Rex bioreactor, figure by https://www.wilsonwolf.com/product-and-order-info/.
(B) Wave bioreactor, figure by https://acmerevival.com/wp-content/uploads/2021/03/GEWaveBioreactorSystem210EHBrochure.pdf.
(C) Quantum cell expansion system, figure by https://www.medicalexpo.com/prod/terumo-bct/product-75244-850081.html.
(D) CliniMACS prodigy, figure by https://www.miltenyibiotec.com/DE-en/products/clinimacs-prodigy.html.

Quantum Cell Expansion System (Terumo Blood and Cell Technologies, Lakewood, CO, USA) is another automated and fully closed platform (Figure 19C). It uses hollow-fiber bioreactors instead of cultivation bags, which permits the cultivation of both suspension and adherent cell types (Coeshott et al., 2019).

CliniMACS Prodigy (Miltenyi Biotec, Bergisch Gladbach, Germany) is an advanced, fully closed system that combines magnetic sorting of T cells, transduction with LV vector or electroporation of non-viral vectors, T-cell activation, and expansion into one instrument (Figure 19D). The all-in-one solution offers reproducibility due to automation, scalability, and flexibility (Lock et al., 2022; Mock et al., 2016).

1.4.5. Cytokines for CAR T cultivation

Cytokine supplementation to the cell culture media is essential for T-cell expansion. This is also referred to as "signal 3". The most widely used cytokine is IL-2, followed by other members of the common γ-chain family cytokines, notably IL-7, IL-15, and IL-21 (Stock et al., 2019). IL-2 strongly supports T-cell proliferation. However, it drives T cells into an effector phenotype that is linked with potent cytotoxic functions, yet the cells are only short-lived (Liao et al., 2013). Although, only high IL-2 concentrations had this phenotypic effect (Kaartinen et al., 2017). Therefore, a combination of cytokines is required to maintain early-differentiated memory T-cell subsets known for their high proliferative capacity and persistence.

Most expansion protocols that omit IL-2 are based on IL-7, often with the addition of IL-15. Indeed, IL-7 + IL-15 is a popular combination that supports the retention of central memory (Kaneko et al., 2009), and stem cell memory phenotype, leading to better T-cell survival (Cieri et al., 2013; Gargett & Brown, 2015; Xu et al., 2014). Compared to IL-2, the cytokine combination IL-7 + IL-15 significantly enhanced the overall anti-tumor effect of CAR T cells (Gargett & Brown, 2015; Xu et al., 2014; J. Zhou et al., 2019) and promoted a superior activation and expansion of CAR T and transgenic TCR T cells (Gargett & Brown, 2015; Gong et al., 2019; Hoffmann et al., 2018). The addition of IL-15 alone supports stem cell memory phenotype by inhibiting mTORC1 and improving the metabolic fitness of T cells (Alizadeh et al., 2019).

An especially interesting cytokine for CAR T-cell cultivation is IL-21. In particular, IL-21 enhances T-cell activation, expansion, and survival (Hinrichs et al., 2008; Moroz et al., 2004; Singh et al., 2011), and strongly promotes cytotoxic functions while maintaining an early-memory phenotype (Hinrichs et al., 2008; Santegoets et al., 2013; Singh et al., 2011). In terms of T-cell phenotype and metabolic programs, IL-21 acts as an IL-2 antagonist, as they both drive T cells into opposite fates (Hinrichs et al., 2008). IL-21 suppresses terminal differentiation of T cells, promotes the metabolism of fatty acid oxidation instead of glycolysis, and increases mitochondrial biogenesis (Loschinski et al., 2018). Furthermore, IL-21 suppresses Tregs (Attridge et al., 2012; Peluso et al., 2007). Cultivating CAR T cells in the presence of IL-21, IL-7, and glycogen synthase-3β inhibitor WTS119 resulted in stem cell memory CAR T cells with improved metabolic fitness, providing a long-term anti-tumor effect in ALL xenograft model (Sabatino et al., 2016). The combination of IL-21 with IL-15 enhanced CAR expression, *in vitro* early-memory phenotype, and CAR T-cell effector functions (Lamers et al., 2014). Our protocol relies on the combination of IL-4, IL-7, and IL-21 (Ptáčková et al., 2018). The IL-4 and IL-7 give strong pro-survival signals, and the addition of IL-21 supports the early differentiated memory phenotype, together with low expression of inhibitory receptors.

After cultivation, the CAR T-cell product is cryopreserved. Before administration and during manufacturing, thorough quality control testing must be carried out (Gee, 2018).

1.5. T-cell phenotype

A set of distinct functional and phenotypic attributes characterizes the T-cell memory phenotype. The functional elements include T-cell trafficking, homing, effector functions, longevity, and durability of the immune response. From the functional spectrum, several T-cell memory subsets are traditionally defined by a combination of surface antigens (Figure 20). From the earliest to the latest differential stage, T cells go from naïve T (T_N) cells to stem cell memory T (T_{SCM}) cells and central memory T (T_{CM}) cells, after which follows effector memory T (T_{EM}) cells, and terminally differentiated effector T (T_{TE}, T_{EMRA} or T_{EFF}) cells (Jameson & Masopust, 2018).

T-cell development finishes in the thymus, resulting in mature T_N cells. They are antigen-inexperienced, and they subsequently migrate into the periphery. T_N cells are characterized by the expression of CD45RA, CD62L (L-selectin), and C-C chemokine receptor 7 (CCR7) (Van Den Broek et al., 2018). The CD45RA is the long CD45 isoform on T_N, T_{SCM}, and T_{TE} cells, and its expression is inverse to the CD45RO short isoform. CD62L and CCR7 are important for homing to secondary lymphoid tissue such as lymph nodes. Moreover, T_N cells are positive for costimulatory molecules CD27 and CD28 and the IL-7 receptor (CD127). Notably, T_N cells are negative for CD95 (Fas receptor); it is expressed after antigen stimulation for the possible negative selection by apoptosis (Gattinoni et al., 2017; Van Den Broek et al., 2018). After antigen stimulation, T_N cells undergo expansion and differentiation to other memory stages. T_{SCM} cells retain the same immunophenotype as T_N cells – CD45RA$^+$CD45RO$^-$CCR7$^+$CD62L$^+$CD27$^+$CD28$^+$, except for the high expression of CD95, chemokine C-X-C motif receptor 3 (CXCR3), CD122 (IL-2Rβ, IL-2 receptor β chain), and LFA-1 (CD11a) (Gattinoni et al., 2011; Lugli et al., 2013). T_{SCM} cells are minimally differentiated and capable of multipotency, incredible self-renewal, and persistence. Thus, they are of great interest to adoptive T-cell therapies. T_{CM} cells represent the next differentiation stage. They are phenotypically similar to T_{SCM} except for the exchange of CD45RA to the short isoform CD45RO (Mahnke et al., 2013). The T_{CM} cells are capable of cytotoxicity to a lower extent than the later effector stages. Nevertheless, their ability to proliferate and persist outperforms the effectors in anti-tumor response (Sallusto et al., 2004). Further differentiation of T_{CM} cells leads to the T_{EM}, accompanied by the loss of the expression of lymph node homing molecules CD62L and CCR7. Therefore, T_{EM} cells migrate toward the periphery (Masopust et al., 2001). Additionally, T_{EM} stepwise stops expressing costimulatory molecules CD27 and CD28, which correlates with a gradual increase in cytotoxic functions (Romero et al., 2007). The CXCR3 and IL-7Rα are also downregulated (Mahnke et al., 2013). Finally, under prolonged antigen exposure, T_{EM} cells differentiate into the terminal effector stage. These T_{TE} cells re-express the CD45RA isoform, hence the term T_{EMRA}. Phenotypically, T_{TE} are CD45RA$^+$CD62L$^-$CCR7$^-$CD28$^-$CD27$^-$ and have the shortest telomeres of all the subsets (Romero et al., 2007). Also, they are prone to T-cell exhaustion and senescence, accompanied by the expression of markers like CD57 and KLRG1 (Mahnke et al., 2013). They are highly cytotoxic but lack longevity and self-renewal. The vast majority of T_{TE} or T_{EMRA}

cells die as a result of antigen clearance (Stock et al., 2019). T-cell early-memory subsets' enhanced persistence and proliferative capacity can improve CAR T-cell products to exert better clinical responses (El Marabti & Abdel-Wahab, 2023; Meyran et al., 2021).

	T_N	T_{SCM}	T_{CM}	T_{EM}	T_{TE}
CD45RA	+	+	−	−	+
CD45RO	−	−	+	+	−
CCR7	+	+	+	−	−
CD62L	+	+	+	−	−
CD28	+	+	+	+/−	−
CD27	+	+	+	+/−	−
IL-7Rα	+	+	+	+/−	−
CXCR3	−	+	+	−	−
CD95	−	+	+	+	+
CD11a	−	+	+	+	+
IL-2Rβ	−	+	+	+	+
CD58	−	+	+	+	+
CD57	−	−	−	+/−	+

Stemness	Senescence
Proliferative potential	Cytotoxicity
Lymphoid homing	Tissue tropism
Antigen independence	Antigen addiction
Lipid metabolism	Glycolytic metabolism
Low $\Delta\psi m$	Oxidative stress
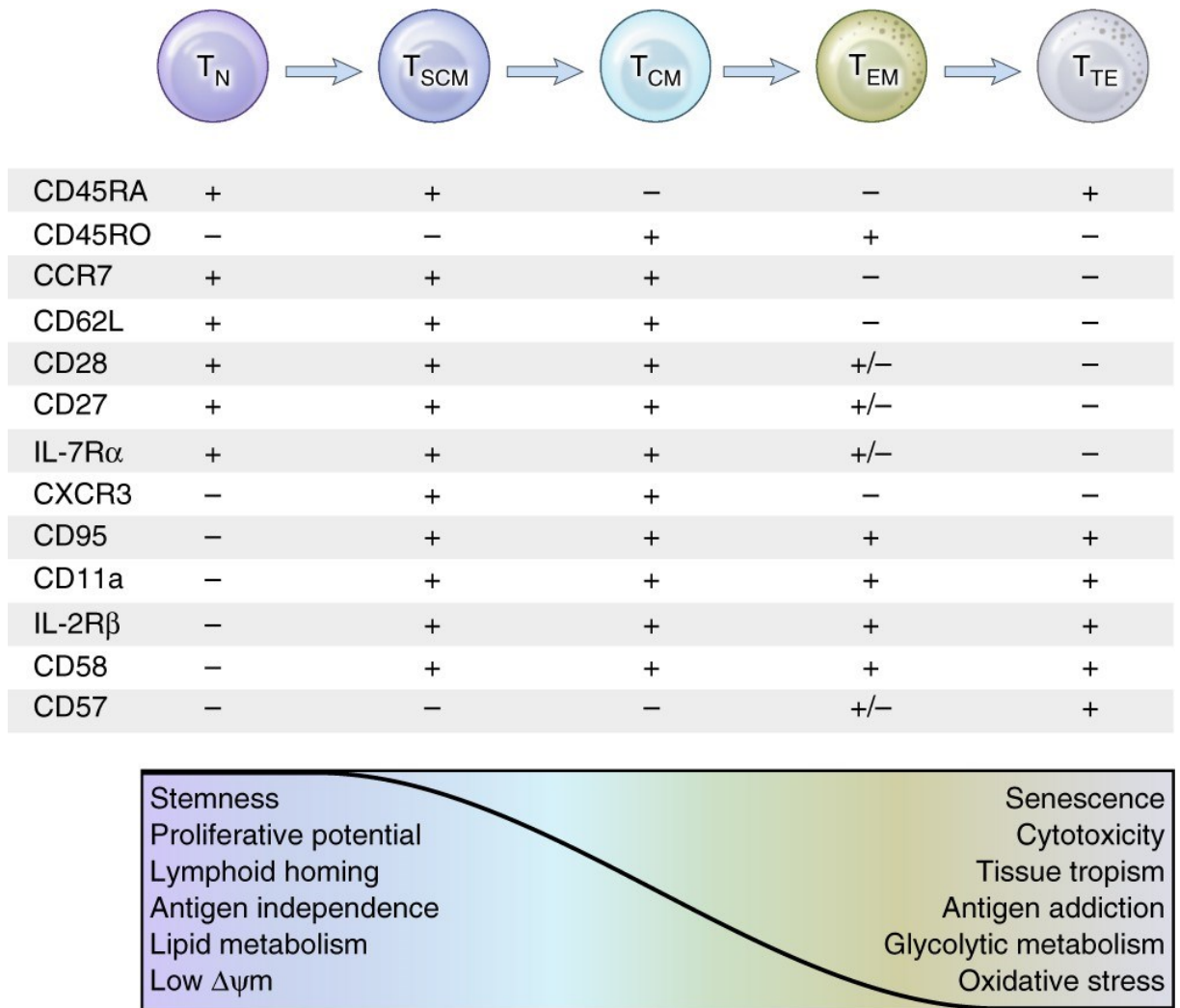
Figure 20. Differentiation of the T-cell memory subsets. T_N – naïve T cells, T_{SCM} – stem cell memory T cells, T_{CM} – central memory T cells, T_{EM} – effector memory T cells, T_{TE}- terminal effector T cells. $\Delta\Psi m$ – mitochondrial membrane potential. Figure by Gattinoni et al., 2017.

1.6. T-cell exhaustion and tonic signaling

Long-term antigen exposure, such as during chronic infections, cancer, and autoimmunities, leads to exhausted T cells (Gallimore et al., 1998; Zander & Cui, 2023). This state is characterized by progressive loss of effector functions and proliferative capacity. It is accompanied by the downregulation of perforin/granzyme B, dampening cytokine secretion, altered metabolism, and epigenetic regulation (Belk et al., 2022; Gumber & Wang, 2022). Exhaustion is additionally marked by the sustained expression of checkpoint inhibitory receptors like PD-1, CTLA-4, T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and LAG-3 (Gumber & Wang, 2022). Additionally, exhausted T cells have short telomeres and express senescence marker CD57 (Brenchley

et al., 2003). There is a spectrum of exhaustion stages; an early subset can be "re-invigorated" by PD-1 blockade, while terminally exhausted T cells are insensitive to it (Beltra et al., 2020).

Due to the functional impotence, T-cell exhaustion is one of the occurring mechanisms of CAR T-cell therapy failure (Fraietta et al., 2018). Besides the general mechanisms of inducing exhaustion, like immunosuppressive TME and inhibitory receptors, cytokines (IL-10, TGF-β), and cells (Tregs, MDSCs) (Gumber & Wang, 2022), a CAR-specific mechanism was found to contribute as well. Particularly, antigen-independent activation, called "tonic CAR signaling", can lead to exhaustion and AICD. It is caused by increased CAR clustering and subsequent persistent signaling (Guedan et al., 2018; Long et al., 2015). The CD28-bearing CAR constructs are more prone to exhaustion than the 4-1BB ones (Frigault et al., 2015; Long et al., 2015). The selection of the expression system, particularly using strong promoters, can cause the overexpression of CAR molecules on the T-cell surface, resulting in tonic signaling (Frigault et al., 2015; Guedan et al., 2018). Gomes-Silva et al. reported that the choice of vector for CAR T-cell manufacturing could influence tonic signaling (Gomes-Silva et al., 2017). In their study, the LTR in a non-self-inactivating γ-RV vector caused a positive feedback loop and enhanced CAR expression, which, in turn, enhanced tonic signaling. Using a self-inactivating LV vector mitigated the tonic signaling (Gomes-Silva et al., 2017). Next, the type and length of the hinge region affected tonic signaling (Watanabe et al., 2016). Another strategy that minimized the tonic signaling was positioning the 4-1BB domain farther away from the cell membrane in a 3rd-generation CAR (Guedan et al., 2018). Additionally, targeting the CAR gene to the TRAC locus using CRISPR/Cas9 averted the tonic signaling by ensuring the optimal CAR expression levels (Eyquem et al., 2017).

1.7. Registered CAR T-cell products

Developing a CAR T-cell product from a preclinical model to clinical trials and finally to a registered product is a highly complex process. Even though the number of clinical trials with CAR T cells against various cancers is enormous and growing yearly, the only registered products are against hematological malignancies. There are six FDA-approved commercial products, all of which are 2nd-generation CAR designs (Figure 21). Four are targeting CD19 – tisagenlecleucel (tisa-cel, Kymriah™ by Novartis), axicabtagene ciloleucel (axi-cel, Yescarta™ by Kite Pharma/Gilead), brexucabtagene autoleucel (brexu-cel, Tecartus™ by Kite Pharma/Gilead), lisocabtagene maraleucel (liso-cel, Breyanzi™ by Juno Therapeutics / BMS); and two are against B-cell maturation antigen (BCMA) – idecabtagene vicleucel (ide-cel, Abcema™ by Bluebird Bio / BMS), and ciltacabtagene autoleucel (cilta-cel, Carvykti™ by Janssen / J&J). All of them are manufactured from autologous T cells transduced by RV/LV vectors.

All four CD19 CAR T products, tisa-cel, axi-cel, brexu-cel, and liso-cel, have their scFv derived from the exact mouse mAb clone FMC63 (Cappell & Kochenderfer, 2023). Axi-cel[4] and brexu-cel[5] designs are identical, incorporating the CD28 hinge, transmembrane, and costimulatory domains; they vary in their production methods. In brexu-cel manufacturing, malignant cells are eliminated from the patient leukapheresis before transduction (M. Wang et al., 2020). Tisa-cel[6] includes CD8α hinge and transmembrane regions and the 4-1BB costimulatory domain. Liso-cel[7] employs IgG4 hinge, CD28 transmembrane, and 4-1BB costimulatory domains. Besides, it further contains separate truncated EGFR as a safety switch[8] (Ogasawara et al., 2022). Additionally, in liso-cel manufacturing, the CD4$^+$ and CD8$^+$ T-cells are separated, and then processed independently, and finally, the CAR T-cell product is administrated at a defined CD4$^+$/CD8$^+$ ratio (Teoh & Brown, 2022). Γ-RV vectors in axi-cel and brexu-cel achieve the transduction of the CAR gene, while in the case of tisa-cel and liso-cel, LV vectors are used (Cappell & Kochenderfer, 2023).

The BCMA CAR T product ide-cel[9], during development known as bb2121, derives its scFv from a mouse mAb clone 11D5-3 (Friedman et al., 2018). The BCMA-binding domain of cilta-cel[10] is composed of two connected camelid heavy-chain-only variable (VHH) fragments against two distinct BCMA epitopes (Chekol Abebe et al., 2022). Both products contain CD8α hinge and transmembrane regions and the 4-1BB costimulatory domain. Likewise, both products are manufactured via LV transduction. They are both indicated for the treatment of adult relapsed refractory multiple myeloma (RRMM) (Anderson, 2022; Chekol Abebe et al., 2022). Axi-cel, tisa-cel, and liso-cel are indicated for treating recurrent large B-cell lymphoma (LBL). Axi-cel and tisa-cel are additionally used for follicular lymphoma (FL). Tisa-cel and brexu-cel have been further approved for recurrent B-cell acute lymphoblastic leukemia (B-ALL), and brexu-cel is mainly indicated for recurrent mantle cell lymphoma (MCL).

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[4] https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/yescarta
[5] https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/tecartus-brexucabtagene-autoleucel
[6] https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/kymriah-tisagenlecleucel
[7] https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/breyanzi-lisocabtagene-maraleucel
[8] https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/breyanzi-lisocabtagene-maraleucel
[9] https://www.fda.gov/vaccines-blood-biologics/abecma-idecabtagene-vicleucel
[10] https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/carvykti

Figure 21. Overview of commercial CAR T-cell products. LBCL – large B cell lymphoma, FL – follicular lymphoma, MCL – mantle cell lymphoma, B-ALL – B-cell acute lymphoblastic leukemia, RRMM – relapsed refractory multiple myeloma. Figure by Cappell & Kochenderfer, 2023.

2. AIMS

The aims of this thesis revolve around CAR T-cell manufacturing and how their phenotype affects anti-tumor functions.

Specific aims were:

1) Determination of the influence of cultivation in IL-4 + IL-7 + IL-21 on the phenotype of CAR T cells

2) Verification of the 4th-generation CAR construct with inducible secretion of IL-21 and characterization of the effect of IL-21 on CAR T-cell immunophenotype and effector functions

3) Implementation of the CAR T-cell production method with transposon vector using electroporation of linear DNA and mRNA

4) Measurement and analysis of the immunophenotype of CAR T cells in a group of patients treated with the product tisagenlecleucel

3. MATERIAL AND METHODS

The material, methods, and flow cytometry panels are described in each publication or their supplementary materials.

3.1. Instruments

Flow cytometer BD LSRFortessa™ (BD Biosciences, San Diego, CA, USA)

Laser Name	Wavelength	Detector	Mirror	Filter	Parameter
UV	355	A	690 LP	740/35 BP	BUV737
		B	570LP	585/15 BP	BUV563
		C		450/50 BP	Indo-1 (Violet)
Violet	405	A	750 LP	780/60	BV786
		B	690 LP	710/50	BV711
		C	630 LP	670/30 BP	BV650
		D	600 LP	610/20 BP	BV605
		E	505 LP	525/50 BP	AmCyan
		F		450/50 BP	Pacific Blue
Blue	488	A	685 LP	710/50 BP	PerCP-Cy5-5
		B	505 LP	530/30 BP	FITC
		C		488/10 BP	SSC
YG	561	A	750 LP	780/60 BP	PE-Cy7
		B	635 LP	670/30 BP	PE-Cy5
		C	600 LP	610/20 BP	PE-Texas Red
		D		585/15 BP	PE
Red	640	A	750 LP	780/60 BP	APC-Cy7
		B	690 LP	730/45 BP	Alexa Fluor 700
		C		670/14 BP	APC

Table 2. Configuration of the flow cytometer BD LSRFortessa.

4. PUBLICATIONS

4.1. Publication 1

Ptáčková P, Musil J, **Štach M**, Lesný P, Němečková Š, Král V, Fábry M, Otáhal P.

A new approach to CAR T-cell gene engineering and cultivation using piggyBac transposon in the presence of IL-4, IL-7, and IL-21.

Author contribution: cell cultivation, cytotoxicity assays, manuscript editing

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T CELL THERAPY

A new approach to CAR T-cell gene engineering and cultivation using piggyBac transposon in the presence of IL-4, IL-7 and IL-21

PAVLÍNA PTÁČKOVÁ[1,★], JAN MUSIL[1,★], MARTIN ŠTACH[1], PETR LESNÝ[1], ŠÁRKA NĚMEČKOVÁ[1], VLASTIMIL KRÁL[2], MILAN FÁBRY[2] & PAVEL OTÁHAL[1,3]

[1]*Institute of Hematology and Blood Transfusion, Prague, Czech Republic,* [2]*Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic, and* [3]*Department of Hematology, First Faculty of Medicine and General University Hospital, Prague, Czech Republic*

Abstract

Background aims. Clinical-grade chimeric antigenic receptor (CAR)19 T cells are routinely manufactured by lentiviral/ retroviral (LV/RV) transduction of an anti-CD3/CD28 activated T cells, which are then propagated in a culture medium supplemented with interleukin (IL)-2. The use of LV/RVs for T-cell modification represents a manufacturing challenge due to the complexity of the transduction approach and the necessity of thorough quality control. *Methods.* We present here a significantly improved protocol for CAR19 T-cell manufacture that is based on the electroporation of peripheral blood mononuclear cells with plasmid DNA encoding the piggyBac transposon/transposase vectors and their cultivation in the presence of cytokines IL-4, IL-7 and IL-21. *Results.* We found that activation of the CAR receptor by either its cognate ligand (i.e., CD19 expressed on the surface of B cells) or anti-CAR antibody, followed by cultivation in the presence of cytokines IL-4 and IL-7, enables strong and highly selective expansion of functional CAR19 T cells, resulting in >90% CAR+ T cells. Addition of cytokine IL-21 to the mixture of IL-4 and IL-7 supported development of immature CAR19 T cells with central memory and stem cell memory phenotypes and expressing very low amounts of inhibitory receptors PD-1, LAG-3 and TIM-3. *Conclusions.* Our protocol provides a simple and cost-effective method for engineering high-quality T cells for adoptive therapies.

Key Words: *CAR T cells, cancer immunotherapy, IL-4, IL-7, IL-21, piggyBac transposon*

Introduction

Current manufacture of clinical-grade CAR T cells is primarily based on lentiviral/retroviral (LV/RV) transduction of T cells followed by anti-CD3/CD28 activation, which enables generation of large numbers of transduced T cells. However, the use of viral vectors for clinical grade manufacturing has to follow a complicated set of rules to minimize biohazards associated with their use. Such a high level of manufacturing complexity leads to high costs and limits the availability of the technology itself. Thus, there is a general need for an easier method of CAR-T manufacture. One such approach is based on the electroporation of transposon vectors such as Sleeping Beauty or piggyBac in the form of plasmid DNA [1,2]. These methods are,

however, considered less efficient than LV/RV because of poor cell growth after electroporation and lower transduction efficiency compared with LV/RV. However, the use of transposons significantly reduces the overall costs of the manufacturing process and reduces the biological risks associated with the use of LV/RV [3]. Additionally, the maximum size of LV/ RV is limited to approximately 10kB base pairs between LTRs, whereas transposons such as piggyBac can easily integrate far larger constructs, and their effectiveness is basically limited only by decreasing efficiency of electroporation of larger DNA molecules [4]. Recent discoveries in the CAR field suggest that there is a need to transfer multiple transgenes simultaneously, such as suicide genes, cytokines, decoy receptors, dominant negative receptors or multiple CARs [5].

Transfer of such large DNA sequences via LV/RV is impossible, which thus justifies the effort to improve transposon-based techniques of T-cell gene engineering.

The manufacture of CAR T cells is usually achieved by anti-CD3/CD28 activation followed by cultivation in the presence of exogenous interleukin (IL)-2 [6]; proliferating T cells are then infected with LV/RV to express the CARs. This is a well-established approach but one that has several limitations (in addition to low capacity of LV/RV); for example, it also induces an expansion of non-transduced T cells, and the anti-CD3/CD28 activation in combination with exogenous IL-2 drives T cells toward terminal effector differentiation at the expense of memory cell formation [7]. Currently, several alternative methods for T-cell expansion have been described; these protocols substituted IL-2 for other cytokines, such as IL-4, IL-7, IL-15 or IL-21, or activators of the Wnt pathway, such as glycogen synthase-3β inhibitor TWS119 [8], which increased the proportion of less-differentiated T cells with central memory/stem cell memory features. Some of these protocols were originally developed for the manufacture of multivirus-specific T cells [9], and they are based on T-cell stimulation with antigenic peptides derived from viral antigens, followed by cultivation in the presence of cytokines IL-4 and IL-7. This cytokine mix supports the retention of a central memory phenotype by T cells and promotes T-cell survival by up-regulating anti-apoptotic molecules. This procedure has been used to manufacture Good Manufacturing Practice (GMP)-grade multivirus-specific T cells, which were then successfully used for the treatment of viral infections such as cytomegalovirus, Epstein-Barr virus and adenovirus in patients after allogeneic bone marrow transplantation [10]. Similar protocols based on IL-7 and IL-21 were also developed for the manufacture of CAR T cells [8,11–13]. These results thus indicate that the differentiation of CAR T cells toward the terminal effector stage can be reduced by cytokines IL-7 and IL-21.

The quality of the manufactured product depends not only on the functional properties of CAR T cells but also on their frequency, which is the result of the transduction efficiency. Because the transduction of larger DNA constructs using LV/RV is inefficient, certain methods have been developed to enrich for transduced T cells. A nice example of this that was tested clinically is a procedure based on the co-transduction of a chimeric IL-4/IL-2 receptor, which delivers a selective mitogenic signal to transduced T cells by culturing them in IL-4 instead of IL-2 [14]. Although this approach works, it prevents the use of alternative cytokine combinations and requires modification with another chimeric cytokine receptor. We present in this article an easier technique that is based

on a selective activation of transduced T cells with anti-CAR antibody or through recognition of the CAR-specific ligand (i.e., CD19 in the case of CAR19 T cells). We show that the activation of CAR either by recognition of CD19⁺ B cells or by anti-CAR antibody in the presence of exogenous IL-4 and IL-7 enables selective proliferation of transduced T cell. Next, we show that IL-21 (in combination with IL-4 and IL-7) prevents terminal differentiation of CAR19 T cells and reduces expression of inhibitory receptors TIM-3 and PD-1 by CAR19 T cells. Our method represents a substantial improvement of currently used electroporation/transposon-based methods of CAR19 manufacturing because it does not require special feeder cells and enables reliable and simple generation of clinical doses of CAR19 T cells.

Methods

Cell source

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy-coats obtained from blood donors or from fresh whole blood samples obtained from lymphoma patients. The use of all human materials was approved by the institutional review boards, and all donors signed an informed consent with the study.

DNA plasmids and sequences

As has been previously described [15], CAR19 contains 4-1BB costimulatory domain and T-cell receptor zeta domain. Prostate specific membrane antigen (PSMA) CAR has been prepared by cloning Vh and Vl chains from antibody J591 followed by Myc epitope into the 4-1BB-zeta CAR backbone via InFusion cloning. Both CARs were cloned into the piggyBac vector behind the UBC promoter via BamHI/EcoRI [1] to generate pPB CAR19 and pPB PSMA CAR. Hyperactive piggyBac transposase is driven by the cytomegalovirus promoter [1]. Plasmids were purified by standard techniques using EndoFree kits (Qiagen).

The amino acid sequence of CAR19 is: MALPVTALLLPLALLLHAARPTREVQLQQSGPE LVKPGASVKMSCKASGYTFTSYVMWVKQKPG QGLEW**IGYVNP**YNDGTKYNEKFKGKATLTSD KSSSTAYMELSSLTSEDSAVYYCARGPYYYGSSP FDYWGQGTTVTVSGGGGSGGGGSGGGGSGG GGSDIVMTQSPLSLPVSLGDQASISCRSSQSLEN SNGNTYLNWYLQKPGQSPQLLIYRVSNRFSGVL DRFSGSGSGTDFTLKISRVEAEDLGVYFCLQVT HVPPTFGAGTKLEIKRTSPTTTPAPRPPTPAPTIA SQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWA PLAGTCGVLLLSLVITLYCNHRNRRRVKRGRKK LLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGG CELRVKFSRSADAPAYQQGQNQLYNELNLGRRE EYDVLDKRRGRDPEMGGKPQRRKNPQEGLYN

ELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ
GLSTATKDTYDALHMQALPPR

The amino acid sequence of PSMA CAR is
MALPVTALLLPLALLLHAARPTREVQLQQSGPE
LVKPGTSVRISCKTSGYTFTEYTIHWVKQSHGK
SLEWIGNINPNNGGTTYNQKFEDKATLTVDKS
SSTAYMELRSLTSEDSAVYYCAAGWNFDYWGQ
GTTVTVSGGGGSGGGGSGGGGSGGGGSDIVM
TQSHKFMSTSVGDRVSIICKASQDVGTAVDWYQ
QKPGQSPKLLIYWASTRHTGVPDRFTGSGSGTD
FTLTITNVQSEDLADYFCQQYNSYPLTFGAGTM
LEIKREQKLISEEDLNGTTSPTTTPAPRPPTPAPT
IASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI
WAPLAGTCGVLLLSLVITLYCNHRNRRRVKRGR
KKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEE
GGCELRVKFSRSADAPAYQQGGQNQLYNELNLG
RREEYDVLDKRRGRDPEMGGKPQRRKNPQEG
LYNELQKDKMAEAYSEIGMKGERRRGKGHDG
LYQGLSTATKDTYDALHMQALPPR

Cell culture, transfections and magnetic bead isolation

For all experiments, we used CellGro medium (CellGenix) supplemented with 10% heat-inactivated 10% fetal calf serum (Gibco) and penicillin/ streptomycin (Gibco).

PBMCs were prepared from buffy coats or fresh blood samples by gradient centrifugation using Ficoll-Paque Premium (GE Healthcare) after separation cells were rested overnight in cell medium. The next day, cells were transfected with Neon electroporator (Thermo Fisher Scientific) similarly to the description by Ramanayake *et al.* [3]: 4×10^6 cells were resuspended in 100 μL buffer T and mixed with 5 μg plasmid DNA (2:1 mixture of piggyBac vector expressing the CAR and a plasmid expressing a hyperactive piggyBac transposase) and were electroporated 1×20 ms/2300V using 100-μL tips (in some experiments, 10-μL tips were used as well). Afterward, cells rested again overnight and were stimulated the next day.

To stimulate T cells with antibodies, anti-CD3 and anti-CD28 mouse mAb (Miltenyi Biotec) or mouse anti-Myc tag Ab (Cell Signaling) were diluted in phosphate-buffered saline (PBS) at a concentration 1 μg/mL and adhered onto a cell culture plastic for 1 h at 37°C; they were then washed with PBS and used for cell activation.

Cell medium was supplemented with cytokines: 1000 U/mL of IL-2 (Proleukin, Roche), 20 ng/mL IL-4, 10 ng/mL IL-7 and 20 ng/mL IL-21 (all Miltenyi Biotec). Cells were fed with fresh medium every 3–4 days according to the growth rate.

HEK293 (ATCC CRL-1573) cells were kept in Dulbecco's Modified Eagle's Medium and transfected with cDNA encoding CD19 (Origene cDNA

clone SC127938) using Metafectene Pro reagent (Biontex). Ramos is a CD19$^+$ Burkitt lymphoma cell line (ATCC CRL-1596), PC3 is prostate carcinoma cell line negative for PSMA (ATCC CRL-1435) and LNCap is prostate carcinoma cell line positive for PSMA (ATCC CRL-1740).

To isolate CAR$^+$ T cells, we stained CAR19 T cells with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (Jackson ImmunoResearch), followed by labeling with anti-FITC magnetic beads (Miltenyi Biotec), and positive cells were isolated on an LS column. To deplete B cells, PBMCs were labeled with biotinylated anti-CD19 antibody. Cells were then incubated with anti-biotin magnetic beads (Miltenyi Biotec) and separated on an LS magnetic column. Negative fraction depleted from B cells was then electroporated with CAR19 transposon using 10-μL tips (4×10^5 cells).

Antibodies, fluorescence-activated cell sorting and functional assays

Mouse anti-human CD3 APC-Cy7, CD45RA Alexa Fluor 488, CD45RO BV786, CCR7 BV605, CD95 PeCy5 and PD1 Pe-Dazzle 594 were obtained from BioLegend. CD4-Pacific Blue, CD8 Alexa Fluor 700 were purchased form Exbio. Live cells were identified using LIVE/DEAD stain kit (Thermo Fisher Scientific).

To detect CAR transgene, cells were stained with Alexa-647, or FITC-labeled FAB$_2$ fragment of Goat anti-Mouse IgG (Jackson ImmunoResearch), this polyclonal antibody reacts with mouse anti-CD19 scFv in the CD19 CAR. To detect PSMA CAR, cells were labeled with unlabeled rabbit anti-Myc tag antibody (Cell Signaling) followed by Alexa 647-labeled goat anti-rabbit IgG (Jackson ImmunoResearch). If CAR T cells were stained for additional surface antigens, cells were first labeled with goat anti-mouse antibody, washed twice and blocked with 10% mouse serum, then were stained with fluorescently labeled mouse mAb. The specificity of staining was extensively tested to rule out possible binding of mouse antibodies to cells via goat anti-mouse Ab used to detect CAR.

To measure cytokine production, HEK293 cells were transfected with CD19 cDNA or control plasmid DNA (empty vector). The next day, CAR T cells were added into the wells with transfected HEK cells, and after 4 h of co-incubation, GolgiPlug (eBiosciences) was added at a concentration of 1 μg/mL. Cell were then left overnight in the incubator (~12 h). After the incubation period was over, cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD Biosciences) and stained with fluorescently labeled mouse mAb antibodies to surface antigens, and cytokines

interferon (IFN)-γamma, tumor necrosis factor (TNF)-α and IL-4 (all eBiosciences).

Cytotoxic test was performed as follows: Ramos cells were labeled with 5 μmol/L carboxyfluorescein succinimidyl ester (Sigma) in cell medium for 5 min at room temperature, washed and mixed with effector T cells in 96-well flat bottom plate. After overnight incubation, DAPI at a concentration of 100 ng/mL (Sigma) was added to cells, and the number of dead Ramos cells was determined by fluorescence-activated cell sorting (FACS).

To visualize cytotoxicity of PSMA CAR T cells, monolayers of PC3 or LNCap cells were incubated with CAR T cells, and after 3 days, cells were imaged with phase-contrast microscope with digital camera (Olympus).

To determine the degranulation of CAR T cells, cells were incubated with or without Ramos B cells at ration of 1:1 for 4 h, phycoerythrin-labeled anti-CD107a Antibody (Exbio) was added to the cell medium at the beginning of co-incubation. After 4 h, cells were harvested and labeled with anti-CD8 and anti-CD4 antibodies and analyzed by FACS.

FACS samples were analyzed with BD Fortessa instrument, and FACS data were processed by FlowJo software. Statistical analysis was performed with GraphPad Prism software using unpaired *t*-test.

Results

Our experience with the manufacture of antiviral T cells shows that a supplementation of cell medium with IL-4 + IL-7 efficiently supports the *in vitro* expansion of antiviral T cells after stimulation with antigen [9]. Thus, we tested this approach to produce CD19-specific CAR T cells. First, PBMCs were electroporated using the Neon device with piggyBac transposon vectors encoding the CAR19 transgene and hyperactive piggyBac transposase (i.e., CAR19 transposon). The next day, cells were stimulated with immobilized anti-CD3+anti-CD28 mAbs or left unstimulated. Next, both stimulated and unstimulated cell cultures were supplemented with cytokines IL-2 or IL-4 + IL-7. After 7 days, cells were analyzed for the expression of CAR19 by staining with goat anti-mouse Ab, which reacts with the scFv portion of CAR19 (Figure 1A). After 14 days, we compared the expansion of non-stimulated cells grown in IL-2 or IL-4 + IL-7 (Figure 1B). The results in Figure 1A show, that the stimulation with anti-CD3+anti-CD28 mAbs induced a polyclonal expansion of T cells, while the cultivation in the presence of cytokines only (i.e. without anti-CD3+anti-CD28 mAbs) resulted in much higher frequency of CAR19⁺ T cells among cultured cells (32% vs. 90%). However, we found that cells grown in IL-4 + IL-7 expanded at later time points

significantly more than cells grown in IL-2 (Figure 1B). This experiment suggested to us that signaling via the CAR19 transgene in the presence of exogenously added IL-4 and IL-7 enables selective expansion of transduced T cells.

To verify this hypothesis, PBMCs were depleted from B cells via anti-CD19 magnetic beads before electroporation with CAR19 transposon. Electroporated T cells were then grown alone in IL-4 + IL-7 or were mixed back with sorted B cells, and then further cultured for 12 days. The data in Figure 1C show that electroporated PBMCs depleted from B cells did not grow significantly, whereas the same cells stimulated with anti-CD3/CD28 efficiently expanded, demonstrating their viability. When we mixed electroporated T cells with sorted autologous B cells, we observed a selective expansion of CAR19 T cells. This experiment thus demonstrates that recognition of B cells by CAR19 T cells in the presence of IL-4 and IL-7 enables their priming and expansion.

We have then determined the proliferation capacity of CAR19 T cells grown in various cytokine combinations: IL-2, IL-4 + IL-7, IL-4 + IL-7 + IL-2 and IL-4 + IL-7 + IL-21 and compared the growth after 24 days with the widely used type of cultivation anti-CD3/CD28 plus IL-2. The data in Figure 2 show that (i) IL-2 alone was not sufficient to initiate good and sustained proliferation of CAR19 T cells, which had to be pre-activated with anti-CD3/28 antibodies to expand; (ii) IL-4 + IL-7 enabled proliferation of CAR19 T cells, and additional cytokine stimulation with IL-2 or IL-21 (or IL-15, data not shown) did not result in significant enhancement of the cell growth; and (iii) CAR19 T cells treated with anti-CD3/CD28 plus IL-2 grew to similar numbers as cells grown in IL-4 + IL-7.

The next key question was to determine the percentage of CAR19 T cells in cultures generated under these conditions. We found that polyclonal activation of electroporated T cells (i.e., anti-CD3/CD28 plus IL-2) resulted in a significantly lower percentage of CAR19 T cells (~30%) than cells grown in IL-4 + IL-7 (~90%), IL-4 + IL-7 + IL-2 (~90%) or IL-4 + IL-7 + IL-21 (>95% of all cells were CAR positive; Figure 3A). The composition of the cytokine cocktails, however, had a significant effect on the ratio of CD4⁺ to CD8⁺ T cells (Figure 3B). We found that the cultivation in the presence of exogenous IL-4 + IL-7 or IL-4 + IL-7 + IL-21 favored the expansion of CD4 T cells (none of them were regulatory T cells; data not shown), whereas expansion induced by anti-CD3/CD28 plus IL-2 treatment resulted in the majority (>80%) of all CAR19 T cells being of CD8⁺ type (Figure 3B). Next, we compared the effector functions of CAR19 T cells prepared under these conditions. The experiment shown in Figure 3C and

7 days after electroporation
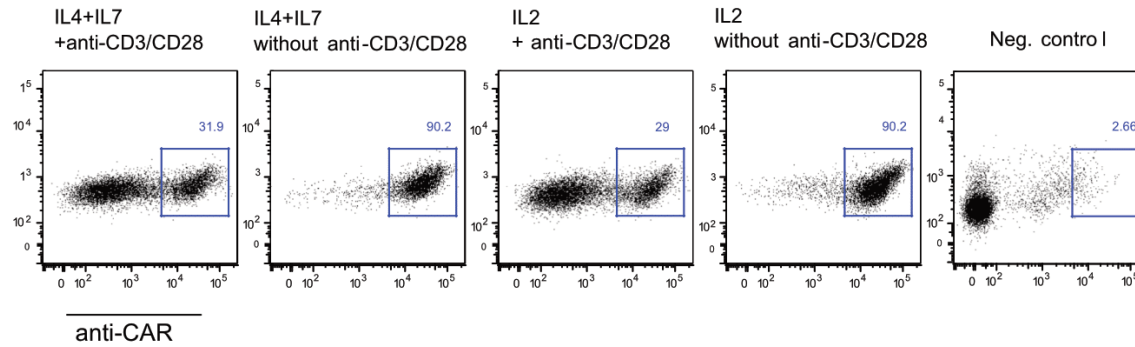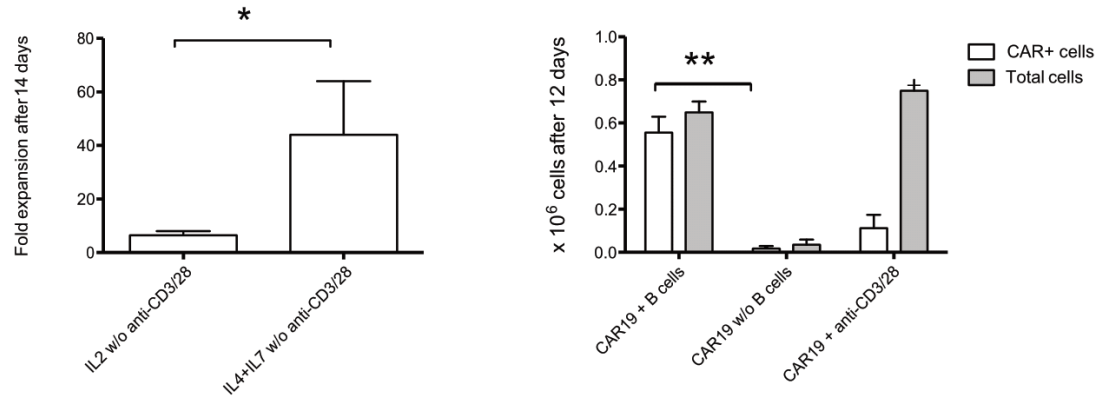
Figure 1. Recognition of CD19^{+} B cells by CAR19 T cells enables their selective expansion in the presence of IL-4 + IL-7. (A) Human PBMCs were electroporated with CAR19 transposon and stimulated with anti-CD3/CD28 Abs or left unstimulated. Cells were then were cultivated in the presence of IL-2 or IL-4 + IL-7. Seven days later, the expression of CAR19 transgene was determined with anti-CAR antibody. The numbers show the percentage of CAR^{+} cells out of all live cells. Negative controls are untransduced cells. (B) The level of expansion of cells from (A) was analyzed after 14 days. (C) To determine whether endogenous B cells initiate CAR19 T-cell priming, PBMCs were first depleted from CD19^{+} cells by magnetic beads and then were electroporated with CAR19 transposon (4×10^5 cells in 10-μL tips). Cells were split into three groups and cultivated in the presence of IL-4 + IL-7: (i) with anti-CD19-sorted B cells, (ii) alone (iii) alone but activated with anti-CD3/28 Abs. After 12 days, live cells were counted, and FACS was performed to identify CAR19 T cells. (C) The graph shows the number of all cells and CAR19 T cells, n = 3. In (A), one representative donor is shown.
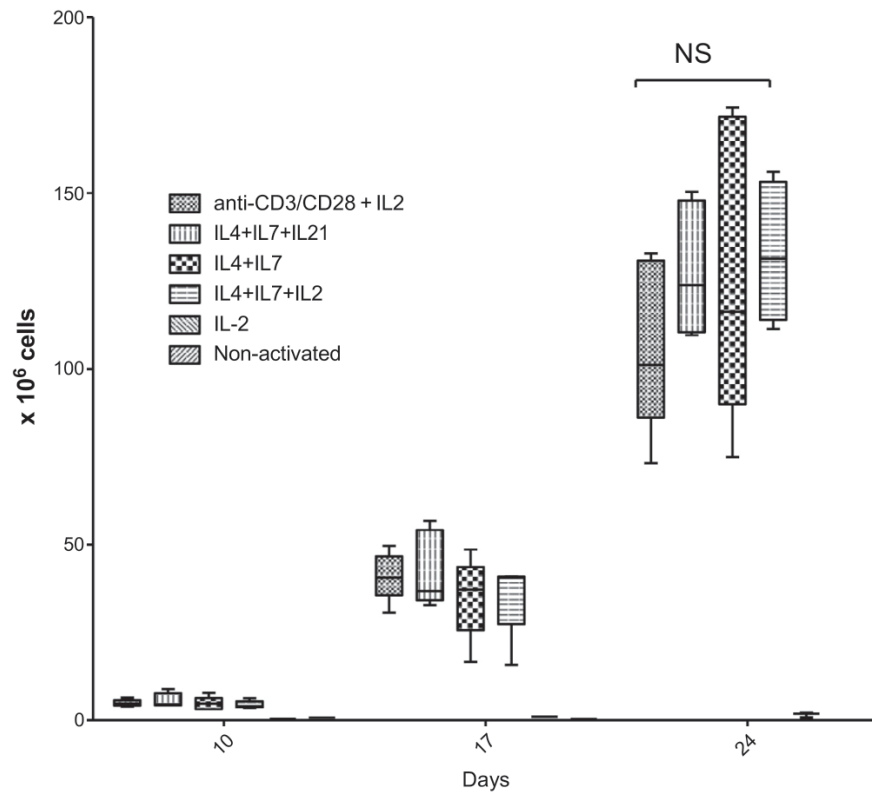
Figure 2. Anti-CD3/CD28 activation is not required for expansion of CAR19 T cells in the presence of IL-4 + IL-7. PBMCs were electroporated with CAR19 transposon (4×10^6 cells in 100-μL tips per sample) and then incubated in the presence of the cytokines as indicated in the legend. The graph shows the total number of cells at indicated time points. The non-activated cells (i.e., without any exogenous cytokines) and cells grown only in the presence of IL-2 did not expand. The differences in the expansion among the samples in the first four groups were not significant (NS), n = 5.

3D demonstrates that all groups of CAR19 T cells were equally cytolytic (Figure 3D) and degranulated to similar levels (Figure 3C) after co-incubation with the Ramos B cell line. These results thus demonstrate that the activation of CAR19 T cells through recognition of CD19 on B cells in the presence of IL-4 + IL-7 leads to an almost pure population of cytotoxic CAR19 T cells.

Next, we determined the production of effector cytokines TNF-alpha and IFN-gamma by CAR19 T cells (Figure 4). To do that, we transfected the HEK293 cell line with cDNA encoding antigen CD19 and used the transfected HEK cells as stimulators for CAR19 T cells, whereas non-transfected HEK cells served as a negative control. The presented data (two out of five representative donors are depicted) show that (i) the response of CAR19 T cells is specific to antigen CD19 because no cytokine production was detected after incubation with HEK cells were transfected with control DNA and (ii) CD4^{+} CAR19 T cells grown in IL-4 + IL-7 produced more TNF-alpha than CAR19 T cells grown under anti-CD3/CD28+IL-2 conditions

and CD8^{+} CAR19 T cells grown under anti-CD3/CD28+IL-2 conditions produced more IFN-gamma than cells from remaining groups. Further, we did not detect any significant production of IL-4 in any group (data not shown). These results further demonstrate acquisition of effector functions by CAR19 T cells.

The experiments presented thus far were done with PBMCs obtained from healthy donors, and therefore we wished to determine whether this technique would also be functional with PBMCs obtained from patients undergoing chemotherapy for B-cell lymphoma. We tested PBMCs from six patients; each patient had a normal complete blood cell count at the time of the experiment, but because of previous treatment with rituximab, they had very low numbers of B cells in the peripheral blood. The data in Figure 5 show that we were able to induce expansion of CAR19 T cells in four of six patients similarly to healthy donors. These cells were also cytotoxic against Ramos cells (results not shown) However, the efficiency of the expansion depends on the presence of B cells in the blood of the donors.
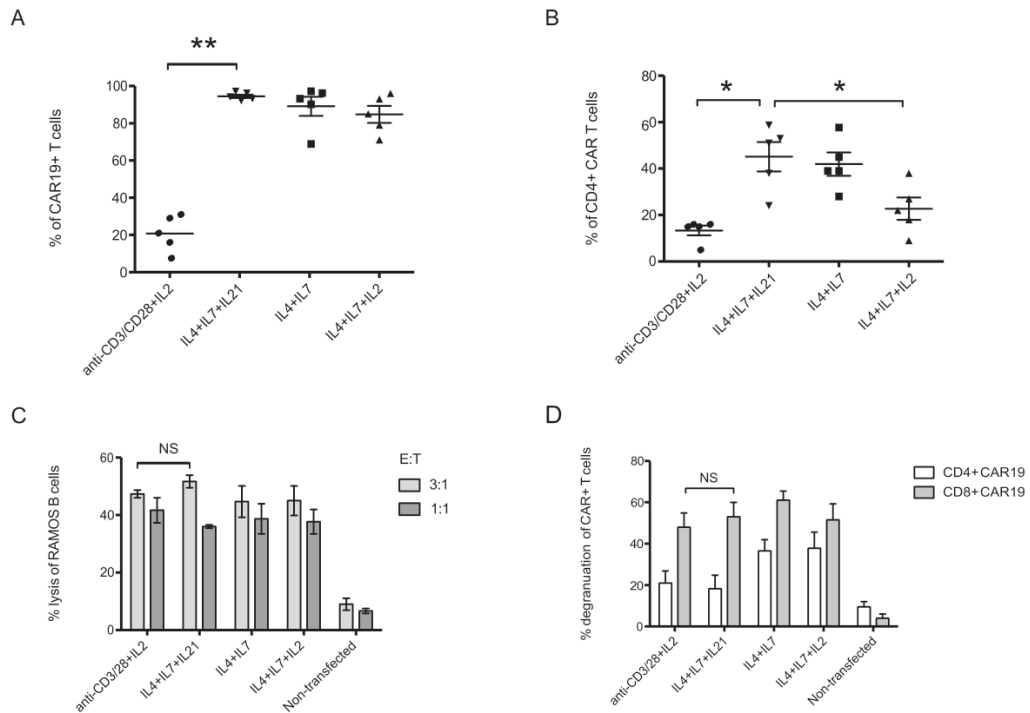
Figure 3. Cultivation in the presence of IL-4 + IL-7 results in almost pure population of cytotoxic CAR19 T cells. Cells prepared in Figure 2 were further analyzed to determine the percentage of CAR19 T cells and their functionality. The graphs show the percentage of CAR19$^+$ cells (A) and the percentage of CD4$^+$ CAR19 T cells (B) out of all live cells. The effector functions of CAR19 T cells were determined by cytotoxic assay against Ramos B cell line (C). Effector to target ratio (E:T) is 3 and 1. (D) The graph shows the level of degranulation of both CD4$^+$ and CD8$^+$ subtypes of CAR19 T cells after co-incubation with Ramos B cells. Before these two assays, CAR$^+$ T cells from anti-CD3/CD28+IL-2 group were sorted with magnetic beads to obtain a homogenous population of pure CAR19 T cells (>90%). Non-transfected control cells are anti-CD3/28 activated T cells grown in the presence of IL-2, n = 5.

Supplementation of culture medium with cytokines such as IL-2, IL-7, IL-15 and IL-21 has been shown to influence the phenotype and expansion of CAR T cells [16,17]. Similarly, we have performed a comprehensive FACS analysis to determine variations in the differentiation status of CAR19 T cells. The central memory T cells (Tcm) were identified as CD45RA$^-$CD45RO$^+$CCR7$^+$, the effector memory T cells (Tem) were CD45RA$^-$CD45RO$^+$ CCR7$^-$, the memory stem cell T cells (Tscm) were CD45RA+CD45RO$^-$CCR7$^+$CD95$^+$CD27$^+$CD28$^+$ and the effector T cells were CD45RA+CD45RO$^-$CCR7-CD95+, (naive CD45RA$^+$CD95$^-$ T cells were not detected; data not shown). Additionally, we determined the expression of inhibitory receptors TIM-3, LAG-3 and PD-1 on CAR19 T cells.

We found that CD8$^+$ CAR19 T cells cultivated in the presence of IL-4 + IL-7 + IL-21 were mostly of Tcm phenotype. In addition, they contained more Tscm and fewer Tem than cells grown under other experimental conditions (Figure 6). When we analyzed the expression of antigens CD27 and CD28 (Figure 7), we found that approximately 75–80% of CD8+CAR19

T cells generated in the presence of IL-4 + IL-7 + IL-21 were highly positive for both antigens, whereas 10-fold fewer cells were similarly positive in cultures generated under anti-CD3/CD28+IL-2 conditions. Most interestingly, we observed (Figure 8) that both CD4$^+$ and CD8+CAR19 T cells in cultures supplemented with IL-21 expressed undetectable amounts of TIM-3, whereas CAR19 T cells grown under anti-CD3/CD28+IL-2 conditions had approximately 40% TIM-3 positive CD8+CAR19 and 20% TIM-3 positive CD4$^+$ CAR19 T cells. A similar, albeit less pronounced, effect was observed for PD-1, but no significant effect was found for LAG-3. We did not detect any CTLA-4$^+$ cells (data not shown).

IL-21 prevents terminal differentiation of CAR19 T cells *in vitro*, helps them maintain an immature phenotype and prevents upregulation of inhibitory receptors TIM-3 and PD-1.

We have demonstrated that CAR19 T cells expand in the presence of autologous B cells. To further analyze the mechanisms of CAR T activation *in vitro*, we have transduced T cells with a CAR specific for an antigen that is not expressed by PBMCs to eliminate the
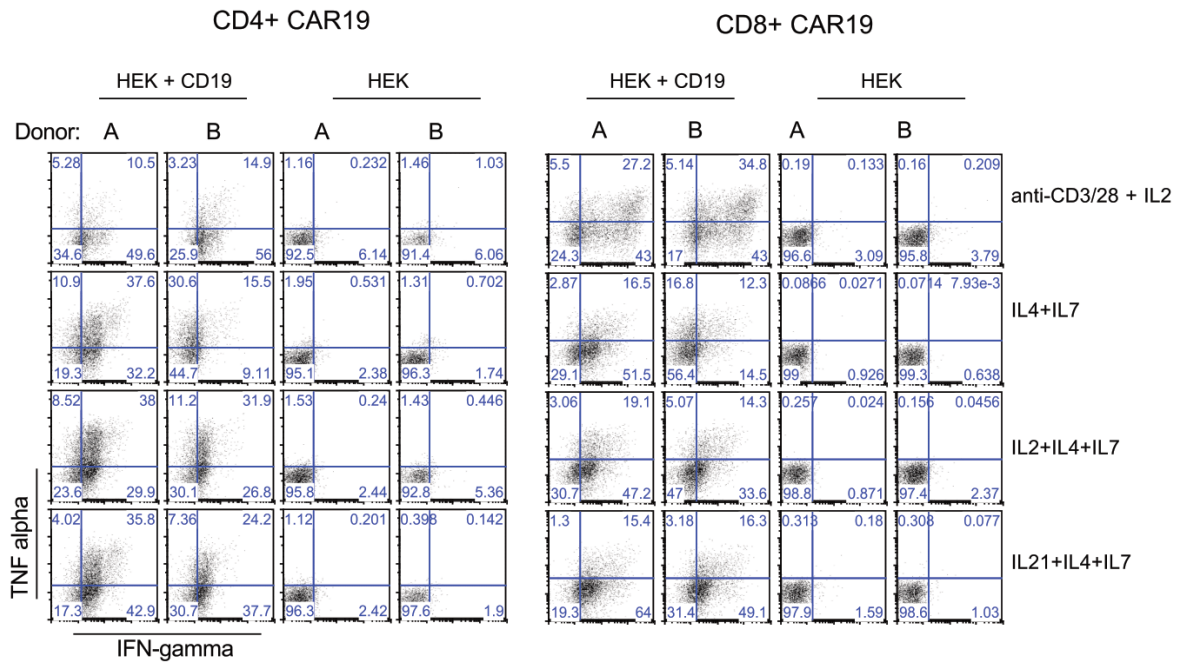
Figure 4. CAR19 T cells produce effector cytokines IFN-γ and TNF-α following activation through CAR. HEK293 cells were transfected with CD19 cDNA and used as stimulators for CAR19 T cells manufactured in the presence of cytokines as indicated in the figure. After overnight incubation in the presence of brefeldin A, cells were stained for CAR, fixed and stained for cytokines CD4 and CD8. HEK293 cells transfected with a control plasmid served as a negative control. The numbers in each quadrant indicate the percentage out of CD4$^+$ or CD8$^+$ CAR19 T cells. Two of five representative donors are shown.
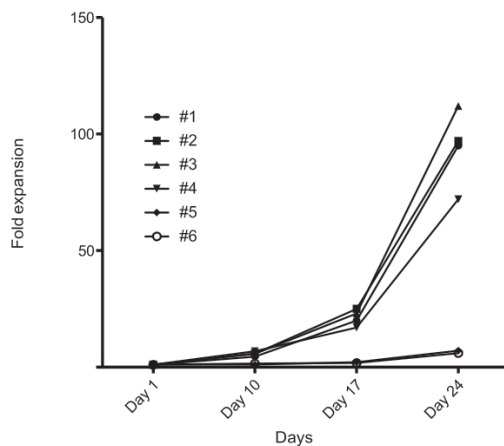
Figure 5. CAR19 T cells can be generated from PBMCs obtained from patients undergoing chemotherapy for non-Hodgkin lymphoma. Fresh blood samples (5 mL) were subjected to a Ficoll gradient centrifugation and electroporated (4×10^5 cells in 10-μL tips) with CAR19 transposon. After electroporation, cells were cultivated in the presence of IL-4 + IL-7 for 24 days. The graph shows the fold expansion of each sample. Two of six samples did not expand, possibly due to the low number B cells due to previous rituximab (anti-CD20 Ab) therapy.

priming by endogenously expressed target cells. We selected the PSMA CAR [18], which is not expressed on the surface of PBMCs. The structure of the PSMA CAR is identical to CAR19 except for the extracellular part encoding the anti-PSMA scFv and a Myc tag located after the scFv. The amino acid sequence of both CARs is provided earlier in the Methods section.

The data in Figure 9 show the results of an experiment in which the PBMCs were electroporated with PSMA CAR transposon followed by activation with immobilized anti-Myc tag antibody that reacts with extracellular part of PSMA CAR or were activated polyclonally with anti-CD3/28 antibodies. After activation, cells were cultivated in the presence of IL-4 + IL-7 for 24 days. We found that anti-Myc-treated cells expanded to significantly higher numbers than anti-CD3/28-treated cells, whereas no significant expansion was detected in the third group of anti-Myc treated cells grown in the absence of exogenous cytokines (Figure 9A). Next, we found that anti-Myc-treated cells contained >95% of PSMA CAR$^+$ T cells, while the anti-CD3/28 treated cells contained only ~10% of PSMA CAR$^+$ T cells (Figure 9B). Both types
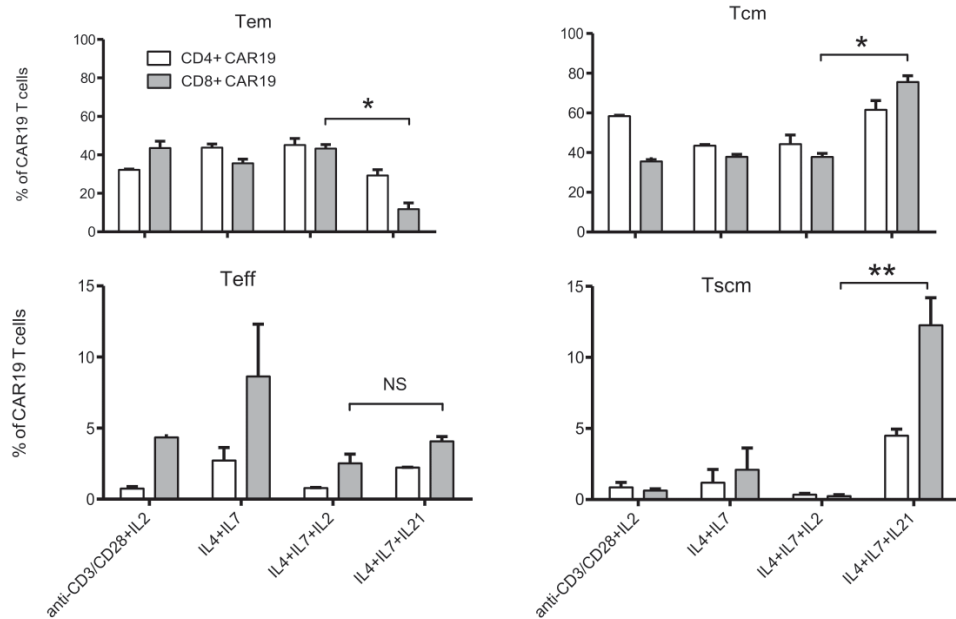
Figure 6. Combination of cytokines IL-4 + IL-7 + IL-21 enhances the expansion of a less differentiated CAR19 T cells with Tcm and Tscm phenotypes. Cells were grown in the presence of indicated cytokines for 24 days and then were analyzed by FACS to determine the differentiation status of CD4$^+$ CAR19 T cells and CD8$^+$ CAR19 T cells. The central Tcm were identified as CD45RA$^-$CD45RO$^+$CCR7$^+$; the Tem were CD45RACD45RO$^+$ CCR7$^-$; the Tscm) were CD45RA$^+$CD45ROCCR7$^+$ CD95$^+$CD27$^+$CD28$^+$, the effector T cells were CD45RA$^+$CD45RO$^-$CCR7$^-$CD95$^+$, naive CD45RA$^+$CD95$^-$ T cells were not detected (data not shown), n = 5.

of PSMA CAR T cells specifically killed PSMA$^+$ target cell line LnCAP (Figure 9C,D) at similar levels.

We thus provide evidence that signaling via the CAR transgene in the presence of IL-4 and IL-7 enables efficient and selective expansion of transduced T cells and their differentiation into cytotoxic CAR T cells. The addition of IL-21 helps maintain their immature phenotype and reduces the expression of inhibitory receptors TIM-3 and PD-1 without negative effects on the cytotoxic functions of CAR T cells.

Discussion

Significant improvements of electroporation-based genetic modification of T cells was achieved in the past through the use of genetically modified feeder cell lines expressing various co-stimulatory and cytokine ligands [2]. The expansion of CAR T in the presence of modified K562 cells expressing CD19, 4-1BB ligand and IL-15 was effective but required many days of culture [2]. For GMP-grade manufacturing, the master cell banks of the artificial antigen presenting cells had to be prepared and extensively characterized, which increases costs and limits the widespread use of the this technique. In contrast, we found that the electroporation of CAR19 transposon triggers a spontaneous activation of transfected T cells through recognition of B cells and results in their proliferation

in the presence of cytokines IL-4 and IL-7. If IL-21 is added with IL-4 + IL-7 to the cell medium, proliferating CAR19 T cells maintain their early memory phenotype and express low amounts of inhibitory receptors TIM-3 and PD-1 compared with cells generated under anti-CD3/28 + IL-2 conditions. This mechanism also works using an anti-CAR antibody to initiate CAR T activation, which makes this technique universally applicable to CARs specific to other antigens. We successfully generated approximately $100-150 \times 10^6$ CAR T cells from one electroporation of 4×10^6 PBMCs after 24 days *in vitro* and this process can be easily scaled up to produce a clinical dose of about 1×10^9 CAR T cells.

There are several approaches to specifically expand transduced CAR T cells without any additional manipulation, such as magnetic bead sorting. An elegant method developed by Ramanayake *et al.* [3] is based on a repeated re-stimulation of CAR19 T cells with irradiated autologous PBMCs, and it most likely works through stimulation of CAR19 T cell through B cells similarly as we have observed. Another nice molecular trick described by Wilkie *et al.* uses a chimeric cytokine receptor composed of extracellular IL-4 fused with the intracellular IL-2 chain that is co-expressed with CAR. This artificial receptor delivers an IL-2–specific mitogenic signal upon culture in IL-4, resulting in expansion of a pure population of CAR T cells [19].
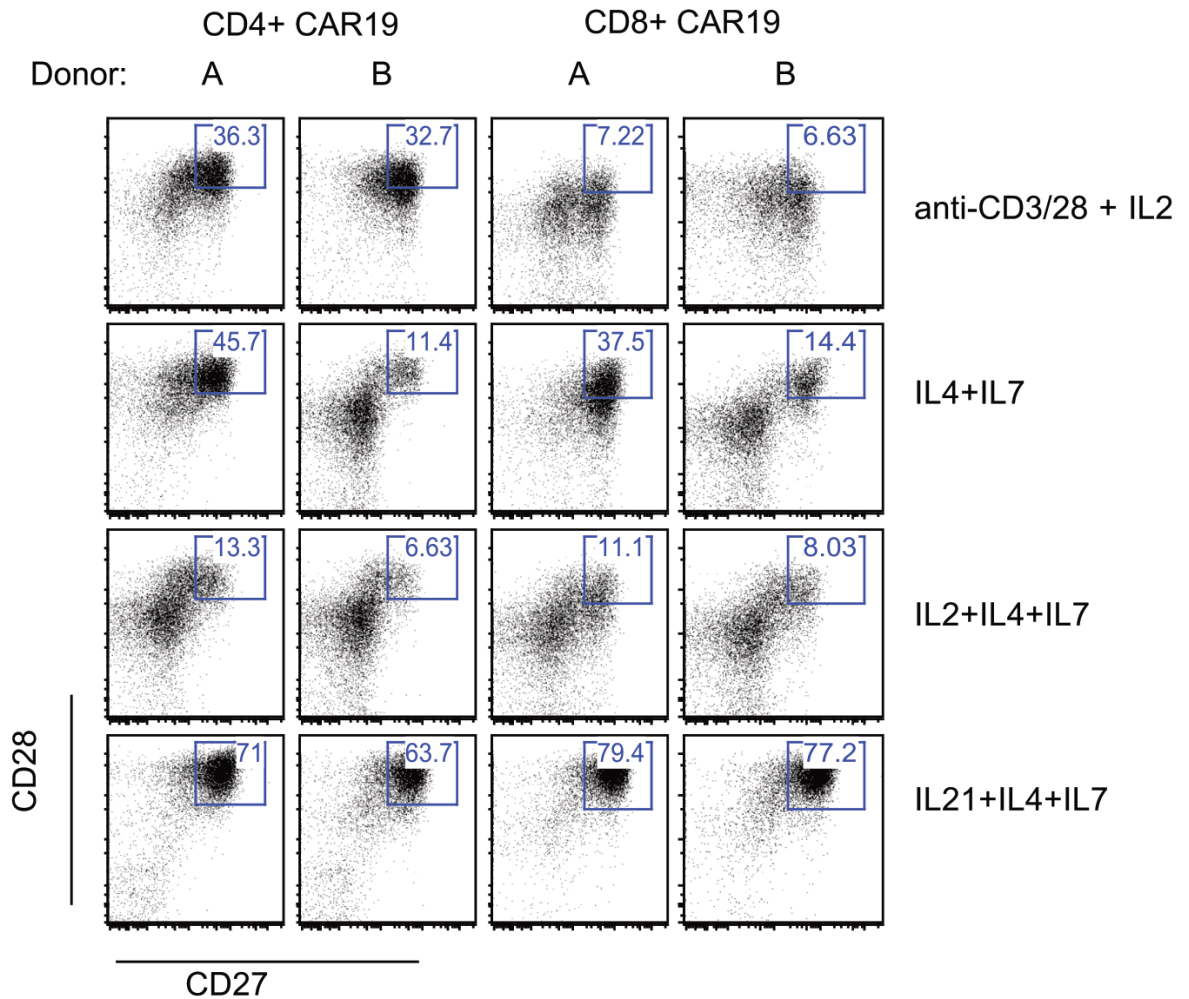
Figure 7. Cultivation of CAR19 T cells in the presence of IL-4 + IL-7 + IL-21 supports the expansion of immature CD27(high)CD28(high) population of CAR19 T cells. The numbers in each dot plot indicate the percentage of cells within the gate out of all CD4+ or CD8+ CAR19 T cells. Two of five representative donors are shown.

Our data show that similar results can be achieved through a simple step that is based on a TCR-like activation of transduced CAR T cells via ligation of the CAR. We constructed a PSMA CAR that can be easily activated with anti-Myc tag antibody and is not spontaneously activated after electroporation into PBMCs. Similar to experiments shown in Figure 1B, we found that anti-Myc tag antibody induces activation of PSMA CAR T cells and their intensive proliferation in the presence of IL-4 + IL-7. Thus, our approach, based on a selective activation of transduced CAR T cells with anti-CAR antibody or, with a physiological ligand (i.e., B cells) in the case of CAR19, also effectively induces expansion of CAR T cells. Interestingly, however, this method works only if CAR T cells are cultivated in the presence of IL-4 + IL-7;

supplementation of cell medium with only IL-2 was insufficient (Figure 2).

The combination of cytokines IL-4 and IL-7 was shown to be superior to IL-2 to manufacture clinical-grade antiviral T cells [9]. IL-7 is mainly produced by non-hematopoietic cells such as specialized lymphoid stromal subsets in lymphoid organs. It is absolutely required for the development of secondary lymphoid tissues and for the development of T cells in the thymus [20]. In addition to its role in lymphoid development, it also promotes the maintenance of activated T cells recruited into the secondary lymphoid organs and has strong pro-survival effects on T cells *in vitro* [21]. IL-4 is produced by T cells, mast cells and basophils and primarily shapes the immune response by inducing Th2 type differentiation

CD4+ CAR19

CD8+ CAR19

Figure 8. Cultivation in the presence of IL-4 + IL-7 + IL-21 reduces the expression of inhibitory receptors PD-1 and TIM-3 on CD4+CAR19 T cells (left) and CD8+ CAR19 T cells (right), n = 5.
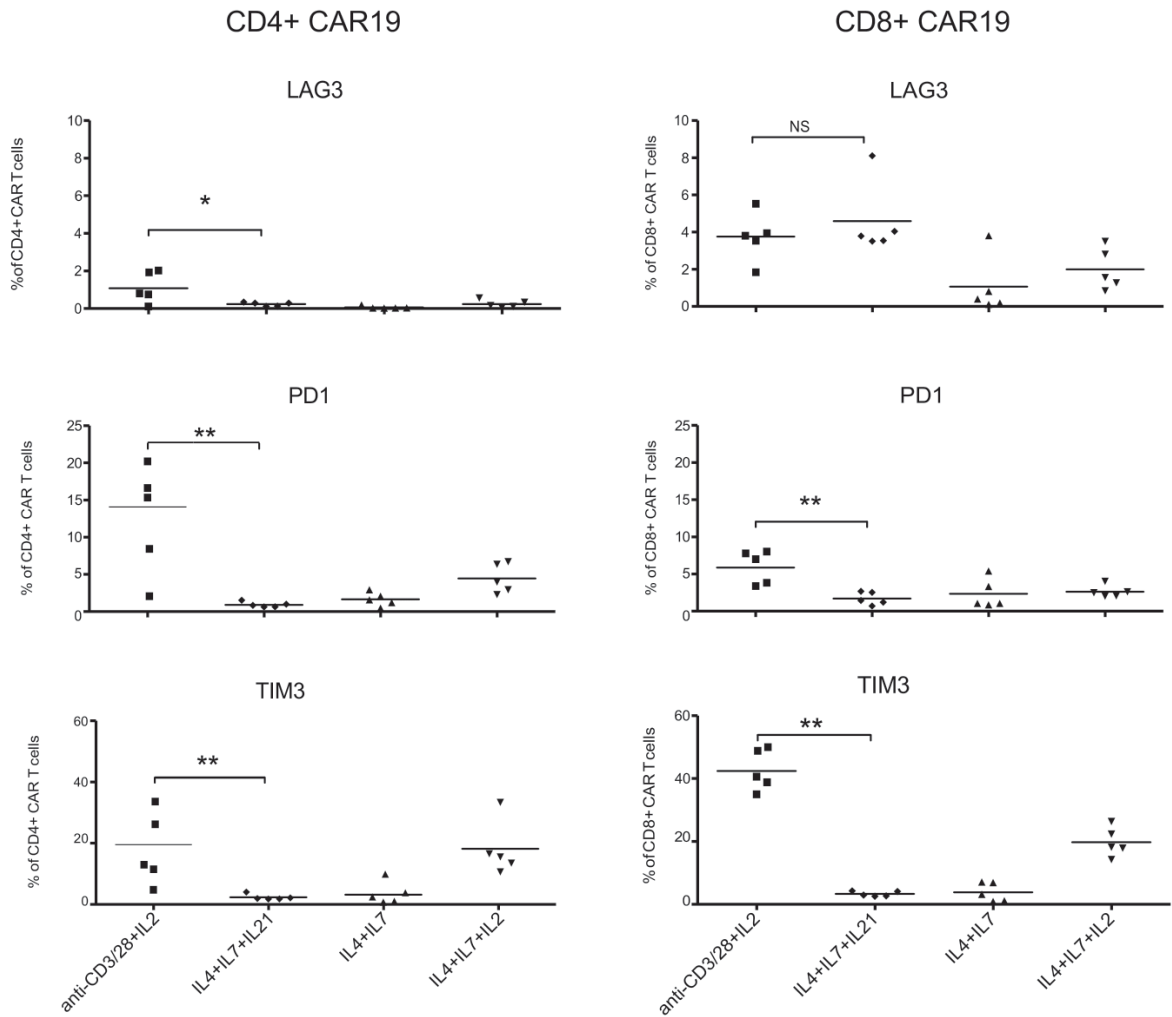
of T cells. In addition to T-cell effects, it stimulates the proliferation of activated B cells accompanied by immunoglobulin class switching and production of immunoglobulin E and G1. Besides its well-known TH2-polarizing ability, IL-4 also delivers a strong pro-survival signal to T cells and can inhibit apoptosis of activated T cells similarly to cytokines IL-7, IL-15 and IL-2 [21,22]. IL-21 does not have such well-defined effects on T cells; it is mainly produced by activated T cells but targets a broad range of lymphoid and myeloid cells of the immune system and therefore is able to regulate innate and acquired immune responses [23]. In contrast to IL-2, it is not a T-cell-specific growth factor, but it seems that IL-21 can block terminal differentiation of T cells (similarly to Wnt pathway activation) and also support T-cell proliferation [8]. The addition of IL-21 to the cocktail

of IL-4 and IL-7 thus inhibits terminal differentiation of rapidly proliferating CAR T cells. In agreement with these observations, our analysis of the phenotypes of CAR19 T cells revealed that cultivation in the presence of IL-4 + IL-7 + IL-21 favors the expansion of Tcm and Tscm and increases the proportion of CD4+CAR19 T cells. The majority of CAR19 T cells generated under these conditions was also highly positive for CD27 and CD28, which suggests that they were of immature phenotype. Similar findings were reported by Kaartinen *et al.* [11], who demonstrated that low IL-2 concentration in combination with anti-CD3/28-based activation favored the development of early memory CAR T cells. This suggest that high IL-2 concentrations drive T cells toward the terminal developmental stages. In accordance with that study, we similarly found that high concentrations
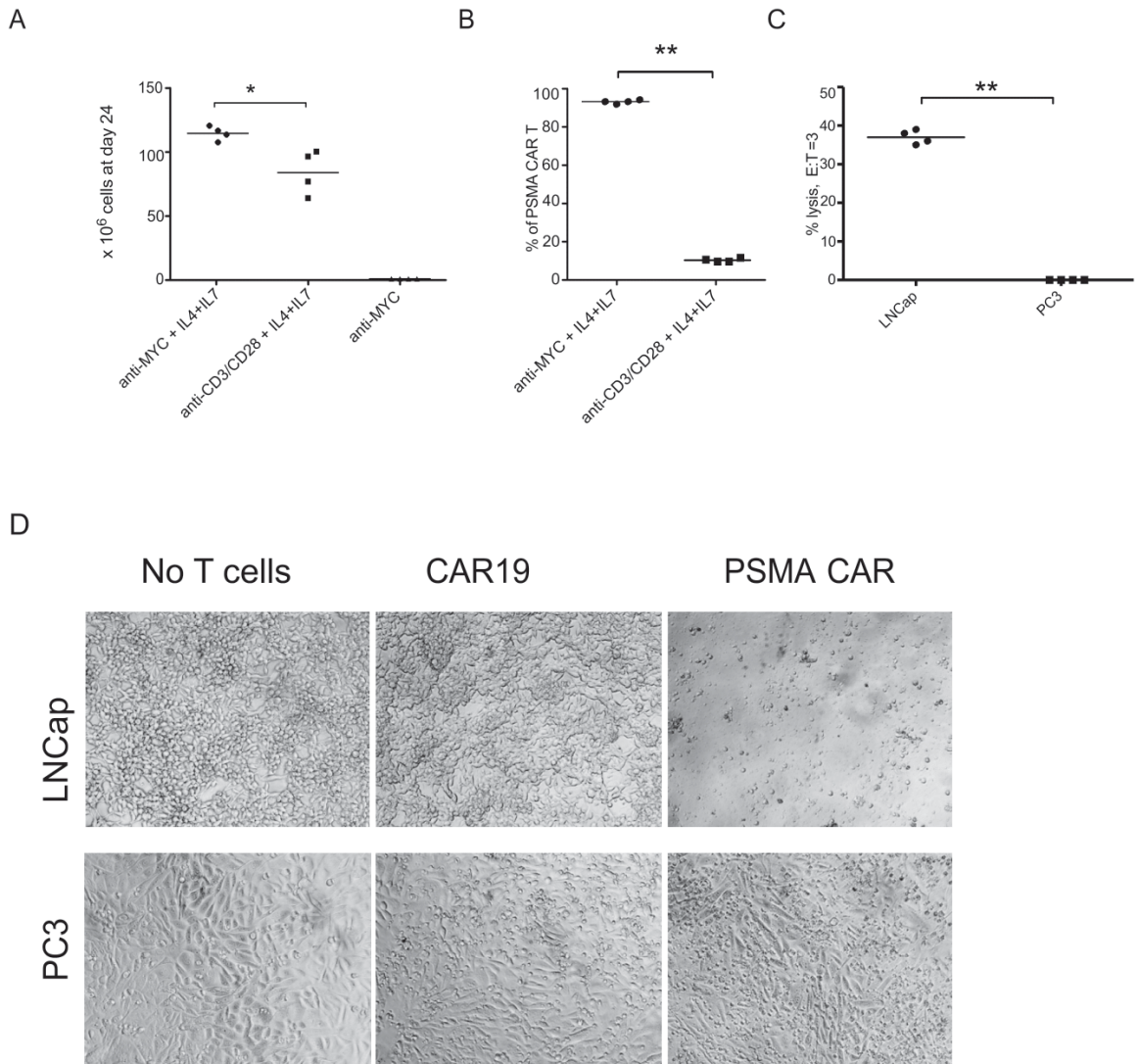
Figure 9. Signaling via CAR in the presence of IL-4 + IL-7 enables selective expansion of PSMA CAR T cells and acquisition of their effector functions. PBMCs were electroporated with PSMA CAR (4×10^6 cells in 100-μL tips per sample) and activated with immobilized anti-Myc or anti-CD3/28 antibodies, followed by cultivation in media supplemented with IL-4 + IL-7, or were activated with anti-Myc antibody but without addition of exogenous cytokines. The graphs show cell number (A) after 24 days of culture and the percentage (B) of PSMA CAR T cells determined by staining with anti-Myc antibody. (C) Cytotoxicity of PSMA CAR T cells was determined against PSMA$^+$ cell line LnCap using carboxyfluorescein succinimidyl ester–based FACS assay, PC3 are PSMA$^-$ cells. (D) Cytotoxicity was similarly determined by a 3-day co-culture with LNCap or PC3 cells. CAR19 T cells were used as a negative control. (D) The images show that PSMA CAR T cells destroyed monolayers of LNCap cells, whereas PC3 cells remained intact (one representative donor is shown), n = 4.

of IL-2 supported terminal differentiation of CAR T cells (Figure 7).

Tscm are thought to better repopulate recipients, survive longer *in vivo* and be less susceptible to tumor suppression mechanisms; therefore, it is believed that adoptive T-cell therapy will be more effective with this T-cell subtype. Singh *et al.* [13] showed that exogenous IL-21 helps to generate CAR T cells with a less differentiated phenotype. Similarly, a recent report [8] showed that the Wnt signaling pathway, in combination with IL-21, is important for Tscm generation. The addition of the glycogen synthase-3β inhibitor TWS119 into *in vitro* cultures of CAR19 T cells together with IL-21 and IL-7 resulted in expansion of Tscm CAR T cells. Our data show that CAR19 T cells generated under IL-4 + IL-7 + IL-21 conditions

contained approximately 10% of CD8+ Tscm CAR19 T cells, while CAR19 T cells generated under IL-4 + IL-7 + IL-2 contained none. Our data thus show similar effects of IL-21 on T-cell development as those described by Sabatino *et al*. We are currently studying the effects of TWS119 on CAR T-cell differentiation using our experimental system. The ability of IL-21 to block differentiation of CAR T cells toward terminal phenotypes is certainly a unique feature of IL-21, and this cytokine will be most likely included in future expansion protocols.

We also found that that the cultivation of CAR19 T cells in the presence of IL-4 and IL-7 supports expansion of CD4+ CAR19 T cells (Figure 3B). The anti-tumor role of CD4+ CAR T has not been clearly determined yet, but it has been shown that CD4+ CAR T cells are cytotoxic [24], and the existence of cytolytic CD4+ T cells is also a well-known fact because they play important role in antiviral cytotoxic T-cell responses. Our data show that both CD4+ and CD8+ CAR19 T cells were cytotoxi.c because they degranulated (Figure 3D) and produced IFN-gamma and TNF-alpha after stimulation with CD19+ target cells. It is believed that CAR T cells should contain both CD4+ and CD8+ subsets because there are reports suggesting that CD4+ CAR T cells might provide help and support the antitumor functions of CD8+ CAR T cells [25].

In conclusion, we have developed a simple method for manufacturing CAR19 T cells based on the electroporation of piggyBac transposon vector and cultivation in the presence of cytokines IL-4, IL-7 and IL-21. The manufacturing process does not require special feeder cells and was specifically designed to be compatible with current GMP rules. We are now preparing a clinical trial to test the efficiency of CAR19 T cells prepared via this protocol.

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Disclosure of interests: The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

References

[1] Yusa K, Zhou L, Li MA, Bradley A, Craig NL. A hyperactive piggyBac transposase for mammalian applications. Proc Natl Acad Sci USA 2011;108:1531–6. https://doi.org/10.1073/pnas.1008322108.

[2] Singh H, Figliola MJ, Dawson MJ, Olivares S, Zhang L, Yang G, et al. Manufacture of clinical-grade CD19-specific T cells stably expressing chimeric antigen receptor using sleeping beauty system and artificial antigen presenting cells. PLoS ONE 2013;8:e64138. https://doi.org/10.1371/journal.pone.0064138.

[3] Ramanayake S, Bilmon IAN, Bishop D, Dubosq M, Blyth E, Clancy L, et al. Low-cost generation of Good Manufacturing Practice e grade CD19-speci fi c chimeric antigen receptor e expressing T cells using piggyBac gene transfer and patient-derived materials. Cytotherapy 2015;17:1251–67. https://doi.org/10.1016/j.jcyt.2015.05.013.

[4] Field A-C, Vink C, Gabriel R, Al-Subki R, Schmidt M, Goulden N, et al. Comparison of lentiviral and sleeping beauty mediated αβ T cell receptor gene transfer. PLoS ONE 2013;8:e68201. https://doi.org/10.1371/journal.pone.0068201.

[5] Kakarla S, Gottschalk S. CAR T cells for solid tumors: armed and ready to go? Cancer J 2014;20:151–5. https://doi.org/10.1097/PPO.0000000000000032.

[6] Duong CPM, Yong CSM, Kershaw MH, Slaney CY, Darcy PK. Cancer immunotherapy utilizing gene-modified T cells: from the bench to the clinic. Mol Immunol 2015;https://doi.org/10.1016/j.molimm.2014.12.009.

[7] Crompton JG, Sukumar M, Restifo NP. Uncoupling T-cell expansion from effector differentiation in cell-based immunotherapy. Immunol Rev 2014;257:264–76. https://doi.org/10.1111/imr.12135.

[8] Sabatino M, Hu J, Sommariva M, Gautam S, Fellowes V, Hocker JD, et al. Generation of clinical-grade CD19-specific CAR-modified CD8+ memory stem cells for the treatment of human B-cell malignancies. Blood 2016;128:519–28. https://doi.org/10.1182/blood-2015-11-683847.

[9] Gerdemann U, Keirnan JM, Katari UL, Yanagisawa R, Christin AS, Huye LE, et al. Rapidly generated multivirus-specific cytotoxic t lymphocytes for the prophylaxis and treatment of viral infections. Mol Ther 2009;20:1622–32. https://doi.org/10.1038/mt.2012.130.

[10] Papadopoulou A, Gerdemann U, Katari UL, Tzannou I, Liu H, Martinez C, et al. Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. Sci Transl Med 2014;6:242ra83. https://doi.org/10.1126/scitranslmed.3008825.

[11] Kaartinen T, Luostarinen A, Maliniemi P, Keto J, Arvas M, Belt H, et al. Low interleukin-2 concentration favors generation of early memory T cells over effector phenotypes during chimeric antigen receptor T-cell expansion. Cytotherapy 2017;19:689–702. https://doi.org/10.1016/j.jcyt.2017.03.067.

[12] Deniger DC, Yu J, Huls MH, Figliola MJ, Mi T, Maiti SN, et al. Sleeping Beauty transposition of chimeric antigen receptors targeting receptor tyrosine kinase-like orphan receptor-1 (ROR1) into diverse memory T-cell populations. PLoS ONE 2015;10:e012815. https://doi.org/10.1371/journal.pone.0128151.

[13] Singh H, Figliola MJ, Dawson MJ, Huls H, Olivares S, Switzer K, et al. Reprogramming CD19-specific T cells with IL-21 signaling can improve adoptive immunotherapy of B-lineage malignancies. Cancer Res 2011;71:3516–27. https://doi.org/10.1158/0008-5472.CAN-10-3843.

[14] Whilding LM, Parente-Pereira AC, Zabinski T, Davies DM, Petrovic RMG, Kao YV, et al. Targeting of aberrant αvβ6 integrin expression in solid tumors using chimeric antigen receptor-engineered T cells. Mol Ther 2017;25:259–73. https://doi.org/10.1016/j.ymthe.2016.10.012.

[15] Otáhal P, Průková D, Král V, Fabry M, Vočková P, Latečková L, et al. Lenalidomide enhances antitumor functions of chimeric antigen receptor modified T cells. Oncoimmunology

2016;5:e1115940. https://doi.org/10.1080/2162402X.2015.1115940.

[16] Gargett T, Brown MP. Different cytokine and stimulation conditions in fl uence the expansion and immune phenotype of third-generation chimeric antigen receptor T cells specific for tumor antigen GD2. Cytotherapy 2015;17:487–95. https://doi.org/10.1016/j.jcyt.2014.12.002.

[17] Xu X-J, Song D-G, Poussin M, Ye Q, Sharma P, Rodríguez-García A, et al. Multiparameter comparative analysis reveals differential impacts of various cytokines on CAR T cell phenotype and function *ex vivo* and *in vivo*. Oncotarget 2014;7:82354–68. https://doi.org/10.18632/oncotarget.10510.

[18] Santoro SP, Kim S, Motz GT, Alatzoglou D, Li C, Irving M, et al. T cells bearing a chimeric antigen receptor against prostate-specific membrane antigen mediate vascular disruption and result in tumor regression. Cancer Immunol Res 2015;3:68–84. https://doi.org/10.1158/2326-6066.CIR-14-0192.

[19] Wilkie S, Van Schalkwyk MCI, Hobbs S, Pereira ACP, Burbridge SE, Box C, et al. Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors engineered to provide complementary signaling. J Clin Immunol 2012;32:1059–70. https://doi.org/10.1007/s10875-012-9689-9.

[20] Perna SK, Pagliara D, Mahendravada A, Liu H, Brenner MK, Savoldo B, et al. Interleukin-7 mediates selective expansion of tumor-redirected cytotoxic T lymphocytes (CTLs) without

enhancement of regulatory T-cell inhibition. Clin Cancer Res 2014;20:131–9. https://doi.org/10.1158/1078-0432.CCR-13-1016.

[21] Vella AT, Dow S, Potter TA, Kappler J, Marrack P. Cytokine-induced survival of activated T cells in vitro and in vivo. Proc Natl Acad Sci USA 1998;95:3810–15. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=19919&tool=pmcentrez&rendertype=abstract.

[22] Ryan JJ, McReynolds LJ, Keegan A, Wang LH, Garfein E, Rothman P, et al. Growth and gene expression are predominantly controlled by distinct regions of the human IL-4 receptor. Immunity 1996;4:123–32. https://doi.org/10.1016/S1074-7613(00)80677-9.

[23] Tian Y, Zajac AJ. IL-21 and T cell differentiation: consider the context. Trends Immunol 2016;https://doi.org/10.1016/j.it.2016.06.001.

[24] Liadi I, Singh H, Romain G, Rey-Villamizar N, Merouane A, Adolacion JRT, et al. Individual motile CD4(+) T cells can participate in efficient multikilling through conjugation to multiple tumor cells. Cancer Immunol Res 2015;3:473–82. https://doi.org/10.1158/2326-6066.CIR-14-0195.

[25] Turtle CJ, Hanafi L-A, Berger C, Hudecek M, Pender B, Robinson E, et al. Immunotherapy of non-Hodgkins lymphoma with a defined ratio of CD8+ and CD4+ CD19-specific chimeric antigen receptor-modified T cells. Sci Transl Med 2016;8:355ra116. https://doi.org/10.1126/scitranslmed.aaf8621.

4.2. Publication 2

Štach M, Ptáčková P, Mucha M, Musil J, Klener P, Otáhal P.

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FULL-LENGTH ARTICLE

Translational Research

Inducible secretion of IL-21 augments anti-tumor activity of piggyBac-manufactured chimeric antigen receptor T cells

Martin Štach[1,3], Pavlína Ptáčková[1,2], Martin Mucha[1,3], Jan Musil[1], Pavel Klener[2], Pavel Otáhal[1,2,*]

[1] Institute of Hematology and Blood Transfusion, Prague, Czech Republic
[2] First Department of Medicine, Department of Haematology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic
[3] Faculty of Natural Sciences, Charles University, Prague, Czech Republic

ARTICLE INFO

ABSTRACT

Background: The efficiency of chimeric antigen receptor (CAR) T-cell-based therapies depends on a sufficient expansion of CAR T cells *in vivo* and can be weakened by intra-tumoral suppression of CAR T cell functions, leading to a failure of therapy. For example, certain B-cell malignancies such as chronic lymphocytic leukemia are weakly sensitive to treatment with CAR T cells. Co-expression of proinflamatory cytokines such as IL-12 and IL-18 by CAR T cells have been shown to enhance their antitumor function. We similarly engineered CAR T cell to co-express IL-21 and studied the effects of IL-21 on CAR T cells specific to CD19 and prostate-specific membrane antigens using an in vitro co-culture model and NSG mice transplanted with B-cell tumors.
Results: IL-21 enhanced the expansion of CAR T cells after antigenic stimulation, reduced the level of apoptosis of CAR T cells during co-culture with tumor cells and prevented differentiation of CAR T cells toward late memory phenotypes. In addition, induced secretion of IL-21 by CAR T cells promoted tumor infiltration by CD19-specific CAR (CAR19) T cells in NSG mice, resulting in reduced tumor growth. By co-culturing CAR19 T cells with bone-marrow fragments infiltrated with CLL cells we demonstrate that IL-21 reduces the immunosupressive activity of CLL cells against CAR19 T cells.
Conclusions: CAR19 T cells armed with IL-21 exhibited enhanced antitumor functions. IL-21 promoted their proliferation and cytotoxicity against chronic lymphocytic leukemia (CLL). The results suggest that arming CAR T cells with IL-21 could boost the effectiveness of CAR T-mediated therapies.

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Introduction

Various strategies are currently being explored to improve the functionality of chimeric antigen receptor (CAR) T cells and enhance their survival *in vivo*. In addition to T-cell receptor engagement and co-stimulatory signaling, cytokines play a critical role in modulating T-cell function. Therefore, an appealing strategy is to engineer CAR T cells to produce cytokines that enhance anti-tumor responses by acting on tumor stroma or influence CAR T cells directly in a paracrine/autocrine manner. Several pro-inflammatory cytokines, including IL-12, IL-15 and IL-18, that are co-expressed by CAR T cells have been shown to modulate their anti-tumor functions [1−3], supporting this strategy. The authors studied another relevant cytokine as a potential enhancer of CAR T cells, IL-21. IL-21 is a pleiotropic cytokine that acts on many lymphocyte subsets but in natural T cells and CAR T cells promotes

expansion and the maintenance of the early memory phenotype [4]. Interestingly, the effects of IL-21 are counteracted by high concentrations of IL-2, which directs CAR T cells toward terminal memory phenotypes and, eventually, activation-induced cell death (AICD) [5]. It is thought that such early memory CAR T cells (CD62L+/CCR7+/CD45RA+/CD28+/CD27+/CD95+/PD-1−) have enhanced *in vivo* persistence and provide greater anti-tumor effects than the more differentiated T-cell subsets [6,7]. These experimental observations were further verified by the outcomes of clinical studies with CD19-specific CAR (CAR19) T cells in patients with B-cell acute lymphoblastic leukemia (B-ALL), showing that more efficient treatment correlated with an increased number of less differentiated CAR T cells with early memory phenotype [8]. Although CAR19 T cells have been shown to be highly effective against B-ALL, their efficiency against indolent B-cell lymphoproliferative diseases, such as chronic lymphocytic leukemia (CLL), is significantly weaker [9]. Recently, CLL cells were shown to directly inhibit proliferation of CAR19 T cells [10] and impede their functions. The efficiency of CAR T cells against solid tumors is even worse, as solid tumors induce

* Correspondence.
E-mail address: otahal@uhkt.cz (P. Otáhal).

abortive activation and apoptosis of infiltrating lymphocytes, which prevents their long-term expansion [11].

These facts led the authors to further study the effects of IL-21 on memory differentiation of CAR T cells. The authors hypothesized that the efficiency of CAR T cells can be augmented by modulating their memory differentiation by inducing the expression of IL-21. The authors constructed an artificial nuclear factor of activated T cells (NFAT) promoter, as described for IL-12 or IL-18 [1,3,12], which becomes activated upon anti-genic stimulation of CAR T cells. The authors prepared human CAR T cells to react to prostate-specific membrane antigen (PSMA) and CD19 antigen co-expressing NFAT-regulated IL-21 (NFAT IL-21). CAR T cells were manufactured via electroporation of piggyBac transposon vector, followed by *in vitro* expansion in a cocktail of cytokines containing IL-4, IL-7 and IL-21, as the authors described previously [6], resulting in a homogeneous population of >90% CAR+ T cells with an early memory phenotype. CAR T cells were then repeatedly restimulated with tumor cells *in vitro* or analyzed in NSG mice transplanted with B-cell lymphoma cells. To study the effects of CAR19 T cells against CLL, the authors developed an *in vitro* model based on co-cultivation of CAR19 T cells with bone marrow (BM) fragments obtained from patients with CLL that were infiltrated by CLL cells.

The authors found that IL-21 inhibited terminal differentiation of CAR T cells after anti-genic restimulation with tumor cells and promoted their expansion and the development of effector functions. By contrast, IL-2 enhanced the expansion of CAR T cells to lower levels, with opposite effects on memory differentiation, producing terminally differentiated CAR T cells expressing significantly more PD-1. CAR T cells engineered with induced secretion of IL-21 had increased anti-tumor effects against B-cell tumors transplanted in NSG mice and enhanced activity against CLL cells compared with non-armed CAR19 T cells. In summary, the authors' results show that IL-21 is an important regulator of T-cell memory development and suggest that modification of CAR19 T cells to secrete IL-21 upon their activation could be another way to enhance their therapeutic potency—for example, against weakly sensitive B-cell malignancies, such as CLL.

Methods

Cell source

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from blood donors. The use of all human materials was approved by the institutional review board, and all donors signed informed consent for the use of biological materials. Ramos is a CD19+ Burkitt lymphoma cell line (American Type Culture Collection CRL-1596). LNCaP is a prostate carcinoma cell line positive for PSMA (American Type Culture Collection CRL-1740). BM fragments were obtained by trephine biopsy from donors with newly diagnosed CLL, and 20–90% infiltration of the BM was verified by fluorescence-activated cell sorting (FACS) and immunohistochemistry.

DNA plasmids and sequences

CAR19 and PSMA CAR constructs have been described previously [6,13]. Both CARs have the same design and contain a 4-1BB co-stimulatory domain and T-cell receptor zeta domain. The hyperactive piggyBac transposase is driven by a cytomegalovirus promoter [14]. To construct the NFAT IL-21 vector, the promoter was synthesized containing ×6 NFAT binding motif, minimal IL-2 promoter, human full-length IL-21 and SV40 polyA. This segment was cloned behind the CAR sequence in the same direction as the CAR gene into CAR19 and PSMA CAR vectors via XhoI-SalI. All CAR constructs were cloned into the piggyBac vector behind the ubiquitin C promoter. For fluorescent labeling of T cells, green fluorescent protein (GFP) was cloned into a piggyBac vector in a manner similar to that used for the CAR

constructs. Plasmids were purified by standard techniques using EndoFree kits (Qiagen, Germany). The sequences of PSMA CAR and NFAT promoter are provided in the supplementary material. The sequence of CAR19 has been published previously [13].

Cell culture, electroporation and antibody stimulation

For all experiments, the authors used CellGro media (CellGenix, Germany) supplemented with 10% heat-inactivated fetal calf serum (Gibco, USA) and the antibiotics penicillin and streptomycin (Gibco, USA). PBMCs were isolated from buffy coats or fresh blood samples by gradient centrifugation using Ficoll-Paque premium (GE Healthcare). After separation, cells were transfected with a Neon electroporator (Thermo Fisher Scientific, USA); $1 \times 10e7$ cells were resuspended in 100 μL buffer T and mixed with 5 μg plasmid DNA (2:1 mixture of piggyBac vector expressing CAR and a plasmid expressing a hyperactive piggyBac transposase) and electroporated for 20 ms at 2300 V using 100-μL tips. Cells then rested overnight in cell media with cytokines. The next day, the cells were stimulated with immobilized antibodies or left unstimulated. To manufacture GFP+ CAR T cells, a 2-μg GFP pPB vector was added to a mixture of plasmids. Virtually all GFP+ cells were positive for CAR and GFP− cells negative for CAR (data not shown).

To stimulate T cells with antibodies, both anti-CD3 and anti-CD28 mouse monoclonal antibodies (Miltenyi Biotec, Germany) and mouse anti-myc-tag antibody clone 9E10 (Exbio, Czech Republic) were diluted in phosphate-buffered saline at a concentration of 1 mg/mL and adhered onto a cell culture plate for 1 h at 37°C. The cells were washed with phosphate-buffered saline and used for cell activation. After 3 days of stimulation, cells were replated onto new culture plates.

Cell media were supplemented with cytokines as follows: 1000 U/mL IL-2 (Proleukin; Roche), 20 ng/mL IL-4 (Miltenyi Biotec), 10 ng/mL IL-7 (Miltenyi Biotec) and 40 ng/mL IL-21 (Miltenyi Biotec). Cells were fed with fresh media containing cytokines every 3–4 days according to growth rate. To sort cells by FACS, the authors used fluorescently labeled antibodies and isolated on a FACSAria instrument (BD Biosciences, USA).

Antibodies, FACS and enzyme-linked immunosorbent assay

Mouse anti-human CD3 APC-Cy7, CD45RA Alexa Fluor 488, CCR7 BV605, CD45RA-BUV737 (clone HI100), CD62L-BV650 (clone DREG-56), CD56-BUV563 (clone NCAM16.2), CD244-APC (clone 2-69), BTLA-BV421 (clone J168-540), CD3-BV786 (clone UCHT1), Tim3-BV480 (clone 7D3), CD160-PE (clone B455), PD1-BB700 (clone EH12.1), TIGIT-BV421 (clone 741182) and LAG3-PE (clone T47-530) were purchased from BD Biosciences (USA). LAG3-PE-eFluor610 (clone 3DS223H) and LAG3-APC (clone 3DS223H) were purchased from eBioscience (USA). TIGIT-PE-Cy7 (clone A151536), CD4-qDOT605 (clone S3.5), CD28-PE-Cy7 (clone CD28.2) and PD1-PE (clone EH12.2H7) were purchased from BioLegend (USA). CD8-AF700 (clone MEM-31), CD27-PE-Dy590 (clone LT27), Myc-FITC (clone 9E10), CD107a-PE (clone H4A3) and Annexin V-Phycoerythrin were purchased from Exbio. CD57-APC-Vio7701 (130-104-197), Myc-FITC (120-003-159), CD28 (clone 15E8) and CD3 (130-093-387) were purchased from Miltenyi Biotec. CD4-PB (clone RPA-T4) was obtained from Sony (USA). Live cells were identified using a fixable blue dead cell stain kit (Thermo Fisher Scientific, USA).

To detect the CAR transgene, cells were stained with Alexa-647- or Alexa-488-labeled Fab2 fragment from goat anti-mouse IgG (Jackson ImmunoResearch, UK). This polyclonal antibody reacts with mouse anti-CD19 scFv in CAR19. To detect PSMA CAR, cells were labeled with anti-myc-tag antibody clone 9E10 (Exbio). Cells were labeled with goat anti-mouse antibody, washed twice and blocked with 10% mouse serum and then stained with fluorescently labeled

mouse monoclonal antibodies. The specificity of staining was extensively tested to rule out possible binding of mouse antibodies to cells due to the goat anti-mouse antibodies used to detect CAR.

To visualize in situ proliferation of CAR19 T cells, Ramos cells were adhered onto polylysine-coated (Merck, USA) chambered glass coverslips (Labtek, USA), and adherent LNCaP cells were directly grown on glass slides. Next, cells were briefly co-incubated with CAR T cells at a low E:T ratio and then overlaid with 0.5% agarose prepared in cell media. Cells were imaged using a fluorescent microscope with a digital camera (Olympus, Japan).

To determine the degranulation of CAR T cells, cells were incubated with or without Ramos B cells at a 1:1 ratio for 4 h. Phycoerythrin-labeled anti-CD107a antibody (Exbio) was added to the cell media at the beginning of the co-incubation. After 4 h, cells were harvested and labeled with anti-CD8 and anti-CD4 antibodies and analyzed by FACS on a FACS LSRFortessa instrument (BD Biosciences). To detect apoptotic cells, fluorescent caspase-3/7 substrate (Life Sciences, USA) was added to the cells for 30 min before FACS analysis.

To detect IL-21, CAR T NFAT 21 cells were stimulated overnight with anti-CD3 antibody or Ramos cells. After incubation, cells were freeze-thawed twice and the supernatants centrifuged to remove cell debris. The samples were then analyzed using an IL-21 enzyme-linked immunosorbent assay kit (BioLegend, USA). Absorbances were determined on an enzyme-linked immunosorbent assay plate reader and concentrations of IL-21 calculated against IL-21 standards provided with the kit using an Excel spreadsheet.

The production of interferon gamma was determined using a cytokine capture kit (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, T cells cultured in the presence of CLL cells for 7 days were restimulated with Ramos B cells for 4 h in the presence of an interferon gamma labeling reagent. The cells were then washed and labeled with an interferon gamma detection reagent and monoclonal antibodies. FACS samples were analyzed using the LSRFortessa instrument (BD Biosciences) and FACS data processed using FlowJo software. Statistical analysis was performed using Prism software (GraphPad). The gating strategy for the phenotype analysis is provided in supplementary Figure 1.

In vitro *co-cultivation assays*

CAR T cells were cultured together with their tumor target cells at a 1:5 E:T ratio without cytokines or with the addition of IL-2 or IL-21. Ramos cells were used for CAR19 and LNCaP cells for PSMA CAR. The CAR T cells were restimulated with cells two to three times every 3−4 days depending on the experiment. To restimulate CAR T cells with BM fragments, a BM specimen obtained by trephine biopsy was cut with a blade into several pieces approximately 2 mm in size. BM fragments were cultured in a suspension of CAR19 T cells (1 million/mL) for 24 h and then gently washed with fresh media and cultured for 12 days in media without cytokines. Microscopic images of GFP+ cells were taken during the culture using a confocal microscope to asses infiltration of the BM. At the end of the experiments, BM fragments were washed with media and dissociated by pipetting and then used for functional assays and FACS analysis. The differences between groups were analyzed by a two-tailed unpaired *t*-test using Prism software (GraphPad).

In vivo *experiments and statistical analysis*

In vivo studies were approved by the Institutional Animal Care and Use Committee. Immunodeficient NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, or NSG, mice (Jackson Laboratory) were maintained in individually ventilated cages under specific pathogen-free conditions. Groups of five or six mice aged 6−12 weeks and of the same sex were injected subcutaneously with $5 \times 10e6$ Ramos B cells. After 12 days, when large macroscopic tumors had formed, the mice

received one intravenous dose of $5 \times 10e6$ CAR T cells. Both CAR19 NFAT IL-21 and control CAR19 T cells were grown in IL-4, IL-7 and IL-21. After another 18 days, the mice were killed, tumors excised and weighed and tumor tissues mechanically dissociated by pipetting and analyzed by flow cytometry for the presence of human CD4 and CD8 T cells. The differences between groups were analyzed by a two-tailed unpaired *t*-test. Mice were also analyzed by one-way analysis of variance with Dunnett's multiple comparison post-test using Prism software (GraphPad). All animal work was performed under approved ethical guidelines.

Results

To characterize the prepared cells, the authors determined the percentage of CAR19 T cells with early memory phenotype or a stem cell memory-like (Tscm-like) phenotype, characterized by expression of antigens CD45RA, CD62L, CD27 and CD28 [7]. The data in Figure 1A show that the majority of CAR19 T cells cultivated in the presence of IL-4, IL-7 and IL-21 were mostly of the early memory phenotype. Both cultivation methods produced CAR19 T cells with an activated phenotype, as the authors detected upregulation of antigen CD95 (Figure 1F). The expansion of CAR19 T cells did not require anti-CD3/CD28 stimulation because the transduced T cells were activated by B cells, which were present in the mixture of electroporated PBMCs. Omitting anti-CD3/CD28 activation enabled selective expansion of the modified T cells, yielding >90% CAR+ T cells [6], and did not significantly influence the phenotype of CAR19 T cells. By contrast, cultivation in the presence of IL-2 plus activation with anti-CD3/CD28 antibodies yielded a population with a very low number of Tscm-like CAR19 T cells (Figure 1A).

CAR19 T cells grown in IL-4, IL-7 and IL-21 had significantly better viability than CAR19 T cells grown in the presence of IL-2 with anti-CD3/CD28 after anti-genic restimulation with Ramos B cells (Figure 1B). The addition of IL-2 or IL-21 during co-cultivation with Ramos B cells improved their expansion, but the effects of IL-21 were stronger (Figure 1C). The authors noted minimal expansion of CAR19 T cells grown in the presence of IL-2 with anti-CD3/CD28 in the absence of exogenously added cytokines. Subsequently, the authors determined the memory phenotype (Figure 1D) and expression of exhaustion marker PD-1 (Figure 1E) on CAR19 T cells after restimulation with Ramos B cells. The addition of IL-21 maintained the Tscm-like phenotype of proliferating cells, whereas the addition of IL-2 induced the expansion of more differentiated CAR19 T cells expressing high levels of PD-1 compared with non-restimulated CAR19 T cells (Figure 1E). These results suggest that IL-21 promotes not only the expansion of CAR19 T cells but also the maintenance of early memory phenotype and improves their survival after anti-genic restimulation with tumor cells.

In the next experiment, the authors sorted CD8+ CAR19 T cells expressing CD62L+ and CD45RA+ (Figure 2A) and restimulated them twice with Ramos B cells in the presence of IL-2 or IL-21. Similar to previous experiments, the addition of IL-21 significantly enhanced expansion more than the addition of IL-2 (Figure 2B), and the CAR19 T cells restimulated in the presence of IL-21 retained their immature phenotype (>90% of cells were CD27+CD28+CD62L+) (Figure 2C). By contrast, the addition of IL-2 during restimulation resulted in a significant loss of expression of CD62L, CD27 and CD28, indicating that although IL-2 promotes the expansion of CAR19 T cells, it induces their differentiation toward effector CD8+ T cells.

Next, the authors assessed the effects of IL-21 on CAR19 T cells grown in the presence of IL-2 and vice versa. Unsorted CAR19 T cells grown in the presence of IL-2 with anti-CD3/CD28 were co-cultured with Ramos B cells in the presence of IL-21, and CAR19 T cells grown in the presence of IL-4, IL-7 and IL-21 were co-cultured with Ramos B cells in the presence of IL-2 (Figure 2D). IL-21 enhanced the expansion of Tscm-like CAR T cells during co-culture, but the upregulation
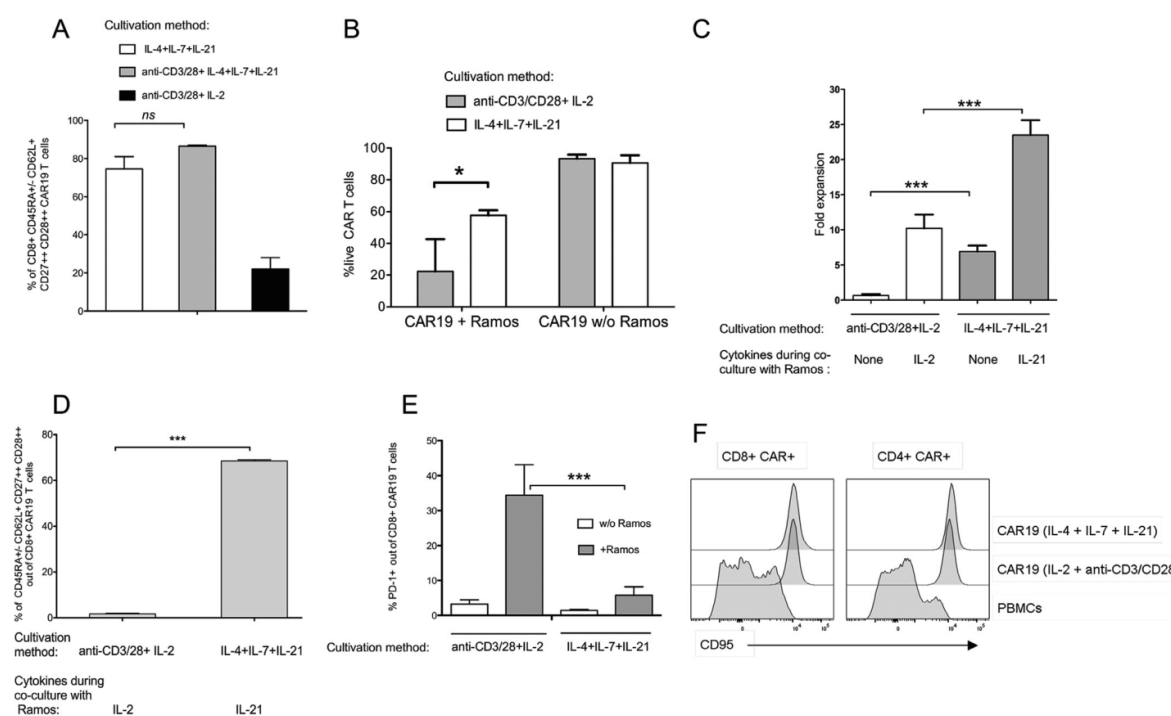
Figure 1. IL-21 promotes expansion of CAR19 T cells and maintenance of early memory phenotype after anti-genic restimulation. (A) Percentage of Tscm-like (CD45RA+/− CD62L+ CD27++ CD28++) CAR19 T cells in the product obtained by cultivating in a mixture of IL-4, IL-7 and IL-21 or a mixture of IL-4, IL-7 and IL-21 plus activation with anti-CD3/28 antibodies or CAR19 T cells manufactured by cultivation in IL-2 activated with anti-CD3/28 antibodies. (B) Percentage of viable (non-apoptotic) CAR T cells after co-cultivation with Ramos cells for 5 days. No exogenous cytokines were added to the cells in this experiment. The viability was determined with fluorescent caspase-3/7 substrate and a DNA dye (DAPI). (C) The fold expansion after two rounds of restimulation with Ramos cells in the presence or absence of IL-2 or IL-21. (D) Percentage of Tscm-like CD8+ CAR T cells after two rounds of restimulation with Ramos cells in the presence of IL-2 or IL-21. (E) Percentage of PD-1+CD8+ CAR T cells after two rounds of restimulation with Ramos cells in the presence or absence of IL-2 or IL-21. The white bar shows the expression of PD-1 on the product without restimulation with Ramos cells. N = 3; error bars indicate SEM. Significance was determined by unpaired *t*-test. (F) Histogram of the expression of CD95 on non-co-cultivated CD4+ and CD8+ CAR T cells in one representative donor compared with CD4+ and CD8+ T cells from a healthy donor. *ns* (not significant) *P* > 0.05 , * *P* ≤ 0.05, *** *P* ≤ 0.001. DAPI, 4',6-diamidino-2-phenylindole; ns, not significant; SEM, standard error of the mean; w/o, without.

of PD-1 depended on the presence of IL-2. If IL-2 was added during the co-culture of CAR T cells prepared in IL-4+IL-7+IL-21, it induced the downregulation of CD62L, CD27 and CD28 but did not induce the upregulation of PD-1 (Figure 2D, middle panel). By contrast, the addition of IL-21 during the co-culture of CAR T cells prepared in IL-2 did not prevent upregulation of PD-1 but enabled expansion and maintenance of the early memory phenotype (CD62L+CD27+CD28+) (Figure 2D, bottom panel). Thus, the upregulation of PD-1 depends on previous exposure of CAR T cells to IL-2.

The authors decided to investigate whether the effects of IL-21 were also observed using CAR T cells targeting tumor cells of non-hematological origin. The authors tested CAR T cells specific for anti-gen PSMA, which specifically recognizes the LNCaP carcinoma cell line. The PSMA CAR construct contains a myc-tag epitope between scFv and a transmembrane domain, and activation of transduced T cells with immobilized myc-tag-specific antibody (clone 9E10) leads to selective expansion of PSMA CAR T cells. Total T-cell expansion was equal regardless of whether cells were activated via anti-myc antibody or anti-CD3/CD28 antibodies or whether IL-2 or a cocktail of IL-4, IL-7 and IL-21 was used during *in vitro* culture (Figure 3A). However, anti-myc activation produced >90% CAR+ cells, whereas poly-clonal activation with anti-CD3/CD28 antibodies produced only about 15% CAR+ T cells (Figure 3C). The phenotype of PSMA CAR T cells also depended on the cytokines used for the expansion, as the authors have shown in previous experiments [6]. Cultivation in IL-2 yielded approximately 5% Tscm-like CD8+ PSMA CAR T cells (CD45RA

+/−CD62L+CD27++CD28++), whereas cultivation in the presence of IL-4, IL-7 and IL-21 yielded approximately 60−70% Tscm-like PSMA CAR T cells after anti-myc activation and approximately 85% Tscm-like CD8+ PSMA CAR T cells after anti-CD3/CD28 activation (Figure 3B). The authors noted a slightly higher number of Tscm-like cells after anti-CD3/CD28 activation in the presence of IL-4, IL-7 and IL-21, similar to what was observed with CAR19 T cells (Figure 1A).

Next, the authors hypothesized that IL-21 may enable the expansion of Tscm-like PSMA CAR T cells after restimulation with LNCaP cells, similar to what has been observed in previous experiments. Instead of using PSMA CAR T cells expanded in IL-4, IL-7 and IL-21, the authors tested whether the small number of Tscm-like cells detected after expansion in IL-2+ anti-myc (Figure 3B) could be "rescued" by IL-21. Thus, the authors determined the phenotype (Figure 3D) and level of expansion (Figure 3E) of PSMA CAR T cells (prepared in IL-2) after two rounds of restimulation with LNCaP in the presence of IL-21 or IL-2 or without any exogenous cytokines. PSMA CAR T cells expanded roughly 2.5 times more in the presence of IL-21 than in the presence of IL-2 (Figure 3E), whereas no expansion was observed in the absence of exogenous cytokines; therefore, it was not possible to reliably determine the phenotype of remaining cells. FACS analysis showed (Figure 3D) that CD8+ PSMA CAR T cells expanded in the presence of IL-2 were late-stage T cells, the majority of which were Temra (CD62L−CD45RA+CD27−CD28−) cells. By contrast, co-cultivation in the presence of IL-21 led to a stronger expansion of PSMA CAR T cells, and a significant proportion of
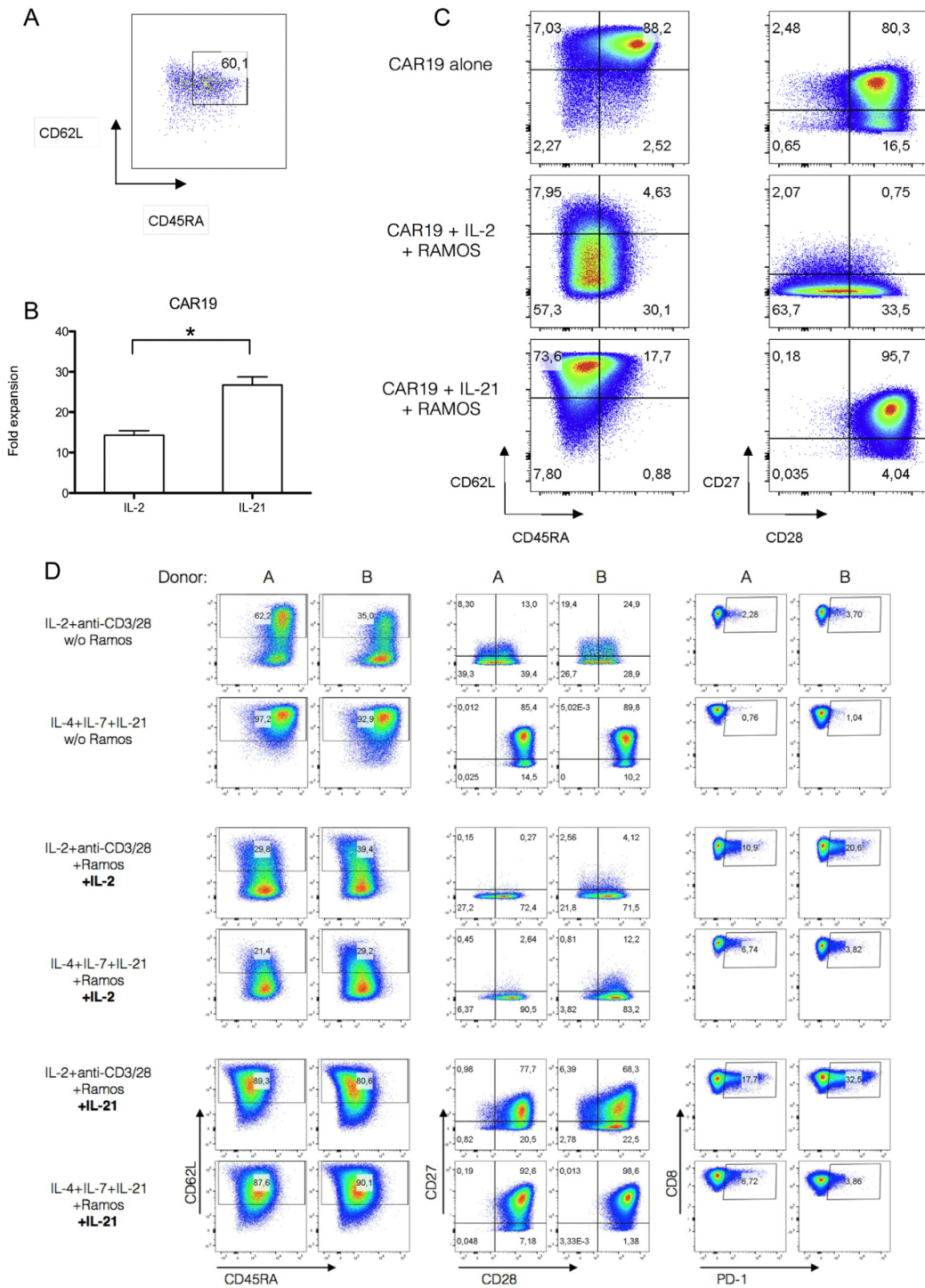
Figure 2. IL-2 supports proliferation of CAR19 T cells but does not promote maintenance of early memory phenotype. (A) CAR19 T cells cultivated in IL-4, IL-7 and IL-21 were sorted by FACS to isolate early memory T cells (CD62L+CD45RA+) and then restimulated with Ramos B cells in the presence of exogenous IL-2 or IL-21 or left in media with cytokines without Ramos cells. After 7 days, CAR19 T cells were counted and their developmental status determined by FACS. (B) Fold expansion of sorted CAR19 T cells after co-cultivation with Ramos cells in the presence of IL-2 or IL-21. N = 2; error bars indicate SEM. Significance was determined by unpaired *t*-test. (C) Dot plot showing the phenotype of CD8+ CAR T cells after restimulation for one representative donor out of two. Both donors had almost identical results. (D) CAR19 T cells were obtained by cultivation in a mixture of IL-4, IL-7 and IL-

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these expanded cells were CD62L+CD28+; some were also positive for CD27. Notably, the initial population contained very low numbers of Tscm-like CD8+ T cells because PSMA CAR T cells were cultured in IL-2.

Next, the authors analyzed the sensitivity of differently expanded PSMA CAR T cells to apoptosis after restimulation with LNCaP cells (Figure 3F). Apoptotic cells were identified by staining cells with a fluorescent caspase-3/7 substrate after 1 day, 2 days or 3 days of restimulation. The data show that the least sensitive cells to apoptosis were PSMA CAR T cells expanded in IL-4, IL-7 and IL-21 and restimulated with LNCaP cells in the presence of exogenous IL-21. Thus, PSMA CAR T cells with Tscm-like phenotype are significantly more resistant to AICD than the more differentiated cells, and exogenous IL-21 can improve their survival. These findings are in agreement with similar data presented in Figure 1B,C.

As the authors' data suggested a positive effect of IL-21 on the viability and expansion of CAR T cells, CAR T cells were engineered to secrete IL-21 after T-cell activation using an NFAT-regulated artificial promoter [3] to drive the expression of IL-21. In T cells, the antigen receptor stimulation activates the Ca^{2++}-calmodulin pathway, resulting in dephosphorylation of NFAT family transcription factors via phosphatase calcineurin, inducing them to translocate to the nucleus, where they bind NFAT response elements and modulate gene expression [15]. Artificial promoters containing NFAT response elements activate transcription similar to endogenous NFAT promoters and are commonly used to regulate gene expression in an activation-dependent manner [3]. The promoter DNA sequence is provided in supplementary Figure 1C,D.

To characterize the functionality of the NFAT IL-21 gene, the authors determined the production of IL-21 by CAR19 NFAT IL-21 T cells after activation via anti-CD3 antibody or Ramos B cells (Figure 4A). IL-21 was produced after anti-CD3 activation and at lower amounts after activation with Ramos cells. The functionality of PSMA CAR NFAT IL-21 T cells was similarly verified by measuring the production of IL-21 after activation (data not shown). To determine whether transgenic production of IL-21 enhances the expansion of CAR T cells, the authors prepared GFP-labeled CAR19 T cells and restimulated them with Ramos cells in a soft agar matrix. The growth of the CAR19 T cells was then monitored under a fluorescent microscope by measuring the size of proliferating cell clusters. The images in Figure 4B show an example of representative GFP+ clusters after 7 days. Estimation of the cell number per cluster shows that the majority of clusters in the CAR19 NFAT IL-21 sample were composed of more than five cells, whereas the clusters in the CAR19 T cell sample were small, with one or two cells per cluster (Figure 4C). The authors performed a similar experiment with GFP-labeled PSMA CAR NFAT IL-21 T cells (see supplementary Figure 2A,B), which similarly showed that expansion was stimulated by endogenously secreted IL-21.

Next, the authors determined the effect of induced IL-21 secretion on the phenotype of CAR19 T cells after restimulation with Ramos B cells (Figure 4D). FACS analysis showed that majority of CAR19 NFAT IL-21 T cells were CD62L+CD27++CD28++ compared with non-armed CAR19 T cells (~56% versus 13%). Repeated anti-genic restimulation may result in inhibition of effector functions; therefore, the authors tested the functionality of co-cultivated CAR19 NFAT IL21 cells via degranulation assay (see supplementary Figure 3B). This experiment revealed that CAR19 NFAT-21 cells were functional. In addition, the authors tested the production of pro-inflammatory cytokines by CAR19 NFAT IL-21 T cells and non-armed CAR19 T cells before and after co-cultivation with Ramos cells using a Bio-Plex assay (see supplementary Figure 4). These results indicate that CAR19 NFAT IL-21 remained functional and retained the ability to secrete pro-inflammatory cytokines upon repeated anti-genic challenge. Thus, the authors demonstrated that induced secretion of IL-21 is biologically active and enhances the expansion of functional early memory CAR T cells.

Next, the authors determined whether CAR19 NFAT IL-21 T cells have enhanced anti-tumor functions using NSG mice transplanted with Ramos B cells (Figure 5A). Transgenic expression of IL-21 enhanced the anti-tumor activity of CAR19 NFAT IL-21 cells (Figure 5B) and significantly enhanced the infiltration of tumors by CD8+ CAR19 NFAT IL-21 (Figure 5D) compared with non-armed CAR19 T cells. The infiltration by CD4+ CAR T cells was approximately 10-fold lower than the infiltration by CD8+ CAR T cells (Figure 5C,E). Analysis of the immunophenotype of T cells recovered from tumors revealed that CAR19 NFAT IL-21 maintained the early memory phenotype (see supplementary Figure 5). These results further demonstrate the enhancement of CAR T-cell functions by induced secretion of IL-21.

The authors' next goal was to try to assess CAR19 NFAT IL-21 T-cell functions in a more physiological manner against directly isolated malignant human B cells. The microscopy images in Figure 6A show the representative samples after 3 days and 10 days of cultivation. The graph in Figure 6B shows the percentage of GFP+ cells in paired samples (the same PBMC donors tested against the same CLL patients). Both the microscopic images and the FACS results show that CAR19 NFAT IL-21 T cells infiltrated the BM to much higher levels than non-armed CAR19 T cells. The FACS analysis of cells recovered from BM fragments showed that induced secretion of IL-21 promoted maintenance of the Tscm-like phenotype (Figure 6C). However, no changes in the CD8:CD4 ratio between CAR19 and CAR19 NFAT IL-21 T cells were detected in these samples (Figure 6F). Interestingly, when testing the functionality of infiltrating CAR19 T cells in degranulation assays, the authors found that the non-armed CAR19 T cells had reduced degranulation, whereas CAR19 NFAT IL-21 T cells efficiently degranulated after challenge with Ramos cells (Figure 6E). In addition, the cytotoxic test against Ramos cells revealed that NFAT IL-21 CAR T cells recovered from BM fragments were functional (Figure 6D), similar to control CAR19 NFAT IL-21 T cells cultivated in media without BM fragments. To further demonstrate the ability of IL-21 to counteract the immunosuppressive effects of CLL cells, the authors determined the production of interferon gamma by CAR T cells co-cultured with CLL cells. CAR19 NFAT IL-21 T cells produced significantly more interferon gamma than CAR19 T cells not armed with IL-21 after anti-genic restimulation (0% versus 53%) (Figure 6G). The basis of this inhibition is not yet known, but the suppressed CAR19 T cells did not express inhibitory receptors PD-1, TIM-3 or LAG-3, and they were not senescent, as they did not express CD57 (data not shown). These data suggest that IL-21 enhances the activity of CAR19 T cells against CLL by stimulating their proliferation.

In conclusion, the data show that IL-21 augments the anti-tumor functions of CAR T cells via modification of their memory differentiation. IL-21 promotes maintenance of the early memory phenotype (CD62L+CD27++CD28++PD-1−) after anti-genic restimulation, which supports the survival and expansion of functional CAR T cells. These findings suggest that arming CAR T cells with inducible secretion of IL-21 could be a reasonable strategy for boosting the effectiveness of CAR T cell-mediated therapies.

21 or in IL-2 plus activation with anti-CD3/28 antibodies. The dot plots show the memory phenotype and expression of PD-1 antigen by CD8+ CAR19 T cells before co-culture with Ramos B cells (top two rows), after co-culture with Ramos B cells in the presence of IL-2 (middle two rows) or in the presence of IL-21 (bottom two rows). Two representative donors are shown. The experiment was repeated once with similar results. *$P < 0.05$. SEM, standard error of the mean; w/o, without. (Color version of figure is available online).
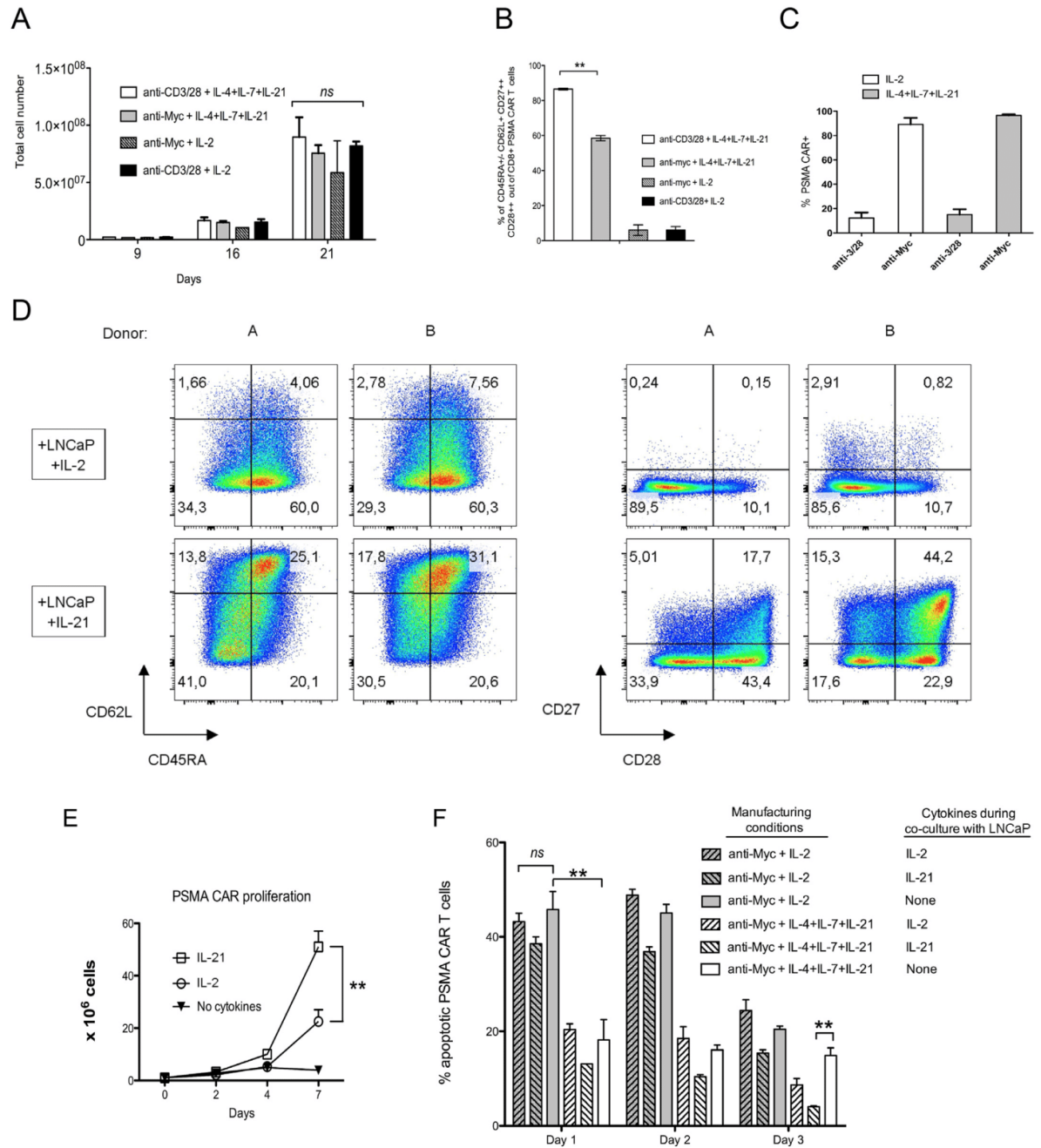
Figure 3. IL-21 enhances expansion of Tscm-like PSMA CAR T cells and reduces their apoptosis after anti-genic restimulation. (A–C) Characterization of manufactured PSMA CAR T cells. PBMCs were electroporated with DNA plasmids and activated with anti-CD3/CD28 antibodies or anti-Myc antibody, followed by expansion in the presence of IL-4, IL-7, IL-21 or IL-2. The expansion of all cells (A), percentage of CD8+ PSMA CAR T cells with Tscm-like phenotype after 21 days (B) and percentage of transduced T cells after 21 days (C) are shown. N = 3. (D) PSMA CAR T cells generated with anti-CD3/28 and IL-2 were co-cultured for 7 days with LNCaP cells in the presence of exogenous IL-2 or IL-21. Two representative donors are shown. The experiment was performed twice with similar outcomes. Importantly, PSMA CAR T cells before co-cultivation were manufactured in IL-2 and contained <10% Tscm-like T cells, as shown in (B). The image shows the memory phenotype. (E) Expansion of PSMA CAR T cells in the presence of IL-2 or IL-21 compared with PSMA CAR T cells co-cultivated with LNCaP without any exogenously added cytokines. N = 3; error bars indicate SEM. Significance was determined by unpaired *t*-test. (F) PSMA CAR T cells were manufactured under various indicated conditions and then restimulated with LNCaP cells in the presence of IL-2, IL-21 or no cytokines. The graph shows the percentage of apoptotic PSMA CAR T cells at the indicated time points. N = 3; error bars indicate SEM. Significance was determined by unpaired *t*-test. *ns* (not significant) $P > 0.05$, $^*P \leq 0.05$, $^{**}P \leq 0.01$; SEM, standard error of the mean. (Color version of figure is available online).
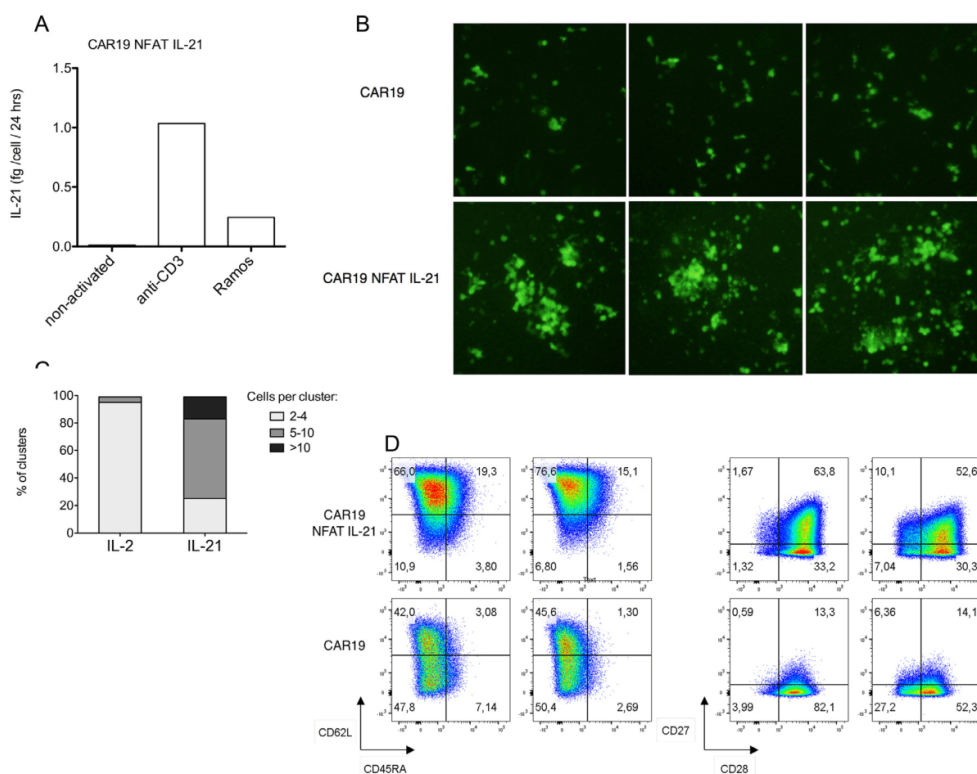
Figure 4. Inducible secretion of IL-21 stimulates the proliferation of CAR T cells after co-culture with tumor cells. (A) CAR19 T cells expressing IL-21 as a transgene under an artificial NFAT promoter produce IL-21 upon T-cell activation by anti-CD3 antibody or after co-cultivation with Ramos B cells. (B) CAR19 T cells and CAR19 NFAT IL-21 T cells were stably transduced with GFP-expressing transposon. CAR T cells were then co-incubated with Ramos B cells immobilized on the coverslip via polylysine and overlaid with soft agarose to immobilize cell clusters. After 7 days, the cell clusters were visualized by microscopy and their size determined by calculating the number of cells per cluster. The image shows examples of three areas containing the largest clusters. (C) The number of clusters containing two to four, five to 10 or more than 10 cells was calculated per bright field, and approximately 50 clusters were analyzed for each CAR19 construct. One representative donor is shown. The experiment was performed twice with similar results. (D) CAR19 T cells were restimulated twice at day 0 and day 4) with Ramos B cells to determine the effect of induced IL-21 on the phenotype of CAR19 T cells. The dot plots show the immunophenotype of CAR19 T cells or CAR19 NFAT IL-21 T cells at day 7. No exogenous cytokines were added to the cells in this experiment. Two representative donors are shown. The experiment was repeated twice with similar results. w/o, without. (Color version of figure is available online).

Discussion

Except for B-cell tumors treated with CD19 CAR T cells, a major limitation of current CAR T-cell approaches is insufficient expansion and persistence of infused T cells [11,16]. IL-21 has been shown to regulate T-cell homeostasis by promoting the proliferation and maintenance of the early memory phenotype of T cells. The rationale for the authors' study is to enhance CAR T-cell proliferation and utilize IL-21 as a growth-promoting cytokine to boost anti-tumor effects toward cancer cells that suppress the activity of CAR T cells.

The significance of the memory phenotype of CAR T cells in therapeutic efficiency is demonstrated by the results of recent clinical studies showing that an increased number of CAR T cells with a Tscm phenotype (CCR7+, CD62L+, CD27+, CD28+, CD45RA+ and PD-1−) correlates with greater persistence and expansion *in vivo* and better efficiency [8]. Various cytokine cocktails containing IL-2, IL-7, IL-15 and IL-21, or the addition of signaling pathway modulators, such as PI3K inhibitor or GSK3-beta inhibitor [7,17], have been shown to increase the number of Tscm CAR T cells during culture. By contrast, cultivation of CAR T cells in a high concentration of IL-2 promotes terminal memory differentiation of CAR T cells [18]. These findings led the authors to optimize the composition of the cytokine cocktail used during *in vitro* expansion, and it was found that a mixture of cytokines (i. e., IL-4, IL-7 and IL-21) enables efficient expansion of early memory CAR

T cells [6]. The authors also found that activation of transduced T cells with antigen (or anti-CAR antibody) instead of polyclonal activation with anti-CD3/CD28 antibodies selectively expands only the transduced T cells, yielding almost a pure population of CAR T cells. A similar approach has been described previously [19].

Because the authors used alternative methods of T-cell activation (via natural antigen or anti-CAR antibodies), as opposed to the commonly used anti-CD3/CD28 Dynabeads, the nature of CAR T-cell activation may influence their function and yield different results than activation via anti-CD3/CD28. The authors' data show that stimulation of PSMA CAR T cells via anti-myc or anti-CD3/CD28 antibodies produced functional CAR T cells with similar immunophenotypes and growth rates (Figure 3B,E). The authors did not detect any significant differences in regard to their cytotoxic functions, and similar findings were observed for CAR19 T cells (Figure 1). However, the authors found that CAR T cells cultivated in the presence of IL-2 performed poorly after antigenic restimulation compared with cells prepared in IL-4, IL-7 and IL-21, most likely because they had a more differentiated memory phenotype (Figures 1A, 3B).

Several published studies have shown similar effects of IL-21. For example, Singh et al.[20] showed that IL-21 enhances the development of less differentiated CAR T cells *in vitro*, but the effects were much weaker than those seen in the current study because Singh
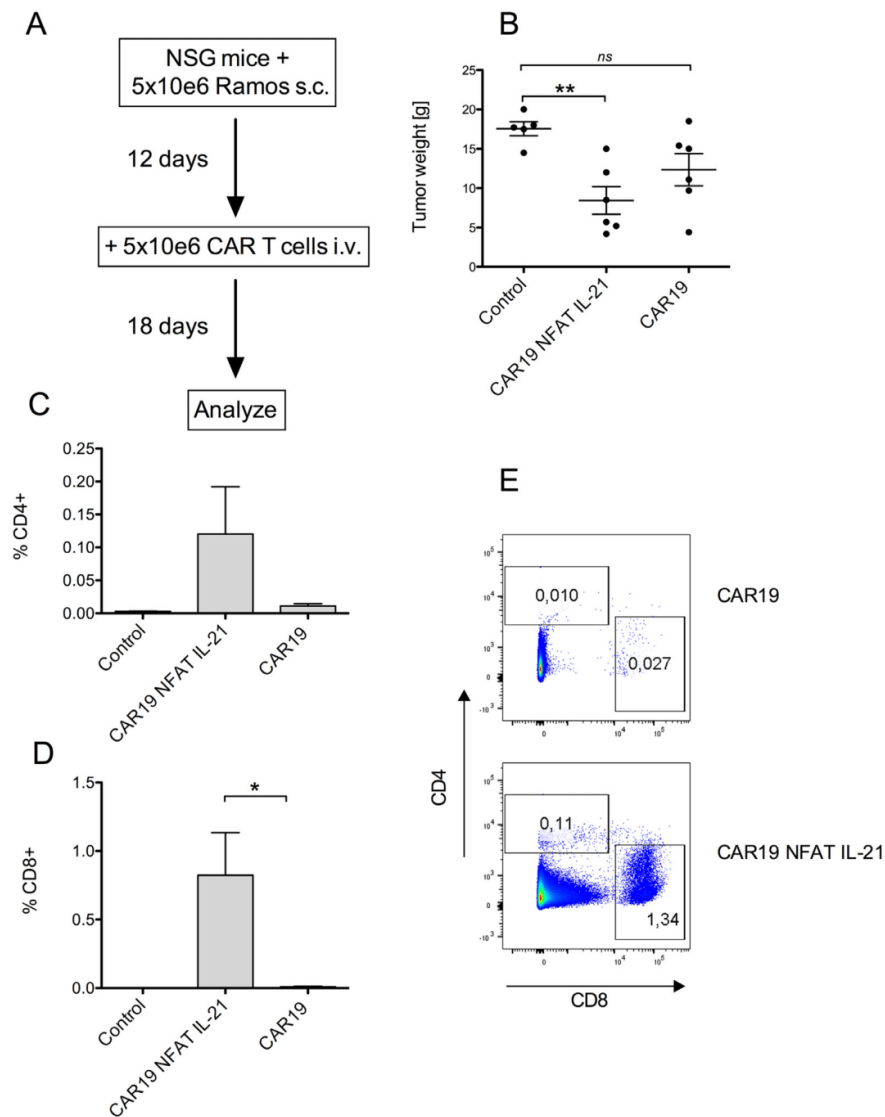
Figure 5. CAR19 T cells armed with IL-21 have enhanced anti-tumor efficacy *in vivo*. (A) Immunodeficient NSG mice were transplanted subcutaneously with 5 million Ramos cells. After 12 days, when macroscopic tumors had formed, mice received 5 million CAR T cells intravenously. (B) The treatment response was assessed by measuring the tumor weight at the end of the experiment and comparing it with a control group. N = 6; error bars indicate SEM. Significance was determined by unpaired *t*-test. (C–D) Level of infiltration of tumors by CD4+ T cells and CD8+ T cells. (E) Representative dot plot of tumors stained for CD4 and CD8. N = 6; error bars indicate SEM. Significance was determined by unpaired *t*-test. $^*P < 0.05$, $^{**}P < 0.01$, *ns* (not significant) $P > 0.05$ i.v., intravenously; s.c., subcutaneously; SEM, standard error of the mean. (Color version of figure is available online).

*et al.*cultured CAR T cells in IL-21 and IL-2, which may have had antagonistic effects. Furthermore, IL-2 and IL-21 have been shown to confer opposing differentiation programs on CD8+ T cells for adoptive immunotherapy, and IL-21 plays a key role in the development and maintenance of central memory T cells by inducing an early differentiation phenotype [5]. Markley *et al.* [21] found that CAR19 T cells co-expressing IL-21 have an enhanced anti-tumor effect in mice compared with CAR T cells co-expressing IL-2, but they accumulated very poorly *in vitro* upon repeated anti-genic stimulation. The major difference between this study and the authors' observations is that Markley *et al.* used CAR19 T cells with a CD28 zeta signaling motif that secreted IL-21 constitutively, whereas the authors' CAR T cells

contained the 4-1BB zeta signaling motif, and IL-21 was inducible after anti-genic stimulation.

The authors compared the effects of IL-2 and IL-21 on sorted CD8+ CAR19 T cells with the Tscm phenotype (CD45RA+CD62L+) after stimulation with CD19+ cells. IL-2 induced downregulation of CD62L, CD27 and CD28, which is consistent with their differentiation toward effector memory T cells. Conversely, IL-21 enabled their expansion, as well as maintenance of the Tscm phenotype, which suggests that IL-21 can block the transition from early to late memory subtypes.

Next, the authors hypothesized that the results of anti-genic stimulation of CAR T cells may depend on the phenotype and origin of the tumor cells. Therefore, several experiments were performed with PSMA CAR T cells challenged with LNCaP cells because this is a
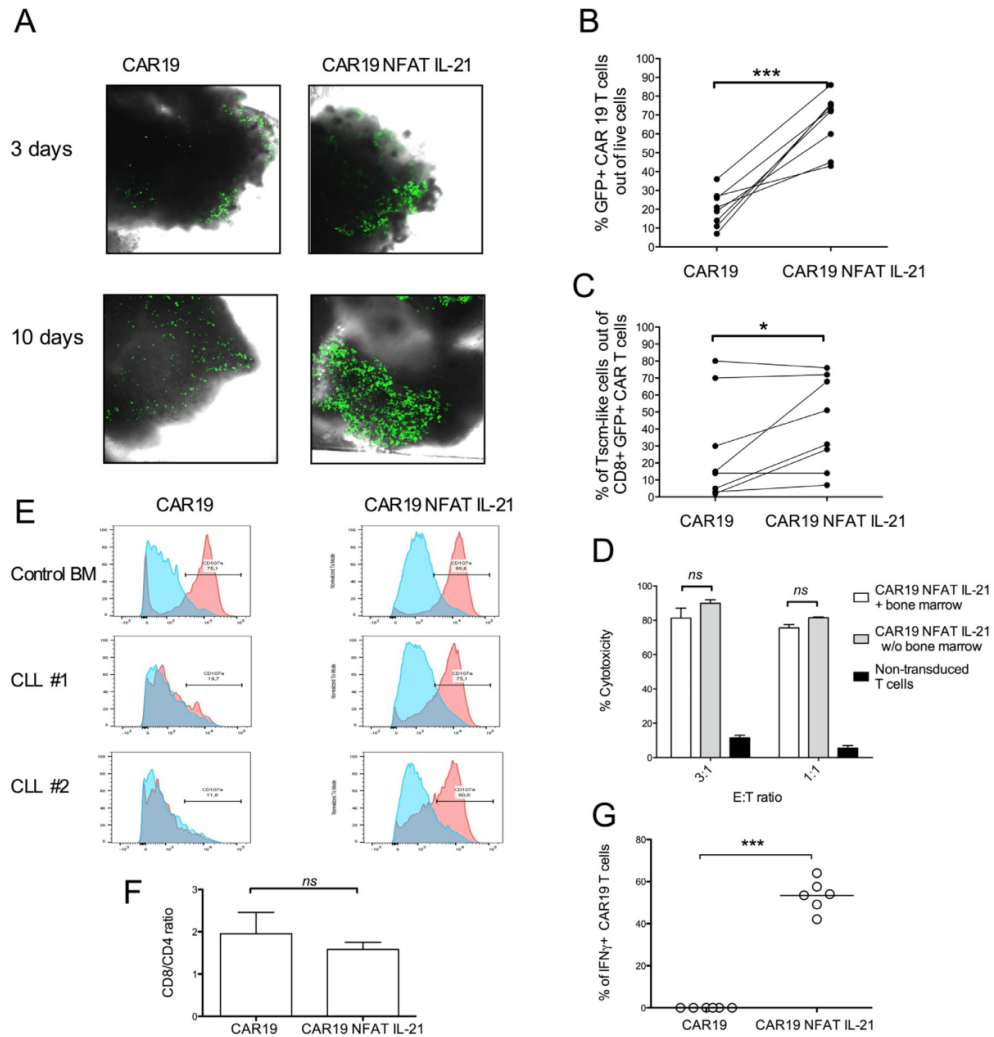
Figure 6. CAR19 T cells armed with IL-21 are resistant to immunosuppression induced by CLL cells. (A) GFP-expressing CAR19 T cells were co-cultivated with BM fragments obtained from CLL patients. The images show infiltration by GFP+ cells after 3 days and 10 days in one representative sample. The images are taken from different areas of the bone fragments ×200 magnification. (B) The level of infiltration by GFP+ CAR T cells in each sample was analyzed by FACS. N = 8; error bars indicate SEM. Significance was determined by paired *t*-test. (C) Percentage of CD8+ CAR T cells with Tscm-like phenotype (CD45RA+/−CD62L+CD27++CD28++). N = 8; error bars indicate SEM. Significance was determined by paired *t*-test. (D) Cytotoxicity of CAR T cells against Ramos cells and comparison of CAR T cells co-cultivated with BM to control CAR T cells grown *in vitro* in the presence of cytokines without BM. N = 3. (E) Histograms showing the level of degranulation of CAR T cells recovered from BM fragments after 10 days. Control BM is a sample obtained from an otherwise healthy patient without significant pathology in the BM. Red histograms indicate results after challenging with Ramos cells. Blue histograms represent non-challenged CAR T cells (negative control). This experiment was repeated once with similar results. (F) CD8:CD4 ratio among CAR19 T cells infiltrating BM fragments. N = 8. (G) CAR19 T cells were co-cultivated with CLL cells and analyzed for the production of IFNγ by cytokine capture assay after anti-genic restimulation with Ramos B cells. The graph shows the percentage of IFNγ+ CAR19 T cells. N = 6; error bars indicate SEM. Significance was determined by paired *t*-test. *ns* (not significant) P > 0.05 , * P ≤ 0.05, *** P ≤ 0.001. IFNγ, interferon gamma; ns, not significant; SEM, standard error of the mean; w/o, without. (Color version of figure is available online).

common CAR T cell model [22]. Furthermore, LNCaP is an adherent carcinoma cell line that does not express any co-stimulatory molecules [23]. Gargett *et al.* [11] similarly studied GD2-specific CAR T cells co-cultured with GD2-positive neuroblastoma cells and observed significant AICD of CAR T cells after repeated antigen stimulation with tumor cells. Natural antigen-specific effector T cells are more prone to apoptosis than memory T cells because of elevated levels of caspase 3, which indicates that sensitivity to apoptosis after anti-genic stimulation differs among various T-cell memory subsets [21]. Accordingly, the authors found that PSMA CAR T cells with effector phenotype (i.e., cultivated in the presence of IL-2) were

significantly more sensitive to apoptosis after anti-genic restimulation than PSMA CAR T cells with a Tscm-like immunophenotype grown in the presence of IL-4, IL-7 and IL-21 (Figure 3F). The beneficial effects of IL-21 on CAR T-cell survival and expansion are further demonstrated by the findings that CAR19 NFAT IL-21 T cells were superior to non-armed CAR19 T cells in NSG mice bearing established human lymphoma tumors.

The authors developed an *in vitro* model of CLL, which was selected mainly because of good availability of tumor tissues and because all patients with lymphocytosis in the blood have highly infiltrated BM. In addition, CLL is far more resistant to CAR19-based

therapies than B-ALL. Recent studies have shown that CLL cells can directly inhibit the proliferation and effector functions of CAR19 T cells [10] and initiate abortive activation of CAR T cells, resulting in very low proliferation upon *in vitro* restimulation with CLL cells [23]. However, the molecular mechanism has not been clearly determined and is currently being studied by several groups, including the authors'. This *in vitro* co-cultivation model using directly isolated human primary CLL cells is a useful biological model for studying CAR19 T-cell functions. The authors' opinion is that it may provide interesting data that are useful for better understanding tumor-induced immunosuppression of CAR T cells, especially when targeting solid tumors, which are mostly refractory to CAR T cells. Moreover, IL-21 potentiated the activity of CAR19 T cells against CLL B cells and significantly enhanced their expansion compared with non-armed CAR19 T cells. In conclusion, the authors' data suggest that arming CAR T cells with inducible IL-21 could be another possible strategy to counteract tolerance mechanisms by which tumor cells evade CAR T-cell therapies.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: PO, JM, PK. Acquisition of data: MS, PP, MM. Analysis and interpretation of data: PO, MS. Drafting or revising the manuscript: PO, MS. All authors have approved the final article.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2020.08.005.

References

[1] Pegram HJ, Lee JC, Hayman EG, Imperato GH, Tedder TF, Sadelain M, et al. Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning. Blood 2012;119:4133–41.

[2] Hoyos V, Savoldo B, Quintarelli C, Mahendravada A, Zhang M, Vera J, et al. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. Leukemia 2010;24:1160–70.

[3] Chmielewski M, Abken H. CAR T Cells Releasing IL-18 Convert to T-Bet^high FoxO1^low Effectors that Exhibit Augmented Activity against Advanced Solid Tumors. Cell Rep 2017;21:3205–19.

[4] Tian Y, Zajac AJ. IL-21 and T Cell Differentiation: Consider the Context. Trends Immunol 2016;37:557–68.

[5] Hinrichs CS, Spolski R, Paulos CM, Gattinoni L, Kerstann KW, Palmer DC, et al. IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy. Blood 2008;111:5326–33.

[6] Ptáčková P, Musil J, Štach M, Lesný P, Němečková Š, Král V, et al. A new approach to CAR T-cell gene engineering and cultivation using piggyBac transposon in the presence of IL-4, IL-7 and IL-21. Cytotherapy 2018;20:507–20.

[7] Sabatino M, Hu J, Sommariva M, Gautam S, Fellowes V, Hocker JD, et al. Generation of clinical-grade CD19-specific CAR-modified CD8+ memory stem cells for the treatment of human B-cell malignancies. Blood 2016;128:519–28.

[8] Xu Y, Zhang M, Ramos CA, Durett A, Liu E, Dakhova O, et al. Closely related T-memory stem cells correlate with *in vivo* expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15. Blood 2014;123:3750–9.

[9] Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med 2011;365:725–33.

[10] Van Bruggen JAC, Martens AWJ, Fraietta JA, Hofland T, Tonino SH, Eldering E, et al. Chronic lymphocytic leukemia cells impair mitochondrial fitness in CD8+ T cells and impede CAR T-cell efficacy. Blood 2019;134:44–58.

[11] Gargett T, Yu W, Dotti G, Yvon ES, Christo SN, Hayball JD, et al. GD2-specific CAR T Cells Undergo Potent Activation and Deletion Following Antigen Encounter but can be Protected from Activation-induced Cell Death by PD-1 Blockade. Mol Ther 2016;24:1135–49.

[12] Pulè MA, Straathof KC, Dotti G, Heslop HE, Rooney CM, Brenner MK. A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. Mol Ther 2005;12:933–41.

[13] Otáhal P, Průková D, Král V, Fabry M, Vočková P, Latečková L, et al. Lenalidomide enhances antitumor functions of chimeric antigen receptor modified T cells. Oncoimmunology 2016;5:e1115940.

[14] Yusa K, Zhou L, Li MA, Bradley A, Craig NL. A hyperactive piggyBac transposase for mammalian applications. Proc Natl Acad Sci U S A 2011;108:1531–6.

[15] Smith-garvin JE, Koretzky GA, Jordan MS. T Cell Activation n.d. doi:10.1146/annurev.immunol.021908.132706

[16] Hege KM, Bergsland EK, Fisher GA, Nemunaitis JJ, Warren RS, McArthur JG, et al. Safety, tumor trafficking and immunogenicity of chimeric antigen receptor (CAR)-T cells specific for TAG-72 in colorectal cancer. J Immunother Cancer 2017;5:22.

[17] Perkins MR, Grande S, Hamel A, Horton HM, Garrett TE, Miller SM, et al. Manufacturing an enhanced car T cell product by inhibition of the PI3K/AKT pathway during T cell expansion results in improved *in vivo* efficacy of anti-BCMA car T cells. Blood 2015;126:1893.

[18] Kaartinen T, Luostarinen A, Maliniemi P, Keto J, Arvas M, Belt H, et al. Low interleukin-2 concentration favors generation of early memory T cells over effector phenotypes during chimeric antigen receptor T-cell expansion. Cytotherapy 2017;19:689–702.

[19] Ramanayake S, Bilmon IAN, Bishop D, Dubosq M, Blyth E, Clancy L, et al. Low-cost generation of Good Manufacturing Practice-grade CD19-specific chimeric antigen receptor-expressing T cells using piggyBac gene transfer and patient-derived materials. Cytotherapy 2015;17:1251–67.

[20] Singh H, Figliola MJ, Dawson MJ, Huls H, Olivares S, Switzer K, et al. Reprogramming CD19-specific T cells with IL-21 signaling can improve adoptive immunotherapy of B-lineage malignancies. Cancer Res 2011;71:3516–27.

[21] Markley JC, Sadelain M. IL-7 and IL-21 are superior to IL-2 and IL-15 in promoting human T cell-mediated rejection of systemic lymphoma in immunodeficient mice. Blood 2010;115:3508–19.

[22] Santoro SP, Kim S, Motz GT, Alatzoglou D, Li C, Irving M, et al. T cells bearing a chimeric antigen receptor against prostate-specific membrane antigen mediate vascular disruption and result in tumor regression. Cancer Immunol Res 2015;3:68–84.

[23] Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, et al. LNCaP model of human prostatic carcinoma. Cancer Res 1983;43:1809–18.

4.3. Publication 3

Kaštánková I, **Štach M**, Žižková H, Ptáčková P, Šmilauerová K, Mucha M, Šroller V, Otáhal P.

Enzymatically produced piggyBac transposon vectors for efficient non-viral manufacturing of CD19-specific CAR T cells.

Author contribution: CAR T-cell phenotype analysis, manuscript editing

Enzymatically produced piggyBac transposon vectors for efficient non-viral manufacturing of CD19-specific CAR T cells

Iva Kaštánková,[1] Martin Štach,[1,2] Hana Žižková,[1] Pavlína Ptáčková,[1] Kristýna Šmilauerová,[1,2] Martin Mucha,[1,2] Vojtěch Šroller,[1] and Pavel Otáhal[1]

[1]Institute of Hematology and Blood Transfusion, 128 00 Prague, Czechia; [2]Faculty of Natural Sciences, Charles University, 128 00 Prague, Czechia

The piggyBac transposon system provides a non-viral alternative for cost-efficient and simple chimeric antigen receptor (CAR) T cell production. The generation of clinical-grade CAR T cells requires strict adherence to current good manufacturing practice (cGMP) standards. Unfortunately, the high costs of commonly used lentiviral or retroviral vectors limit the manufacturing of clinical-grade CAR T cells in many non-commercial academic institutions. Here, we present a manufacturing platform for highly efficient generation of CD19-specific CAR T cells (CAR19 T cells) based on co-electroporation of linear DNA transposon and mRNA encoding the piggyBac transposase. The transposon is prepared enzymatically *in vitro* by PCR and contains the CAR transgene flanked by piggyBac 3′ and 5′ arms. The mRNA is similarly prepared via *in vitro* transcription. CAR19 T cells are expanded in the combination of cytokines interleukin (IL)-4, IL-7, and IL-21 to prevent terminal differentiation of CAR T cells. The accurate control of vector copy number (VCN) is achieved by decreasing the concentration of the transposon DNA, and the procedure yields up to 1×10^8 CAR19 T cells per one electroporation of 1×10^7 peripheral blood mononuclear cells (PBMCs) after 21 days of *in vitro* culture. Produced cells contain >60% CAR+ cells with VCN < 3. In summary, the described manufacturing platform enables a straightforward cGMP certification, since the transposon and transposase are produced abiotically *in vitro* via enzymatic synthesis. It is suitable for the cost-effective production of highly experimental, early-phase CAR T cell products.

INTRODUCTION

Chimeric antigen receptors (CARs) are artificial proteins that can redirect the specificity of T cells to any surface antigen. One of these antigens is the B cell antigen CD19, a well-validated target of already commercially available CD19-specific CAR (CAR19) T cells approved against refractory B cell malignancies. The CAR consists of a single-chain antibody segment (scFv) that defines the specificity, a short linker sequence, a transmembrane domain, and an intracellular signaling domain. Upon recognizing the antigen, the CAR initiates a T cell activation and expansion similarly as an endogenous T cell receptor and subsequent elimination of tumor cells.[1] The development of novel and experimental CAR-T cell therapies requires a cost-effective and rapid production chain.[2] The most complicated step in the manufacture of good manufacturing practice (GMP)-grade CAR T cells is the transfection of T cells, in a majority of cases performed via recombinant lentiviral or retroviral vectors (LV/RV). To meet the current quality standards, these viral vectors must be similarly produced at GMP quality, which is expensive and very slow.[3] Several alternative non-viral approaches were thus developed, including transposons such as piggyBac (PB) or Sleeping Beauty (SB) or genome editing via CRISPR-Cas9.[3–5] Transposons are usually used as two plasmids; the first one encodes the CAR19 transgene, and the second one encodes the PB transposase. Both plasmids are electroporated into T cells; the transposase excises the transposon from the first plasmid and randomly integrates it into the genome.[6] Notably, the transposase can be introduced into T cells as mRNA to reduce the risk of random integration of the second plasmid.[7] Several clinical trials demonstrated the efficiency of both PB and SB transposons for generating CAR T cells and validated this approach as safe and feasible.[8] Other non-viral gene engineering techniques such as CRISPR-Cas9-based DNA editing enable transgenesis into a precise DNA locus within the genome. However, this unique feature is impaired by a highly increased risk of chromosomal translocations resulting from the off-target effect of the CRISPR-Cas9 enzyme complex.[5] In contrast, PB transposon integrates its cargo randomly within the genome without producing such severe genomic mutations.[9]

It will be of value to develop a universal and straightforward T cell transfection technology to overcome such barriers. Here, we show that abiogenically (i.e., by *in vitro* enzymatic synthesis) prepared PB transposon/transposase can be used in this manner to manufacture CD19-specific CAR T cells efficiently. The transposon was prepared by preparative PCR as a linear dsDNA containing the CAR19

A

B

transgene flanked with PB 3′ and 5′ arms. The PB transposase is encoded by mRNA and was prepared by *in vitro* transcription. According to the already established protocol, the transposon/transposase were co-electroporated into T cells, followed by *in vitro* expansion.[10] By titrating down the amount of transposon DNA, it was possible to efficiently produce >60% CAR+ T cells containing less than three copies of CAR19 transgene per transduced cell. Generated CAR19 T cells were of early memory immunophenotype and displayed efficient effector functions. This manufacturing platform reduces the complexity of currently used viral-based gene transfection techniques and supports novel immunotherapeutic product development and rapid clinical testing.

RESULTS

CAR19 T cells can be generated with enzymatically *in vitro* prepared linear DNA transposon and mRNA transposase

A second-generation CAR19 construct previously generated by us[11]þ was used as a template for the amplification of the transposon by preparative PCR (Figure 1A). The CAR19 construct contains anti-CD19 scFv derived from hybridoma B-D3 and has the following structure: CD8 leader sequence, anti-CD19 scFv, CD8 hinge domain, CD8 transmembrane domain, 4-1BB intracellular domain, TCR zeta intracellular domain; its full sequence is provided in Materials and methods. The manufacturing of the transposon DNA was performed commercially as described in Materials and methods, the purity of DNA was >99%, and there were no mutations within the coding sequence. Next, to manufacture the transposase mRNA, a plasmid pST containing a hyperactive PB transposase (hyPBase) under the T7 promoter was used as a template for the mRNA production by

in vitro transcription. As a control reagent for initial experiments, an mRNA encoding GFP was similarly produced. First, we determined the electroporation efficiency of mRNA in T cells under conditions (voltage and pulse length) optimized for the electroporation of DNA by electroporating increasing amounts of GFP mRNA. The results in Figure 1B show that an amount between 10 and 15 μg of mRNA per electroporation should transduce a sufficient number of cells. Based on this estimate, we decided always to use 12 μg of transposase mRNA per one electroporation of 1×10^7 peripheral blood mononuclear cells (PBMCs) in a volume of 100 μL, since our goal was to transduce a sufficient number of cells with a lesser amount of transposase to ensure that hyPBase remains active intracellularly only for a short time.

To determine the optimal concentration of the transposon, PBMCs were electroporated with decreasing amounts of DNA (from 9 to 0.1 μg/100 μL). The transfection efficiency was compared to the procedure based on the electroporation of plasmids encoding the transposon/transposase. The data in Figure 2A show that the viability at day 1 and day 4 post electroporation was slightly higher upon electroporation of PCR CAR19 + mRNA hyPBase than the control PBMCs electroporated with plasmids (4 μg transposon plasmid + 2 μg transposase plasmid). As expected, the transfection efficiency depended on the DNA concentration (Figure 2B). The percentage of CAR+ T cells was initially low at day 7 post electroporation, but at later time points (day 14, day 21), we observed an increase in CAR+ cells reaching ~60%–70%. The efficient generation of CAR19 T cells was possible in the range of 3–0.3 μg of DNA per one (100 μL) electroporation. A further decrease below 0.3 μg/
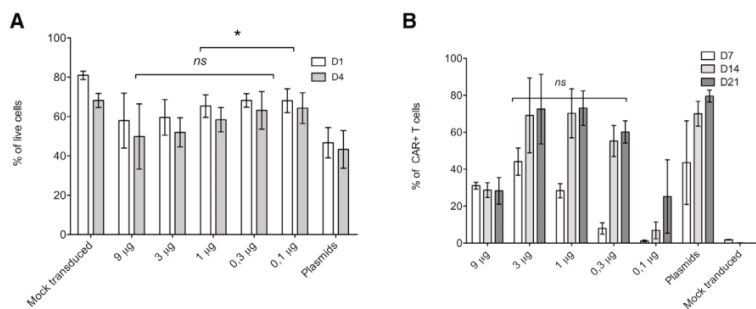
A

B

Figure 3. PCR DNA/mRNA approach enables efficient generation of CAR19 T cells with low VCN

(A) The integration efficiency of the transposon was determined by quantifying the number of integrated CAR19 transgenes per transduced T cell by digital PCR. The graph shows the correlation of VCN with the amount of electroporated transposon. The control group included CAR19 T cells prepared via a plasmid approach and mock-transduced T cells. The bar indicates the mean value. (B) The efficiency of generation of CAR19 T cells with decreasing amounts of transposon was determined by calculating the yield of produced CAR19 T cells 7, 14, and 21 days post-electroporation and was compared to the CAR19 T cells generated via plasmids. n = 4. ns, Not significant.

100 μL resulted in a substantial reduction of the percentage of transfected cells.

Low VCN can be achieved by transfection with minimal amount of transposon DNA using both PCR DNA/mRNA and plasmid/plasmid approach

The vector copy number (VCN) is a critical parameter for the production of clinical-grade CAR T cells. Thus, we quantified the number of integrated CAR19 transposons (i.e., VCN) per cell by digital PCR (ddPCR) and corrected the value to the percentage of CAR+ cells within the sample, which was determined by fluorescence-activated cell sorting (FACS). The data in Figure 3A show that the VCN of CAR19 transposon correlated with the decreasing amount of electroporated PCR CAR19 (the amount of mRNA was constant for all samples). Importantly, the generation of CAR19 T cells with as low as 1–3 copies of transgene was possible by reducing the PCR CAR19 concentration to 0.3 μg/100 μL. In contrast, control electroporation with a high amount of transposon/transposase plasmids (4 μg CAR19 + 2 μg PBASE) produced CAR19 T cells with approximately VCN = 23.

Next, we determined the maximal number of CAR19 T cells generated by this protocol by large-scale cultivations in G-Rex bottles (Figure 3B). Electroporation of DNA in the range 3–0.3 μg, which yields CAR T cells with an acceptable VCN (<10), produced equal numbers of CAR T cells compared to electroporation of plasmids. At day 14 the CAR19 T cell yield was ∼1 × 10^7 CAR+ cells per one electroporation, and at day 21 the CAR-T yield was ∼1 × 10^8 CAR+ cells per one electroporation. Control PBMCs electroporated with the transposon DNA without transposase mRNA did not expand *in vitro* and therefore were not further analyzed (data not shown). These data demonstrate that such a transposon dilution technique enables accurate titration of transposon DNA and reliably generates high numbers of CD19-specific CAR T cells with VCN < 5.

Similarly to the manufacturing of CAR19 T cells via PCR-made transposon DNA, we also used this transposon dilution technique to generate CAR19 T cells via electroporation of transposon/transpo-

sase plasmids. PBMCs were electroporated with a constant amount of transposase plasmid (1 μg) plus decreasing amounts of CAR19 transposon plasmids. The transfection and CAR19 T cell yield efficiency decreased with the reduced concentration of transposon plasmid, similarly to the PCR DNA/mRNA approach (Figure 4A). The optimal amount of transposon plasmid that enabled an efficient generation of CAR19 T cells with approximately VCN = 2-5 was in the range of 0.125–0.25 μg DNA per electroporation (Figure 4B). Interestingly, the comparison of the plasmid/plasmid approach with the PCR DNA/mRNA approach (Figure 3A versus Figure 4B) showed that the transfection with 1 μg of plasmid transposon produced CAR19 T cells with ∼9 copies of transgene per cell and the transfection with PCR DNA/mRNA produced CAR19 T cells with ∼3 copies of transgene per cell, while the overall yield of cultivation was similar under both conditions. This finding suggests that the transposition via the plasmid/plasmid approach is more efficient. Next, to determine any possible changes of VCN during the *in vitro* expansion, CAR-T cells were generated using 1 μg of transposon/transposase plasmids per electroporation and VCN was measured on day 14 and at the end (day 21) of *in vitro* expansion. The results presented in (Figure 4C) show that VCN did not significantly change between day 14 and day 21, since ∼7 versus 6 copies per cell were detected.

Functional assays of CAR T cells and immunophenotype of CAR19 T cells

To examine the effector functions of CAR19 T cells engineered with PCR DNA/mRNA, their cytotoxic activity and interferon-gamma (IFN-γ) production was measured after antigenic challenge with CD19+ B cell line Ramos and compared to CAR19 T cells generated via DNA plasmids. The amount of transposon used for transfection was the lowest concentration that effectively produced CAR19 T cells with VCN < 5, i.e., 0.3 μg PCR DNA/12 μg mRNA and 0.25 μg transposon plasmid/1 μg transposase plasmid. Both methods produced CAR19 T cells with similar effector functions, as the cytotoxicity (Figure 5A) and the production of IFN-γ (Figure 5B) were not significantly different. Next, we determined the immunophenotype of generated CAR19 T cells via flow cytometry. The representative dot plots in (Figure 5D) demonstrate that

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both methods produced CAR T cells with very similar immunophenotype, characterized by the high level of expression of early memory antigens CD62L, CD27, and CD28 and absence of expression of the exhaustion marker PD-1. The graph in (Figure 5C) shows the immunophenotype of all donors analyzed. In summary, we found no significant differences in immunophenotype or the cytotoxic activity of CAR19 T cells prepared by these two approaches.

DISCUSSION

The manufacturing of clinical-grade CAR T cells via transfection by LV/RV is a complicated multi-step process with high costs. Additionally, difficulties in obtaining commercially produced GMP-grade viral vectors within a reasonable time window create challenging obstacles, especially for academic centers not equipped with virus production facilities.[12,13]

The presented work demonstrates as proof of principle that CAR19 cells can be efficiently manufactured via transfection with abiogenically prepared transposon/transposase vectors. The described procedure enables accurate control of VCN per cell while providing sufficient yield of CAR19 T cells for therapeutic purposes. The control of VCN is achieved by a "transposon dilution technique" that enables CAR19 T cell generation with VCN below 3, not only after modification via PCR DNA/mRNA but also after traditional modification with plasmids encoding the transposon and transposase. The *in vitro* expansion of CAR19 T cells is performed in cytokines interleukin (IL)-4, IL-7, and IL-21, similarly, as we have already described[11,14]

to preserve an early memory phenotype to enable cultivation for up to 21 days. The use of PCR DNA/mRNA has no significant adverse effects on the CAR T cell memory phenotype or effector activity compared to the transfection with plasmids. Notably, the generation of transposon DNA by preparative PCR and the generation of transposase mRNA are both rapid and straightforward procedures with no relevant biohazard issues. The simplicity and the speed of vector preparation via this method might enable faster clinical testing of novel types of CAR T cells.

A similar method of T cell transfection was tested by Bishop et al.[15] The authors attempted to generate CAR T cells by electroporation of *in vitro*-amplified "doggy bone" DNA (dbDNA) combined with dbDNA encoding the PB transposase. Our data similarly demonstrate that enzymatically prepared genetic vectors represent a feasible alternative. The transposon copy number can be accurately controlled by a simple dilution of the PCR DNA without adverse effects on the cultivation yield.

Another experimental non-viral technique used to produce CAR T cells is based on CRISPR-Cas9 genome editing. CRISPR-Cas9 enables the integration of the transgene into a precise locus within the genome. It has been shown that the insertion of CAR transgene into the TCR locus disrupts the expression of endogenous TCR, which leads to improved function of CAR T cells by reducing the tonic TCR signaling.[16] However, CRISPR-Cas9 has significant off-target effects and produces chromosomal translocations.[6] Additionally, the procedure for T cell editing via CRISPR-Cas9 is far more complex than a modification via transposons and cannot be as quickly GMP certified.[17]

Lentiviral vectors are currently the gold standard for genetic engineering of T cells, and their safety was reliably proven in thousands of patients. This type of vector is especially suited for large-scale pharmaceutical processes where costs, biohazard, and time are not an
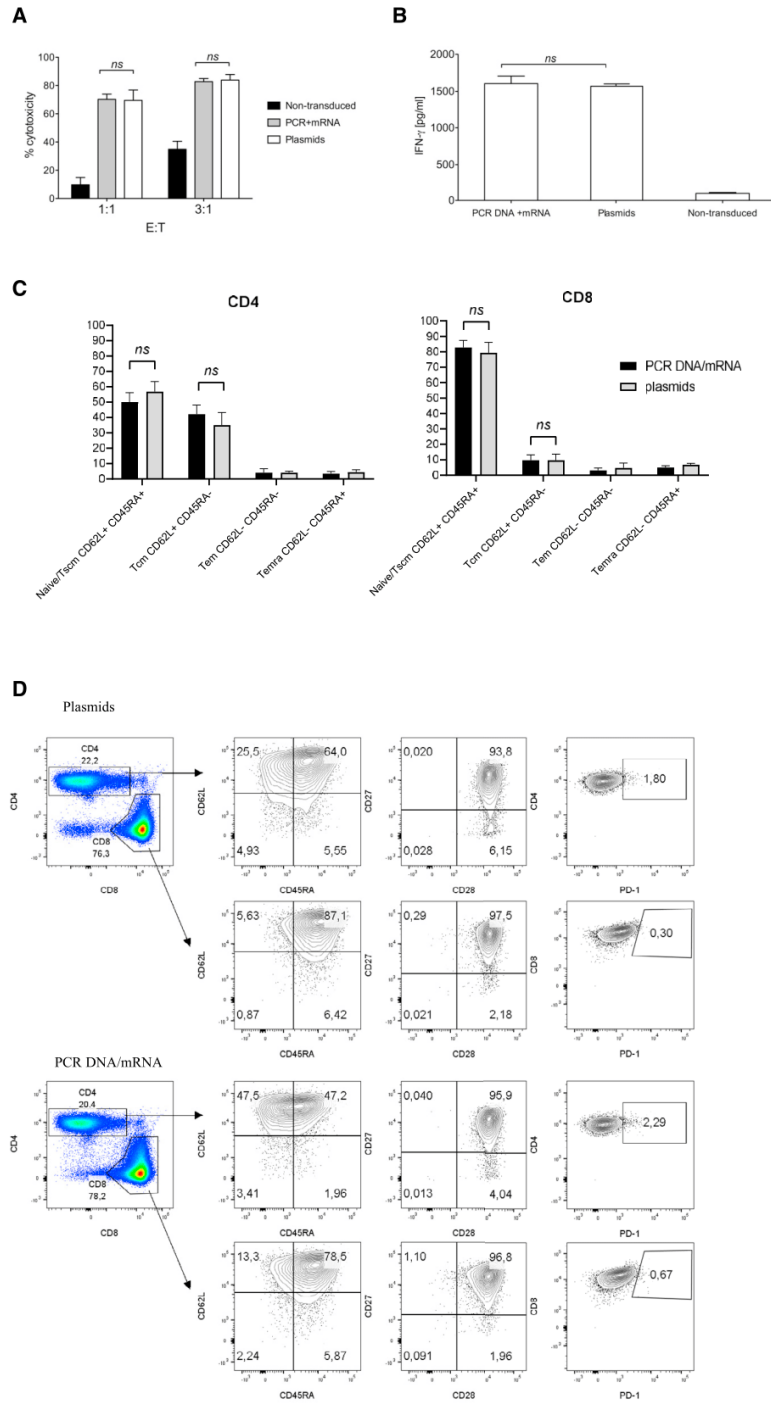
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issue. However, their use for small-scale generation of experimental phase 1 products is problematic. The presented technique might fill this gap and provide a rapid methodology for clinical testing of novel types of CAR constructs with unproven medical efficiency. Undeniably, the clinical experience with transposon vectors is insufficient, and thus the likelihood of oncogenesis induced by transposons cannot be reliably determined at this moment.

Moreover, animal studies can hardly answer such complex questions. On the other hand, mature T cells are inherently resistant to malignant transformation, compared to, for example, undifferentiated hematopoietic precursors, or B cells. In our experience, pharmaceutical regulatory agencies require additional quality control (QC) tests such as mapping of integration sites and monitoring of T cell clonality for approval of clinical trials with transposon-engineered T cells.

In summary, the manufacture of clinical-grade CAR T cells via lentiviral or retroviral transfection is a complicated and expensive task primarily resulting from GMP rules regulating the preparation of viral vectors. Currently, the major bottleneck is the difficulty in obtaining the GMP-grade viral vectors within a reasonable time. Enzymatically prepared transposon vectors are surprisingly highly efficient for small-scale manufacturing of this type of therapeutical product. In our opinion, the development of an easily certifiable GMP-grade method of T cell transfection is critical for the rapid development of T cell-based therapies in the future.

MATERIALS AND METHODS

Preparation of DNA and mRNA amplicons and plasmid cloning

The mRNA encoding the hyperactive PB transposase (hyPbase) was prepared by *in vitro* transcription with the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs). To increase the stability of mRNA, we used 3′-O-Me-m7G (5′) PPP(5′) G RNA cap structure analog at a concentration of 8 mM (New England Biolabs). Before an *in vitro* transcription, the pSTI plasmid template encoding the hyPBase, or GFP, was linearized via BspQI (New England Biolabs) and gel-purified. The reaction was carried out for 3 h at 37°C using 1 μg of linearized plasmid template per 50-μL reaction. Prepared mRNA was purified with the MEGAclear Transcription Clean-Up Kit (Thermo Fisher Scientific) and analyzed with the Experion Automated Electrophoresis System (Bio-Rad Laboratories). The yield was ~100 μg per one 50-μL reaction.

The transposon containing the CAR19 transgene was prepared commercially by preparative PCR (LinearX, USA) using M13 fwd and M13 rev primers and a Taq DNA polymerase, followed by a pu-

rification step on a chromatographic column. The declared purity of the PCR product was >99%. Furthermore, no mutations in the coding sequence were detected by Sanger sequencing. Plasmids were amplified in *E. coli* and purified by QIAGEN plasmid kits.

T cell cultivation and electroporation

PBMCs were separated from buffy coats obtained from healthy donors with Ficoll density gradient centrifugation (Ficoll-Paque, GE Healthcare). All donors gave written informed consent in accordance with Helsinki ethical guidelines. Cells were cultivated at 37°C in 5% CO$_2$ in CellGenix GMP DC medium (CellGenix) supplemented with heat-inactivated 10% fetal bovine serum (Thermo Fisher Scientific), penicillin/streptomycin 100 U/mL (Thermo Fisher Scientific), and cytokines IL-4 (20 ng/mL), IL-7 (10 ng/mL), and IL-21 (40 ng/ml), all from Miltenyi Biotec. For large-scale expansion, we used G-Rex10 flasks (Wilson Wolf). For electroporations, 1×10^7 PBMCs were resuspended in 100 μL of buffer T containing plasmid DNA or linear transposon+ mRNA. Cells were then electroporated with the Neon Electroporation System (Thermo Fisher Scientific, USA) by a single 20-ms/2,300-V pulse and then were transferred to complete cell media. Next day, cells were polyclonally stimulated with TransAct reagent (anti-CD3/CD28, Miltenyi Biotec, Germany) and expanded for 21 days in the presence of IL-4, IL-7, and IL-21, identically as already described.[13]

Functional assays and flow cytometry

The functionality of CAR19 T cells was tested by FACS-based cytotoxic assay against Ramos B cellsþ. Briefly, Ramos cells were labeled with 0.5 μM CFSE (Thermo Fisher Scientific) in PBS for 10 min at room temperature, washed in CellGenix medium, and mixed with target cells at a ratio of 3:1 and 1:1. After 24-h incubation, cells were harvested, labeled with DAPI, and analyzed by FACS to determine the percentage of dead Ramos cells.

To determine the production of IFN-γ, we similarly co-cultivated CAR19 T cells with Ramos B cells at a 1:1 ratio overnight. The production of IFN-γ was determined in supernatants with the ELISA MAX Deluxe Set Kit (BioLegend). The absorbance at 450 nm was measured on an ELISA reader (Tecan Infinite 200 Microplate Reader). The IFN-γ concentration was calculated according to the diluted IFN-γ standard calibration curve in the GraphPad Prism software.

To determine the immunophenotype of CAR19 T cells, the cells were washed in 1× PBS, stained with fluorochrome-conjugated monoclonal antibodies (mAbs) in FACS buffer (1% BSA, 0.1% sodium azide, 1× PBS) for 30 min on ice, washed with PBS, and resuspended

Figure 5. Generated CAR19 T cells are functional and express early memory surface antigens
The graph shows the cytotoxicity of CAR19 T cells generated with PCR DNA/mRNA or with plasmids against target Ramos cells at ratios of 1:1 and 3:1; black bars show the cytotoxicity of control non-transduced T cells. n = 4. (B) The production of IFN-γ was measured after restimulation of CAR19 T cells with Ramos cells at 1:1 ratio after 24 h. n = 4. ns, Not significant. (C) The immunophenotype of generated CAR19 T cells was determined by multiparameter flow cytometry. The graphs show the percentages of individual T cell memory subsets among CAR+ T cells. n = 5. ns, Not significant. (D) The dotplots show the expression of antigens CD4, CD8, CD45RA, CD62L, CD27, CD28, and PD-1 on either CD4+ or CD8+ CAR19 T cells. One representative matched sample out of five donors is shown.

Table 1. The sequences of primers and probes

CAR19	forward	5′ atcggatatcgtgatgacac 3′
	reverse	5′ gactagacctgcaagagatg 3′
	probe	5′ FAM-ctctccctgcctgtcagtcttgga-BHQ1 3′
PBASE	forward	5′ CCCTGAACATCGTGAGAA 3′
	reverse	5′ ATCTCGTCGGTGAAGAAC 3′
	probe	5′ FAM-CCCACCAGGATG TGCAGGAACATCT-BHQ1 3′
Albumin	forward	5′ TGAAACATACGTTCCCAAAGAGTTT 3′
	reverse	5′ CTCTCCTTCTCAGAAAGTGTGCATAT 3′
	probe	5′ HEX-TGCTGAAAC-ZEN- ATTCACCTTCCATGCAGAT-IABkFQ 3′

in FACS buffer containing DAPI 100 ng/mL (Merck). In the case of indirect labeling, the primary antibody was washed out with PBS, and cells were then stained with fluorochrome-conjugated secondary antibody for 30 min on ice. The expression of CAR19 transgene was determined with Alexa Fluor 647-conjugated F(ab′)2 fragment of Goat Anti-Mouse immunoglobulin G (IgG) (Jackson ImmunoResearch). This polyclonal antibody reacts with mouse anti-CD19 scFv. The immunophenotype of CAR T cell was determined similarly as already described.[13] First, cells were stained either with Alexa Fluor 647- or Alexa Fluor 488-labeled F(ab′)2 fragment of Goat Anti-Mouse IgG (Jackson ImmunoResearch, UK) to detect the CAR transgene. Cells were then washed twice and blocked with 10% mouse serum, followed by staining with fluorescently labeled mouse mAbs specific to human antigens: CD45RA-BUV737 (clone HI100), CD62L-BV650 (clone DREG-56), and CD3-BV786 (clone UCHT1) were purchased from BD Biosciences (USA). CD4-qDOT605 (clone S3.5) and CD28-PE-Cy7 (clone CD28.2) were from BioLegend (USA). CD8-AF700 (clone MEM-31) and CD27-PE-Dy590 (clone LT27) were from EXBIO. Live cells were identified with the Fixable Blue Dead Cell Stain Kit (Thermo Fischer Scientific, USA). Staining specificity was extensively tested to rule out possible binding of mouse antibodies to cells via goat anti-mouse Ab used to detect CAR. Cells were analyzed with BD LSRFortessa (BD Biosciences), and the data were processed with FlowJo software.

Duplex digital droplet PCR

To determine the copy number of CAR19 transgene, the genomic DNA was isolated with the QIAamp DNA Mini Kit (QIAGEN). The PrimerQuest Tool online software (ITD, USA) was used to design primers and probes listed in Table 1. Sequences of primers and double-quenched probes for the human albumin gene that served as a reference gene were previously described.[18] Duplex PCR reactions contained ddPCR Supermix for Probes (no dUTP) (cat. no. 186-3024, Bio-Rad Laboratories, USA), 900 nM of each primer pair, 250 nM of each FAM- and HEX-labeled probe, and 30–40 ng of DNA template. The reaction mix was split into ~20,000 droplets with a QX200 Droplet Generator (Bio-Rad Laboratories). The PCR was performed on a C1000 Touch Thermal Cycler (Bio-Rad) using the following amplification conditions:

10 min at 95°C, 45 cycles of a two-step of 30 s at 94°C and 60 s at 54°C, ending with a final hold of 10 min at 98°C for droplet stabilization and cooling to 4°C. Droplets were counted on/divided by a QX200 droplet reader based on their fluorescence amplitude into positive or negative. Data were analyzed with QuantaSoft Analysis Pro version 1.0.596 software (Bio-Rad), including automatic Poisson distribution. The VCN was determined as the ratio of CAR copies/albumin copies × 2 and divided by the fraction of CAR+ T cells in the sample.

DNA sequence of the CAR19 transposon

(M13F)GTAAAACGACGGCCAGTGAGCGCGCGTAATACGAC
TCACTATAGGGCGAATTGGGGCGCGCCATTCTAGATTAAC
CCTAGAAAGATAGTCTGCGTAAAATTGACGCATGCATTCT
TGAAATATTGCTCTCTCTTTCTAAATAGCGCGAATCCGTCG
CTGTGCATTTAGGACATCTCAGTCGCCGCTTGGAGCTCCCG
TGAGGCGTGCTTGTCAATGCGGTAAGTGTCACTGATTTTGA
ACTATAACGACCGCGTGAGTCAAAATGACGCATGATTATC
TTTTACGTGACTTTTAAGATTTAACTCATACGATAATTATA
TTGTTATTTCATGTTCTACTTACGTGATAACTTATTATATAT
ATATTTTCTTGTTATAGATATCAACTAGAATGCTAGCCTCG
AGGGCCTCCGCGCCGGGTTTTGGCGCCTCCCGCGGGCGCC
CCCCTCCTCACGGCGAGCGCTGCCACGTCAGACGAAGGGC
GCAGCGAGCGTCCTGATCCTTCCGCCCGGACGCTCAGGAC
AGCGGCCCGCTGCTCATAAGACTCGGCCTTAGAACCCCAG
TATCAGCAGAAGGACATTTTAGGACGGGACTTGGGTGACT
CTAGGGCACTGGTTTTCTTTCCAGAGAGCGGAACAGGCGA
GGAAAAGTAGTCCCTTCTCGGCGATTCTGCGGAGGGATCT
CCGTGGGGCGGTGAACGCCGATGATTATATAAGGACGCGC
CGGGTGTGGCACAGCTAGTTCCGTCGCAGCCGGGATTTGG
GTCGCAGTTCTTGTTTGTGGATCGCTGTGATCGTCACTTGG
TGAGTAGCGGGCTGCTGGGCTGGCCGGGGCTTTCGTGGCC
GCCGGGCCGCTCGGTGGGACGGAGGCGTGTGGAGAGACCG
CCAAGGGCTGTAGTCTGGGTCCGCGAGCAAGGTTGCCCTG
AACTGGGGGTTGGGGGGAGCGCAGCAAAATGGCGGCTGTT
CCCGAGTCTTGAATGGAAGACGCTTGTGAGGCGGGCTGTG
AGGTCGTTGAAACAAGGTGGGGGGCATGGTGGGCGGCAAG
AACCCAAGGTCTTGAGGCCTTCGCTAATGCGGGAAAGCTC
TTATTCGGGTGAGATGGGCTGGGGCACCATCTGGGGACCC
TGACGTGAAGTTTGTCACTGACTGGAGAACTCGGTTTGTCG
TCTGTTGCGGGGGCGGCAGTTATGGCGGTGCCGTTGGGCA
GTGCACCCGTACCTTTGGGAGCGCGCGCCCTCGTCGTGTC
GTGACGTCACCCGTTCTGTTGGCTTATAATGCAGGGTGGG
GCCACCTGCCGGTAGGTGTGCGGTAGGCTTTTCTCCGTCG
CAGGACGCAGGGTTCGGGCCTAGGGTAGGCTCTCCTGAAT
CGACAGGCGCCGGACCTCTGGTGAGGGGAGGGATAAGTGA
GGCGTCAGTTTCTCTGGTCGGTTTTATGTACCTATCTTCTT
AAGTAGCTGAAGCTCCGGTTTTGAACTATGCGCTCGGGGTT
GGCGAGTGTGTTTTGTGAAGTTTTTTAGGCACCTTTTGAAA
TGTAATCATTTGGGTCAATATGTAATTTTCAGTGTTAGACT
AGTAAATTGTCCGCTAAATTCTGGCCGTTTTTGGCTTTTTT
GTTAGACGGATCCATGAATTCGCCAGCatggccctgcctgtgacagccc
tgctgctgcctctggctctgctgctgcatgccgctagacccacgcgtgaagtgcagctgcagcagtc
tggacctgagctggtaaagcctggggcttcagtgaagatgtcctgcaaggcttctggatacacattc

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actagctatgttatgcactgggtgaagcagaagcctgggcagggccttgagtggattggatatgtta
atccttacaatgatggtactaagtacaatgagaagttcaaaggcaaggccacactgacttcagaca
aatcctccagcacagcctacatggagctcagcagcctgacctctgaggactctgcggtctattactg
tgcaagagggccttattactacggtagtagccccttgactactggggccaagggaccacggtcac
cgtctccggaggtggcggttcaggcggttggcggatccggcggtggcggatccggcggtggcgga
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ttgcaggtctagtcagagccttgaaaacagtaatggaaacacctatttgaactggtacctccagaaa
ccaggccagtctccacagctcctgatctacagggtttccaacctgatttct gggtcctagacaggt
tcagtggtagtggatcagggacagatttcacactgaaaatcagcagagtggaggctgaggatttgg
gagtttatttctgcctccaagttacacatgtccctcccacgttcggtgctgggaccaagctcgagatc
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cagcctctgtctctgaggcccgaggcttgtagacctgctgcaggcggagccgtgcacaccagagg
actggatttcgcctgcgacatctacatctgggcccctctggccggcacatgcggagtgctgctgctg
agcctcgtgatcaccctgtactgcaaccaccggaaccggcggagagtgaagcggggcagaaaga
agctgctgtacatcttcaagcagcccttcatgcggcccgtgcagaccacccaggaagaggacggc
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cgccgacgcccctgcctaccagcagggacagaaccagctgtacaacgagctgaacctgggcaga
cgggaagagtacgacgtgctggacaagcggagaggcagggacctgagatgggcggaaagccc
cagcggagaaagaacccccaggaaggcctgtataacgaactgcagaaagacaagatggccgag
gcctacagcgagatcggaatgaagggcgagcggagaagaggcaagggccacgatggactgtat
cagggcctgagcaccgccaccaaggacacctatgacgccctgcacatgcaggccctgcccccca
gatgaTGAGAATTCCGACTGTGCCTTCTAGTTGCCAGCCATCT
GTTGTTTGCCCCTCCCCGTGCCTTCCTTGACCCTGGAAGG
TGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTG
CATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGT
GGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAC
AATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGGTC
GACATACTAGTTAAAAGTTTTGTTACTTTATAGAAGAAATT
TTGAGTTTTTGTTTTTTTTTAATAAATAAATAAACATAAAT
AAATTGTTTGTTGAATTTATTATTAGTATGTAAGTGTAAAT
ATAATAAAACTTAATATCTATTCAAATTAATAAATAAACCT
CGATATACAGACCGATAAAACACATGCGTCAATTTTACGC
ATGATTATCTTTAACGTACGTCACAATATGATTATCTTTCT
AGGGTTAATCTAGTATACGCGTATGCGGCCGCTTAATTAAT
CCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTG
GCGTAATCATG<u>GTCATAGCTGTTTCCTG</u> (M13R)

Amino acid sequence of CAR19 construct

MALPVTALLLPLALLLHAARPTREVQLQQSGPELVKPGASVKM
SCKASGYTFTSYVMHWVKQKPGQGLEWIGYVNPYNDGTKYN
EKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGPYYYGSS
PFDYWGQGTTVTVSGGGGSGGGGSGGGGSGGGGSDIVMTQS
PLSLPVSLGDQASISCRSSQSLENSNGNTYLNWYLQKPGQSPQL
LIYRVSNRFSGVLDRFSGSGSGTDFTLKISRVEAEDLGVYFCLQV
THVPPTFGAGTKLEIKRTSPTTTPAPRPPTPAPTIASQPLSLRPEA
CRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYC
NHRNRRRVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEE
EEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVL
DKRRGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIG
MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR*

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AUTHOR CONTRIBUTIONS

I.K., P.P., and M.Š. designed and performed experiments; H.Ž., V.Š., and K.Š. analyzed data; P.O. conceptualized the study and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Shi, H., Sun, M., Liu, L., and Wang, Z. (2014). Chimeric antigen receptor for adoptive immunotherapy of cancer: latest research and future prospects. Mol. Cancer *13*, 219.

2. Li, Y., Huo, Y., Yu, L., and Wang, J. (2019). Quality Control and Nonclinical Research on CAR-T Cell Products: General Principles and Key Issues. Engineering (Beijing) *5*, 122–131.

3. Ausubel, L.J., Hall, C., Sharma, A., Shakeley, R., Lopez, P., Quezada, V., Couture, S., Laderman, K., McMahon, R., Huang, P., et al. (2012). Production of CGMP-grade lentiviral vectors. Bioprocess Int. *10*, 32–43.

4. Singh, H., Manuri, P.R., Olivares, S., Dara, N., Dawson, M.J., Huls, H., Hackett, P.B., Kohn, D.B., Shpall, E.J., Champlin, R.E., and Cooper, L.J. (2008). Redirecting specificity of T-cell populations for CD19 using the Sleeping Beauty system. Cancer Res. *68*, 2961–2971.

5. Manuri, P.V.R., Wilson, M.H., Maiti, S.N., Mi, T., Singh, H., Olivares, S., Dawson, M.J., Huls, H., Lee, D.A., Rao, P.H., et al. (2010). piggyBac transposon/transposase system to generate CD19-specific T cells for the treatment of B-lineage malignancies. Hum. Gene Ther. *21*, 427–437.

6. Stadtmauer, E.A., Fraietta, J.A., Davis, M.M., Cohen, A.D., Weber, K.L., Lancaster, E., Mangan, P.A., Kulikovskaya, I., Gupta, M., Chen, F., et al. (2020). CRISPR-engineered T cells in patients with refractory cancer. Science *367*, eaba7365.

7. Huang, X., Guo, H., Tammana, S., Jung, Y.C., Mellgren, E., Bassi, P., Cao, Q., Tu, Z.J., Kim, Y.C., Ekker, S.C., et al. (2010). Gene transfer efficiency and genome-wide integration profiling of Sleeping Beauty, Tol2, and piggyBac transposons in human primary T cells. Mol. Ther. *18*, 1803–1813.

8. Jin, Z., Maiti, S., Huls, H., Singh, H., Olivares, S., Mátés, L., Izsvák, Z., Ivics, Z., Lee, D.A., Champlin, R.E., and Cooper, L.J. (2011). The hyperactive Sleeping Beauty transposase SB100X improves the genetic modification of T cells to express a chimeric antigen receptor. Gene Ther. *18*, 849–856.

9. Deniger, D.C., Yu, J., Huls, M.H., Figliola, M.J., Mi, T., Maiti, S.N., Widhopf, G.F., 2nd, Hurton, L.V., Thokala, R., Singh, H., et al. (2015). Sleeping Beauty transposition of chimeric antigen receptors targeting receptor tyrosine kinase-like orphan receptor-1 (ROR1) into diverse memory T-cell populations. PLoS ONE *10*, e0128151.

10. Yusa, K., Zhou, L., Li, M.A., Bradley, A., and Craig, N.L. (2011). A hyperactive piggyBac transposase for mammalian applications. Proc. Natl. Acad. Sci. USA *108*, 1531–1536.

11. Ptáčková, P., Musil, J., Štach, M., Lesný, P., Němečková, Š., Král, V., Fábry, M., and Otáhal, P. (2018). A new approach to CAR T-cell gene engineering and cultivation using piggyBac transposon in the presence of IL-4, IL-7 and IL-21. Cytotherapy *20*, 507–520.

12. Poorebrahim, M., Sadeghi, S., Fakhr, E., Abazari, M.F., Poortahmasebi, V., Kheirollahi, A., Askari, H., Rajabzadeh, A., Rastegarpanah, M., Linē, A., and Cid-Arregui, A. (2019). Production of CAR T-cells by GMP-grade lentiviral vectors: latest advances and future prospects. Crit. Rev. Clin. Lab. Sci. *56*, 393–419.

13. Wiesinger, M., März, J., Kummer, M., Schuler, G., Dörrie, J., Schuler-Thurner, B., and Schaft, N. (2019). Clinical-Scale Production of CAR-T Cells for the Treatment of Melanoma Patients by mRNA Transfection of a CSPG4-Specific CAR under Full GMP Compliance. Cancers (Basel) *11*, 1198.

14. Štach, M., Ptáčková, P., Mucha, M., Musil, J., Klener, P., and Otáhal, P. (2020). Inducible secretion of IL-21 augments anti-tumor activity of piggyBac-manufactured chimeric antigen receptor T cells. Cytotherapy *22*, 744–754.

15. Bishop, D.C., Caproni, L., Gowrishankar, K., Legiewicz, M., Karbowniczek, K., Tite, J., Gottlieb, D.J., and Micklethwaite, K.P. (2020). CAR T Cell Generation by *piggyBac*

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Transposition from Linear Doggybone DNA Vectors Requires Transposon DNA-Flanking Regions. Mol. Ther. Methods Clin. Dev. *17*, 359–368.

16. Eyquem, J., Mansilla-Soto, J., Giavridis, T., van der Stegen, S.J.C., Hamieh, M., Cunanan, K.M., Odak, A., Gönen, M., and Sadelain, M. (2017). Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature *543*, 113–117.

17. Basar, R., Daher, M., Uprety, N., Gokdemir, E., Alsuliman, A., Ensley, E., Ozcan, G., Mendt, M., Hernandez Sanabria, M., Kerbauy, L.N., et al. (2020). Large-scale GMP-compliant CRISPR-Cas9-mediated deletion of the glucocorticoid receptor in multi-virus-specific T cells. Blood Adv. *4*, 3357–3367.

18. van der Velden, V.H.J., Cazzaniga, G., Schrauder, A., Hancock, J., Bader, P., Panzer-Grumayer, E.R., Flohr, T., Sutton, R., Cave, H., Madsen, H.O., et al.; European Study Group on MRD detection in ALL (ESG-MRD-ALL) (2007). Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. Leukemia *21*, 604–611.

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4.4. Publication 4

Štach M, Pytlík R, Šmilauerová K, Rychlá J, Mucha M, Musil J, Koladiya A, Nemec M, Petráčková M, Kaštánková I, Pecherková P, Šrámková L, Polgárová K, Trněný M, Lesný P, Vydra J, Otáhal P.

Characterization of the input material quality for the production of tisagenlecleucel by multiparameter flow cytometry and its relation to the clinical outcome.

Author contribution: sample processing and measurement, data analysis, manuscript writing

Characterization of the input material quality for the production of tisagenlecleucel by multiparameter flow cytometry and its relation to the clinical outcome

Martin Štach[1,2], Robert Pytlík[1,3], Kristýna Šmilauerová[1,2],
Jana Rychlá[1], Martin Mucha[1,2], Jan Musil[1], Abhishek Koladiya[1],
Matěj Nemec[1], Martina Petráčková[1], Iva Kaštánková[1],
Pavla Pecherková[1], Lucie Šrámková[4], Kamila Polgárová[3,5],
Marek Trněný[3,5], Petr Lesný[1], Jan Vydra[1] and Pavel Otáhal[1,3]*

[1]Institute of Hematology and Blood Transfusion, Praha, Czechia, [2]Faculty of Science, Charles
University, Praha, Czechia, [3]First Faculty of Medicine, Charles University, Praha, Czechia, [4]Second
Faculty of Medicine (2. LF UK), University Hospital in Motol, Praha, Czechia, [5]General University Hospital
in Prague, Praha, Czechia

Tisagenlecleucel (tisa-cel) is a CD19-specific CAR-T cell product approved for
the treatment of relapsed/refractory (r/r) DLBCL or B-ALL. We have followed a
group of patients diagnosed with childhood B-ALL ($n = 5$), adult B-ALL ($n = 2$),
and DLBCL ($n = 25$) who were treated with tisa-cel under non-clinical trial
conditions. The goal was to determine how the intensive pretreatment of
patients affects the produced CAR-T cells, their *in vivo* expansion, and the
outcome of the therapy. Multiparametric flow cytometry was used to analyze
the material used for manufacturing CAR-T cells (apheresis), the CAR-T cell
product itself, and blood samples obtained at three timepoints after
administration. We present the analysis of memory phenotype of CD4/
CD8 CAR-T lymphocytes (CD45RA, CD62L, CD27, CD28) and the expression
of inhibitory receptors (PD-1, TIGIT). In addition, we show its relation to the
patients' clinical characteristics, such as tumor burden and sensitivity to prior
therapies. Patients who responded to therapy had a higher percentage of
CD8+CD45RA+CD27+ T cells in the apheresis, although not in the produced
CAR-Ts. Patients with primary refractory aggressive B-cell lymphomas had the
poorest outcomes which was characterized by undetectable CAR-T cell
expansion *in vivo*. No clear correlation of the outcome with the
immunophenotypes of CAR-Ts was observed. Our results suggest that an
important parameter predicting therapy efficacy is CAR-Ts' level of
expansion *in vivo* but not the immunophenotype. After CAR-T cells'
administration, measurements at several timepoints accurately detect their
proliferation intensity *in vivo*. The outcome of CAR-T cell therapy largely
depends on biological characteristics of the tumors rather than on the
immunophenotype of produced CAR-Ts.

Introduction

Tisagenlecleucel (tisa-cel, Kymriah®) is CD19-specific CAR-T-cell product approved for the treatment of relapsed/refractory (r/r) DLBCL or B-ALL. First, an input material is obtained *via* apheresis from individual patients to produce CAR-T cells. Collected peripheral blood mononuclear cells (PBMCs) are then cryopreserved and supplied to the manufacturer. Followingly, PBMCs are transduced with lentiviral vector, expanded *in vitro*, and cryopreserved. The generated CAR-T cells are usually within a month supplied back to the hospital site. However, such a highly complex process utilizes materials obtained from patients who received multiple lines of intensive chemotherapeutic regimens and have active disease. Additionally, the variability within differentiation/memory subsets of T cells in the apheresis might affect the quality of produced CAR-T cells and subsequently limit the efficacy of the treatment (1, 2).

Treatment of DLBCL with CD19-specific CAR-T cells such as tisa-cel is slightly less effective than identical approach to patients with B-ALL (2–6). Generally, two types of treatment failure are encountered. The first type is non-responsiveness—the patients do not achieve even a partial remission (PR) within 2–3 months post-treatment. The second type is late relapse after achievement of good clinical response following CAR-T cell treatment (7). Detailed analysis of the input material and the produced CAR-T cells could help identify factors responsible for these types of treatment failures (8–10).

The first goal of our study was to perform a detailed FACS analysis of the apheretic material used for CAR-T cells' production to determine present leukocyte subsets and the immunophenotype of T cells. Secondly, the manufactured product was analyzed to determine the percentage of CAR+ cells and their memory phenotype. Thirdly, CAR-T cells' expansion kinetics and their differentiation status were measured in samples of peripheral blood after treatment. Collected measurements were compared with the treatment efficiency and patients' survival.

In summary, this study describes the analysis of apheresis, manufactured CAR-T cells and samples of patients treated with tisa-cel. Based on obtained measurements and clinical data, our results suggest that a specific phenotype of starting material (i.e., apheresis) influences possible success of the therapy. The results also show that undetectable CAR-T cell expansion at D+14 is linked to early treatment failure ($p < 0.05$) which might provide a chance to effectively indicate the patient enrollment into clinical trials.

Results

Detection of CAR-T cells and gating strategy for their phenotyping

To identify CAR-T cells, we used recombinant PE-conjugated CD19 protein for the first eight patients due to the unavailability of anti-FMC63 antibody, which was then used for the remaining patients. Example of staining is presented in Supplementary Figure S1. CAR-T cells were further stained with a multicolor antibody panel to determine their differentiation immunophenotype using antibodies against antigens CD3, CD4, CD8, CD14, CD45RA, CD62L, CD27, CD28, PD-1, and TIGIT (11). Additionally, samples were analyzed with a second antibody panel to determine the composition of all significant leukocyte subsets using antibodies against antigens CD3, CD4, CD8, CD14, CD16, CD19, CD45, CD56, and TCRgd. Figure 1 shows an example of typical CAR detection. Gating strategy of CAR-T memory subsets is similarly presented in Figure 2. Pre-gating on CD3+ cells is shown in Supplementary Figure S2. Controls for CAR staining (healthy donor) and immunophenotype staining (FMOs, healthy donor) are presented in Supplementary Figures S3, S4, respectively. Threshold for the detection of CAR+ cells was set at 0.1% out of CD3 T cells, and the percentage of CAR-T cells was further converted to the absolute counts per microliter using the total white blood cell count values obtained from hematology analyzer. The antibody panels are presented in Supplementary Table S1.

Analysis of apheresis and CAR-T cell products

The first goal was to characterize patients' apheresis and to determine whether differences in their T cell memory subsets are associated with stronger *in vivo* expansion of CAR-T cells and improved clinical outcome. For each patient, the apheretic product samples were cryopreserved, as well as from five healthy donors functioning as control samples. Prior to flow cytometry analysis, the cells were thawed and let to rest in media overnight. We have found out that 27 out of 32 subjects had undetectable B-cells in apheresis due to preceding treatment by rituximab (Figure 3A, Supplementary Figure S2). To characterize the T cells, we determined their immunophenotype with the differentiation antibody panel (omitting the anti-FMC63 antibody). The patients' T cells were characterized by highly variable numbers of Tem/Tcm/Temra CD8+ and CD4+ memory subsets, similarly to what was detected in healthy donors

FIGURE 1
Detection of CAR-T cells in the product and in blood at three timepoints: T1 early after infusion (day 2−4), T2 at the expected peak of expansion (days 10−14), and T3 at the predicted contraction phase (days 30−60). Percentages of CAR$^-$ (left gate) and CAR$^+$ (right gate) cells of CD3$^+$ cells are shown for one representative patient. Gates were set based on measurements of healthy donor control.

FIGURE 2
Gating strategy for immunophenotyping of CD4$^+$/CD8$^+$ CAR-T cells using antibodies against antigens CD45RA, CD62L, CD27, CD28, PD-1 and TIGIT. Memory subsets were defined as: SCM, stem cell memory (CD45RA$^+$CD62L$^+$); CM, central memory (CD45RA$^-$CD62L$^+$); EM, effector memory (CD45RA$^-$CD62L$^-$); EMRA, terminally differentiated effector memory (CD45RA$^+$CD62L$^-$). One representative patient is shown. Percentages of populations of CD4$^+$CAR$^+$ or CD8$^+$CAR$^+$ shown in respective quadrants. Gates were set based on measurements of healthy donor control and FMO controls.

(Figure 3B, Supplementary Figure S6). Nevertheless, the patients' T cells contained more exhausted T cells expressing PD-1 receptor compared to healthy donors—50% vs. 12% CD4$^+$ (p = <0.001) and 35% vs. 14% CD8$^+$ (p = 0.046). In addition, the non-responders were characterized by significantly reduced numbers of CD8$^+$ CD45RA$^+$ CD27$^+$ T cells (Figure 3C) in the apheresis, in agreement with report by Fraietta et al. (12).

Subsequently, the manufactured CAR-T cells were analyzed using aforementioned antibody panel with added anti-FMC63 antibody. The samples for measurements were obtained from

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FIGURE 3

Analysis of apheresis. **(A)** Composition of individual leukocyte subsets in the apheresis. **(B)** Immunophenotype of CD4+ and CD8+ T cells in apheresis of responders, non-responders and healthy donors. SCM, stem cell memory; CM, central memory; EM, effector memory; EMRA, terminally differentiated effector memory. **(C)** Representative immunophenotypes of T cells (CD8+CD27+DC45RA+) as percentages of CD8+ cells in four subjects: CR, complete response; PR, partial response; NR, non-responder; HD, healthy donor. Unpaired *t*-test **p* < 0.05, ns-not significant.

FIGURE 4

Analysis of the product. **(A)** Manufactured products cells were analyzed to determine the percentage of CAR+ in CD4+ and CD8+ T cells out of all CD3+ T cells. ND, not done; N*, product not meeting specifications. **(B)** Statistical analysis of the percentages of CD4+CAR+ (left) and CD8+CAR+ (right) in product of responders and non-responders. **(C)** Immunophenotypes of CD4+CAR+ and CD8+CAR+ cells in product of responders and non-responders. SCM, stem cell memory; CM, central memory; EM, effector memory; EMRA, terminally differentiated effector memory. Unpaired *t*-test; ns, not significant, **p* < 0.05.

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FIGURE 5
Expansion of CAR-T cells *in vivo*. **(A)** The level of expansion of CAR-T cells was determined in peripheral blood samples at three timepoints after administration: early after infusion (day 2–4), at the expected peak of expansion (days 10–14), and at the predicted contraction phase (days 30–60). The left panels show the expansion kinetics of CD4+CAR+ cells and right panels shows CD8+CAR+ cells as a percentage of CD3+ T cells. **(B)** Statistical analysis of the level of expansion in responders (*n* = 13) vs. non-responders (*n* = 10) at indicated timepoints shown as a total number of CAR-T cells in the blood and as a fold expansion of absolute CAR-T cell counts in the blood between timepoint 1 (T1) and timepoint 2 (T2). Unpaired *t*-test *p* < 0.05, **p* < 0.005.

discarded infusion bags after administration to the patients. The infusion bags and their filters were thoroughly washed with PBS to acquire the remaining cells. In some cases, we were unable to measure administered products for technical reasons. We

observed that the products contained a highly variable percentage of CAR+ cells (range 1%–40%) and that the majority of CAR-T cells were CD4 positive (Figure 4A). There was no significant difference of percentage of CD4+ or CD8+

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TABLE 1 Patients' characteristics.

Patient ID	Age	Dg	Primary refractory disease	Sensitivity to bridge treatment before CAR-T	Clinical stage before CAR-T	Response to CAR-T at 3 m/6 m	Lymphodepletion regimen	Number of lines of treatment before CAR-T	T1 % CD4+CAR+	T2% CD4	T1 % CD8	T2 % CD8	CAR-T expansion
1	25	ALL	No	Yes	CR	CR/CR	Flu/Cy	3+alloSCT	0	1.06	0	6.17	Yes
2	27	ALL	No	Yes	CR	CR/relapse	Flu/Cy	5+alloSCT	3.11	0.15	0.21	0.36	Yes
3	69	DLBCL	No	Yes	CR	CR/CR	Flu/Cy	4+ASCT	0	4.85	0.81	5.14	Yes
4	67	DLBCL	No	Yes	2	progression	None	4+ASCT	1.15	1.13	0.35	0	No
5	43	DLBCL	No	Yes	1	CR/CR	Flu/Cy	4	1.39	3.47	0.4	8.25	Yes
6	53	DLBCL	Yes	No	4	PR/CR	bendamustine	4	0.35	0.16	0	0.22	Yes
7	74	DLBCL	No	Yes	1	CR/CR	Flu/Cy	5+ASCT	2.87	2.95	1.13	1.32	Yes
8	77	DLBCL	No	Yes	4	CR/CR	Flu/Cy	3	1.46	3.68	0.55	0.62	Yes
9	34	DLBCL	No	No	4	PR/progression	Flu/Cy	3+ASCT	0.3	8.9	0.59	1.07	Yes
10	61	DLBCL	Yes	No	4 (bulky)	progression	Flu/Cy	4	ND	1.19	ND	0.35	Yes
11	72	DLBCL	No	Yes	CR	CR/CR	bendamustine	3+ASCT	ND	1.1	ND	0.14	Yes
12	39	DLBCL	No	Yes	2	CR/CR	Flu/Cy	3+ASCT	1	0	0.16	0	No
13	57	DLBCL	Yes	No	4	progression	bendamustine	4	0	0	0	0	No
14	41	FL/DLBCL	No	Yes	4	CR/CR	bendamustine	3+ASCT	1.65	0	0	0	No
15	54	DLBCL	No	No	3	progression	bendamustine	3+ASCT	0.56	0	0	0	No
16	49	DLBCL	No	Yes	4	progression	bendamustine	2	0.18	0	0	0.11	No
17	10	ALL	No	Yes	4	CR/CR	Flu/Cy	1	ND	1.19	ND	4.84	Yes
18	15	ALL	No	Yes	2	CR/CR	Flu/Cy	1	0.18	0.14	1.79	2.56	Yes
19	5	ALL	No	Yes	1	CR/CR	Flu/Cy	1	0	0.18	0.36	0.64	Yes
20	3	ALL	No	Yes	2	CR/CR	Flu/Cy	1	ND	0	ND	5.14	Yes
21	74	DLBCL	Yes	No	4	progression	Flu/Cy	3	0.10	0.12	0	0.23	Yes
22	72	DLBCL	No	No	2	CR/CR	bendamustine	3	11.10	6.31	1.43	13.20	Yes
23	71	FL/DLBCL	No	Yes	1	CR/CR	bendamustine	4	1.49	0.51	0.27	0.73	Yes
24	70	DLBCL	Yes	Yes	2	progression	bendamustine	2	0.22	0.14	0	0	No
25	10	ALL	No	Yes	PR	CR/CR	Flu/Cy	1	0.45	0.95	0	9.23	Yes
26	44	DLBCL	Yes	No	4	progression	None	2	0	0	0	0	No
27	76	DLBCL	No	No	4	progression	bendamustine	2	0.36	0.31	0.10	0.42	Yes
28	23	DLBCL	No	Yes	4	PR/---	pixantron+ benda	5	0.12	1.57	0	11.30	Yes
29	49	DLBCL	No	Yes	4	CR/CR	Flu/Cy	4+ASCT	0.49	1.20	0.22	0.12	Yes
30	64	DLBCL	Yes	No	4 (bulky)	progression	Flu/Cy	3	0.13	0	0	0	No
31	56	DLBCL	No	Yes	4 (bulky)	PR/progression	Flu/Cy	3	0.10	0.32	0.14	1.50	Yes
32	70	FL/DLBCL	No	Yes	3	progression	Flu/Cy	3	0	0.16	0	0	No

FIGURE 6
Immunophenotype of CAR-T cells *in vivo*. **(A)** Expanded CD4^{+} and CD8^{+} CAR^{+} T cells were analyzed for each patient by multiparameter flow cytometry to determine the expression of major surface antigens reflecting their differentiation status. Phenotypes with significant difference between responders and non-responders are shown. Percentages of phenotype subsets are of CD8^{+} CAR^{+} T cells. CM, central memory (CD45RA^{−}CD62L^{+}). Mann-Whitney test; ns, not significant, *$p < 0.05$. **(B)** Cluster analysis of detected CAR^{+} T cells at the first timepoint for indicated antigens, all identified CAR-T cells from all responders and non-responders at the first timepoint were analyzed together. EmbedSOM algorithm was used. The scale indicates relative intensity of detected antigens.
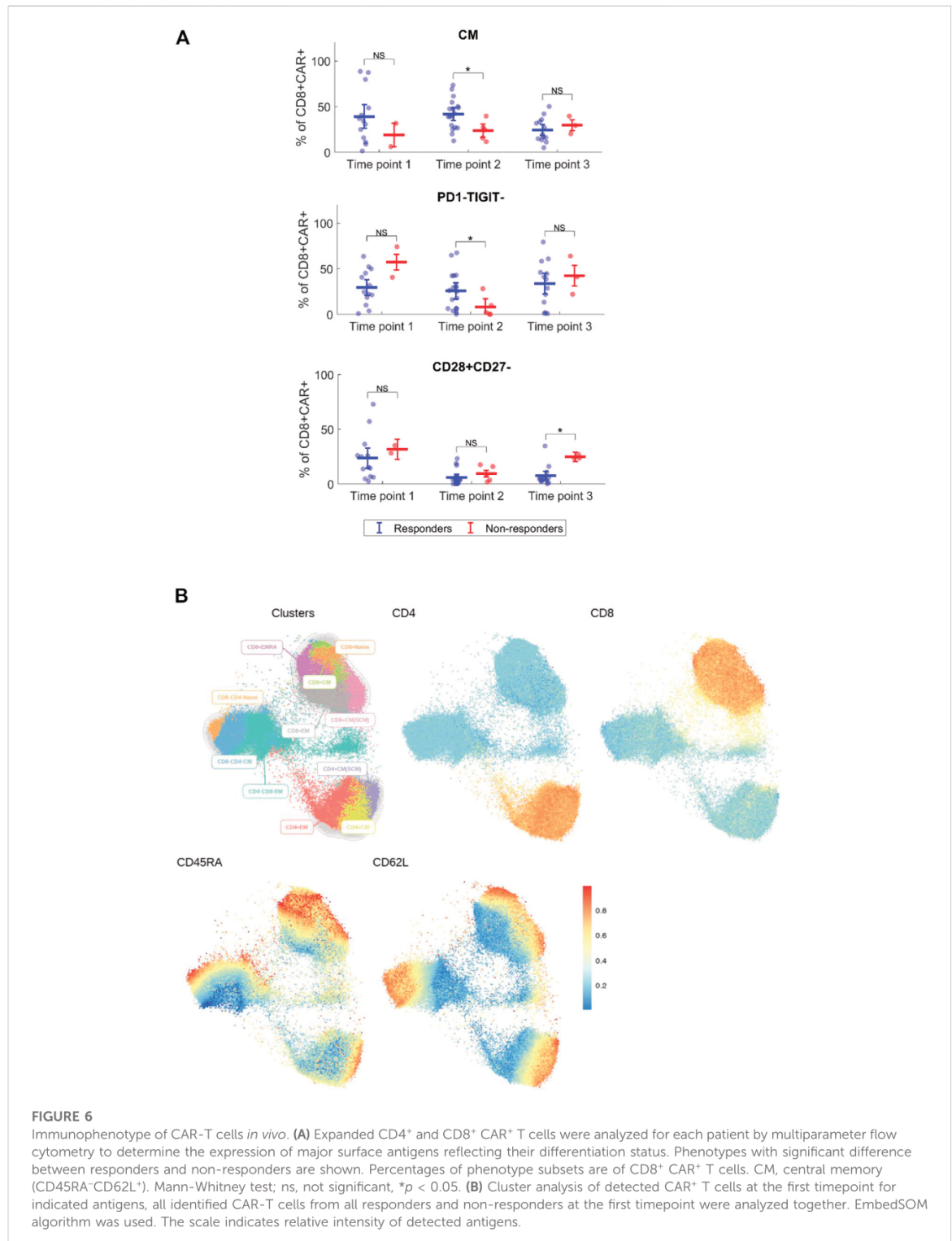
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TABLE 2 Characteristics of DLBCL patients.

	Expansion of CAR-T	Primary refractory disease	Response to bridge treatment before CAR-T	Median clinical stage before CAR-T
Responders	87.5% (12/14)	7.1% (1/14)	78.5% (11/14)	3
Non-responders	27.3% (3/11)	54.5% (6/11)	36.4% (4/11)	4

CAR-T cells in product between responders and non-responders (Figure 4B). The immunophenotype of CAR-T cells was likewise highly variable between individual patients in regards to the proportion of Tscm/Tcm/Tem/Temra, and CD27/CD28/PD-1/TIGIT positive cells (Figure 4C, Supplementary Figure S7). There was a slightly higher PD1+TIGIT− percentage of CD4+CAR+ T cells in responders ($p = 0.039$, Figure 4C). CAR+ T cells in the product had almost identical immunophenotype as the CAR− population (data not shown), suggesting that their differentiation during manufacturing was driven more by polyclonal anti-CD3/CD28 activation than by CAR signaling. One product (P31) did not meet the entry specifications since it contained only 1% of CAR-T cells. Despite such a low percentage of CAR-T cells, this patient responded to the therapy and achieved PR by PET/CT (nonetheless relapsed later). Conversely to apheresis, we did not detect a significant difference in the numbers of CD8+ CD45RA+ CD27+ T cells in the product (Figure 4C).

Fate of CAR-T cells *in vivo*

Expansion and immunophenotype of CAR-T cells were determined in the patients' blood samples after administration at three timepoints—between days 2–4 (T1), days 10–14 (T2), and days 30–60 (T3). Since the study was performed in a non-clinical trial setting, the samples could be obtained only during regular medical examinations including signing of the informed consent form by the patient. To determine the kinetics of CAR-T cells' expansion, we measured percentage of CD4+/CD8+ CAR-T out of CD3+ T cells and calculated their absolute counts in blood. The CAR-T cell percentages were compared between T1 and T2 to assess CAR-T cells' expansion kinetics. If the percentage of either CD4+ or CD8+ CAR+ at T2 was higher than their percentage at T1, the patient was categorized as showing a detectable expansion. Figure 5A shows the kinetics of CAR-T cell expansion for each patient. In addition, we calculated the absolute number of CAR-T cells per μl of blood in the responders and the non-responders at T1 and T2 (Figure 5B). Table 1 shows the measured values of CAR-T cells in blood for each patient and demonstrates that only 27% (3/11) of non-responders had a detectable expansion of CAR-T cells and that 90% (19/21) of responders had detectable expansion (Table 2). Out of 25 patients with DLBCL, 10 subjects had no expansion of CAR-T cells (all patients with B-ALL were responders with detectable CAR-T cell
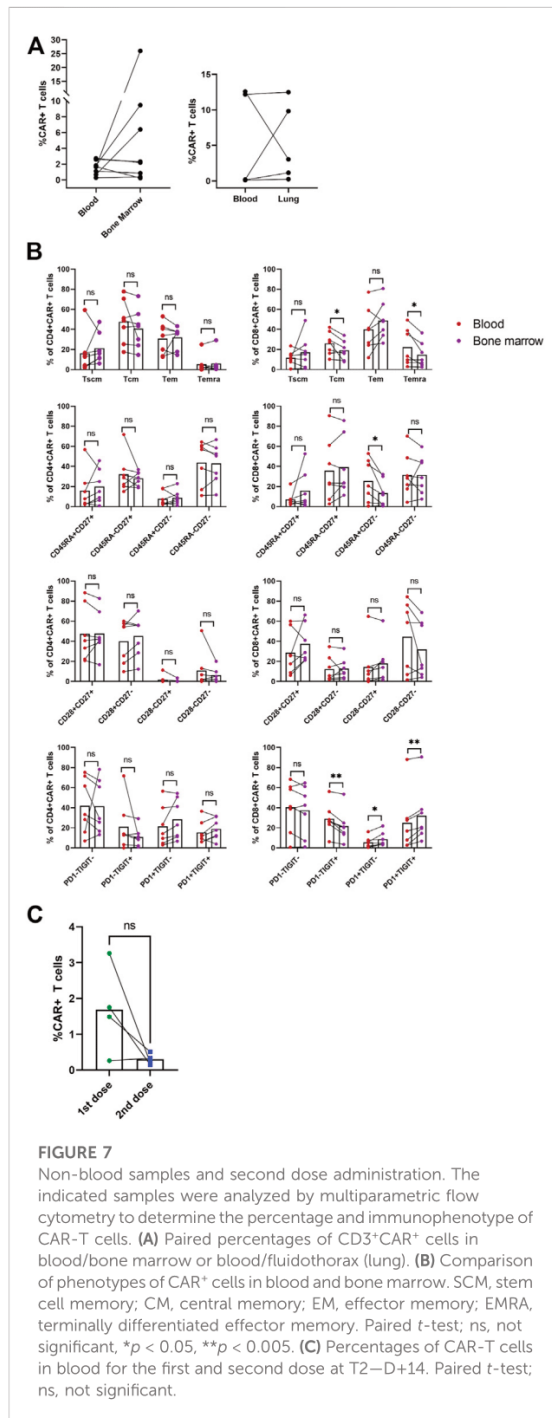
expansion). 80% of these "non-expanders" were also non-responders, but only 40% of them had primary refractory disease. Following this, we determined the immunophenotype of detected CAR-T cells in the blood similarly as was done for the product (Supplementary Figure S8). Threshold for the detection of CAR-T cells was set at 0.1% of CD3+ T cells, samples with CAR+ percentage below this value were not analyzed. Significant differences between responders and non-responders are presented in Figure 6A. At time point 2, responders had higher numbers of CD8+CAR+ Tcm ($p = 0.030$) and PD1−TIGIT− than non-responders ($p = 0.048$). At time point 3, CD28+CD27− percentage was higher in non-responders ($p = 0.025$), although only 3 samples were used for the analysis. Clustering by EmbedSOM algorithm (13) shows relative distribution of memory subsets of CAR-T cells for time point 1 (Figure 6B).

Non-blood samples and second dose administration

In addition to blood samples, we analyzed samples obtained from bone marrow and in three subjects with lung infiltration also from the malignant pleural effusion. Figure 7A shows percentages of CAR-T cells detected at a single time point in blood with corresponding bone marrow or pleural effusion (lung) sample. Interestingly, we observed effective migration of CAR-T cells into the tumor sites, demonstrated by high CAR-T percentage in the pleural effusion. We also detected long-term persisting CAR-T cells in the bone marrow of several subjects.

Immunophenotype of CAR-T cells in bone marrow was compared to paired blood samples (Figure 7B). CD8+ CAR-T cells in blood had elevated Tcm ($p = 0.038$), Temra ($p = 0.046$), and CD45RA+CD27− ($p = 0.032$) phenotypes compared to bone marrow. CD8+ CAR-T cells in bone marrow had slightly higher percentage of PD1+TIGIT+ ($p = 0.003$), PD1+TIGIT− ($p = 0.028$) populations, and lower PD1−TIGIT+ ($p = 0.004$) percentage. Phenotype of CAR-T cells in pleural effusion was not statistically analyzed due to insufficient number of paired blood samples with detectable CAR-T cells (Supplementary Figure S9).

Four patients from the selected study group were administered a second dose of CAR-T cells after they relapsed; the source of the

FIGURE 7
Non-blood samples and second dose administration. The indicated samples were analyzed by multiparametric flow cytometry to determine the percentage and immunophenotype of CAR-T cells. **(A)** Paired percentages of CD3+CAR+ cells in blood/bone marrow or blood/fluidothorax (lung). **(B)** Comparison of phenotypes of CAR+ cells in blood and bone marrow. SCM, stem cell memory; CM, central memory; EM, effector memory; EMRA, terminally differentiated effector memory. Paired *t*-test; ns, not significant, *$p < 0.05$, **$p < 0.005$. **(C)** Percentages of CAR-T cells in blood for the first and second dose at T2—D+14. Paired *t*-test; ns, not significant.

CAR-T cells was a remaining cryopreserved bag of the original product. Subject P09 received the second dose simultaneously with anti-PD-1 antibody pembrolizumab. Subject P16 received the

second dose concurrently with lenalidomide treatment. Subjects P18 and P19 were pediatric B-ALL patients in clinical remission, although B-cell presence in the peripheral blood was established, suggesting the therapy's failure. Therefore, the second CAR-T cells' dose was used successfully as a bridging therapy before allo-HSCT. Furthermore, we observed that the subjects P16, P18, and P19 had a non-detectable expansion of the second CAR-T cells' dose. In contrast, in the subject P09, we observed high levels of CAR-T cells in the pleural effusion even though they were almost undetectable in the blood sample. Figure 7C shows percentage of CAR-T cells at D+14 for first and second administration. The immunophenotype of these CAR-T cells was very similar to the immunophenotype of CAR-T cells detected in the patient's blood sample following the administration of the first dose. Unfortunately, despite such a very efficient expansion of CAR-T cells, subject P09 did not achieve complete remission (CR) and the disease continually progressed.

Discussion

In this report, we analyzed a group of 32 patients diagnosed with either B-ALL or DLBCL and treated in a real-world setting with tisa-cel, CD19·specific CAR-T cell product. The high costs of commercial CAR-T cell therapy and extended production time emphasize the need to search for clinical and laboratory parameters that will enable selection of patients with the highest chances to respond to therapy and determine what prior therapies might adversely affect the efficiency of the production of CAR-T cells. Thus, the main goals of our study were to correlate the efficacy of the therapy with the immunophenotype of input material used for the production of CAR-T cells (i.e., the apheresis) and with the immunophenotype of the produced CAR-T cells. Additionally, we observed CAR-T cell expansion kinetics after administration in the blood and measured their immunophenotype. The analysis presents a comprehensive data set from 32 subjects, including detailed clinical parameters such as prior treatments and complications associated with CAR-T cell therapy.

We observed a similar efficiency of the treatment in comparison to reported results of other real-world experiences (13). A noticeable subset of patients were non-responders who were characterized by continuously progressing disease after CAR-T cell therapy without achieving even a PR at 3-month restaging by PET/CT or by flow cytometry. In all cases, these patients were diagnosed with DLBCL and have shown no detectable expansion of CAR-T cells. Unfortunately, several patients with promising CR relapsed at later time points. The highly variable numbers and ratios of CD4+ and CD8+ T cells and the depletion of central memory (Tcm)/stem cell memory T cell (Tscm) subsets due to prior chemotherapies is a challenging issue for cellular therapy approaches. These subsets are essential for durable anti-tumor responses in adoptive cell therapy (14). For example, B-cell lymphoma patients have an increased percentage of terminal effector memory CD8+ T cells (15).

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Comparably to published reports (12), we found that an increased number of stem-cell-like memory CD45RA$^+$CD27$^+$ CD8$^+$ T cells in the apheresis correlated with enhanced efficacy of CAR-T therapy, suggesting that the quality of the input material influences outcome of the treatment. However, the responders did not have a significantly higher number of CD45RA$^+$CD27$^+$ CAR-T cells in the product in comparison to the non-responders.

Next, the product analysis showed an interesting, highly variable CD4/CD8 ratio of CAR-T cells between products. Moreover, the percentage of CAR$^+$ T cells in the product did not correlate with the response to the treatment suggesting that out-of-specification products might be equally efficient and worth administering (Figure 4B). For example, patient 32 had a bulky tumor and achieved near CR despite the administered product containing only 2% of CAR$^+$ cells. These findings indicate that an efficient response probably requires a relatively small number of CAR$^+$ cells with stem-cell memory phenotype, which can be reliably achieved in most patients despite significant differences in the T cell subsets between products.

Immunophenotypic analysis of expanded CAR-T cells *in vivo* revealed higher numbers of central memory and PD1−TIGIT− CD8$^+$CAR$^+$ cells in responders at time point 2 (Figure 6A). This might contribute to the hypothesis that early memory phenotypes are persisting longer, and thus leading to a better response. However, more data is needed to further support these findings.

Performed studies analyzing factors associated with responsiveness to CAR-T cell therapy among patients with DLBCL showed that one of the most significant negative factors is tumor mass volume (5, 7). We similarly observed the lowest efficacy of the treatment among patients with primary refractory DLBCL who frequently were at clinical stage IV with a large tumor burden. Furthermore, these patients continuously undergo intensive chemotherapy, negatively impacting CAR-T cells' quality. Thus, a combination of these factors might severely impair the treatment outcome among patients with primary refractory DLBCL. Any solution to these problems would be complicated – for example, such patients could be considered for clinical trials with enhanced next-generation CAR-T cell products, which can be rapidly manufactured from the original apheresis for commercial CAR-T cell products (16). Hence, patients with primary refractory DLBCL without detectable CAR-T cell expansion would be such candidates. Therapeutic options for relapsed patients after CAR-T cell therapy are purely experimental; a frequently used option is the second dose of CAR-T cell infusion (17). We have analyzed four of these subjects. However, we observed, similarly to already reported data, a significantly reduced expansion of CAR-T cells compared to the first treatment except for subject P09 (who also received an anti-PD1 antibody). Analysis of non-blood samples such as bone marrow and malignant pleural effusion showed efficient infiltration of CAR-T cells into these compartments

without apparent differences in their immunophenotype. This data suggests that undetectable or very low expansion of CAR-T cells in the blood might falsely indicate a treatment failure as the majority of expanding CAR-Ts *in vivo* might be localized in tumor or other tissues. In summary, our results suggest that the outcome of CAR-T cell therapy largely depends on the biological characteristics of the tumors rather than on the immunophenotype of produced CAR-T cells.

Methods

Patient samples

Samples were acquired from patients diagnosed with DLBCL and B-ALL who were treated with tisagenlecleucel at the Institute of Hematology and Blood Transfusion, General University Hospital in Prague, and University Hospital in Motol. All patients (or their parents/guardians) signed informed consent form. Following samples were collected from each patient: 1) apheresis used for CAR-T cell manufacturing, 2) CAR-T-cell product from infusion bag, 3) whole blood sample from patients following CAR-T infusion at three time points—first early after administration (days 2–4), second at the time of expected maximal CAR-T-cell expansion (days 10–14), and third after the retraction of the immune response (days 30–60). The control samples were obtained from age-matched healthy volunteers. In addition to FACS analysis, a total blood cell count was determined as part of a regular medical examination. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples by gradient centrifugation using Ficoll-Paque premium (GE Healthcare). After isolation, PBMCs were resuspended in PBS and were either stained immediately or frozen in CryoStor CS10 (StemCell Technologies) for further processing. For unfreezing, cells were thawed and cultivated in cell culture media overnight. For all experiments, CellGro media (CellGenix, Germany) supplemented with 10% heat-inactivated fetal calf serum (Gibco, United States) was used with addition of antibiotics penicillin and streptomycin (Gibco, United States).

Patients' characteristics

The study group includes patients with pediatric B-ALL (n = 5, age 3–10), adult B-ALL (n = 2, age 25–27), or with DLBCL (n = 25, age 34–77) who were eligible for treatment with tisa-cel (Table 1). In total, 32 patients received the treatment under non-clinical trial settings according to recommended clinical practice. All patients with B-ALL were in complete remission before infusion of CAR-T cells. In contrast, 24 out of 25 (96%) of the remaining patients with DLBCL had active disease with a significant tumor burden. After administration of CAR-T cells,

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the patients were monitored, and when necessary, treated for CRS/neurotoxicity symptoms with corticosteroids or tocilizumab according to recommended CAR-T cells' medication protocols. A grade 2 CRS was observed in 5 patients, which required hemodynamic support. However, no patient required mechanical ventilation, all rapidly recovered from CRS symptoms. The effects of CAR-T cell therapy were determined at three and six-months' time points *via* PET/CT (18) and by flow cytometry. Those patients who either progressed or failed to achieve at least a PR at 3 months were classified as non-responders. The remaining individuals were classified as responders and were further followed. All relevant pretreatment and post-treatment parameters are presented in Table 1. Patient P28 had a significant CAR-T expansion, so we included them as a responder; however, this patient died within a month due to complications connected with primary disease. In summary, the patients' responses to CAR-T cell treatment and associated complications were similar to the currently known clinical experience with tisa-cel (19).

Antibody panels and staining

Approximately five million freshly isolated PBMCs per sample were used for staining. For the first eight patients, we used PE-Labeled Human CD19 (20–291) Protein (Acro Biosystems). Later, anti-FMC63-FITC antibody by Acro Biosystems was used. Comparison of staining with protein CD19 and anti-FMC63 antibody is shown in Supplementary Figure S1. Besides detection of CAR-T cells the samples were stained with antibodies against antigens CD3, CD4, CD8, CD45RA, CD62L, CD27, CD28, CD57, PD-1, TIM-3, and TIGIT. A second detection panel against antigens CD3, CD4, CD8, CD14, CD16, CD19, CD45, CD56, and TCRgd was used to determine significant leukocyte subsets in the samples. Antibody panels used in this study were used previously (20) and can be found in Supplementary Tables S1, S2. All antibodies were titrated before use, and fluorescence-minus-one controls for selected antibodies were measured. Firstly, PBMCs were stained using a fixable blue dead cell stain kit (Thermo Fisher Scientific, United States), washed with FACS buffer, and then stained with antibody mix in Brilliant Stain Buffer (BD) at room temperature for 30 min. The samples were washed with FACS buffer prior to measurement.

Flow cytometry

Sample data were acquired on a five-laser BD LSRFortessa instrument (BD Biosciences). All measurements were standardized using 8-peak Rainbow beads (Spherotech, Lake Forest, IL). BD CompBeads (BD, anti-rat #552844, and anti-mouse #552843) were used for compensation.

Data analysis

Manual analysis of cytometry data was performed using FlowJo software (TreeStar).

Statistical analysis was carried out using GraphPad Prism version 9 (GraphPad Software) and MATLAB (R2022b, The MathWorks Inc.).

Key Clinical Message

Non-responders to therapy with CD19-specific CAR-T cells (tisagenlecleucel) are characterized by undetectable expansion of CAR-T cells and in the majority of cases are diagnosed with primary refractory aggressive B-cell lymphomas..

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the patients to publish this report in accordance with the journal's patient consent policy.

Author contributions

MŠ and AK: data analysis, sample measurements, and manuscript writing. RP, LŠ, PL, and JV: clinical data analysis and management. MP, JR, and IK: sample processing and data administration. KŠ and MM: data analysis and sample measurements. JM: flow-cytometry analysis and assay development. PO: manuscript writing and data analysis. PP: statistical analysis.

Funding

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.por-journal.com/articles/10.3389/pore.2023.1610914/full#supplementary-material

References

1. Siddiqi T, Abramson JS, Li D, Brown W, Devries T, Dave K, et al. Patient characteristics and pre-infusion biomarkers of inflammation correlate with clinical outcomes after treatment with the defined composition, CD19-targeted CAR T cell product, JCAR017. Blood (2017) 130:193. doi:10.1182/blood.V130.Suppl_1.193.193

2. Zhang X, Yang JF, Li JJ, Li WQ, Song D, Lu XA, et al. Factors associated with treatment response to CD19 CAR-T therapy among a large cohort of B cell acute lymphoblastic leukemia. Cancer Immunol Immunother (2022) 71(3):689–703. doi:10.1007/s00262-021-03009-z

3. Martino M, Alati C, Canale FA, Musuraca G, Martinelli G, Cerchione C. A Review of clinical outcomes of CAR T-Cell therapies for B-Acute lymphoblastic leukemia. Int J Mol Sci (2021) 22:2150. doi:10.3390/ijms22042150

4. Schuster SJ, Bishop MR, Tam CS, Waller EK, Borchmann P, McGuirk JP, et al. Tisagenlecleucel in adult relapsed or refractory diffuse large B-cell lymphoma. N Engl J Med (2019) 380:45–56. doi:10.1056/NEJMoa1804980

5. Vercellino L, Di Blasi R, Kanoun S, Tessoulin B, Rossi C, D'Aveni-Piney M, et al. Predictive factors of early progression after CAR T-cell therapy in relapsed/refractory diffuse large B-cell lymphoma. Blood Adv (2020) 4(22):5607–15. doi:10.1182/bloodadvances.2020003001

6. Riedell PA, Walling C, Nastoupil LJ, Pennisi M, Maziarz RT, McGuirk JP, et al. A multicenter retrospective analysis of clinical outcomes, toxicities, and patterns of use in institutions utilizing commercial axicabtagene ciloleucel and tisagenlecleucel for relapsed/refractory aggressive B-cell lymphomas. Blood (2019) 134:1599. doi:10.1182/blood-2019-127490

7. Dean EA, Mhaskar RS, Lu H, Mousa MS, Krivenko GS, Lazaryan A, et al. High metabolic tumor volume is associated with decreased efficacy of axicabtagene ciloleucel in large B-cell lymphoma. Blood Adv (2020) 4(14):3268–76. doi:10.1182/bloodadvances.2020001900

8. Monfrini C, Stella F, Aragona V, Magni M, Ljevar S, Vella C, et al. Phenotypic composition of commercial anti-CD19 CAR-T cells affects in vivo expansion and disease response in large B-cell lymphoma patients. Clin Cancer Res (2022) 28: 3378–86. doi:10.1158/1078-0432.ccr-22-0164

9. Cuffel A, Allain V, Faivre L, Di Blasi R, Morin F, Vercellino L, et al. Real-world characteristics of T-cell apheresis and clinical response to tisagenlecleucel in B-cell lymphoma. Blood Adv (2022) 6:4657–60. doi:10.1182/bloodadvances.2022007057

10. Byrne M, Oluwole OO, Savani B, Majhail NS, Hill BT, Locke FL. Understanding and managing large B cell lymphoma relapses after chimeric antigen receptor T cell therapy. Biol Blood Marrow Transplant (2019) 25: e344–e351. doi:10.1016/j.bbmt.2019.06.036

11. Blache U, Weiss R, Boldt A, Kapinsky M, Blaudszun AR, Quaiser A, et al. Advanced flow cytometry assays for immune monitoring of CAR-T cell applications. Front Immunol (2021) 12:658314. doi:10.3389/fimmu.2021.658314

12. Fraietta JA, Lacey SF, Orlando EJ, Pruteanu-Malinici I, Gohil M, Lundh S, et al. Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. Nat Med (2018) 24(5): 563–71. doi:10.1038/s41591-018-0010-1

13. Schuster SJ, Svoboda J, Chong EA, Nasta SD, Mato AR, Anak O, et al. Chimeric antigen receptor T cells in refractory B-cell lymphomas. N Engl J Med (2017) 377:2545–54. doi:10.1056/NEJMoa1708566

14. Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, et al. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. J Clin Invest (2005) 115(6):1616–26. doi:10.1172/JCI24480

15. Sommermeyer D, Hudecek M, Kosasih PL, Gogishvili T, Maloney DG, Turtle CJ, et al. Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity in vivo. Leukemia (2016) 30(2):492–500. doi:10.1038/leu.2015.247

16. An L, Lin Y, Deng B, Yin Z, Zhao D, Ling Z, et al. Humanized CD19 CAR-T cells in relapsed/refractory B-ALL patients who relapsed after or failed murine CD19 CAR-T therapy. BMC Cancer (2022) 22(1):393. doi:10.1186/s12885-022-09489-1

17. Park JH, Riviere I, Gonen M, Wang X, Senechal B, Curran KJ, et al. Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. N Engl J Med (2018) 378(5):449–59. doi:10.1056/NEJMoa1709919

18. Štach M, Ptáčková P, Mucha M, Musil J, Klener P, Otahal P. Inducible secretion of IL-21 augments anti-tumor activity of piggyBac-manufactured chimeric antigen receptor T cells. Cytotherapy (2020) 22(12):744–54. doi:10.1016/j.jcyt.2020.08.005

19. Cheson BD, Fisher RI, Barrington SF, Cavalli F, Schwartz LH, Zucca E, et al. Recommendations for initial evaluation, staging, and response assessment of hodgkin and non-hodgkin lymphoma: The lugano classification. J Clin Oncol (2014) 32(27):3059–68. doi:10.1200/JCO.2013.54.8800

20. Kratochvíl M, Koladiya A, Vondrášek J. Generalized EmbedSOM on quadtree-structured self-organizing maps. F1000Res (2020) 8:2120. doi:10.12688/f1000research.21642.2

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5. DISCUSSION

CAR T cells are becoming a well-established treatment method. They are particularly effective for hematological B-cell malignancies and are currently approved for use in later lines of treatment. Despite the indisputable revolutionary accomplishments in the field of cancer immunotherapy, this type of therapy still suffers from several pitfalls. Namely, there is a need to enhance efficacy, prevent relapses, increase safety, broaden the target specificity, or decrease its astronomical production costs. This thesis addresses several of these topics of improvement in CAR T-cell therapy. The first aim was to test the cultivation of CAR T cells using cytokines IL-4, IL-7, and IL-21 to increase yield and efficacy by increasing the early-memory phenotype. Second, employing the qualities of IL-21 led us to develop a 4th-generation CAR construct with inducible IL-21 production. It was further used to investigate the IL-21 effects on enhancing CAR T-cell functions. The third aim was to implement a transfection method based on electroporating CAR vector in piggyBac transposon vector as linear DNA and piggyBac transposase as mRNA to further simplify the manufacturing procedure regarding regulatory requirements. Fourth, the access to samples of tias-cel-treated patients and their CAR T-cell products allowed us to study whether there is a link between the parameters of the apheresis or product and the outcome of the treatment, together with observing the CAR T-cell expansion kinetics *in vivo*.

5.1. Improving cytokine composition in CAR T-cell manufacturing protocol

Chapter 1.4.5 reviewed the most common cytokines used for CAR T-cell cultivation. All of the effort is aimed at a better CAR T-cell product in terms of proliferation, efficacy, and persistence, all of which can be influenced by the T-cell differentiation phenotype. It has been shown that early memory CAR T cells correlated with better treatment outcomes due to their enhanced persistence and proliferative capacity (Gattinoni et al., 2005; Klebanoff et al., 2005; Xu et al., 2014). Besides choosing the costimulatory domain in a CAR construct, the cytokines used in the cell culture significantly impact these parameters. First, the cytokines used in CAR T-cell cultivation contributing to this early differentiated phenotype will be discussed.

Our protocol for CAR T-cell cultivation (Ptáčková et al., 2018) was based on the study of Gerdemann et al., who used the cytokine combination IL-4 + IL-7 for multi-virus-specific T cells (Gerdemann et al., 2012). They tested various anti-apoptotic and pro-proliferative cytokines, and they showed that this combination outperformed IL-2 in T-cell growth and IL-2 or IL-15 in IFN- γ production. The choice of IL-4 might seem controversial, as it is a prototypic Th2-polarizing or Th1-suppressive cytokine. Notwithstanding, they reported that the T cells cultivated in the presence of IL-4 + IL-7 produced signature Th1 cytokines – IFN- γ, GM-CSF, IL-2, and TNFα. Moreover, the T cells did not produce a significant amount of Th2-specific cytokines IL-5 and IL-13. Furthermore, the combination of IL-4 + IL-7 did not stimulate the growth of Tregs (Gerdemann et al., 2012).

In our study, we first replicated this protocol on our model anti-CD19 CAR (CAR19) T cells (Ptáčková et al., 2018). We confirmed the superior effect of IL-4 + IL-7 on CAR T-cell expansion to IL-2. In addition, we tested activating the cells with anti-CD3/CD28 mAbs or leaving cells unstimulated. We observed that without stimulation, CAR19 cells expanded up to 90% of the CAR$^+$ population. This was due to the recognition of endogenous B cells in the electroporated PBMCs, which we verified by B-cell depletion via cell sorting. This method elegantly and efficiently solves specific CAR T-cell activation via autologous feeder cells. Nonetheless, the limitations of this method represent pretreated patients with B-cell aplasia and that this works only against targets available on PBMCs. Irradiated autologous or donor-derived PBMCs or aAPCs could address this; however, it adds more complexity to the manufacturing process (chapter 1.4.3.). Another universally applicable solution for any CAR construct is the stimulation of the transduced cells via CAR-specific mAbs. These could either be anti-idiotypic or tag-specific. In our case, we engineered the Myc tag into a PSMA-specific CAR. Activation with anti-Myc mAb led to a selective CAR T cell expansion compared to polyclonal stimulation, where CAR T cells were a minority (Ptáčková et al., 2018).

Then, in our protocol, we have further added IL-21 to this mixture of IL-4 and IL-7 (Ptáčková et al., 2018). Our data showed that CAR T cells cultivated in the cytokine cocktail of IL-4 + IL-7 + IL-21 efficiently expanded, had antigen-specific cytotoxicity, and produced IFN-γ and TNFα upon activation. According to expectations, adding IL-21 resulted in an elevated percentage of T_{SCM}-like and T_{CM} cells. In addition, these cells were double positive on costimulatory markers CD27 and CD28, corresponding to the early differentiated phenotype. Moreover, the expression of inhibitory receptors PD-1, TIM-3, and LAG-3 was lower than in those cultivated in IL-2. Another difference was the preferential growth of CD4+ CAR T cells and cultivation in IL-2-supported CD8$^+$ CAR T cells. Nonetheless, there was no difference in cytotoxicity against Ramos cells. We further showed that even CD4$^+$ CAR T cells degranulate, although to a lower extent than CD8$^+$ (Ptáčková et al., 2018).

The classical method of manufacturing CAR T cells uses polyclonal anti-CD3/CD28 activation, transduction via RV/LV vectors, and IL-2 supplementation (Duong et al., 2015). IL-2 is a well-known T-cell growth factor. Nevertheless, the IL-2-mediated expansion is physiologically coupled with effector differentiation. The effector T-cells are highly cytotoxic but have a limited lifespan (Liao et al., 2013). However, only high IL-2 concentrations drove T-cells into the effector phenotype (Kaartinen et al., 2017). Additionally, IL-2 can be responsible for the induction of AICD. The mechanism of this coupling lies in the IL-2 signaling (Crompton et al., 2014). The IL-2 receptor is comprised of the common γ-chain receptor (γ$_c$), which is shared with the γc family cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21), and the IL-2R subunit (CD122, IL-2Rβ) as the low-affinity heterodimer or plus CD25 (IL-2Rα), the high-affinity non-signaling chain as a heterotrimeric receptor on activated T and B cells. The signal is transduced by JAK3 associated with the γ$_c$ and JAK1 associated with the IL-2Rβ chain. This activates the JAK/STAT, PI3K/Akt, and MAPK pathways (Boyman & Sprent, 2012). The effector differentiation

part is driven by the JAK/STAT and PI3K/Akt ones. These pathways lead to the activation of corresponding downstream transcription factors, STAT5 and FOX01, respectively. Phosphorylated STAT5 dimerizes and directly stimulates the expression of effector molecules, while FOX01 stimulates and inhibits other transcription factors. The proliferation signal is mediated by the RAS/RAF/MAPK pathway, ending with activation of the cMYC transcription factor. cMYC directly stimulates the expression of cyclins and represses the expression of anti-apoptotic molecules Bcl-2 and cyclin-dependent kinase inhibitor p21 (Crompton et al., 2014).

IL-4 is a pleiotropic cytokine particularly known for differentiating Th2 T cells, supporting B-cell expansion and isotype switch, activating mast cells, and supporting M2 macrophage differentiation. IL-4 is produced by T-cells, eosinophils, NKT cells, basophils, and M2 macrophages (Keegan et al., 2021). IL-7 is an essential cytokine for the development of the immune system by stimulating the lymphoid progenitors and lymphoid tissue. It is produced mainly by non-hematopoietic cells such as bone marrow stroma cells, thymus epithelial cells, liver and gut cells. IL-7 promotes the homeostasis of T, B, and NK cells. In contrast to IL-2, IL-7 supports T-cell survival without inducing AICD (D. Chen et al., 2021). The IL-4 and IL-7 receptors contain the γc and their corresponding subunits, IL-4Rα or IL-7Rα. The signaling pathways activated by IL-4 and IL-7 are PI3K/Akt, MAPK, and IL-4 activates STAT-6 and IL-7 STAT-5 (J. X. Lin & Leonard, 2018). Therefore, both IL-4 and IL-7 strongly support the proliferation and survival of T cells. This includes the elevation of anti-apoptotic proteins of the Bcl-2 family, such as Bcl-2, Bcl-XL, and Mcl-1, together with the downregulation of pro-apoptotic proteins BAX and BAD (Aronica et al., 2000; D. Chen et al., 2021; Keegan et al., 2021; J. X. Lin & Leonard, 2018; Vella et al., 1997).

IL-21 is produced mainly by T cells and NKT cells. IL-21 receptor consists of the γc and IL-21Rα, which signals via PI3K/Akt, MAPK, and STAT3 (J. X. Lin & Leonard, 2018). STAT3 is crucial to maintaining memory T cells (Siegel et al., 2011). IL-21 has pleiotropic functions on T and B cells, monocytes, DCs, and macrophages. In T cells, IL-21 stimulates cytotoxicity, proliferation, anti-tumor activity, Th17 and follicular helper T-cell (T_{FH}) differentiation, and importantly, prevents the terminal differentiation of T cells (Hinrichs et al., 2008; Leonard et al., 2016; Moroz et al., 2004; Santegoets et al., 2013; Singh et al., 2011). Regarding differentiation programs, IL-21 acts as an IL-2 antagonist (Hinrichs et al., 2008). This also corresponds to T-cell metabolism; IL-21 supports oxidative phosphorylation and mitochondrial biogenesis, while IL-2 stimulates glycolysis (Loschinski et al., 2018). Another important note is that IL-2 supports Treg expansion, while IL-21 inhibits it (Attridge et al., 2012; Peluso et al., 2007).

It has been described that IL-21 is synergistic with IL-7, IL-15, and IL-18 (Du et al., 2021; Lamers et al., 2014; S. Liu et al., 2007; Pouw et al., 2010; Strengell et al., 2003; Zeng et al., 2005). Li et al. have shown that IL-21 and IL-7 together further enhanced the expansion of T cells, production of Th1

and inflammatory cytokines, cytotoxic and anti-tumor functions of CD8^{+} T cells, and increased the expression of IL-7Rα (Du et al., 2021; S. Liu et al., 2007; Zeng et al., 2005). The combination of IL-21 and IL-15 supported the proliferation of CD8^{+} T cells, early-memory phenotype maintenance, and effector functions such as cytotoxicity, IFN-γ production, and anti-tumor effect (Du et al., 2021; Lamers et al., 2014; Pouw et al., 2010; Zeng et al., 2005). IL-21 and IL-18 enhanced IFN production, albeit to a lower extent than with IL-15 (Strengell et al., 2003). Finally, IL-21 and IL-2 do not have a synergistic effect, as they act antagonistically (Du et al., 2021; Hinrichs et al., 2008; Singh et al., 2011; Zeng et al., 2005).

Other approaches to achieve a high percentage of memory phenotype CAR T cells can vary. For example, low IL-2 concentrations and shorter cultivation stimulated the retention of the T_{SCM}-like cells, while high concentrations caused the opposite differentiation (Kaartinen et al., 2017). Next, Sabatino et al. developed a protocol for generating CAR T cells with T_{SCM} phenotype based on cytokines IL-7 + IL-21 with glycogen synthase-3β (GSK-3β) inhibitor TWS119 (Sabatino et al., 2016). The inhibition of GSK-3β activates the WNT/β-catenin pathway, which is crucial for maintaining stemness. It was also shown to prevent T-cell differentiation from memory into the effector phenotype (Gattinoni et al., 2009). These T_{SCM} anti-CD19 CAR T cells exhibited enhanced anti-tumor effects in xenograft ALL models to standard CAR T cells cultivated in IL-2 with T_{EM} (Sabatino et al., 2016). Another way of successfully increasing the T_{SCM} percentage in CAR T-cell products was by PI3K inhibitors (Funk et al., 2022; Perkins et al., 2015). The underlying reasoning was that the T cells were shown to divide asymmetrically, and the one with more PI3K/mTOR signaling differentiated into effectors and vice versa (W. H. W. Lin et al., 2015; Nish et al., 2017).

Currently, IL-7 + IL-15 is one of the most used combinations of cytokines for CAR T-cell cultivation. This combination strongly supports T-cell expansion, survival, and retention of the $T_{SCM/CM}$ phenotype (Cieri et al., 2013; Gargett & Brown, 2015; Kaneko et al., 2009; Xu et al., 2014). The IL-15 receptor comprises the low-affinity heterodimeric IL-2R (γc and IL-2Rβ) and IL-15Rα subunit. The high-affinity non-signaling IL-15Rα chain is expressed mainly on monocytes and DCs, which *trans*-presents IL-15 to IL-15R-bearing cells. Its signaling is mediated via PI3K/Akt, MAPK, and STAT5. IL-15 serves for NK-cell development, maintenance, and proliferation; in T cells, it stimulates proliferation and CD8^{+} memory development and opposes Th17 differentiation (J. X. Lin & Leonard, 2018). The difference in signaling between IL-2 and IL-15 is that IL-2 binds the preformed high-affinity receptors, and when binding IL-2, there is closer interaction between the subunits. Also, IL-15 signaling is mainly mediated in the context of *trans*-presenting by APCs (Waldmann, 2006). IL-15 signaling triggers the production of anti-apoptotic proteins of the Bcl-2 family (Shenoy et al., 2014). In a study by Alizadeh et al., IL-15 alone preserved the T_{SCM} phenotype in CAR T cells by reducing mTORC1 activity, similar to the PI3K inhibitors. Interestingly, the addition of IL-7 or IL-21 reduced this effect (Alizadeh et al., 2019).

Strategies to improve the memory phenotype composition of CAR T-cell products are primarily based on the cytokine composition during the cultivation. Another approach is manufacturing the CAR T cells directly from the preselected memory T-cell subsets (Meyran et al., 2021).

5.2. Secretory IL-21 for augmenting CAR T-cell functions

The previous chapter discussed the cytokine composition used in CAR T-cell manufacturing. Ideally, the result would be invincible CAR T cells that eliminate all tumor cells without causing severe adverse reactions, which would persist *in vivo* to prevent potential relapses. However, after the CAR T-cell administration, *in vivo*, there can be a lack of stimulatory cytokines and costimulatory signals, and the presence of inhibitory ones may result in CAR T-cell dysfunction and therapy failure. The 4th-generation CAR-T cells are engineered to tackle these problems, as described in the introductory chapter 1.3.4. Here, the focus is on IL-21-armed CAR T cells and their potential against a CAR-resistant malignancy like chronic lymphocytic leukemia (CLL).

Based on our previous research (Ptáčková et al., 2018), we have further investigated the effect of IL-21 on the model CAR19 T cells during co-culture with their target cells. Subsequently, we constructed an inducibly IL-21-producing CAR (i.e., TRUCK) and tested it in various contexts (Štach et al., 2020). As described before, IL-21 promotes T-cell proliferation and the retention of early memory phenotype. Initially, we showed that our cultivation method using IL-4 + IL-7 + IL-21 resulted in the majority of $CD62L^+CD27^+CD28^+CD95^-$ population, termed T_{SCM}-like, contrary to using IL-2, where these were only in the minority (i.e., "effector-like"). By using a simple *in vitro* co-culture model with Burkitt lymphoma cell line Ramos, we compared CAR T cells manufactured this way, either without adding exogenous cytokines or with adding either IL-2 or IL-21. CAR T cells grown in IL-4 + IL-7 + IL-21 survived and expanded more, even without exogenous cytokines. However, the addition of IL-21 caused much higher CAR T-cell expansion and retainment of the T_{SCM}-like phenotype and much lower expression of PD-1 in $CD8^+$ CAR T cells than the addition of IL-2 to cells initially expanded with IL-2. Next, we looked at the phenotype of CAR T cells cultivated in IL-4 + IL-7 + IL-21 or IL-2 (i.e., were T_{SCM}-like or effector-like) after co-culture with Ramos cells and with either IL-2 or IL-21 added. The addition of IL-2 caused differentiation into effector phenotype in both groups, while IL-21 caused upregulation of the memory markers in both groups. Therefore, IL-2 differentiated T_{SCM}-like cells into effectors, and IL-21 reinvigorated effectors into the early memory phenotype. We have thus confirmed the opposing effect of these cytokines. These experiments were repeated with solid tumor antigen (PSMA) CAR T cells with similar results. We have also shown that IL-21 inhibited apoptosis of CAR T cells during a multiple-day co-culture.

This data persuaded us to construct CAR19 with IL-21 under an inducible NFAT promotor that activates expression after T-cell activation. These CAR19 NFAT-IL21 T cells could benefit from IL-21 after recognizing their target. First, we successfully tested the inducible IL-21 expression. Second,

the functional test in repeated antigenic re-stimulation with Ramos cells proved enhanced proliferation and retainment of the T_{SCM}-like phenotype in CAR19 NFAT-IL21 T cells. Furthermore, even after three rounds of re-stimulation, these CAR19 NFAT-IL21 T cells were still found functional by degranulation assay. Furthermore, in a Ramos cell-induced tumor mouse model, CAR19 NFAT-IL21 T cells infiltrated tumors and reduced the tumor weight more than CAR19-control cells.

Finally, we tested these IL-21-armed CAR T cells on an *in vitro* model of a CAR T-cell-resistant malignancy, CLL. The average complete response (CR) rate of CAR19 treatment of CLL is only about 30% (Todorovic et al., 2022). It has been described that the CLL cells cause T-cell dysfunction via extracellular vesicles (Cox et al., 2021). Analysis of T cells from CLL patients revealed that they have impaired metabolism in terms of reduced glucose uptake and mitochondrial fitness (Van Bruggen et al., 2019). In addition, they have hampered synapse formation (Ramsay et al., 2012). Moreover, the chronic antigenic stimulation results in an exhausted phenotype of T cells accompanied by high expression of inhibitory receptors (Vlachonikola et al., 2021). Thus, we have hypothesized that autologous secretion of IL-21 could overcome this inhibition. We developed a physiological *in vitro* model of CLL by cultivating the primary CLL cells within the bone marrow fragments they infiltrated. In this model, we co-cultured CLL cells with GFP-labeled regular CAR19 and CAR19 NFAT-IL21 T cells via plasmid co-electroporation. This way, we could observe the CAR T-cell infiltration into the bone marrow fragments, which IL-21 enhanced. After 10 days, at the end of the co-culture, we measured the percentage of GFP$^+$ cells and their immunophenotype. The CAR19 NFAT-IL21 T cells expanded significantly more than regular CAR19, and IL-21 helped maintain the T_{SCM}-like phenotype. Then, we tested the functionality of these CAR T cells after the co-culture with CLL cells by degranulation assay, and interestingly, CAR19 cells did not degranulate. In contrast, the CAR19 NFAT-IL21 T cells degranulated and killed Ramos cells. Moreover, the CAR19 cells did not produce IFN-γ, while in CAR19 NFAT-IL21 T cells, the IFN-γ production remained unaffected. Interestingly, the CAR19 T cells did not express inhibitory receptors PD-1, TIM-3, or LAG-3, or the senescent marker CD57, which suggests a dysfunction in metabolism or in synapse formation. Overall, we demonstrated that the production of IL-21 by CAR T cells helped overcome the inhibitive mechanisms of CLL *in vitro*.

Other groups have published similar effects of IL-21 on T cells. First, Markley and Sadelain developed CD19-specific CAR T cells constitutively expressing either IL-2, IL-7, IL-15, or IL-21 and tested them on eradicating systemic B-cell tumors in mice (Markley & Sadelain, 2010). Expression of each cytokine increased CAR efficacy but on a different level. CAR T cells producing IL-2 and IL-15 exerted the highest effector functions *in vitro*, although IL-7 and IL-21 outclassed them *in vivo* by providing the longest tumor-free survival. By repeated antigenic stimulation *in vitro*, the most expanding CAR T cells were those producing IL-7, albeit they did not sustain this persistence *in vivo*. CAR T cells secreting IL-15 or IL-21 could be detected in the spleen of tumor-free mice even up to 100+ days post-

infusion (Markley & Sadelain, 2010). Batra et al. studied the effect of secretory IL-15, IL-21, or both on CAR T cells in a solid tumor HCC model (Batra et al., 2020). They reported that *in vivo*, the combination of IL-15 + IL-21 exhibited the highest efficacy in anti-tumor effect, expansion, and persistence compared to either cytokine expressed alone. A recent study on IL-21-secreting CAR-iNKT cells reported that IL-21 promoted the memory phenotype of the CAR-iNKT cells, their persistence in solid tumor-bearing mice model, and overall anti-tumor activity (Y. Liu et al., 2024). In another recent study, the authors first evaluated the exogenous addition of IL-21 on TCR T cells *in vitro* and *in vivo* for the treatment of HCC (Zhu et al., 2024). As expected, IL-21 increased T-cell expansion, the percentage of memory phenotype, and downregulated PD-1. Further, they engineered a novel mutant constitutively active IL-21 receptor, which promotes STAT3 signaling without binding an exogenous ligand. This receptor is a homodimer of two IL-21Rα chains connected with a disulfide bond in the transmembrane domain. To allow detection, the extracellular domains were swapped for those of CD34. TCR T cells with this novel receptor exhibited similar superior functions as with exogenous IL-21 *in vitro* after three rounds of co-culture with tumor cells and *in vivo* in a mouse model. Moreover, the engineered IL-21 receptor mitigated TCR T apoptosis and exhaustion and stimulated memory phenotype maintenance after repeated stimulations (Zhu et al., 2024).

Altogether, multiple groups have published very similar results to ours on augmenting the anti-tumor effects and promoting the memory phenotype of T-cell-based therapies by IL-21. With its unique properties on T cells, IL-21 seems severely underappreciated in the current CAR T-cell manufacturing protocols. Thus, this approach seems promising in developing products against resistant diseases and potentially solid tumors.

5.3. Non-viral vector platform to meet regulatory requirements for CAR T-cell production

Chapter 1.4.2 described vectors for genetic alteration of T cells. Currently, the most widely used vectors for manufacturing clinical-grade CAR T cells are based on RV/LV transduction, which is an efficient and reliable technology (Cappell & Kochenderfer, 2023). However, developing novel and experimental constructs with GMP certification and their subsequent approval takes a substantial amount of time (1-3 years) and represents astronomically high expenditures. Moreover, it requires a biosafety level 2 facility (Morgan & Boyerinas, 2016; Poorebrahim et al., 2019). Aside from that, point-of-care CAR T-cell production in local medical facilities could immensely improve the accessibility of this treatment. Therefore, there is a need for an easily-approvable and more economical way of genetically modifying T cells.

Transposon vectors represent only nucleic acids and, thus, do not require special biosafety, and the generation of new constructs is quick and inexpensive. However, they are considered less efficacious to RV/LV vectors due to lower transduction rate and high initial cell mortality

due to electroporation. Additionally, there is a risk of genotoxicity by a more random integration pattern, and the amount of introduced transgene could be high (Balke-Want et al., 2023).

In our previous work (Ptáčková et al., 2018), we have shown that producing CAR T cells with a piggyBac transposon via electroporation of plasmids is feasible. Later, in 2021, the Czech regulatory agency SÚKL approved this protocol for the GMP-certified manufacture of CAR19-T cells and a phase I clinical trial (NCT05054257). In our following study (Kaštánková et al., 2021), we have further updated this system to make it completely plasmid- and bacteria-free, making it easier for the regulatory agency to approve its use in clinics. Our method is based on the electroporation of CAR-encoding linear DNA transposon and mRNA encoding the piggyBac transposase. The transient mRNA expression circumvents even a slight chance of transposase-carrying plasmid integration. The linear transposon DNA was prepared commercially via preparative PCR by LinearX, USA. The mRNA was prepared by *in vitro* transcription using a linearized plasmid as a template.

One of the main requests by regulatory agencies regarding CAR T-cell product quality is a low vector copy number (VCN). Currently, most agencies in the EU require a VCN below 5. Using our regular protocol of 4 µg of CAR plasmid and 2 µg of transposase plasmid, the average VCN was about 23. To reduce the VCN, we titrated the amount of linear DNA per electroporation and assessed the VCN by digital droplet PCR (ddPCR). We aimed to introduce VCN between 1 and 3, which would stay safely below the upper maximal limit of 5 copies per cell. We have found that this "transposon dilution" method works with both linear DNA/mRNA and plasmid/plasmid settings. The optimal concentration was 0.3 µg DNA per electroporation reaction while still obtaining over 60% CAR^{+} T cells at day 21 post-electroporation. We have also shown that the VCN is stable during the *in vitro* cultivation. Next, we compared the functionality, i.e., cytotoxicity and IFN-γ production, and differentiation phenotype of the CAR T cells produced by linear DNA/mRNA and those produced by the classical plasmid/plasmid method. We have found them both functional and with T_{SCM}-like phenotype, corresponding to the cytokines used, without any significant differences. Therefore, we present our protocol as a feasible, easily-approvable alternative to other vectors in CAR T-cell manufacturing.

Bishop et al. shared our goal of removing the bacterial element from piggyBac vector manufacturing (Bishop et al., 2020). As the transposon vector, they enzymatically synthesized linear covalently closed DNA termed "doggybone". After optimizing the flanking regions, this approach allowed the successful transposition of the CAR gene (Bishop et al., 2020). This illustrates that abiotically produced vectors can reduce costs and facilitate approval. Minicircle DNA is another possibility of a transposon vector form containing no bacterial elements. It is created from a plasmid by recombination and works similarly with a transposase such as SB (Prommersberger et al., 2021, 2022).

Currently, there are many clinical trials on CAR T cells produced by transposon vectors of both piggyBac (Costello et al., 2021; Nishio et al., 2021; Slovin et al., 2022; Y. Zhang, Zhang, et al., 2021)

and *Sleeping Beauty* systems (T. Chan et al., 2020; Kebriaei et al., 2016; Magnani et al., 2020; Prommersberger et al., 2021; Sallman et al., 2021; Singh et al., 2022). Therefore, this technology is being accepted as a viable alternative to RV/LV vectors.

In one clinical trial with allogeneic donor-derived anti-CD19 CAR T cells produced by piggyBac (ACTRN12617001579381), the CAR T-cell product developed into a malignant lymphoma in 2 patients. The malignant CAR T cells had a high transgene copy number (>20 copies/cell), although the number was similar in non-malignant CAR T cells and with no known oncogenic insertions. However, changes were found in genomic copy numbers and insertion-unrelated point mutations. Additionally, due to transgenic promotor-driven readthroughs, CAR-adjacent genes were overexpressed. The malignant CAR T cells did not expand *in vitro* after CD19 antigenic stimulation, indicating that the malignant transformation was CAR-signaling independent. The authors suggest it was a multifactorial process with no unveiled mechanism (Micklethwaite et al., 2021). Even as a solitary incident, it raises a precedent in the safety question. Moving forward, there will probably be a call for more stringent quality control of CAR T-cell production, which may include mapping of integration sites.

5.4. Characterization of the CAR T cells of tisa-cel-treated patients

Chapter 1.7 describes commercially approved CAR T-cell products. Tisagenlecleucel (tisa-cel, Kymriah® by Novartis) has been used in the Czech Republic since 2019 as the first commercially available CAR T-cell product. It is an anti-CD19 CAR T-cell product approved for treating r/r DLBCL and B-ALL. The product is autologous and is manufactured from intensively pretreated patients' cells. Chemotherapy, particularly bendamustine, negatively affects the quality of T cells (Iacoboni et al., 2024). For an expensive therapy like CAR T cells, there is a desperate search for traits correlating with the response. Knowing the parameters predicting the treatment outcome could better stratify the patients into those likely to respond to standard CAR T cells and those eligible for alternative treatment, such as next-generation CAR T cells. In our study, we have tried to find a connection between the differentiation phenotype of the (CAR) T cells and the treatment outcome. The early-memory phenotype was shown to be beneficial for successful CAR T-cell treatment (Gattinoni et al., 2005; Klebanoff et al., 2005; Xu et al., 2014). Additionally, we monitored the CAR T-cell expansion kinetics (Sarikonda et al., 2021).

There are two types of CAR T-cell treatment failure. The first is an early failure when the administered CAR T cells do not cause even a partial response (PR), and the disease continues progressing, so-called non-responders (NR). The second is a late relapse, which occurs after an initial response and a period of remission. It is either caused by an escape variant of the tumor or by the extinction of CAR T cells.

Our study followed 32 patients treated with tisa-cel for either B-ALL, DLBCL, or pediatric B-ALL (Štach et al., 2023). We used multiparametric flow cytometry to assess the differentiation phenotype

and the percentage of CAR T cells in a set of patient samples. Following the manufacturing protocol, the first was the starting material, apheresis. Next was the CAR T-cell product obtained by washing out the discarded effusion bag after the product administration. Finally, samples of peripheral blood (or bone marrow) were measured at three time points post-infusion: an early time point (day 2-4), a point of predicted peak CAR T-cell expansion (day 10-14), and a late time point (day 30+). Then, we compared the results of the patients based on their response state at 3 months post-infusion – complete response (CR), PR, and NR (evaluated by PET/CT scan).

The T-cell phenotype analysis of apheretic material revealed an elevated percentage of the $CD8^+CD45RA^+CD27^+$ in the CR group to the NR. This population corresponds to a non-naïve resting memory population with a high proliferative capacity, which obtains effector functions after antigen re-exposure (Fraietta et al., 2018; Lécuroux et al., 2009). This is in agreement with the hypothesis that these memory-like phenotype cells hold greater anti-tumor ability. This population was similarly described in another CAR T-cell study on CLL patients (Fraietta et al., 2018). The T cells of patients were generally lower percent in the T_{SCM}-like compartment than those of healthy donors, corresponding to the effect of disease and chemotherapies. The CAR T-cell product analysis first showed a variable percentage of CAR^+ cells (10-40%) and mostly $CD4^+$ CAR T cells. Phenotypically, most represented T_{CM} and T_{EM} cells, with only a few T_{SCM} cells and mostly CD27 negative cells. This more effector-like phenotype could be attributed to IL-2 during cultivation. However, no major significant differences were found between the CR and NR groups.

Next, the *in vivo* CAR T-cell expansion kinetics showed the peak expansions at the second time point. The most evident difference between the CR and NR groups was the fold expansion between the first two time points. In the peripheral blood of responders, the CAR T cells expanded up to 150-fold, while there was little to no observable expansion in NRs. This might be a useful tool for clinicians to predict treatment outcomes. The differences in immunophenotype were found at the second time point; the CR group had more T_{CM} and PD1–TIGIT– CAR T cells in peripheral blood. Again, this supports the early-memory phenotype hypothesis. We received a bone marrow sample in several cases. Compared to the corresponding peripheral blood samples, there was a much higher percentage of CAR T cells in the bone marrow in a couple of cases. This might explain some responders' very low observable peripheral blood expansion when most CAR T-cell expansion occurs at the tumor site. Unfortunately, obtaining such samples, apart from some exceptions, is not possible. The immunophenotype comparison showed only minor differences.

Another highly important parameter affecting the response is the disease's attributes. The NR group had mostly primary-refractory aggressive B-cell lymphomas and large tumor burdens.

To summarize, we did not observe a correlation between the product's immunophenotype and treatment outcome. Our data suggested that responders to the CAR T-cell therapy had a higher percentage

of the early-memory T cells in the apheresis. The measurable expansion of CAR T cells in peripheral blood correlated with the response rate and could be an important prognostic factor.

Many other groups have researched the factors contributing to the CAR T-cell response rate, which includes the parameters of expansion, effector functions, persistence, and length of the anti-tumor effect. In a preclinical *in vitro* and *in vivo* setting, experiments with anti-CD19 CAR T cells made from sorted $CD4^+$ or $CD8^+$ memory subsets. They revealed that the cytokine-producing ability, expansion, and anti-tumor ability were highest in the T_N then T_{CM}-derived CAR T cells and lowest in the T_{EM}-derived ones, both for $CD4^+$ and $CD8^+$ cells. Moreover, the $CD4^+$ and $CD8^+$ CAR T cells showed synergistic activity (Sommermeyer et al., 2015). Fraietta et al. unveiled some of the response determinants for CAR T-cell therapy of CLL. The memory-like phenotype of T cells in the starting material and the CAR T-cell product correlated with a favorable therapeutic response. And vice versa, non-responding patients' CAR T cells upregulated genes linked with effector phenotype, exhaustion, glycolytic metabolism, and pro-apoptotic pathways (Fraietta et al., 2018). A study by Finney et al., similar to ours, reported attenuated CAR T-cell expansion and effector functions in the initial non-responding patients. In contrast, the CAR T-cell products of all patients were phenotypically and functionally comparable. Additionally, they reported an increased percentage of the $LAG\text{-}3^+TNF\alpha^{lo}$ population in the aphereses of non-responders and increased expression of exhaustion markers after CAR T-cell administration (O. C. Finney et al., 2019). In a solid tumor setting, Klaver et al. described that the patients that had more $CD8^+$ T_N cells in the apheresis and $CD8^+$ central effectors ($CD45RA^+CD45RO^+CD27/CD28^-$) in the product exhibited higher fold of *in vivo* CAR T-cell expansion (Klaver et al., 2016). In a clinical trial with CAR T cells against glioblastoma, a link was found between the percentage of $CD4^+$ and T_{CM} cells in the product and the prolonged duration of CAR T-cell persistence (Louis et al., 2011). Clinical trials with TILs likewise connected responsiveness and either early-memory parameters like longer telomere length and $CD8^+CD27^+$ percentage (Rosenberg et al., 2011) or $CD8^+$ stem-like $CD39^-CD69^-$ phenotype in the infused cells (Krishna et al., 2020). Detailed analysis done by Wang et al. showed that a higher population of $CD8^+$ T_{SCM}-like cells in the premanufacture material was key to establishing a durable anti-tumor response in DLBCL patients treated by anti-CD19 CAR T cells (Y. Wang et al., 2023). They also suggested that the variability of clinical outcomes reflected the heterogeneity of the starting material, which was similar to what we observed.

On a side note, as of 2024, tisa-cel has been mostly outclassed by axi-cel due to better treatment outcomes despite higher associated toxicities (Bachy et al., 2022; Gagelmann et al., 2024).

All of the mentioned studies, including ours, underline the importance of the early-memory T-cell phenotype for successful CAR T-cell treatment. Unfortunately, the outlooks of patients not responding to CAR T-cell treatment or relapsing at a later time point are dismal. At this point, the only available

therapy is purely experimental (Negishi et al., 2023). In the case of a target-antigen-negative relapse, there is the possibility of administering CAR T cells specific to another antigen (e.g., CD20 or CD22 in a CD19-negative relapse). However, this would require a pre-prepared second product, as the manufacturing process is lengthy and the patients face a time constraint. The second administration of the same CAR T-cell product is often not particularly effective (Gauthier et al., 2021), as we have seen in the case of four patients, where it prolonged the remission of one patient by a year (Štach et al., 2023). Other salvage therapies include lenalidomide, bispecific antibodies, checkpoint inhibitors, molecular target drugs, chemotherapy, immunochemotherapy, or radiotherapy, all with discouraging results (Negishi et al., 2023). Another option could be manufacturing a 4th- or next-generation CAR T from the same apheresis. Additionally, one of the possible solutions to promote CAR T-cell efficacy by preventing T-cell deterioration by chemotherapy would be acquiring the apheresis at the time of diagnosis before starting traditional treatment. Nonetheless, even a CAR T-cell treatment leading only to a temporary remission can be successfully used as a bridging therapy before a curative HSCT (X. Y. Cao et al., 2023; Qiu et al., 2023).

6. CONCLUSION

This thesis is based on four publications that reflect the CAR T-cell manufacturing pipeline, from vector design to optimizing the cytokine composition during cultivation and testing a 4th-generation construct or *in vivo* CAR T-cell monitoring in real-world usage. While the topic mainly focuses on applied research, it delved into the basic biology of cytokine functions and T-cell memory subset characteristics.

First, the protocol of CAR T-cell manufacturing based on electroporating piggyBac vector and cultivating the cells in the presence of cytokines IL-4 + IL-7 + IL-21 showed the potential of a non-viral vector and the feasible production of adequate numbers of CAR T cells with superior early-memory T_{SCM}-like phenotype.

Second, the positive effect of IL-21 on CAR T-cell differentiation, expansion, and effector functions was thoroughly tested. CAR T cells engineered to produce IL-21 inducibly were shown to be functionally superior to regular ones and could present a solution to treating CAR-resistant diseases like B-CLL.

Third, the piggyBac transposon vector for CAR T-cell production was tested as enzymatically produced linear DNA and mRNA. The CAR T cells were functionally comparable to those produced using plasmids. Moreover, the vector copy number per cell was optimized by lowering the amount of DNA used. This bacteria- and plasmid-free method makes regulatory agency approval easier.

Fourth, the samples of pre-manufacture material, the CAR T-cell product tisagenlecleucel, and patients' samples after CAR T-cell administration were characterized by multiparameter flow cytometry. The absence of significant CAR T-cell expansion characterized the non-responding patients. Moreover, the non-responders had a lower percentage of early-memory phenotype in their apheresis.

In conclusion, this thesis presents the data on which a CAR T-cell manufacturing protocol of an ongoing phase I clinical trial with CD19-specific CAR T cells (NCT05054257) is based. This shows the great importance of this research, which is affecting patients' lives today and in the future.

7. REFERENCES

Adachi, K., Kano, Y., Nagai, T., Okuyama, N., Sakoda, Y., & Tamada, K. (2018). IL-7 and CCL19 expression in CAR-T cells improves immune cell infiltration and CAR-T cell survival in the tumor. *Nature Biotechnology 2018 36:4*, *36*(4), 346–351. https://doi.org/10.1038/nbt.4086

Agarwal, S., Weidner, T., Thalheimer, F. B., & Buchholz, C. J. (2019). In vivo generated human CAR T cells eradicate tumor cells. *Oncoimmunology*, *8*(12). https://doi.org/10.1080/2162402X.2019.1671761

Agliardi, G., Liuzzi, A. R., Hotblack, A., De Feo, D., Núñez, N., Stowe, C. L., Friebel, E., Nannini, F., Rindlisbacher, L., Roberts, T. A., Ramasawmy, R., Williams, I. P., Siow, B. M., Lythgoe, M. F., Kalber, T. L., Quezada, S. A., Pule, M. A., Tugues, S., Straathof, K., & Becher, B. (2021). Intratumoral IL-12 delivery empowers CAR-T cell immunotherapy in a pre-clinical model of glioblastoma. *Nature Communications 2021 12:1*, *12*(1), 1–11. https://doi.org/10.1038/s41467-020-20599-x

Alabanza, L. M., Xiong, Y., Vu, B., Webster, B., Wu, D., Hu, P., Zhu, Z., Dropulic, B., & Dash, P. (2022). Armored BCMA CAR T Cells Eliminate Multiple Myeloma and Are Resistant to the Suppressive Effects of TGF-β. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/FIMMU.2022.832645/FULL

Alishah, K., Birtel, M., Masoumi, E., Jafarzadeh, L., Mirzaee, H. R., Hadjati, J., Voss, R. H., Diken, M., & Asad, S. (2021). CRISPR/Cas9-mediated TGFβRII disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells in vitro. *Journal of Translational Medicine*, *19*(1), 482. https://doi.org/10.1186/S12967-021-03146-0

Alizadeh, D., Wong, R. A., Yang, X., Wang, D., Pecoraro, J. R., Kuo, C. F., Aguilar, B., Qi, Y., Ann, D. K., Starr, R., Urak, R., Wang, X., Forman, S. J., & Brown, C. E. (2019). IL15 Enhances CAR-T Cell Antitumor Activity by Reducing mTORC1 Activity and Preserving Their Stem Cell Memory Phenotype. *Cancer Immunology Research*, *7*(5), 759–772. https://doi.org/10.1158/2326-6066.CIR-18-0466

Allen, M. E., Zhou, W., Thangaraj, J., Kyriakakis, P., Wu, Y., Huang, Z., Ho, P., Pan, Y., Limsakul, P., Xu, X., & Wang, Y. (2019). An AND-Gated Drug and Photoactivatable Cre- loxP System for Spatiotemporal Control in Cell-Based Therapeutics. *ACS Synthetic Biology*, *8*(10), 2359–2371. https://doi.org/10.1021/ACSSYNBIO.9B00175/SUPPL_FILE/SB9B00175_SI_002.XLSX

Almeida, A. M., Queiroz, J. A., Sousa, F., & Sousa, Â. (2020). Minicircle DNA: The Future for DNA-Based Vectors? *Trends in Biotechnology*, *38*(10), 1047–1051. https://doi.org/10.1016/J.TIBTECH.2020.04.008

Amirache, F., Lévy, C., Costa, C., Mangeot, P. E., Torbett, B. E., Wang, C. X., Nègre, D., Cosset, F. L., & Verhoeyen, E. (2014). Mystery solved: VSV-G-LVs do not allow efficient gene transfer into unstimulated T cells, B cells, and HSCs because they lack the LDL receptor. *Blood*, *123*(9), 1422–1424. https://doi.org/10.1182/BLOOD-2013-11-540641

Anderson, L. D. (2022). Idecabtagene vicleucel (ide-cel) CAR T-cell therapy for relapsed and refractory multiple myeloma. *Future Oncology*, *18*(3), 277–289. https://doi.org/10.2217/FON-2021-1090/ASSET/IMAGES/LARGE/FIGURE1.JPEG

Ankri, C., Shamalov, K., Horovitz-Fried, M., Mauer, S., & Cohen, C. J. (2013). Human T Cells Engineered To Express a Programmed Death 1/28 Costimulatory Retargeting Molecule Display Enhanced Antitumor Activity. *The Journal of Immunology*, *191*(8), 4121–4129. https://doi.org/10.4049/JIMMUNOL.1203085

Aoyama, S., Da, S. Y., Li, H., Watanabe, D., Umezawa, Y., Okada, K., Nogami, A., Miura, O., & Kawamata, N. (2022). A novel chimeric antigen receptor (CAR) system using an exogenous protease, in which activation of T cells is controlled by expression patterns of cell-surface

proteins on target cells. *International Journal of Molecular Medicine*, *49*(4). https://doi.org/10.3892/IJMM.2022.5097

Arensburger, P., Hice, R. H., Zhou, L., Smith, R. C., Tom, A. C., Wright, J. A., Knapp, J., O'Brochta, D. A., Craig, N. L., & Atkinson, P. W. (2011). Phylogenetic and functional characterization of the hAT transposon superfamily. *Genetics*, *188*(1), 45–57. https://doi.org/10.1534/GENETICS.111.126813

Argyros, O., Wong, S. P., & Harbottle, R. P. (2011). Non-viral episomal modification of cells using S/MAR elements. *Expert Opinion on Biological Therapy*, *11*(9), 1177–1191. https://doi.org/10.1517/14712598.2011.582035

Arndt, C., Fasslrinner, F., Loureiro, L. R., Koristka, S., Feldmann, A., & Bachmann, M. (2020). Adaptor CAR Platforms—Next Generation of T Cell-Based Cancer Immunotherapy. *Cancers 2020, Vol. 12, Page 1302*, *12*(5), 1302. https://doi.org/10.3390/CANCERS12051302

Aronica, M. A., Goenka, S., & Boothby, M. (2000). IL-4-dependent induction of BCL-2 and BCL-X(L)IN activated T lymphocytes through a STAT6- and pi 3-kinase-independent pathway. *Cytokine*, *12*(6), 578–587. https://doi.org/10.1006/CYTO.1999.0603

Attridge, K., Wang, C. J., Wardzinski, L., Kenefeck, R., Chamberlain, J. L., Manzotti, C., Kopf, M., & Walker, L. S. K. (2012). IL-21 inhibits T cell IL-2 production and impairs Treg homeostasis. *Blood*, *119*(20), 4656–4664. https://doi.org/10.1182/BLOOD-2011-10-388546

Bachmann, M. (2019). The UniCAR system: A modular CAR T cell approach to improve the safety of CAR T cells. *Immunology Letters*, *211*, 13–22. https://doi.org/10.1016/J.IMLET.2019.05.003

Bachy, E., Le Gouill, S., Di Blasi, R., Sesques, P., Manson, G., Cartron, G., Beauvais, D., Roulin, L., Gros, F. X., Rubio, M. T., Bories, P., Bay, J. O., Llorente, C. C., Choquet, S., Casasnovas, R. O., Mohty, M., Guidez, S., Joris, M., Loschi, M., … Morschhauser, F. (2022). A real-world comparison of tisagenlecleucel and axicabtagene ciloleucel CAR T cells in relapsed or refractory diffuse large B cell lymphoma. *Nature Medicine*, *28*(10), 2145–2154. https://doi.org/10.1038/S41591-022-01969-Y

Balakrishnan, A., Rajan, A., Salter, A. I., Kosasih, P. L., Wu, Q., Voutsinas, J., Jensen, M. C., Pluckthun, A., & Riddell, S. R. (2019). Multispecific targeting with synthetic ankyrin repeat motif chimeric antigen receptors. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *25*(24), 7506. https://doi.org/10.1158/1078-0432.CCR-19-1479

Balke-Want, H., Keerthi, V., Cadinanos-Garai, A., Fowler, C., Gkitsas, N., Brown, A. K., Tunuguntla, R., Abou-el-Enein, M., & Feldman, S. A. (2023). Non-viral chimeric antigen receptor (CAR) T cells going viral. *Immuno-Oncology and Technology*, *18*. https://doi.org/10.1016/J.IOTECH.2023.100375

Batra, S. A., Rathi, P., Guo, L., Courtney, A. N., Fleurence, J., Balzeau, J., Shaik, R. S., Nguyen, T. P., Wu, M. F., Bulsara, S., Mamonkin, M., Metelitsa, L. S., & Heczey, A. (2020). Glypican-3-specific CAR T cells coexpressing IL15 and IL21 have superior expansion and antitumor activity against hepatocellular carcinoma. *Cancer Immunology Research*, *8*(3), 309–320. https://doi.org/10.1158/2326-6066.CIR-19-0293/470915/AM/GLYPICAN-3-SPECIFIC-CAR-T-CELLS-CO-EXPRESSING-IL15

Baus, J., Liu, L., Heggestad, A. D., Sanz, S., & Fletcher, B. S. (2005). Hyperactive transposase mutants of the Sleeping Beauty transposon. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *12*(6), 1148–1156. https://doi.org/10.1016/J.YMTHE.2005.06.484

Belk, J. A., Daniel, B., & Satpathy, A. T. (2022). Epigenetic regulation of T cell exhaustion. *Nature Immunology*, *23*(6), 848. https://doi.org/10.1038/S41590-022-01224-Z

Bell, M., & Gottschalk, S. (2021). Engineered Cytokine Signaling to Improve CAR T Cell Effector Function. *Frontiers in Immunology*, *12*, 684642. https://doi.org/10.3389/FIMMU.2021.684642/BIBTEX

Beltra, J. C., Manne, S., Abdel-Hakeem, M. S., Kurachi, M., Giles, J. R., Chen, Z., Casella, V., Ngiow, S. F., Khan, O., Huang, Y. J., Yan, P., Nzingha, K., Xu, W., Amaravadi, R. K., Xu, X., Karakousis, G. C., Mitchell, T. C., Schuchter, L. M., Huang, A. C., & Wherry, E. J. (2020). Developmental Relationships of Four Exhausted CD8+ T Cell Subsets Reveals Underlying Transcriptional and Epigenetic Landscape Control Mechanisms. *Immunity*, *52*(5), 825. https://doi.org/10.1016/J.IMMUNI.2020.04.014

Benmebarek, M. R., Karches, C. H., Cadilha, B. L., Lesch, S., Endres, S., & Kobold, S. (2019). Killing Mechanisms of Chimeric Antigen Receptor (CAR) T Cells. *International Journal of Molecular Sciences*, *20*(6). https://doi.org/10.3390/IJMS20061283

Berger, C., Flowers, M. E., Warren, E. H., & Riddell, S. R. (2006). Analysis of transgene-specific immune responses that limit the in vivo persistence of adoptively transferred HSV-TK–modified donor T cells after allogeneic hematopoietic cell transplantation. *Blood*, *107*(6), 2294. https://doi.org/10.1182/BLOOD-2005-08-3503

Berger, C., Jensen, M. C., Lansdorp, P. M., Gough, M., Elliott, C., & Riddell, S. R. (2008). Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *The Journal of Clinical Investigation*, *118*(1), 294. https://doi.org/10.1172/JCI32103

Bielamowicz, K., Fousek, K., Byrd, T. T., Samaha, H., Mukherjee, M., Aware, N., Wu, M. F., Orange, J. S., Sumazin, P., Man, T. K., Joseph, S. K., Hegde, M., & Ahmed, N. (2018). Trivalent CAR T cells overcome interpatient antigenic variability in glioblastoma. *Neuro-Oncology*, *20*(4), 506. https://doi.org/10.1093/NEUONC/NOX182

Bire, S., Ley, D., Casteret, S., Mermod, N., Bigot, Y., & Rouleux-Bonnin, F. (2013). Optimization of the piggyBac transposon using mRNA and insulators: toward a more reliable gene delivery system. *PloS One*, *8*(12). https://doi.org/10.1371/JOURNAL.PONE.0082559

Bishop, D. C., Caproni, L., Gowrishankar, K., Legiewicz, M., Karbowniczek, K., Tite, J., Gottlieb, D. J., & Micklethwaite, K. P. (2020). CAR T Cell Generation by piggyBac Transposition from Linear Doggybone DNA Vectors Requires Transposon DNA-Flanking Regions. *Molecular Therapy - Methods and Clinical Development*, *17*, 359–368. https://doi.org/10.1016/j.omtm.2019.12.020

Bonini, C., Bondanza, A., Perna, S. K., Kaneko, S., Traversari, C., Ciceri, F., & Bordignon, C. (2007). The Suicide Gene Therapy Challenge: How to Improve a Successful Gene Therapy Approach. *Molecular Therapy*, *15*(7), 1248–1252. https://doi.org/10.1038/SJ.MT.6300190

Bonini, C., Ferrari, G., Verzeletti, S., Servida, P., Zappone, E., Ruggieri, L., Ponzoni, M., Rossini, S., Mavilio, F., Traversari, C., & Bordignon, C. (1997). HSV-TK Gene Transfer into Donor Lymphocytes for Control of Allogeneic Graft-Versus-Leukemia. *Science*, *276*(5319), 1719–1724. https://doi.org/10.1126/SCIENCE.276.5319.1719

Boomer, J. S., & Green, J. M. (2010). An Enigmatic Tail of CD28 Signaling. *Cold Spring Harbor Perspectives in Biology*, *2*(8). https://doi.org/10.1101/CSHPERSPECT.A002436

Boulch, M., Cazaux, M., Cuffel, A., Guerin, M. V., Garcia, Z., Alonso, R., Lemaître, F., Beer, A., Corre, B., Menger, L., Grandjean, C. L., Morin, F., Thieblemont, C., Caillat-Zucman, S., & Bousso, P. (2023). Tumor-intrinsic sensitivity to the pro-apoptotic effects of IFN-γ is a major determinant of CD4+ CAR T-cell antitumor activity. *Nature Cancer 2023 4:7*, *4*(7), 968–983. https://doi.org/10.1038/s43018-023-00570-7

Boulch, M., Cazaux, M., Loe-Mie, Y., Thibaut, R., Corre, B., Lemaître, F., Grandjean, C. L., Garcia, Z., & Bousso, P. (2021). A cross-talk between CAR T cell subsets and the tumor microenvironment is essential for sustained cytotoxic activity. *Science Immunology*, *6*(57). https://doi.org/10.1126/SCIIMMUNOL.ABD4344/SUPPL_FILE/ABD4344_TABLE_S1.XLSX

Bourbon, E., Ghesquières, H., & Bachy, E. (2021). CAR-T cells, from principle to clinical applications. *Bulletin Du Cancer*, *108*(10), S4–S17. https://doi.org/10.1016/J.BULCAN.2021.02.017

Boyman, O., & Sprent, J. (2012). The role of interleukin-2 during homeostasis and activation of the immune system. *Nature Reviews Immunology 2012 12:3*, *12*(3), 180–190. https://doi.org/10.1038/nri3156

Bozza, M., De Roia, A., Correia, M. P., Berger, A., Tuch, A., Schmidt, A., Zörnig, I., Jäger, D., Schmidt, P., & Harbottle, R. P. (2021). A nonviral, nonintegrating DNA nanovector platform for the safe, rapid, and persistent manufacture of recombinant T cells. *Science Advances*, *7*(16), 1333–1347. https://doi.org/10.1126/SCIADV.ABF1333

Bozza, M., Green, E. W., Espinet, E., De Roia, A., Klein, C., Vogel, V., Offringa, R., Williams, J. A., Sprick, M., & Harbottle, R. P. (2020). Novel Non-integrating DNA Nano-S/MAR Vectors Restore Gene Function in Isogenic Patient-Derived Pancreatic Tumor Models. *Molecular Therapy. Methods & Clinical Development*, *17*, 957. https://doi.org/10.1016/J.OMTM.2020.04.017

Brenchley, J. M., Karandikar, N. J., Betts, M. R., Ambrozak, D. R., Hill, B. J., Crotty, L. E., Casazza, J. P., Kuruppu, J., Migueles, S. A., Connors, M., Roederer, M., Douek, D. C., & Koup, R. A. (2003). Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+T cells. *Blood*, *101*(7), 2711–2720. https://doi.org/10.1182/BLOOD-2002-07-2103

Brentjens, R. J., Davila, M. L., Riviere, I., Park, J., Wang, X., Cowell, L. G., Bartido, S., Stefanski, J., Taylor, C., Olszewska, M., Borquez-Ojeda, O., Qu, J., Wasielewska, T., He, Q., Bernal, Y., Rijo, I. V., Hedvat, C., Kobos, R., Curran, K., … Sadelain, M. (2013). CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Science Translational Medicine*, *5*(177), 177ra38. https://doi.org/10.1126/SCITRANSLMED.3005930

Brown, C. E., Badie, B., Barish, M. E., Weng, L., Ostberg, J. R., Chang, W. C., Naranjo, A., Starr, R., Wagner, J., Wright, C., Zhai, Y., Bading, J. R., Ressler, J. A., Portnow, J., D'Apuzzo, M., Forman, S. J., & Jensen, M. C. (2015). Bioactivity and Safety of IL13Rα2-Redirected Chimeric Antigen Receptor CD8+ T cells in Patients with Recurrent Glioblastoma. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *21*(18), 4062. https://doi.org/10.1158/1078-0432.CCR-15-0428

Bui, K. C., Ho, V. H., Nguyen, H. H., Dang, T. C., Ngo, T. H., Nguyen, T. M. L., Nguyen, L. T., Dang, T. L., Tran, T. T., Le, Q. H., Pham, H. L., Nguyen, V. B., & Can, V. M. (2023). X-ray-irradiated K562 feeder cells for expansion of functional CAR-T cells. *Biochemistry and Biophysics Reports*, *33*, 101399. https://doi.org/10.1016/J.BBREP.2022.101399

Cao, X., Leonard, K., Collins, L. I., Cai, S. F., Mayer, J. C., Payton, J. E., Walter, M. J., Piwnica-Worms, D., Schreiber, R. D., & Ley, T. J. (2009). Interleukin 12 stimulates IFN-γ-mediated inhibition of tumor-induced regulatory T-cell proliferation and enhances tumor clearance. *Cancer Research*, *69*(22), 8700–8709. https://doi.org/10.1158/0008-5472.CAN-09-1145/655408/P/INTERLEUKIN-12-STIMULATES-IFN-MEDIATED-INHIBITION

Cao, X. Y., Zhang, J. P., Zhao, Y. L., Xiong, M., Zhou, J. R., Lu, Y., Sun, R. J., Wei, Z. J., Liu, D. Y., Zhang, X., Yang, J. F., & Lu, P. (2023). Analysis benefits of a second Allo-HSCT after CAR-T cell therapy in patients with relapsed/refractory B-cell acute lymphoblastic leukemia who relapsed after transplant. *Frontiers in Immunology*, *14*, 1191382. https://doi.org/10.3389/FIMMU.2023.1191382/BIBTEX

Cao, Y., Rodgers, D. T., Du, J., Ahmad, I., Hampton, E. N., Ma, J. S. Y., Mazagova, M., Choi, S. H., Yun, H. Y., Xiao, H., Yang, P., Luo, X., Lim, R. K. V., Pugh, H. M., Wang, F., Kazane, S. A., Wright, T. M., Kim, C. H., Schultz, P. G., & Young, T. S. (2016). Design of Switchable Chimeric Antigen Receptor T Cells Targeting Breast Cancer. *Angewandte Chemie (International Ed. in English)*, *55*(26), 7520. https://doi.org/10.1002/ANIE.201601902

Cappell, K. M., & Kochenderfer, J. N. (2023). Long-term outcomes following CAR T cell therapy: what we know so far. *Nature Reviews Clinical Oncology 2023 20:6*, *20*(6), 359–371. https://doi.org/10.1038/s41571-023-00754-1

Car, B. D., Eng, V. M., Lipman, J. M., & Anderson, T. D. (1999). The toxicology of interleukin-12: A review. *Toxicologic Pathology*, *27*(1), 58–63. https://doi.org/10.1177/019262339902700112

Caratelli, S., Arriga, R., Sconocchia, T., Ottaviani, A., Lanzilli, G., Pastore, D., Cenciarelli, C., Venditti, A., Del Principe, M. I., Lauro, D., Landoni, E., Du, H., Savoldo, B., Ferrone, S., Dotti, G., & Sconocchia, G. (2020). In vitro elimination of epidermal growth factor receptor-overexpressing cancer cells by CD32A-chimeric receptor T cells in combination with cetuximab or panitumumab. *International Journal of Cancer*, *146*(1), 236–247. https://doi.org/10.1002/IJC.32663

Cartellieri, M., Feldmann, A., Koristka, S., Arndt, C., Loff, S., Ehninger, A., von Bonin, M., Bejestani, E. P., Ehninger, G., & Bachmann, M. P. (2016). Switching CAR T cells on and off: a novel modular platform for retargeting of T cells to AML blasts. *Blood Cancer Journal*, *6*(8), e458. https://doi.org/10.1038/BCJ.2016.61

Cary, L. C., Goebel, M., Corsaro, B. G., Wang, H. G., Rosen, E., & Fraser, M. J. (1989). Transposon mutagenesis of baculoviruses: analysis of Trichoplusia ni transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology*, *172*(1), 156–169. https://doi.org/10.1016/0042-6822(89)90117-7

Cavazzana-Calvo, M., Hacein-Bey, S., De Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J. L., Bousso, P., Le Deist, F., & Fischer, A. (2000). Gene Therapy of Human Severe Combined Immunodeficiency (SCID)-X1 Disease. *Science*, *288*(5466), 669–672. https://doi.org/10.1126/SCIENCE.288.5466.669

Cesana, D., Ranzani, M., Volpin, M., Bartholomae, C., Duros, C., Artus, A., Merella, S., Benedicenti, F., Sergi Sergi, L., Sanvito, F., Brombin, C., Nonis, A., Serio, C. Di, Doglioni, C., Von Kalle, C., Schmidt, M., Cohen-Haguenauer, O., Naldini, L., & Montini, E. (2014). Uncovering and dissecting the genotoxicity of self-inactivating lentiviral vectors in vivo. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *22*(4), 774–785. https://doi.org/10.1038/MT.2014.3

Chan, A. C., Irving, B. A., Fraser, J. D., & Weiss, A. (1991). The zeta chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(20), 9166. https://doi.org/10.1073/PNAS.88.20.9166

Chan, J. D., Harrison, A. J., Darcy, P. K., Kershaw, M. H., & Slaney, C. Y. (2020). Chimeric antigen receptor T cell therapies for thoracic cancers— challenges and opportunities. *Journal of Thoracic Disease*, *12*(8), 4510–4515. https://doi.org/10.21037/JTD.2020.03.34

Chang, H. H. Y., Pannunzio, N. R., Adachi, N., & Lieber, M. R. (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nature Reviews. Molecular Cell Biology*, *18*(8), 495. https://doi.org/10.1038/NRM.2017.48

Chekol Abebe, E., Yibeltal Shiferaw, M., Tadele Admasu, F., & Asmamaw Dejenie, T. (2022). Ciltacabtagene autoleucel: The second anti-BCMA CAR T-cell therapeutic armamentarium of relapsed or refractory multiple myeloma. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/FIMMU.2022.991092

Chen, D., Tang, T. X., Deng, H., Yang, X. P., & Tang, Z. H. (2021). Interleukin-7 Biology and Its Effects on Immune Cells: Mediator of Generation, Differentiation, Survival, and Homeostasis. *Frontiers in Immunology*, *12*, 747324. https://doi.org/10.3389/FIMMU.2021.747324/BIBTEX

Chen, K. H., Wada, M., Pinz, K. G., Liu, H., Shuai, X., Chen, X., Yan, L. E., Petrov, J. C., Salman, H., Senzel, L., Leung, E. L. H., Jiang, X., & Ma, Y. (2018). A compound chimeric antigen receptor

strategy for targeting multiple myeloma. *Leukemia*, *32*(2), 402–412. https://doi.org/10.1038/LEU.2017.302

Chen, N., Morello, A., Tano, Z., & Adusumilli, P. S. (2017). CAR T-cell intrinsic PD-1 checkpoint blockade: A two-in-one approach for solid tumor immunotherapy. *Oncoimmunology*, *6*(2). https://doi.org/10.1080/2162402X.2016.1273302

Chen, Z., Liu, Y., Chen, N., Xing, H., Tian, Z., Tang, K., Rao, Q., Xu, Y., Wang, Y., Wang, M., & Wang, J. (2023). Loop CD20/CD19 CAR-T cells eradicate B-cell malignancies efficiently. *Science China Life Sciences*, *66*(4), 754–770. https://doi.org/10.1007/S11427-022-2173-9/METRICS

Cherkassky, L., Morello, A., Villena-Vargas, J., Feng, Y., Dimitrov, D. S., Jones, D. R., Sadelain, M., & Adusumilli, P. S. (2016). Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition. *The Journal of Clinical Investigation*, *126*(8), 3130. https://doi.org/10.1172/JCI83092

Chmielewski, M., & Abken, H. (2015). TRUCKs: the fourth generation of CARs. *Https://Doi.Org/10.1517/14712598.2015.1046430*, *15*(8), 1145–1154. https://doi.org/10.1517/14712598.2015.1046430

Chmielewski, M., & Abken, H. (2017). CAR T Cells Releasing IL-18 Convert to T-Bethigh FoxO1low Effectors that Exhibit Augmented Activity against Advanced Solid Tumors. *Cell Reports*, *21*(11), 3205–3219. https://doi.org/10.1016/J.CELREP.2017.11.063

Chmielewski, M., Kopecky, C., Hombach, A. A., & Abken, H. (2011). IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression. *Cancer Research*, *71*(17), 5697–5706. https://doi.org/10.1158/0008-5472.CAN-11-0103

Cho, J. H., Collins, J. J., & Wong, W. W. (2018). Universal Chimeric Antigen Receptors for Multiplexed and Logical Control of T Cell Responses. *Cell*, *173*(6), 1426-1438.e11. https://doi.org/10.1016/J.CELL.2018.03.038

Cho, J. H., Okuma, A., Al-Rubaye, D., Intisar, E., Junghans, R. P., & Wong, W. W. (2018). Engineering Axl specific CAR and SynNotch receptor for cancer therapy. *Scientific Reports*, *8*(1). https://doi.org/10.1038/S41598-018-22252-6

Choe, J. H., Watchmaker, P. B., Simic, M. S., Gilbert, R. D., Li, A. W., Krasnow, N. A., Downey, K. M., Yu, W., Carrera, D. A., Celli, A., Cho, J., Briones, J. D., Duecker, J. M., Goretsky, Y. E., Dannenfelser, R., Cardarelli, L., Troyanskaya, O., Sidhu, S. S., Roybal, K. T., … Lim, W. A. (2021). SynNotch-CAR T cells overcome challenges of specificity, heterogeneity, and persistence in treating glioblastoma. *Science Translational Medicine*, *13*(591), 7378. https://doi.org/10.1126/SCITRANSLMED.ABE7378

Choi, B. D., Yu, X., Castano, A. P., Bouffard, A. A., Schmidts, A., Larson, R. C., Bailey, S. R., Boroughs, A. C., Frigault, M. J., Leick, M. B., Scarfò, I., Cetrulo, C. L., Demehri, S., Nahed, B. V., Cahill, D. P., Wakimoto, H., Curry, W. T., Carter, B. S., & Maus, M. V. (2019). CAR-T cells secreting BiTEs circumvent antigen escape without detectable toxicity. *Nature Biotechnology 2019 37:9*, *37*(9), 1049–1058. https://doi.org/10.1038/s41587-019-0192-1

Cieri, N., Camisa, B., Cocchiarella, F., Forcato, M., Oliveira, G., Provasi, E., Bondanza, A., Bordignon, C., Peccatori, J., Ciceri, F., Lupo-Stanghellini, M. T., Mavilio, F., Mondino, A., Bicciato, S., Recchia, A., & Bonini, C. (2013). IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors. *Blood*, *121*(4), 573–584. https://doi.org/10.1182/BLOOD-2012-05-431718

Ciuffi, A. (2008). Mechanisms governing lentivirus integration site selection. *Current Gene Therapy*, *8*(6), 419–429. https://doi.org/10.2174/156652308786848021

Clackson, T., Yang, W., Rozamus, L. W., Hatada, M., Amara, J. F., Rollins, C. T., Stevenson, L. F., Magari, S. R., Wood, S. A., Courage, N. L., Lu, X., Cerasoli, F., Gilman, M., & Holt, D. A.

(1998). Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(18), 10437–10442. https://doi.org/10.1073/PNAS.95.18.10437/ASSET/EF9427D3-FCC7-4434-BF5D-146F7B7F7975/ASSETS/GRAPHIC/PQ1781568005.JPEG

Coeshott, C., Vang, B., Jones, M., & Nankervis, B. (2019). Large-scale expansion and characterization of CD3+ T-cells in the Quantum® Cell Expansion System. *Journal of Translational Medicine*, *17*(1), 258. https://doi.org/10.1186/S12967-019-2001-5

Costello, C., Derman, B. A., Kocoglu, M. H., Deol, A., Ali, A. A., Gregory, T., Dholaria, B., Berdeja, J. G., Cohen, A. D., Patel, K. K., Siegel, D. S., Nath, R., McArthur, K., McCaigue, J., Martin, C. E., Ghoddusi, M., Namini, H., Ostertag, E. M., Spear, M. A., … Shah, N. (2021). Clinical Trials of BCMA-Targeted CAR-T Cells Utilizing a Novel Non-Viral Transposon System. *Blood*, *138*(Supplement 1), 3858–3858. https://doi.org/10.1182/BLOOD-2021-151672

Cox, M. J., Lucien, F., Sakemura, R., Boysen, J. C., Kim, Y., Horvei, P., Manriquez Roman, C., Hansen, M. J., Tapper, E. E., Siegler, E. L., Forsman, C., Crotts, S. B., Schick, K. J., Hefazi, M., Ruff, M. W., Can, I., Adada, M., Bezerra, E., Kankeu Fonkoua, L. A., … Kenderian, S. S. (2021). Leukemic extracellular vesicles induce chimeric antigen receptor T cell dysfunction in chronic lymphocytic leukemia. *Molecular Therapy*, *29*(4), 1529–1540. https://doi.org/10.1016/j.ymthe.2020.12.033

Crompton, J. G., Sukumar, M., & Restifo, N. P. (2014). Uncoupling T-cell expansion from effector differentiation in cell-based immunotherapy. *Immunological Reviews*, *257*(1), 264–276. https://doi.org/10.1111/IMR.12135

Cronin, J., Zhang, X.-Y., & Reiser, J. (2005). Altering the Tropism of Lentiviral Vectors through Pseudotyping. *Current Gene Therapy*, *5*(4), 387. https://doi.org/10.2174/1566523054546224

Cueto, F. J., & Sancho, D. (2021). The Flt3L/Flt3 Axis in Dendritic Cell Biology and Cancer Immunotherapy. *Cancers*, *13*(7). https://doi.org/10.3390/CANCERS13071525

Cullen, S. P., & Martin, S. J. (2007). Mechanisms of granule-dependent killing. *Cell Death & Differentiation 2008 15:2*, *15*(2), 251–262. https://doi.org/10.1038/sj.cdd.4402244

Dacek, M. M., Kurtz, K. G., Wallisch, P., Pierre, S. A., Khayat, S., Bourne, C. M., Gardner, T. J., Vogt, K. C., Aquino, N., Younes, A., & Scheinberg, D. A. (2023). Potentiating antibody-dependent killing of cancers with CAR T cells secreting CD47-SIRPα checkpoint blocker. *Blood*, *141*(16), 2003–2015. https://doi.org/10.1182/BLOOD.2022016101

Desnoyers, L. R., Vasiljeva, O., Richardson, J. H., Yang, A., Menendez, E. E. M., Liang, T. W., Wong, C., Bessette, P. H., Kamath, K., Moore, S. J., Sagert, J. G., Hostetter, D. R., Han, F., Gee, J., Flandez, J., Markham, K., Nguyen, M., Krimm, M., Wong, K. R., … Lowman, H. B. (2013). Tumor-specific activation of an EGFR-targeting probody enhances therapeutic index. *Science Translational Medicine*, *5*(207). https://doi.org/10.1126/SCITRANSLMED.3006682

Dimitri, A., Herbst, F., & Fraietta, J. A. (2022). Engineering the next-generation of CAR T-cells with CRISPR-Cas9 gene editing. *Molecular Cancer 2022 21:1*, *21*(1), 1–13. https://doi.org/10.1186/S12943-022-01559-Z

Ding, S., Wu, X., Li, G., Han, M., Zhuang, Y., & Xu, T. (2005). Efficient Transposition of the piggyBac (PB) Transposon in Mammalian Cells and Mice. *Cell*, *122*(3), 473–483. https://doi.org/10.1016/J.CELL.2005.07.013

Dolgin, E. (2021). The tangled history of mRNA vaccines. *Nature*, *597*(7876), 318–324. https://doi.org/10.1038/D41586-021-02483-W

Drent, E., Poels, R., Mulders, M. J., van de Donk, N. W. C. J., Themeli, M., Lokhorst, H. M., & Mutis, T. (2018). Feasibility of controlling CD38-CAR T cell activity with a Tet-on inducible CAR design. *PLOS ONE*, *13*(5), e0197349. https://doi.org/10.1371/JOURNAL.PONE.0197349

Drent, E., Poels, R., Ruiter, R., Van De Donk, N. W. C. J., Zweegman, S., Yuan, H., De Bruijn, J., Sadelain, M., Lokhorst, H. M., Groen, R. W. J., Mutis, T., & Themeli, M. (2019). Combined CD28 and 4–1BB costimulation potentiates affinity-tuned Chimeric Antigen Receptor-engineered T cells. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *25*(13), 4014. https://doi.org/10.1158/1078-0432.CCR-18-2559

Du, L., Nai, Y., Shen, M., Li, T., Huang, J., Han, X., Wang, W., Pang, D., & Jin, A. (2021). IL-21 Optimizes the CAR-T Cell Preparation Through Improving Lentivirus Mediated Transfection Efficiency of T Cells and Enhancing CAR-T Cell Cytotoxic Activities. *Frontiers in Molecular Biosciences*, *8*. https://doi.org/10.3389/FMOLB.2021.675179/FULL

Duong, C. P. M., Yong, C. S. M., Kershaw, M. H., Slaney, C. Y., & Darcy, P. K. (2015). Cancer immunotherapy utilizing gene-modified T cells: From the bench to the clinic. *Molecular Immunology*, *67*(2), 46–57. https://doi.org/10.1016/J.MOLIMM.2014.12.009

Dupuy, A. J., Clark, K., Carlson, C. M., Fritz, S., Davidson, A. E., Markley, K. M., Finley, K., Fletcher, C. F., Ekker, S. C., Hackett, P. B., Horn, S., & Largaespada, D. A. (2002). Mammalian germ-line transgenesis by transposition. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(7), 4495–4499. https://doi.org/10.1073/PNAS.062630599

El Marabti, E., & Abdel-Wahab, O. (2023). Enhancing CD19 Chimeric Antigen Receptor T Cells Through Memory-Enriched T Cells. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *29*(4), 694–696. https://doi.org/10.1158/1078-0432.CCR-22-3232/711594/AM/ENHANCING-CD19-CHIMERIC-ANTIGEN-RECEPTOR-T-CELLS

Elick, T., Bauser, C., & Fraser, M. (1996). Excision of the piggyBac transposable element in vitro is a precise event that is enhanced by the expression of its encoded transposase. *Genetica*, *98*(1), 33–41. https://doi.org/10.1007/BF00120216

Elion, G. B., Furman, P. A., Fyfe, J. A., de Miranda, P., Beauchamp, L., & Schaeffer, H. J. (1977). Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. *Proceedings of the National Academy of Sciences of the United States of America*, *74*(12), 5716. https://doi.org/10.1073/PNAS.74.12.5716

Ellis, J. (2005). Silencing and variegation of gammaretrovirus and lentivirus vectors. *Human Gene Therapy*, *16*(11), 1241–1246. https://doi.org/10.1089/HUM.2005.16.1241

Emery, V., Zuckerman, M., Jackson, G., Aitken, C., Osman, H., Pagliuca, A., Potter, M., Peggs, K., & Clark, A. (2013). Management of cytomegalovirus infection in haemopoietic stem cell transplantation. *British Journal of Haematology*, *162*(1), 25–39. https://doi.org/10.1111/BJH.12363

Ericson, S. G., Guyre, C. A., Benoit, N. E., Meehan, K. R., Mills, L. E., & Fanger, M. W. (1995). Anti-body-dependent cellular cytotoxicity (ADCC) function of peripheral blood polymorphonuclear neutrophils (PMN) after autologous bone marrow transplantation (ABMT). *Bone Marrow Transplantation*, *16*(6), 787–791. https://europepmc.org/article/med/8750271

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 γ or ζ subunits of the immunoglobulin and T-cell receptors. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(2), 720–724. https://doi.org/10.1073/PNAS.90.2.720

Eyquem, J., Mansilla-Soto, J., Giavridis, T., Van Der Stegen, S. J. C., Hamieh, M., Cunanan, K. M., Odak, A., Gönen, M., & Sadelain, M. (2017). Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature*, *543*(7643), 113–117. https://doi.org/10.1038/NATURE21405

Fedorov, V. D., Themeli, M., & Sadelain, M. (2013). PD-1– and CTLA-4–Based Inhibitory Chimeric Antigen Receptors (iCARs) Divert Off-Target Immunotherapy Responses. *Science Translational Medicine*, *5*(215), 215ra172. https://doi.org/10.1126/SCITRANSLMED.3006597

Feldmann, A., Hoffmann, A., Bergmann, R., Koristka, S., Berndt, N., Arndt, C., Rodrigues Loureiro, L., Kittel-Boselli, E., Mitwasi, N., Kegler, A., Lamprecht, C., González Soto, K. E., & Bachmann, M. (2020). Versatile chimeric antigen receptor platform for controllable and combinatorial T cell therapy. *OncoImmunology*, *9*(1). https://doi.org/10.1080/2162402X.2020.1785608

Ferlazzo, G., Pack, M., Thomas, D., Paludan, C., Schmid, D., Strowig, T., Bougras, G., Muller, W. A., Moretta, L., & Münz, C. (2004). Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(47), 16606–16611. https://doi.org/10.1073/PNAS.0407522101

Field, A. C., Vink, C., Gabriel, R., Al-Subki, R., Schmidt, M., Goulden, N., Stauss, H., Thrasher, A., Morris, E., & Qasim, W. (2013). Comparison of Lentiviral and Sleeping Beauty Mediated αβ T Cell Receptor Gene Transfer. *PLoS ONE*, *8*(6), 68201. https://doi.org/10.1371/JOURNAL.PONE.0068201

Fink, E. C., & Ebert, B. L. (2015). The novel mechanism of lenalidomide activity. *Blood*, *126*(21), 2366. https://doi.org/10.1182/BLOOD-2015-07-567958

Finney, H. M., Akbar, A. N., & Lawson, A. D. G. (2004). Activation of Resting Human Primary T Cells with Chimeric Receptors: Costimulation from CD28, Inducible Costimulator, CD134, and CD137 in Series with Signals from the TCR Chain. *The Journal of Immunology*, *172*, 104–113. http://journals.aai.org/jimmunol/article-pdf/172/1/104/1178355/104.pdf

Finney, H. M., Lawson, A. D. G., Bebbington, C. R., & Weir, A. N. C. (1998). Chimeric Receptors Providing Both Primary and Costimulatory Signaling in T Cells from a Single Gene Product. *The Journal of Immunology*, *161*(6), 2791–2797. https://doi.org/10.4049/JIMMUNOL.161.6.2791

Finney, O. C., Brakke, H., Rawlings-Rhea, S., Hicks, R., Doolittle, D., Lopez, M., Futrell, B., Orentas, R. J., Li, D., Gardner, R., & Jensen, M. C. (2019). CD19 CAR T cell product and disease attributes predict leukemia remission durability. *The Journal of Clinical Investigation*, *129*(5), 2123. https://doi.org/10.1172/JCI125423

Foster, A. E., Dotti, G., Lu, A., Khalil, M., Brenner, M. K., Heslop, H. E., Rooney, C. M., & Bollard, C. M. (2008). Antitumor Activity of EBV-specific T Lymphocytes Transduced With a Dominant Negative TGF-β Receptor. *Journal of Immunotherapy (Hagerstown, Md. : 1997)*, *31*(5), 500. https://doi.org/10.1097/CJI.0B013E318177092B

Foster, A. E., Mahendravada, A., Shinners, N. P., Chang, W. C., Crisostomo, J., Lu, A., Khalil, M., Morschl, E., Shaw, J. L., Saha, S., Duong, M. L. T., Collinson-Pautz, M. R., Torres, D. L., Rodriguez, T., Pentcheva-Hoang, T., Bayle, J. H., Slawin, K. M., & Spencer, D. M. (2017). Regulated Expansion and Survival of Chimeric Antigen Receptor-Modified T Cells Using Small Molecule-Dependent Inducible MyD88/CD40. *Molecular Therapy*, *25*(9), 2176–2188. https://doi.org/10.1016/J.YMTHE.2017.06.014

Fousek, K., Watanabe, J., Joseph, S. K., George, A., An, X., Byrd, T. T., Morris, J. S., Luong, A., Martínez-Paniagua, M. A., Sanber, K., Navai, S. A., Gad, A. Z., Salsman, V. S., Mathew, P. R., Kim, H. N., Wagner, D. L., Brunetti, L., Jang, A., Baker, M. L., … Ahmed, N. (2020). CAR T-cells that target acute B-lineage leukemia irrespective of CD19 expression. *Leukemia 2020 35:1*, *35*(1), 75–89. https://doi.org/10.1038/s41375-020-0792-2

Fraietta, J. A., Lacey, S. F., Orlando, E. J., Pruteanu-Malinici, I., Gohil, M., Lundh, S., Boesteanu, A. C., Wang, Y., O'connor, R. S., Hwang, W. T., Pequignot, E., Ambrose, D. E., Zhang, C., Wilcox, N., Bedoya, F., Dorfmeier, C., Chen, F., Tian, L., Parakandi, H., … Melenhorst, J. J. (2018). Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell

therapy of chronic lymphocytic leukemia. *Nature Medicine 2018 24:5*, *24*(5), 563–571. https://doi.org/10.1038/s41591-018-0010-1

Fraser, M. J., Cary, L., Boonvisudhi, K., & Wang, H. G. H. (1995). Assay for movement of Lepidopteran transposon IFP2 in insect cells using a baculovirus genome as a target DNA. *Virology*, *211*(2), 397–407. https://doi.org/10.1006/VIRO.1995.1422

Fraser, M. J., Smith, G. E., & Summers, M. D. (1983). Acquisition of Host Cell DNA Sequences by Baculoviruses: Relationship Between Host DNA Insertions and FP Mutants of Autographa californica and Galleria mellonella Nuclear Polyhedrosis Viruses. *Journal of Virology*, *47*(2), 287. https://doi.org/10.1128/JVI.47.2.287-300.1983

Friedman, K. M., Garrett, T. E., Evans, J. W., Horton, H. M., Latimer, H. J., Seidel, S. L., Horvath, C. J., & Morgan, R. A. (2018). Effective Targeting of Multiple B-Cell Maturation Antigen–Expressing Hematological Malignances by Anti-B-Cell Maturation Antigen Chimeric Antigen Receptor T Cells. *Human Gene Therapy*, *29*(5), 585. https://doi.org/10.1089/HUM.2018.001

Frigault, M. J., Lee, J., Basil, M. C., Carpenito, C., Motohashi, S., Scholler, J., Kawalekar, O. U., Guedan, S., McGettigan, S. E., Posey, A. D., Ang, S., Cooper, L. J. N., Platt, J. M., Johnson, F. B., Paulos, C. M., Zhao, Y., Kalos, M., Milone, M. C., & June, C. H. (2015). Identification of chimeric antigen receptors that mediate constitutive or inducible proliferation of T cells. *Cancer Immunology Research*, *3*(4), 356. https://doi.org/10.1158/2326-6066.CIR-14-0186

Fry, T. J., Shah, N. N., Orentas, R. J., Stetler-Stevenson, M., Yuan, C. M., Ramakrishna, S., Wolters, P., Martin, S., Delbrook, C., Yates, B., Shalabi, H., Fountaine, T. J., Shern, J. F., Majzner, R. G., Stroncek, D. F., Sabatino, M., Feng, Y., Dimitrov, D. S., Zhang, L., … Mackall, C. L. (2018). CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. *Nature Medicine*, *24*(1), 20–28. https://doi.org/10.1038/NM.4441

Fu, Q., Fu, T. M., Cruz, A. C., Sengupta, P., Thomas, S. K., Wang, S., Siegel, R. M., Wu, H., & Chou, J. J. (2016). Structural Basis and Functional Role of Intramembrane Trimerization of the Fas/CD95 Death Receptor. *Molecular Cell*, *61*(4), 602. https://doi.org/10.1016/J.MOLCEL.2016.01.009

Fujii, S. I., Shimizu, K., Shimizu, T., & Lotze, M. T. (2001). Interleukin-10 promotes the maintenance of antitumor CD8+ T-cell effector function in situ. *Blood*, *98*(7), 2143–2151. https://doi.org/10.1182/BLOOD.V98.7.2143

Funk, C. R., Wang, S., Chen, K. Z., Waller, A., Sharma, A., Edgar, C. L., Gupta, V. A., Chandrakasan, S., Zoine, J. T., Fedanov, A., Raikar, S. S., Koff, J. L., Flowers, C. R., Coma, S., Pachter, J. A., Ravindranathan, S., Spencer, H. T., Shanmugam, M., & Waller, E. K. (2022). PI3Kδ/γ inhibition promotes human CART cell epigenetic and metabolic reprogramming to enhance antitumor cytotoxicity. *Blood*, *139*(4), 523. https://doi.org/10.1182/BLOOD.2021011597

Gagelmann, N., Bishop, M., Ayuk, F., Bethge, W., Glass, B., Sureda, A., Pasquini, M. C., & Kröger, N. (2024). Axicabtagene Ciloleucel versus Tisagenlecleucel for Relapsed or Refractory Large B Cell Lymphoma: A Systematic Review and Meta-Analysis. *Transplantation and Cellular Therapy*, *30*(6), 584.e1-584.e13. https://doi.org/10.1016/j.jtct.2024.01.074

Gallimore, A., Glithero, A., Godkin, A., Tissot, A. C., Plückthun, A., Elliott, T., Hengartner, H., & Zinkernagel, R. (1998). Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *The Journal of Experimental Medicine*, *187*(9), 1383–1393. https://doi.org/10.1084/JEM.187.9.1383

Gargett, T., & Brown, M. P. (2015). Different cytokine and stimulation conditions influence the expansion and immune phenotype of third-generation chimeric antigen receptor T cells specific for tumor antigen GD2. *Cytotherapy*, *17*(4), 487–495. https://doi.org/10.1016/J.JCYT.2014.12.002

Gargett, T., Ebert, L. M., Truong, N. T. H., Kollis, P. M., Sedivakova, K., Yu, W., Yeo, E. C. F., Wittwer, N. L., Gliddon, B. L., Tea, M. N., Ormsby, R., Poonnoose, S., Nowicki, J., Vittorio, O., Ziegler, D. S., Pitson, S. M., & Brown, M. P. (2022). GD2-targeting CAR-T cells enhanced by transgenic IL-15 expression are an effective and clinically feasible therapy for glioblastoma. *BioRxiv*, 2022.05.01.490250. https://doi.org/10.1101/2022.05.01.490250

Garrido, F., Aptsiauri, N., Doorduijn, E. M., Garcia Lora, A. M., van Hall, T., & Hall, van. (2016). The urgent need to recover MHC class I in cancers for effective immunotherapy. *Current Opinion in Immunology*, *39*, 44–51. https://doi.org/10.1016/j.coi.2015.12.007

Gasiunas, G., Barrangou, R., Horvath, P., & Siksnys, V. (2012). Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(39), E2579–E2586. https://doi.org/10.1073/PNAS.1208507109/SUPPL_FILE/PNAS.201208507SI.PDF

Gattinoni, L., Klebanoff, C. A., Palmer, D. C., Wrzesinski, C., Kerstann, K., Yu, Z., Finkelstein, S. E., Theoret, M. R., Rosenberg, S. A., & Restifo, N. P. (2005). Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *Journal of Clinical Investigation*, *115*(6), 1616. https://doi.org/10.1172/JCI24480

Gattinoni, L., Lugli, E., Ji, Y., Pos, Z., Paulos, C. M., Quigley, M. F., Almeida, J. R., Gostick, E., Yu, Z., Carpenito, C., Wang, E., Douek, D. C., Price, D. A., June, C. H., Marincola, F. M., Roederer, M., & Restifo, N. P. (2011). A human memory T cell subset with stem cell-like properties. *Nature Medicine*, *17*(10), 1290–1297. https://doi.org/10.1038/nm.2446

Gattinoni, L., Speiser, D. E., Lichterfeld, M., & Bonini, C. (2017). T memory stem cells in health and disease. *Nature Medicine*, *23*(1), 18–27. https://doi.org/10.1038/NM.4241

Gattinoni, L., Zhong, X. S., Palmer, D. C., Ji, Y., Hinrichs, C. S., Yu, Z., Wrzesinski, C., Boni, A., Cassard, L., Garvin, L. M., Paulos, C. M., Muranski, P., & Restifo, N. P. (2009). Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nature Medicine*, *15*(7), 808. https://doi.org/10.1038/NM.1982

Gaud, G., Lesourne, R., & Love, P. E. (2018). Regulatory mechanisms in T cell receptor signalling. *Nature Reviews Immunology 2018 18:8*, *18*(8), 485–497. https://doi.org/10.1038/s41577-018-0020-8

Gauthier, J., Bezerra, E. D., Hirayama, A. V., Fiorenza, S., Sheih, A., Chou, C. K., Kimble, E. L., Pender, B. S., Hawkins, R. M., Vakil, A., Phi, T. D., Steinmetz, R. N., Jamieson, A. W., Bar, M., Cassaday, R. D., Chapuis, A. G., Cowan, A. J., Green, D. J., Kiem, H. P., … Turtle, C. J. (2021). Factors associated with outcomes after a second CD19-targeted CAR T-cell infusion for refractory B-cell malignancies. *Blood*, *137*(3), 323. https://doi.org/10.1182/BLOOD.2020006770

Gee, A. P. (2018). GMP CAR-T cell production. *Best Practice & Research Clinical Haematology*, *31*(2), 126–134. https://doi.org/10.1016/J.BEHA.2018.01.002

Georgiadis, C., Rasaiyaah, J., Gkazi, S. A., Preece, R., Etuk, A., Christi, A., & Qasim, W. (2021). Base-edited CAR T cells for combinational therapy against T cell malignancies. *Leukemia 2021 35:12*, *35*(12), 3466–3481. https://doi.org/10.1038/s41375-021-01282-6

Gerdemann, U., Keirnan, J. M., Katari, U. L., Yanagisawa, R., Christin, A. S., Huye, L. E., Perna, S. K., Ennamuri, S., Gottschalk, S., Brenner, M. K., Heslop, H. E., Rooney, C. M., & Leen, A. M. (2012). Rapidly generated multivirus-specific cytotoxic T lymphocytes for the prophylaxis and treatment of viral infections. *Molecular Therapy*, *20*(8), 1622–1632. https://doi.org/10.1038/mt.2012.130

Geurts, A. M., Yang, Y., Clark, K. J., Liu, G., Cui, Z., Dupuy, A. J., Bell, J. B., Largaespada, D. A., & Hackett, P. B. (2003). Gene transfer into genomes of human cells by the sleeping beauty transposon system. *Molecular Therapy*, *8*(1), 108–117. https://doi.org/10.1016/S1525-0016(03)00099-6

Gocheva, V., Wang, H. W., Gadea, B. B., Shree, T., Hunter, K. E., Garfall, A. L., Berman, T., & Joyce, J. A. (2010). IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes & Development*, *24*(3), 241. https://doi.org/10.1101/GAD.1874010

Gogol-Doring, A., Ammar, I., Gupta, S., Bunse, M., Miskey, C., Chen, W., Uckert, W., Schulz, T. F., Izsvak, Z., & Ivics, Z. (2016). Genome-wide Profiling Reveals Remarkable Parallels Between Insertion Site Selection Properties of the MLV Retrovirus and the piggyBac Transposon in Primary Human CD4+ T Cells. *Molecular Therapy*, *24*(3), 592. https://doi.org/10.1038/MT.2016.11

Gomes, K., Aguiar, G., Mulia, G., Stavrou, E., Figueiredo, M., Athanassiadou, A., Covas, D., & Castro, V. (2021). USING A NONVIRAL AND NONINTEGRATING DNA VECTOR TO GENERATE SAFE CAR-T CELLS. *Hematology, Transfusion and Cell Therapy*, *43*, S272–S273. https://doi.org/10.1016/J.HTCT.2021.10.461

Gomes-Silva, D., Mukherjee, M., Srinivasan, M., Krenciute, G., Dakhova, O., Zheng, Y., Cabral, J. M. S., Rooney, C. M., Orange, J. S., Brenner, M. K., & Mamonkin, M. (2017). Tonic 4-1BB Costimulation in Chimeric Antigen Receptors Impedes T Cell Survival and Is Vector Dependent. *Cell Reports*, *21*(1), 17. https://doi.org/10.1016/J.CELREP.2017.09.015

Gong, W., Hoffmann, J. M., Stock, S., Wang, L., Liu, Y., Schubert, M. L., Neuber, B., Hückelhoven-Krauss, A., Gern, U., Schmitt, A., Müller-Tidow, C., Shiku, H., Schmitt, M., & Sellner, L. (2019). Comparison of IL-2 vs IL-7/IL-15 for the generation of NY-ESO-1-specific T cells. *Cancer Immunology, Immunotherapy : CII*, *68*(7), 1195–1209. https://doi.org/10.1007/S00262-019-02354-4

Gossen, M., & Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(12), 5547–5551. https://doi.org/10.1073/PNAS.89.12.5547

Grabundzija, I., Irgang, M., Mátés, L., Belay, E., Matrai, J., Gogol-Döring, A., Kawakami, K., Chen, W., Ruiz, P., Chuah, M. K. L., Vandendriessche, T., Izsvák, Z., & Ivics, Z. (2010). Comparative Analysis of Transposable Element Vector Systems in Human Cells. *Molecular Therapy*, *18*(6), 1200. https://doi.org/10.1038/MT.2010.47

Grada, Z., Hegde, M., Byrd, T., Shaffer, D. R., Ghazi, A., Brawley, V. S., Corder, A., Schönfeld, K., Koch, J., Dotti, G., Heslop, H. E., Gottschalk, S., Wels, W. S., Baker, M. L., & Ahmed, N. (2013). TanCAR: A novel bispecific chimeric antigen receptor for cancer immunotherapy. *Molecular Therapy Nucleic Acids*, *2*. https://doi.org/10.1038/mtna.2013.32

Griffioen, M., Van Egmond, E. H. M., Kester, M. G. D., Willemze, R., Falkenburg, J. H. F., & Heemskerk, M. H. M. (2009). Retroviral transfer of human CD20 as a suicide gene for adoptive T-cell therapy. *Haematologica*, *94*(9), 1316. https://doi.org/10.3324/HAEMATOL.2008.001677

Gross, G., Waks, T., & Eshhar, Z. (1989). Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity (chimeric genes/antibody variable region). In *Immunology* (Vol. 86).

Gu, X., He, D., Li, C., Wang, H., & Yang, G. (2018). Development of inducible cd19-car t cells with a tet-on system for controlled activity and enhanced clinical safety. *International Journal of Molecular Sciences*, *19*(11). https://doi.org/10.3390/IJMS19113455

Guedan, S., Calderon, H., Posey, A. D., & Maus, M. V. (2019). Engineering and Design of Chimeric Antigen Receptors. *Molecular Therapy. Methods & Clinical Development*, *12*, 145. https://doi.org/10.1016/J.OMTM.2018.12.009

Guedan, S., Posey, A. D., Shaw, C., Wing, A., Da, T., Patel, P. R., McGettigan, S. E., Casado-Medrano, V., Kawalekar, O. U., Uribe-Herranz, M., Song, D., Melenhorst, J. J., Lacey, S. F., Scholler, J., Keith, B., Young, R. M., & June, C. H. (2018). Enhancing CAR T cell persistence

through ICOS and 4-1BB costimulation. *JCI Insight*, *3*(1). https://doi.org/10.1172/JCI.INSIGHT.96976

Guest, R. D., Hawkins, R. E., Kirillova, N., Cheadle, E. J., Arnold, J., O'Neill, A., Irlam, J., Chester, K. A., Kemshead, J. T., Shaw, D. M., Embleton, M. J., Stern, P. L., & Gilham, D. E. (2005). The role of extracellular spacer regions in the optimal design of chimeric immune receptors: Evaluation of four different scFvs and antigens. *Journal of Immunotherapy*, *28*(3), 203–211. https://doi.org/10.1097/01.CJI.0000161397.96582.59

Gumber, D., & Wang, L. D. (2022). Improving CAR-T immunotherapy: Overcoming the challenges of T cell exhaustion. *EBioMedicine*, *77*, 103941. https://doi.org/10.1016/J.EBIOM.2022.103941

Guo, T., Ma, D., & Lu, T. K. (2022). Sense-and-Respond Payload Delivery Using a Novel Antigen-Inducible Promoter Improves Suboptimal CAR-T Activation. *ACS Synthetic Biology*, *11*(4), 1440–1453. https://doi.org/10.1021/ACSSYNBIO.1C00236/SUPPL_FILE/SB1C00236_SI_001.PDF

Guo, X., Jiang, H., Shi, B., Zhou, M., Zhang, H., Shi, Z., Du, G., Luo, H., Wu, X., Wang, Y., Sun, R., & Li, Z. (2018). Disruption of PD-1 enhanced the anti-tumor activity of chimeric antigen receptor T cells against hepatocellular carcinoma. *Frontiers in Pharmacology*, *9*(OCT), 412895. https://doi.org/10.3389/FPHAR.2018.01118/BIBTEX

Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M. P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C. S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J. I., De Saint Basile, G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., … Cavazzana-Calvo, M. (2003). LMO2-Associated Clonal T Cell Proliferation in Two Patients after Gene Therapy for SCID-X1. *Science*, *302*(5644), 415–419. https://doi.org/10.1126/SCIENCE.1088547/SUPPL_FILE/HACEIN.SOM.PDF

Hamada, M., Nishio, N., Okuno, Y., Suzuki, S., Kawashima, N., Muramatsu, H., Tsubota, S., Wilson, M. H., Morita, D., Kataoka, S., Ichikawa, D., Murakami, N., Taniguchi, R., Suzuki, K., Kojima, D., Sekiya, Y., Nishikawa, E., Narita, A., Hama, A., … Takahashi, Y. (2018). Integration Mapping of piggyBac-Mediated CD19 Chimeric Antigen Receptor T Cells Analyzed by Novel Tagmentation-Assisted PCR. *EBioMedicine*, *34*, 18. https://doi.org/10.1016/J.EBIOM.2018.07.008

Han, J., Gao, F., Geng, S., Ye, X., Wang, T., Du, P., Cai, Z., Fu, Z., Zhao, Z., Shi, L., Li, Q., & Cai, J. (2020). Minicircle DNA-engineered CAR T cells suppressed tumor growth in mice. *Molecular Cancer Therapeutics*, *19*(1), 178–186. https://doi.org/10.1158/1535-7163.MCT-19-0204/87929/AM/MINICIRCLE-DNA-ENGINEERED-CAR-T-CELLS-SUPPRESSED

Han, X., Bryson, P. D., Zhao, Y., Cinay, G. E., Li, S., Guo, Y., Siriwon, N., & Wang, P. (2017). Masked Chimeric Antigen Receptor for Tumor-Specific Activation. *Molecular Therapy*, *25*(1), 274. https://doi.org/10.1016/J.YMTHE.2016.10.011

He, C., Zhou, Y., Li, Z., Farooq, M. A., Ajmal, I., Zhang, H., Zhang, L., Tao, L., Yao, J., Du, B., Liu, M., & Jiang, W. (2020). Co-Expression of IL-7 Improves NKG2D-Based CAR T Cell Therapy on Prostate Cancer by Enhancing the Expansion and Inhibiting the Apoptosis and Exhaustion. *Cancers 2020, Vol. 12, Page 1969*, *12*(7), 1969. https://doi.org/10.3390/CANCERS12071969

Hegde, M., Corder, A., Chow, K. K., Mukherjee, M., Ashoori, A., Kew, Y., Zhang, Y. J., Baskin, D. S., Merchant, F. A., Brawley, V. S., Byrd, T. T., Krebs, S., Wu, M. F., Liu, H., Heslop, H. E., Gottachalk, S., Yvon, E., & Ahmed, N. (2013). Combinational Targeting offsets antigen escape and enhances effector functions of adoptively transferred T cells in glioblastoma. *Molecular Therapy*, *21*(11), 2087–2101. https://doi.org/10.1038/mt.2013.185

Hegde, M., Mukherjee, M., Grada, Z., Pignata, A., Landi, D., Navai, S. A., Wakefield, A., Fousek, K., Bielamowicz, K., Chow, K. K. H., Brawley, V. S., Byrd, T. T., Krebs, S., Gottschalk, S., Wels, W. S., Baker, M. L., Dotti, G., Mamonkin, M., Brenner, M. K., … Ahmed, N. (2016). Tandem CAR T cells targeting HER2 and IL13Rα2 mitigate tumor antigen escape. *The Journal of Clinical Investigation*, *126*(8), 3036. https://doi.org/10.1172/JCI83416

Hege, K. M., Bergsland, E. K., Fisher, G. A., Nemunaitis, J. J., Warren, R. S., McArthur, J. G., Lin, A. A., Schlom, J., June, C. H., & Sherwin, S. A. (2017). Safety, tumor trafficking and immunogenicity of chimeric antigen receptor (CAR)-T cells specific for TAG-72 in colorectal cancer. *Journal for Immunotherapy of Cancer*, *5*(1). https://doi.org/10.1186/S40425-017-0222-9

Hinrichs, C. S., Borman, Z. A., Gattinoni, L., Yu, Z., Burns, W. R., Huang, J., Klebanoff, C. A., Johnson, L. A., Kerkar, S. P., Yang, S., Muranski, P., Palmer, D. C., Scott, C. D., Morgan, R. A., Robbins, P. F., Rosenberg, S. A., & Restifo, N. P. (2011). Human effector CD8+ T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy. *Blood*, *117*(3), 808. https://doi.org/10.1182/BLOOD-2010-05-286286

Hinrichs, C. S., Spolski, R., Paulos, C. M., Gattinoni, L., Kerstann, K. W., Palmer, D. C., Klebanoff, C. A., Rosenberg, S. A., Leonard, W. J., & Restifo, N. P. (2008). IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy. *Blood*, *111*(11), 5326–5333. https://doi.org/10.1182/BLOOD-2007-09-113050

Hoffmann, J. M., Schubert, M. L., Wang, L., Hückelhoven, A., Sellner, L., Stock, S., Schmitt, A., Kleist, C., Gern, U., Loskog, A., Wuchter, P., Hofmann, S., Ho, A. D., Müller-Tidow, C., Dreger, P., & Schmitt, M. (2018). Differences in expansion potential of naive chimeric antigen receptor T cells from healthy donors and untreated chronic lymphocytic leukemia patients. *Frontiers in Immunology*, *8*(JAN). https://doi.org/10.3389/FIMMU.2017.01956/FULL

Hombach, A., Barden, M., Hannappel, L., Chmielewski, M., Rappl, G., Sachinidis, A., & Abken, H. (2022). IL12 integrated into the CAR exodomain converts CD8+ T cells to poly-functional NK-like cells with superior killing of antigen-loss tumors. *Molecular Therapy*, *30*(2), 593–605. https://doi.org/10.1016/j.ymthe.2021.10.011

Hombach, A., Köhler, H., Rappl, G., & Abken, H. (2006). Human CD4+ T Cells Lyse Target Cells via Granzyme/Perforin upon Circumvention of MHC Class II Restriction by an Antibody-Like Immunoreceptor. *The Journal of Immunology*, *177*(8), 5668–5675. https://doi.org/10.4049/JIMMUNOL.177.8.5668

Hong, M., Clubb, J. D., & Chen, Y. Y. (2020). Engineering CAR-T Cells for Next-Generation Cancer Therapy. *Cancer Cell*, *38*(4), 473–488. https://doi.org/10.1016/J.CCELL.2020.07.005

Hoyos, V., Savoldo, B., Quintarelli, C., Mahendravada, A., Zhang, M., Vera, J., Heslop, H. E., Rooney, C. M., Brenner, M. K., & Dotti, G. (2010). Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. *Leukemia 2010 24:6*, *24*(6), 1160–1170. https://doi.org/10.1038/leu.2010.75

Hu, B., Ren, J., Luo, Y., Keith, B., Young, R. M., Scholler, J., Zhao, Y., & June, C. H. (2017). Augmentation of Antitumor Immunity by Human and Mouse CAR T Cells Secreting IL-18. *Cell Reports*, *20*(13), 3025–3033. https://doi.org/10.1016/J.CELREP.2017.09.002

Hu, B., Zou, Y., Zhang, L., Tang, J., Niedermann, G., Firat, E., Huang, X., & Zhu, X. (2019). Nucleofection with Plasmid DNA for CRISPR/Cas9-Mediated Inactivation of Programmed Cell Death Protein 1 in CD133-Specific CAR T Cells. *Human Gene Therapy*, *30*(4), 446–458. https://doi.org/10.1089/HUM.2017.234/ASSET/IMAGES/LARGE/FIGURE7.JPEG

Hu, J., Yang, Q., Zhang, W., Du, H., Chen, Y., Zhao, Q., Dao, L., Xia, X., Natalie Wall, F., Zhang, Z., Mahadeo, K., Gorlick, R., Kopetz, S., Dotti, G., & Li, S. (2022). Cell membrane-anchored and tumor-targeted IL-12 (attIL12)-T cell therapy for eliminating large and heterogeneous solid tumors. *Journal for ImmunoTherapy of Cancer*, *10*(1), e003633. https://doi.org/10.1136/JITC-2021-003633

Hu, W., Zi, Z., Jin, Y., Li, G., Shao, K., Cai, Q., Ma, X., & Wei, F. (2019). CRISPR/Cas9-mediated PD-1 disruption enhances human mesothelin-targeted CAR T cell effector functions. *Cancer Immunology, Immunotherapy*, *68*(3), 365–377. https://doi.org/10.1007/S00262-018-2281-2/FIGURES/6

Huang, X., Guo, H., Tammana, S., Jung, Y. C., Mellgren, E., Bassi, P., Cao, Q., Tu, Z. J., Kim, Y. C., Ekker, S. C., Wu, X., Wang, S. M., & Zhou, X. (2010). Gene Transfer Efficiency and Genome-Wide Integration Profiling of Sleeping Beauty, Tol2, and PiggyBac Transposons in Human Primary T Cells. *Molecular Therapy*, *18*(10), 1803. https://doi.org/10.1038/MT.2010.141

Huang, X., Haley, K., Wong, M., Guo, H., Lu, C., Wilber, A., & Zhou, X. (2010). Unexpectedly High Copy Number of Random Integration but Low Frequency of Persistent Expression of the Sleeping Beauty Transposase After Trans Delivery in Primary Human T Cells. *Human Gene Therapy*, *21*(11), 1577. https://doi.org/10.1089/HUM.2009.138

Huang, Z., Wu, Y., Allen, M. E., Pan, Y., Kyriakakis, P., Lu, S., Chang, Y. J., Wang, X., Chien, S., & Wang, Y. (2020). Engineering light-controllable CAR T cells for cancer immunotherapy. *Science Advances*, *6*(8). https://doi.org/10.1126/SCIADV.AAY9209

Huckaby, J. T., Landoni, E., Jacobs, T. M., Savoldo, B., Dotti, G., & Lai, S. K. (2021). Original research: Bispecific binder redirected lentiviral vector enables in vivo engineering of CAR-T cells. *Journal for Immunotherapy of Cancer*, *9*(9), 2737. https://doi.org/10.1136/JITC-2021-002737

Hudecek, M., Gogishvili, T., Monjezi, R., Wegner, J., Shankar, R., Kruesemann, C., Miskey, C., Ivics, Z., Schmeer, M., & Schleef, M. (2016). Minicircle-Based Engineering of Chimeric Antigen Receptor (CAR) T Cells. *Recent Results in Cancer Research. Fortschritte Der Krebsforschung. Progres Dans Les Recherches Sur Le Cancer*, *209*, 37–50. https://doi.org/10.1007/978-3-319-42934-2_3

Hudecek, M., Sommermeyer, D., Kosasih, P. L., Silva-Benedict, A., Liu, L., Rader, C., Jensen, M. C., & Riddell, S. R. (2015). The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for in vivo antitumor activity. *Cancer Immunology Research*, *3*(2), 125–135. https://doi.org/10.1158/2326-6066.CIR-14-0127

Hurton, L. V., Singh, H., Najjar, A. M., Switzer, K. C., Mi, T., Maiti, S., Olivares, S., Rabinovich, B., Huls, H., Forget, M. A., Datar, V., Kebriaei, P., Lee, D. A., Champlin, R. E., & Cooper, L. J. N. (2016). Tethered IL-15 augments antitumor activity and promotes a stem-cell memory subset in tumor-specific T cells. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(48), E7788–E7797. https://doi.org/10.1073/PNAS.1610544113/SUPPL_FILE/PNAS.1610544113.SAPP.PDF

Iacoboni, G., Navarro, V., Martín-López, A. Á., Rejeski, K., Kwon, M., Jalowiec, K. A., Amat, P., Reguera-Ortega, J. L., Gallur, L., Blumenberg, V., Gutiérrez-Herrero, S., Roddie, C., Benzaquén, A., Delgado-Serrano, J., Sánchez-Salinas, M. A., Bailén, R., Carpio, C., López-Corral, L., Hernani, R., … Barba, P. (2024). Recent Bendamustine Treatment Before Apheresis Has a Negative Impact on Outcomes in Patients With Large B-Cell Lymphoma Receiving Chimeric Antigen Receptor T-Cell Therapy. *Journal of Clinical Oncology*, *42*(2), 205–217. https://doi.org/10.1200/JCO.23.01097/SUPPL_FILE/DS_JCO.23.01097.PDF

Imai, C., Mihara, K., Andreansky, M., Nicholson, I. C., Pui, C.-H., Geiger, T. L., & Campana, D. (2004). Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. *Leukemia*, *18*, 676–684. https://doi.org/10.1038/sj.leu.2403302

Irving, B. A., & Weiss, A. (1991). The cytoplasmic domain of the T cell receptor ζ chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell*, *64*(5), 891–901. https://doi.org/10.1016/0092-8674(91)90314-O

Ivics, Z., Hackett, P. B., Plasterk, R. H., & Izsvák, Z. (1997). Molecular Reconstruction of Sleeping Beauty, a Tc1-like Transposon from Fish, and Its Transposition in Human Cells. *Cell*, *91*(4), 501–510. https://doi.org/10.1016/S0092-8674(00)80436-5

Izsvák, Z., & Ivics, Z. (2004). Sleeping Beauty Transposition: Biology and Applications for Molecular Therapy. *Molecular Therapy*, *9*(2), 147–156. https://doi.org/10.1016/J.YMTHE.2003.11.009

Izsvák, Z., Ivics, Z., & Plasterk, R. H. (2000). Sleeping Beauty, a wide host-range transposon vector for genetic transformation in vertebrates. *Journal of Molecular Biology*, *302*(1), 93–102. https://doi.org/10.1006/JMBI.2000.4047

Jameson, S. C., & Masopust, D. (2018). Understanding subset diversity in T cell memory. *Immunity*, *48*(2), 214. https://doi.org/10.1016/J.IMMUNI.2018.02.010

Jan, M., Scarfò, I., Larson, R. C., Walker, A., Schmidts, A., Guirguis, A. A., Gasser, J. A., Słabicki, M., Bouffard, A. A., Castano, A. P., Kann, M. C., Cabral, M. L., Tepper, A., Grinshpun, D. E., Sperling, A. S., Kyung, T., Sievers, Q. L., Birnbaum, M. E., Maus, M. V., & Ebert, B. L. (2021). Reversible ON- and OFF-switch chimeric antigen receptors controlled by lenalidomide. *Science Translational Medicine*, *13*(575). https://doi.org/10.1126/SCITRANSLMED.ABB6295

Jaspers, J. E., Khan, J. F., Godfrey, W. D., Lopez, A. V., Ciampricotti, M., Rudin, C. M., & Brentjens, R. J. (2023). IL-18–secreting CAR T cells targeting DLL3 are highly effective in small cell lung cancer models. *The Journal of Clinical Investigation*, *133*(9). https://doi.org/10.1172/JCI166028

Jiang, W., Bikard, D., Cox, D., Zhang, F., & Marraffini, L. A. (2013). RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nature Biotechnology 2013 31:3*, *31*(3), 233–239. https://doi.org/10.1038/nbt.2508

Jin, C., Fotaki, G., Ramachandran, M., Nilsson, B., Essand, M., & Yu, D. (2016). Safe engineering of CAR T cells for adoptive cell therapy of cancer using long-term episomal gene transfer. *EMBO Molecular Medicine*, *8*(7), 702–711. https://doi.org/10.15252/EMMM.201505869

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, *337*(6096), 816–821. https://doi.org/10.1126/SCIENCE.1225829/SUPPL_FILE/JINEK.SM.PDF

Jo, S., Das, S., Williams, A., Chretien, A. S., Pagliardini, T., Le Roy, A., Fernandez, J. P., Le Clerre, D., Jahangiri, B., Chion-Sotinel, I., Rozlan, S., Dessez, E., Gouble, A., Dusséaux, M., Galetto, R., Duclert, A., Marcenaro, E., Devillier, R., Olive, D., … Valton, J. (2022). Endowing universal CAR T-cell with immune-evasive properties using TALEN-gene editing. *Nature Communications 2022 13:1*, *13*(1), 1–16. https://doi.org/10.1038/s41467-022-30896-2

Juillerat, A., Marechal, A., Filhol, J. M., Valogne, Y., Valton, J., Duclert, A., Duchateau, P., & Poirot, L. (2017). An oxygen sensitive self-decision making engineered CAR T-cell. *Scientific Reports*, *7*. https://doi.org/10.1038/SREP39833

Juillerat, A., Marechal, A., Filhol, J. M., Valton, J., Duclert, A., Poirot, L., & Duchateau, P. (2016). Design of chimeric antigen receptors with integrated controllable transient functions. *Scientific Reports 2016 6:1*, *6*(1), 1–7. https://doi.org/10.1038/srep18950

Jung, I. Y., Kim, Y. Y., Yu, H. S., Lee, M., Kim, S., & Lee, J. (2018). CRISPR/Cas9-Mediated Knockout of DGK Improves Antitumor Activities of Human T Cells. *Cancer Research*, *78*(16), 4692–4703. https://doi.org/10.1158/0008-5472.CAN-18-0030

Kaartinen, T., Luostarinen, A., Maliniemi, P., Keto, J., Arvas, M., Belt, H., Koponen, J., Loskog, A., Mustjoki, S., Porkka, K., Ylä-Herttuala, S., & Korhonen, M. (2017). Low interleukin-2 concentration favors generation of early memory T cells over effector phenotypes during chimeric antigen receptor T-cell expansion. *Cytotherapy*, *19*(6), 689–702. https://doi.org/10.1016/J.JCYT.2017.03.067

Kagoya, Y., Tanaka, S., Guo, T., Anczurowski, M., Wang, C. H., Saso, K., Butler, M. O., Minden, M. D., & Hirano, N. (2018). A novel chimeric antigen receptor containing a JAK–STAT signaling domain mediates superior antitumor effects. *Nature Medicine 2018 24:3*, *24*(3), 352–359. https://doi.org/10.1038/nm.4478

Kaneko, S., Mastaglio, S., Bondanza, A., Ponzoni, M., Sanvito, F., Aldrighetti, L., Radrizzani, M., Simona La, S. C., Provasi, E., Mondino, A., Nagasawa, T., Fleischhauer, K., Russo, V., Traversari, C., Ciceri, F., Bordignon, C., & Bonini, C. (2009). IL-7 and IL-15 allow the

generation of suicide gene-modified alloreactive self-renewing central memory human T lymphocytes. *Blood*, *113*(5), 1006–1015. https://doi.org/10.1182/BLOOD-2008-05-156059

Kaniecki, K., De Tullio, L., & Greene, E. C. (2017). A change of view: homologous recombination at single-molecule resolution. *Nature Reviews Genetics 2017 19:4*, *19*(4), 191–207. https://doi.org/10.1038/nrg.2017.92

Karches, C. H., Benmebarek, M. R., Schmidbauer, M. L., Kurzay, M., Klaus, R., Geiger, M., Rataj, F., Cadilha, B. L., Lesch, S., Heise, C., Murr, R., Vom Berg, J., Jastroch, M., Lamp, D., Ding, J., Duewell, P., Niederfellner, G., Sustmann, C., Endres, S., … Kobold, S. (2019). Bispecific antibodies enable synthetic agonistic receptor-transduced T cells for tumor immunotherapy. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *25*(19), 5890. https://doi.org/10.1158/1078-0432.CCR-18-3927

Karsi, A., Moav, B., Hackett, P., & Liu, Z. (2001). Effects of insert size on transposition efficiency of the sleeping beauty transposon in mouse cells. *Marine Biotechnology (New York, N.Y.)*, *3*(3), 241–245. https://doi.org/10.1007/S101260000072

Kaštánková, I., Štach, M., Žižková, H., Ptáčková, P., Šmilauerová, K., Mucha, M., Šroller, V., & Otáhal, P. (2021). Enzymatically produced piggyBac transposon vectors for efficient non-viral manufacturing of CD19-specific CAR T cells. *Molecular Therapy - Methods and Clinical Development*, *23*. https://doi.org/10.1016/j.omtm.2021.08.006

Kawalekar, O. U., O'Connor, R. S., Fraietta, J. A., Guo, L., McGettigan, S. E., Posey, A. D., Patel, P. R., Guedan, S., Scholler, J., Keith, B., Snyder, N., Blair, I., Milone, M. C., & June, C. H. (2016). Distinct Signaling of Coreceptors Regulates Specific Metabolism Pathways and Impacts Memory Development in CAR T Cells. *Immunity*, *44*(2), 380–390. https://doi.org/10.1016/J.IMMUNI.2016.01.021

Keegan, A. D., Leonard, W. J., & Zhu, J. (2021). Recent advances in understanding the role of IL-4 signaling. *Faculty Reviews*, *10*(71). https://doi.org/10.12703/R/10-71

Kieback, E., Charo, J., Sommermeyer, D., Blankenstein, T., & Uckert, W. (2008). A safeguard eliminates T cell receptor gene-modified autoreactive T cells after adoptive transfer. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(2), 623–628. https://doi.org/10.1073/PNAS.0710198105/SUPPL_FILE/10198FIG7.JPG

Kim, M. Y., Jayasinghe, R., Devenport, J. M., Ritchey, J. K., Rettig, M. P., O'Neal, J., Staser, K. W., Kennerly, K. M., Carter, A. J., Gao, F., Lee, B. H., Cooper, M. L., & DiPersio, J. F. (2022). A long-acting interleukin-7, rhIL-7-hyFc, enhances CAR T cell expansion, persistence, and anti-tumor activity. *Nature Communications 2022 13:1*, *13*(1), 1–17. https://doi.org/10.1038/s41467-022-30860-0

Kim, Y. G., Cha, J., & Chandrasegaran, S. (1996). Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proceedings of the National Academy of Sciences*, *93*(3), 1156–1160. https://doi.org/10.1073/PNAS.93.3.1156

Klaver, Y., van Steenbergen, S. C. L., Sleijfer, S., Debets, R., & Lamers, C. H. J. (2016). T cell maturation stage prior to and during GMP processing informs on CAR T cell expansion in patients. *Frontiers in Immunology*, *7*(DEC), 236018. https://doi.org/10.3389/FIMMU.2016.00648/BIBTEX

Klebanoff, C. A., Gattinoni, L., Torabi-Parizi, P., Kerstann, K., Cardones, A. R., Finkelstein, S. E., Palmer, D. C., Antony, P. A., Hwang, S. T., Rosenberg, S. A., Waldmann, T. A., & Restifo, N. P. (2005). Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. *Proceedings of the National Academy of Sciences*, *102*(27), 9571–9576. https://doi.org/10.1073/PNAS.0503726102

Klesmith, J. R., Su, L., Wu, L., Schrack, I. A., Dufort, F. J., Birt, A., Ambrose, C., Hackel, B. J., Lobb, R. R., & Rennert, P. D. (2019). Retargeting CD19 Chimeric Antigen Receptor T Cells via Engineered CD19-Fusion Proteins. *Molecular Pharmaceutics*, *16*(8), 3544–3558.

https://doi.org/10.1021/ACS.MOLPHARMACEUT.9B00418/SUPPL_FILE/MP9B00418_SI_00
1.PDF

Kloss, C. C., Lee, J., Zhang, A., Chen, F., Melenhorst, J. J., Lacey, S. F., Maus, M. V., Fraietta, J. A., Zhao, Y., & June, C. H. (2018). Dominant-Negative TGF-β Receptor Enhances PSMA-Targeted Human CAR T Cell Proliferation And Augments Prostate Cancer Eradication. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *26*(7), 1855–1866. https://doi.org/10.1016/J.YMTHE.2018.05.003

Kobelt, D., Schleef, M., Schmeer, M., Aumann, J., Schlag, P. M., & Walther, W. (2013). Performance of high quality minicircle DNA for in vitro and in vivo gene transfer. *Molecular Biotechnology*, *53*(1), 80–89. https://doi.org/10.1007/S12033-012-9535-6/FIGURES/6

Kobold, S., Grassmann, S., Chaloupka, M., Lampert, C., Wenk, S., Kraus, F., Rapp, M., Düwell, P., Zeng, Y., Schmollinger, J. C., Schnurr, M., Endres, S., & Rothenfußer, S. (2015). Impact of a New Fusion Receptor on PD-1–Mediated Immunosuppression in Adoptive T Cell Therapy. *JNCI Journal of the National Cancer Institute*, *107*(8), 146. https://doi.org/10.1093/JNCI/DJV146

Koga, A., Suzuki, M., Inagaki, H., Bessho, Y., & Hori, H. (1996). Transposable element in fish. *Nature*, *383*(6595), 30. https://doi.org/10.1038/383030A0

Kokalaki, E., Ma, B., Ferrari, M., Grothier, T., Hazelton, W., Manzoor, S., Costu, E., Taylor, J., Bulek, A., Srivastava, S., Gannon, I., Jha, R., Gealy, R., Stanczuk, L., Rizou, T., Robson, M., El-Kholy, M., Baldan, V., Righi, M., … Pule, M. (2023). Dual targeting of CD19 and CD22 against B-ALL using a novel high-sensitivity aCD22 CAR. *Molecular Therapy*, *31*(7), 2089–2104. https://doi.org/10.1016/J.YMTHE.2023.03.020

Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A., & Liu, D. R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*, *533*(7603), 420–424. https://doi.org/10.1038/NATURE17946

Koneru, M., Purdon, T. J., Spriggs, D., Koneru, S., & Brentjens, R. J. (2015). IL-12 secreting tumor-targeted chimeric antigen receptor T cells eradicate ovarian tumors in vivo. *OncoImmunology*, *4*(3), 1–11. https://doi.org/10.4161/2162402X.2014.994446

Koristka, S., Cartellieri, M., Arndt, C., Bippes, C. C., Feldmann, A., Michalk, I., Wiefel, K., Stamova, S., Schmitz, M., Ehninger, G., Bornhäuser, M., & Bachmann, M. (2013). Retargeting of regulatory T cells to surface-inducible autoantigen La/SS-B. *Journal of Autoimmunity*, *42*, 105–116. https://doi.org/10.1016/J.JAUT.2013.01.002

Koristka, S., Ziller-Walter, P., Bergmann, R., Arndt, C., Feldmann, A., Kegler, A., Cartellieri, M., Ehninger, A., Ehninger, G., Bornhäuser, M., & Bachmann, M. P. (2019). Anti-CAR-engineered T cells for epitope-based elimination of autologous CAR T cells. *Cancer Immunology, Immunotherapy*, *68*(9), 1401. https://doi.org/10.1007/S00262-019-02376-Y

Kosicki, M., Tomberg, K., & Bradley, A. (2018). Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. *Nature Biotechnology 2018 36:8*, *36*(8), 765–771. https://doi.org/10.1038/nbt.4192

Kosti, P., Opzoomer, J. W., Larios-Martinez, K. I., Henley-Smith, R., Scudamore, C. L., Okesola, M., Taher, M. Y. M., Davies, D. M., Muliaditan, T., Larcombe-Young, D., Woodman, N., Gillett, C. E., Thavaraj, S., Maher, J., & Arnold, J. N. (2021). Hypoxia-sensing CAR T cells provide safety and efficacy in treating solid tumors. *Cell Reports Medicine*, *2*(4), 100227. https://doi.org/10.1016/J.XCRM.2021.100227

Krishna, S., Lowery, F. J., Copeland, A. R., Bahadiroglu, E., Mukherjee, R., Jia, L., Anibal, J. T., Sachs, A., Adebola, S. O., Gurusamy, D., Yu, Z., Hill, V., Gartner, J. J., Li, Y. F., Parkhurst, M., Paria, B., Kvistborg, P., Kelly, M. C., Goff, S. L., … Rosenberg, S. A. (2020). Stem-like CD8 T cells mediate response of adoptive cell immunotherapy against human cancer. *Science*, *370*(6522), 1328–1334. https://doi.org/10.1126/SCIENCE.ABB9847

Kubin, M., Kamoun, M., & Trinchieri, G. (1994). Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *Journal of Experimental Medicine*, *180*(1), 211–222. https://doi.org/10.1084/JEM.180.1.211

Kudo, K., Imai, C., Lorenzini, P., Kamiya, T., Kono, K., Davidoff, A. M., Chng, W. J., & Campana, D. (2014). T lymphocytes expressing a CD16 signaling receptor exert antibody-dependent cancer cell killing. *Cancer Research*, *74*(1), 93–102. https://doi.org/10.1158/0008-5472.CAN-13-1365

Kueberuwa, G., Kalaitsidou, M., Cheadle, E., Hawkins, R. E., & Gilham, D. E. (2018). CD19 CAR T Cells Expressing IL-12 Eradicate Lymphoma in Fully Lymphoreplete Mice through Induction of Host Immunity. *Molecular Therapy - Oncolytics*, *8*, 41–51. https://doi.org/10.1016/J.OMTO.2017.12.003

Kumar, M., Keller, B., Makalou, N., & Sutton, R. E. (2001). Systematic determination of the packaging limit of lentiviral vectors. *Human Gene Therapy*, *12*(15), 1893–1905. https://doi.org/10.1089/104303401753153947

Kuwana, Y., Asakura, Y., Utsunomiya, N., Nakanishi, M., Arata, Y., Itoh, S., Nagase, F., & Kurosawa, Y. (1987). Expression of chimeric receptor composed of immunoglobulin-derived V resions and T-cell receptor-derived C regions. *Biochemical and Biophysical Research Communications*, *149*(3), 960–968. https://doi.org/10.1016/0006-291X(87)90502-X

Labbé, R. P., Vessillier, S., & Rafiq, Q. A. (2021). Lentiviral Vectors for T Cell Engineering: Clinical Applications, Bioprocessing and Future Perspectives. *Viruses*, *13*(8). https://doi.org/10.3390/V13081528

Lamers, C. H. J., Sleijfer, S., Vulto, A. G., Kruit, W. H. J., Kliffen, M., Debets, R., Gratama, J. W., Stoter, G., & Oosterwijk, E. (2006). Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *24*(13). https://doi.org/10.1200/JCO.2006.05.9964

Lamers, C. H. J., Van Steenbergen-Langeveld, S., Van Brakel, M., Groot-Van Ruijven, C. M., Van Elzakker, P. M. M. L., Van Krimpen, B., Sleijfer, S., & Debets, R. (2014). T Cell Receptor-Engineered T Cells to Treat Solid Tumors: T Cell Processing Toward Optimal T Cell Fitness. *Human Gene Therapy Methods*, *25*(6), 345. https://doi.org/10.1089/HGTB.2014.051

Landgraf, K. E., Williams, S. R., Steiger, D., Gebhart, D., Lok, S., Martin, D. W., Roybal, K. T., & Kim, K. C. (2020). convertibleCARs: A chimeric antigen receptor system for flexible control of activity and antigen targeting. *Communications Biology 2020 3:1*, *3*(1), 1–13. https://doi.org/10.1038/s42003-020-1021-2

Lécuroux, C., Girault, I., Urrutia, A., Doisne, J. M., Deveau, C., Goujard, C., Meyer, L., Sinet, M., & Venet, A. (2009). Identification of a particular HIV-specific CD8+ T-cell subset with a CD27+ CD45RO−/RA+ phenotype and memory characteristics after initiation of HAART during acute primary HIV infection. *Blood*, *113*(14), 3209–3217. https://doi.org/10.1182/BLOOD-2008-07-167601

Lee, E. H. J., Cullen, C., Murad, J. P., Gumber, D., Park, A. K., Yang, J., Stern, L. A., Adkins, L. N., Dhapola, G., Gittins, B., Chung-Chang, W., Martinez, C., Woo, Y., Cristea, M., Rodriguez-Rodriguez, L., Ishihara, J., Lee, J. K., Forman, S. J., Wang, L. D., & Priceman, S. J. (2023). Antigen-dependent IL-12 signaling in CAR T cells promotes regional to systemic disease targeting. *BioRxiv*, 2023.01.06.522784. https://doi.org/10.1101/2023.01.06.522784

Lee, Y. G., Chu, H., Lu, Y., Leamon, C. P., Srinivasarao, M., Putt, K. S., & Low, P. S. (2019). Regulation of CAR T cell-mediated cytokine release syndrome-like toxicity using low molecular weight adapters. *Nature Communications 2019 10:1*, *10*(1), 1–11. https://doi.org/10.1038/s41467-019-10565-7

Leen, A. M., Sukumaran, S., Watanabe, N., Mohammed, S., Keirnan, J., Yanagisawa, R., Anurathapan, U., Rendon, D., Heslop, H. E., Rooney, C. M., Brenner, M. K., & Vera, J. F.

(2014). Reversal of Tumor Immune Inhibition Using a Chimeric Cytokine Receptor. *Molecular Therapy*, *22*(6), 1211–1220. https://doi.org/10.1038/MT.2014.47

Lei, W., Zhao, A., Liu, H., Yang, C., Wei, C., Guo, S., Chen, Z., Guo, Q., Li, L., Zhao, M., Wu, G., Ouyang, G., Liu, M., Zhang, J., Gao, J., & Qian, W. (2024). Safety and feasibility of anti-CD19 CAR T cells expressing inducible IL-7 and CCL19 in patients with relapsed or refractory large B-cell lymphoma. *Cell Discovery 2023 10:1*, *10*(1), 1–14. https://doi.org/10.1038/s41421-023-00625-0

Leonard, W. J., Wan, C.-K., Young, H., Tangye, S. G., & Klein, C. (2016). IL-21 Signaling in Immunity. *F1000Research 2016 5:224*, *5*, 224. https://doi.org/10.12688/f1000research.7634.1

Leung, W. H., Gay, J., Martin, U., Garrett, T. E., Horton, H. M., Certo, M. T., Blazar, B. R., Morgan, R. A., Gregory, P. D., Jarjour, J., & Astrakhan, A. (2019). Sensitive and adaptable pharmacological control of CAR T cells through extracellular receptor dimerization. *JCI Insight*, *4*(11). https://doi.org/10.1172/JCI.INSIGHT.124430

Levine, B. L. (2015). Performance-enhancing drugs: design and production of redirected chimeric antigen receptor (CAR) T cells. *Cancer Gene Therapy 2015 22:2*, *22*(2), 79–84. https://doi.org/10.1038/cgt.2015.5

Levine, B. L., Bernstein, W. B., Connors, M., Craighead, N., Lindsten, T., Thompson, C. B., & June, C. H. (1997). Effects of CD28 costimulation on long-term proliferation of CD4+ T cells in the absence of exogenous feeder cells. *The Journal of Immunology*, *159*(12), 5921–5930. https://doi.org/10.4049/JIMMUNOL.159.12.5921

Lewis, P. F., & Emerman, M. (1994). Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *Journal of Virology*, *68*(1), 510–516. https://doi.org/10.1128/JVI.68.1.510-516.1994

Li, C., Mei, H., & Hu, Y. (2020). Applications and explorations of CRISPR/Cas9 in CAR T-cell therapy. *Briefings in Functional Genomics*, *19*(3), 175–182. https://doi.org/10.1093/BFGP/ELZ042

Li, G., Boucher, J. C., Kotani, H., Park, K., Zhang, Y., Shrestha, B., Wang, X., Guan, L., Beatty, N., Abate-Daga, D., & Davila, M. L. (2018). 4-1BB enhancement of CAR T function requires NF-κB and TRAFs. *JCI Insight*, *3*(18). https://doi.org/10.1172/JCI.INSIGHT.121322

Li, H., Yang, Y., Hong, W., Huang, M., Wu, M., & Zhao, X. (2020). Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. *Signal Transduction and Targeted Therapy 2020 5:1*, *5*(1), 1–23. https://doi.org/10.1038/s41392-019-0089-y

Li, K., Xu, J., Wang, J., Lu, C., Dai, Y., Dai, Q., Zhang, W., Xu, C., Wu, S., & Kang, Y. (2023). Dominant-negative transforming growth factor-β receptor-armoured mesothelin-targeted chimeric antigen receptor T cells slow tumour growth in a mouse model of ovarian cancer. *Cancer Immunology, Immunotherapy*, *72*(4), 917–928. https://doi.org/10.1007/S00262-022-03290-6/FIGURES/5

Li, L., Li, Q., Yan, Z. X., Sheng, L. S., Fu, D., Xu, P., Wang, L., & Zhao, W. L. (2022). Transgenic expression of IL-7 regulates CAR-T cell metabolism and enhances in vivo persistence against tumor cells. *Scientific Reports 2022 12:1*, *12*(1), 1–14. https://doi.org/10.1038/s41598-022-16616-2

Li, M. A., Turner, D. J., Ning, Z., Yusa, K., Liang, Q., Eckert, S., Rad, L., Fitzgerald, T. W., Craig, N. L., & Bradley, A. (2011). Mobilization of giant piggyBac transposons in the mouse genome. *Nucleic Acids Research*, *39*(22), e148. https://doi.org/10.1093/NAR/GKR764

Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A. K. L., & Flavell, R. A. (2006). Transforming growth factor-beta regulation of immune responses. *Annual Review of Immunology*, *24*, 99–146. https://doi.org/10.1146/ANNUREV.IMMUNOL.24.021605.090737

Li, R., Zhuang, Y., Han, M., Xu, T., & Wu, X. (2013). PiggyBac as a high-capacity transgenesis and gene-therapy vector in human cells and mice. *DMM Disease Models and Mechanisms*, *6*(3), 828–833. https://doi.org/10.1242/DMM.010827/-/DC1

Li, S., Siriwon, N., Zhang, X., Yang, S., Jin, T., He, F., Kim, Y. J., Mac, J., Lu, Z., Wang, S., Han, X., & Wang, P. (2017). Enhanced cancer immunotherapy by chimeric antigen receptor–modified T cells engineered to secrete checkpoint inhibitors. *Clinical Cancer Research*, *23*(22), 6982–6992. https://doi.org/10.1158/1078-0432.CCR-17-0867/116398/AM/ENHANCED-CANCER-IMMUNOTHERAPY-BY-CHIMERIC-ANTIGEN

Li, X., Harrell, R. A., Handler, A. M., Beam, T., Hennessy, K., & Fraser, M. J. (2005). piggyBac internal sequences are necessary for efficient transformation of target genomes. *Insect Molecular Biology*, *14*(1), 17–30. https://doi.org/10.1111/J.1365-2583.2004.00525.X

Liadi, I., Singh, H., Romain, G., Rey-Villamizar, N., Merouane, A., Adolacion, J. R. T., Kebriaei, P., Huls, H., Qiu, P., Roysam, B., Cooper, L. J. N., & Varadarajan, N. (2015). Individual motile CD4+ T cells can participate in efficient multikilling through conjugation to multiple tumor cells. *Cancer Immunology Research*, *3*(5), 473–482. https://doi.org/10.1158/2326-6066.CIR-14-0195/470299/AM/INDIVIDUAL-MOTILE-CD4-T-CELLS-CAN-PARTICIPATE-IN

Liao, W., Lin, J. X., & Leonard, W. J. (2013). Interleukin-2 at the Crossroads of Effector Responses, Tolerance, and Immunotherapy. *Immunity*, *38*(1), 13. https://doi.org/10.1016/J.IMMUNI.2013.01.004

Lin, J. X., & Leonard, W. J. (2018). The Common Cytokine Receptor γ Chain Family of Cytokines. *Cold Spring Harbor Perspectives in Biology*, *10*(9). https://doi.org/10.1101/CSHPERSPECT.A028449

Lin, R. J., Nager, A. R., Park, S., Sutton, J., Lay, C., Melton, Z., Zhang, Y., Boldajipour, B., Van Blarcom, T. J., Panowski, S. H., Sasu, B. J., & Chaparro-Riggers, J. (2022). Design and Validation of Inducible TurboCARs with Tunable Induction and Combinatorial Cytokine Signaling. *Cancer Immunology Research*, *10*(9), 1069–1083. https://doi.org/10.1158/2326-6066.CIR-21-0253/707272/AM/DESIGN-AND-VALIDATION-OF-INDUCIBLE-TURBOCARSTM

Lin, W. H. W., Adams, W. C. C., Nish, S. A. A., Chen, Y. H., Yen, B., Rothman, N. J. J., Kratchmarov, R., Okada, T., Klein, U., & Reiner, S. L. L. (2015). Asymmetric PI3K Signaling Driving Developmental and Regenerative Cell Fate Bifurcation. *Cell Reports*, *13*(10), 2203. https://doi.org/10.1016/J.CELREP.2015.10.072

Lindner, S. E., Johnson, S. M., Brown, C. E., & Wang, L. D. (2020). Chimeric antigen receptor signaling: Functional consequences and design implications. *Science Advances*, *6*(21). https://doi.org/10.1126/SCIADV.AAZ3223

Liu, S., Lizée, G., Lou, Y., Liu, C., Overwijk, W. W., Wang, G., & Hwu, P. (2007). IL-21 synergizes with IL-7 to augment expansion and anti-tumor function of cytotoxic T cells. *International Immunology*, *19*(10), 1213–1221. https://doi.org/10.1093/INTIMM/DXM093

Liu, X., Zhang, Y., Cheng, C., Cheng, A. W., Zhang, X., Li, N., Xia, C., Wei, X., Liu, X., & Wang, H. (2017). CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells. *Cell Research*, *27*(1), 154. https://doi.org/10.1038/CR.2016.142

Liu, X., Zhang, Y., Li, K., Liu, Y., Xu, J., Ma, J., An, L., Wang, H., & Chu, X. (2021). A novel dominant-negative PD-1 armored anti-CD19 CAR T cell is safe and effective against refractory/relapsed B cell lymphoma. *Translational Oncology*, *14*(7), 101085. https://doi.org/10.1016/J.TRANON.2021.101085

Liu, Y., Dang, Y., Zhang, C., Liu, L., Cai, W., Li, L., Fang, L., Wang, M., Xu, S., Wang, G., Zheng, J., & Li, H. (2024). IL-21-armored B7H3 CAR-iNKT cells exert potent antitumor effects. *IScience*, *27*(1), 108597. https://doi.org/10.1016/J.ISCI.2023.108597

Liu, Y., Di, S., Shi, B., Zhang, H., Wang, Y., Wu, X., Luo, H., Wang, H., Li, Z., & Jiang, H. (2019). Armored Inducible Expression of IL-12 Enhances Antitumor Activity of Glypican-3–Targeted Chimeric Antigen Receptor–Engineered T Cells in Hepatocellular Carcinoma. *The Journal of Immunology*, *203*(1), 198–207. https://doi.org/10.4049/JIMMUNOL.1800033

Lock, D., Monjezi, R., Brandes, C., Bates, S., Lennartz, S., Teppert, K., Gehrke, L., Karasakalidou-Seidt, R., Lukic, T., Schmeer, M., Schleef, M., Werchau, N., Eyrich, M., Assenmacher, M., Kaiser, A., Prommersberger, S., Schaser, T., & Hudecek, M. (2022). Automated, scaled, transposon-based production of CAR T cells. *Journal for Immunotherapy of Cancer*, *10*(9). https://doi.org/10.1136/JITC-2022-005189

Loew, R., Heinz, N., Hampf, M., Bujard, H., & Gossen, M. (2010). Improved Tet-responsive promoters with minimized background expression. *BMC Biotechnology*, *10*. https://doi.org/10.1186/1472-6750-10-81

Lohmueller, J. J., Ham, J. D., Kvorjak, M., & Finn, O. J. (2018). mSA2 affinity-enhanced biotin-binding CAR T cells for universal tumor targeting. *Oncoimmunology*, *7*(1). https://doi.org/10.1080/2162402X.2017.1368604

Long, A. H., Haso, W. M., Shern, J. F., Wanhainen, K. M., Murgai, M., Ingaramo, M., Smith, J. P., Walker, A. J., Kohler, M. E., Venkateshwara, V. R., Kaplan, R. N., Patterson, G. H., Fry, T. J., Orentas, R. J., & Mackall, C. L. (2015). 4-1BB Costimulation Ameliorates T Cell Exhaustion Induced by Tonic Signaling of Chimeric Antigen Receptors. *Nature Medicine*, *21*(6), 581. https://doi.org/10.1038/NM.3838

Loschinski, R., Böttcher, M., Stoll, A., Bruns, H., Mackensen, A., & Mougiakakos, D. (2018). IL-21 modulates memory and exhaustion phenotype of T-cells in a fatty acid oxidation-dependent manner. *Oncotarget*, *9*(17), 13125. https://doi.org/10.18632/ONCOTARGET.24442

Louis, C. U., Savoldo, B., Dotti, G., Pule, M., Yvon, E., Myers, G. D., Rossig, C., Russell, H. V., Diouf, O., Liu, E., Liu, H., Wu, M. F., Gee, A. P., Mei, Z., Rooney, C. M., Heslop, H. E., & Brenner, M. K. (2011). Antitumor activity and long-term fate of chimeric antigen receptor–positive T cells in patients with neuroblastoma. *Blood*, *118*(23), 6050–6056. https://doi.org/10.1182/BLOOD-2011-05-354449

Ludwig, J., & Hirschel, M. (2020). Methods and Process Optimization for Large-Scale CAR T Expansion Using the G-Rex Cell Culture Platform. *Methods in Molecular Biology*, *2086*, 165–177. https://doi.org/10.1007/978-1-0716-0146-4_12/FIGURES/3

Lugli, E., Dominguez, M. H., Gattinoni, L., Chattopadhyay, P. K., Bolton, D. L., Song, K., Klatt, N. R., Brenchley, J. M., Vaccari, M., Gostick, E., Price, D. A., Waldmann, T. A., Restifo, N. P., Franchini, G., & Roederer, M. (2013). Superior T memory stem cell persistence supports long-lived T cell memory. *The Journal of Clinical Investigation*, *123*(2), 594. https://doi.org/10.1172/JCI66327

Luke, J. M., Carnes, A. E., & Williams, J. A. (2014). Development of antibiotic-free selection system for safer DNA vaccination. *Methods in Molecular Biology*, *1143*, 91–111. https://doi.org/10.1007/978-1-4939-0410-5_6/FIGURES/3

Luo, G., Ivics, Z., Izsvák, Z., & Bradley, A. (1998). Chromosomal transposition of a Tc1/mariner-like element in mouse embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(18), 10769–10773. https://doi.org/10.1073/PNAS.95.18.10769

Luo, H., Su, J., Sun, R., Sun, Y., Wang, Y., Dong, Y., Shi, B., Jiang, H., & Li, Z. (2020). Coexpression of IL7 and CCL21 increases efficacy of CAR-T cells in solid tumors without requiring preconditioned lymphodepletion. *Clinical Cancer Research*, *26*(20), 5494–5505. https://doi.org/10.1158/1078-0432.CCR-20-0777/16115/AM/COEXPRESSION-OF-IL-7-AND-CCL21-INCREASES-EFFICACY

Luo, H., Wu, X., Sun, R., Su, J., Wang, Y., Dong, Y., Shi, B., Sun, Y., Jiang, H., & Li, Z. (2019). Target-Dependent Expression of IL12 by synNotch Receptor-Engineered NK92 Cells Increases

the Antitumor Activities of CAR-T Cells. *Frontiers in Oncology*, *9*, 1448. https://doi.org/10.3389/FONC.2019.01448/FULL

Luo, Y., Chen, Z., Sun, M., Li, B., Pan, F., Ma, A., Liao, J., Yin, T., Tang, X., Huang, G., Zhang, B., Pan, H., Zheng, M., & Cai, L. (2022). IL-12 nanochaperone-engineered CAR T cell for robust tumor-immunotherapy. *Biomaterials*, *281*. https://doi.org/10.1016/J.BIOMATERIALS.2021.121341

Luther, S. A., Bidgol, A., Hargreaves, D. C., Schmidt, A., Xu, Y., Paniyadi, J., Matloubian, M., & Cyster, J. G. (2002). Differing Activities of Homeostatic Chemokines CCL19, CCL21, and CXCL12 in Lymphocyte and Dendritic Cell Recruitment and Lymphoid Neogenesis. *The Journal of Immunology*, *169*(1), 424–433. https://doi.org/10.4049/JIMMUNOL.169.1.424

Ma, G., Shen, J., Pinz, K., Wada, M., Park, J., Kim, S., Togano, T., & Tse, W. (2019). Targeting T Cell Malignancies Using CD4CAR T-Cells and Implementing a Natural Safety Switch. *Stem Cell Reviews and Reports*, *15*(3), 443–447. https://doi.org/10.1007/S12015-019-09876-5/FIGURES/3

Ma, J. S. Y., Kim, J. Y., Kazane, S. A., Choi, S. H., Yun, H. Y., Kim, M. S., Rodgers, D. T., Pugh, H. M., Singer, O., Sun, S. B., Fonslow, B. R., Kochenderfer, J. N., Wright, T. M., Schultz, P. G., Young, T. S., Kim, C. H., & Cao, Y. (2016). Versatile strategy for controlling the specificity and activity of engineered T cells. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(4), E450–E458. https://doi.org/10.1073/PNAS.1524193113/-/DCSUPPLEMENTAL

Ma, X., Chow, J. M., Gri, G., Carra, G., Gerosa, F., Wolf, S. F., Dzialo, R., & Trinchieri, G. (1996). The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *The Journal of Experimental Medicine*, *183*(1), 147–157. https://doi.org/10.1084/JEM.183.1.147

MacKall, C. L., Fry, T. J., & Gress, R. E. (2011). Harnessing the biology of IL-7 for therapeutic application. *Nature Reviews. Immunology*, *11*(5), 330. https://doi.org/10.1038/NRI2970

Madison, B. B., Patil, D., Richter, M., Li, X., Tong, M., Cranert, S., Wang, X., Martin, R., Xi, H., Tan, Y., Weiss, L., Marquez, K., Coronella, J., Shedlock, D. J., & Ostertag, E. M. (2022). Cas-CLOVER is a novel high-fidelity nuclease for safe and robust generation of TSCM-enriched allogeneic CAR-T cells. *Molecular Therapy. Nucleic Acids*, *29*, 979. https://doi.org/10.1016/J.OMTN.2022.06.003

Magnani, C. F., Gaipa, G., Lussana, F., Belotti, D., Gritti, G., Napolitano, S., Matera, G., Cabiati, B., Buracchi, C., Borleri, G., Fazio, G., Zaninelli, S., Tettamanti, S., Cesana, S., Colombo, V., Quaroni, M., Cazzaniga, G., Rovelli, A., Biagi, E., … Biondi, A. (2020). Sleeping Beauty–engineered CAR T cells achieve antileukemic activity without severe toxicities. *The Journal of Clinical Investigation*, *130*(11), 6021. https://doi.org/10.1172/JCI138473

Maher, J., Brentjens, R. J., Gunset, G., Rivière, I., & Sadelain, M. (2002). Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRζ /CD28 receptor. *Nature Biotechnology 2002 20:1*, *20*(1), 70–75. https://doi.org/10.1038/nbt0102-70

Mahnke, Y. D., Brodie, T. M., Sallusto, F., Roederer, M., & Lugli, E. (2013). The who's who of T-cell differentiation: Human memory T-cell subsets. *European Journal of Immunology*, *43*(11), 2797–2809. https://doi.org/10.1002/EJI.201343751

Manetti, R., Parronchi, P., Giudizi, M. G., Piccinni, M. P., Maggi, E., Trinchieri, G., & Romagnani, S. (1993). Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *The Journal of Experimental Medicine*, *177*(4), 1199. https://doi.org/10.1084/JEM.177.4.1199

Manuri, P. V. R., Wilson, M. H., Maiti, S. N., Mi, T., Singh, H., Olivares, S., Dawson, M. J., Huls, H., Lee, D. A., Rao, P. H., Kaminski, J. M., Nakazawa, Y., Gottschalk, S., Kebriaei, P., Shpall, E. J., Champlin, R. E., & Cooper, L. J. N. (2010). piggyBac Transposon/Transposase System to

Generate CD19-Specific T Cells for the Treatment of B-Lineage Malignancies. *Human Gene Therapy*, *21*(4), 427. https://doi.org/10.1089/HUM.2009.114

Marin, V., Cribioli, E., Philip, B., Tettamanti, S., Pizzitola, I., Biondi, A., Biagi, E., & Pule, M. (2012). Comparison of different suicide-gene strategies for the safety improvement of genetically manipulated T cells. *Human Gene Therapy Methods*, *23*(6), 376–386. https://doi.org/10.1089/HGTB.2012.050

Marin, V., Pizzitola, I., Agostoni, V., Attianese, G. M. P. G., Finney, H., Lawson, A., Pule, M., Rousseau, R., Biondi, A., & Biagi, E. (2010). Cytokine-induced killer cells for cell therapy of acute myeloid leukemia: improvement of their immune activity by expression of CD33-specific chimeric receptors. *Haematologica*, *95*(12), 2144–2152. https://doi.org/10.3324/HAEMATOL.2010.026310

Markley, J. C., & Sadelain, M. (2010). IL-7 and IL-21 are superior to IL-2 and IL-15 in promoting human T cell–mediated rejection of systemic lymphoma in immunodeficient mice. *Blood*, *115*(17), 3508. https://doi.org/10.1182/BLOOD-2009-09-241398

Masopust, D., Vezys, V., Marzo, A. L., & Lefrançois, L. (2001). Preferential localization of effector memory cells in nonlymphoid tissue. *Science (New York, N.Y.)*, *291*(5512), 845–849. https://doi.org/10.1126/SCIENCE.1058867

Mata, M., Gerken, C., Nguyen, P., Krenciute, G., Spencer, D. M., & Gottschalk, S. (2017). Inducible Activation of MyD88 and CD40 in CAR T Cells Results in Controllable and Potent Antitumor Activity in Preclinical Solid Tumor Models. *Cancer Discovery*, *7*(11), 1306. https://doi.org/10.1158/2159-8290.CD-17-0263

Mátés, L., Chuah, M. K. L., Belay, E., Jerchow, B., Manoj, N., Acosta-Sanchez, A., Grzela, D. P., Schmitt, A., Becker, K., Matrai, J., Ma, L., Samara-Kuko, E., Gysemans, C., Pryputniewicz, D., Miskey, C., Fletcher, B., Vandendriessche, T., Ivics, Z., & Izsvák, Z. (2009). Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. *Nature Genetics*, *41*(6), 753–761. https://doi.org/10.1038/NG.343

Maus, M. V., Haas, A. R., Beatty, G. L., Albelda, S. M., Levine, B. L., Liu, X., Zhao, Y., Kalos, M., & June, C. H. (2013). T cells expressing chimeric antigen receptors can cause anaphylaxis in humans. *Cancer Immunology Research*, *1*(1), 26–31. https://doi.org/10.1158/2326-6066.CIR-13-0006/471028/P/T-CELLS-EXPRESSING-CHIMERIC-ANTIGEN-RECEPTORS-CAN

Meir, Y. J. J., Lin, A., Huang, M. F., Lin, J. R., Weirauch, M. T., Chou, H. C., Lin, S. J. A., & Wu, S. C. Y. (2013). A versatile, highly efficient, and potentially safer piggyBac transposon system for mammalian genome manipulations. *The FASEB Journal*, *27*(11), 4429–4443. https://doi.org/10.1096/FJ.12-223586

Meiraz, A., Garber, O. G., Harari, S., Hassin, D., & Berke, G. (2009). Switch from perforin-expressing to perforin-deficient CD8+ T cells accounts for two distinct types of effector cytotoxic T lymphocytes in vivo. *Immunology*, *128*(1), 69. https://doi.org/10.1111/J.1365-2567.2009.03072.X

Meng, Y., Sun, J., Hu, T., Ma, Y., Du, T., Kong, C., Zhang, G., Yu, T., & Piao, H. (2018). Rapid expansion in the WAVE bioreactor of clinical scale cells for tumor immunotherapy. *Human Vaccines & Immunotherapeutics*, *14*(10), 2516. https://doi.org/10.1080/21645515.2018.1480241

Meyran, D., Terry, R. L., Zhu, J. J., Haber, M., Ziegler, D. S., Ekert, P. G., Trapani, J. A., Darcy, P. K., & Neeson, P. J. (2021). Early-phenotype CAR-T cells for the treatment of pediatric cancers. *Annals of Oncology*, *32*(11), 1366–1380. https://doi.org/10.1016/J.ANNONC.2021.07.018

Micklethwaite, K. P., Gowrishankar, K., Gloss, B. S., Li, Z., Street, J. A., Moezzi, L., Mach, M. A., Sutrave, G., Clancy, L. E., Bishop, D. C., Louie, R. H. Y., Cai, C., Foox, J., MacKay, M., Sedlazeck, F. J., Blombery, P., Mason, C. E., Luciani, F., Gottlieb, D. J., & Blyth, E. (2021). Investigation of product-derived lymphoma following infusion of piggyBac-modified CD19

chimeric antigen receptor T cells. *Blood*, *138*(16), 1391–1405.
https://doi.org/10.1182/BLOOD.2021010858

Minagawa, K., Al-Obaidi, M., & Di Stasi, A. (2019). Generation of suicide gene-modified chimeric antigen receptor-redirected T-cells for cancer immunotherapy. *Methods in Molecular Biology*, *1895*, 57–73. https://doi.org/10.1007/978-1-4939-8922-5_5/FIGURES/6

Minutolo, N. G., Sharma, P., Poussin, M., Shaw, L. C., Brown, D. P., Hollander, E. E., Smole, A., Rodriguez-Garcia, A., Hui, J. Z., Zappala, F., Tsourkas, A., & Powell, D. J. (2020). Quantitative Control of Gene-Engineered T-Cell Activity through the Covalent Attachment of Targeting Ligands to a Universal Immune Receptor. *Journal of the American Chemical Society*, *142*(14), 6554–6568. https://doi.org/10.1021/JACS.9B11622/SUPPL_FILE/JA9B11622_SI_007.AVI

Mitsuyasu, R. T., Anton, P. A., Deeks, S. G., Scadden, D. T., Connick, E., Downs, M. T., Bakker, A., Roberts, M. R., June, C. H., Jalali, S., Lin, A. A., Pennathur-Das, R., & Hege, K. M. (2000). Prolonged survival and tissue trafficking following adoptive transfer of CD4ζ gene-modified autologous CD4+ and CD8+ T cells in human immunodeficiency virus–infected subjects. *Blood*, *96*(3), 785–793. https://doi.org/10.1182/BLOOD.V96.3.785

Mock, U., Nickolay, L., Philip, B., Cheung, G. W. K., Zhan, H., Johnston, I. C. D., Kaiser, A. D., Peggs, K., Pule, M., Thrasher, A. J., & Qasim, W. (2016). Automated manufacturing of chimeric antigen receptor T cells for adoptive immunotherapy using CliniMACS prodigy. *Cytotherapy*, *18*(8), 1002–1011. https://doi.org/10.1016/J.JCYT.2016.05.009

Moghimi, B., Muthugounder, S., Jambon, S., Tibbetts, R., Hung, L., Bassiri, H., Hogarty, M. D., Barrett, D. M., Shimada, H., & Asgharzadeh, S. (2021). Preclinical assessment of the efficacy and specificity of GD2-B7H3 SynNotch CAR-T in metastatic neuroblastoma. *Nature Communications*, *12*(1). https://doi.org/10.1038/S41467-020-20785-X

Mohammed, S., Sukumaran, S., Bajgain, P., Watanabe, N., Heslop, H. E., Rooney, C. M., Brenner, M. K., Fisher, W. E., Leen, A. M., & Vera, J. F. (2017). Improving Chimeric Antigen Receptor-Modified T Cell Function by Reversing the Immunosuppressive Tumor Microenvironment of Pancreatic Cancer. *Molecular Therapy*, *25*(1), 249–258. https://doi.org/10.1016/J.YMTHE.2016.10.016

Monjezi, R., Miskey, C., Gogishvili, T., Schleef, M., Schmeer, M., Einsele, H., Ivics, Z., & Hudecek, M. (2016). Enhanced CAR T-cell engineering using non-viral Sleeping Beauty transposition from minicircle vectors. *Leukemia 2017 31:1*, *31*(1), 186–194. https://doi.org/10.1038/leu.2016.180

Moretti, A., Ponzo, M., Nicolette, C. A., Tcherepanova, I. Y., Biondi, A., & Magnani, C. F. (2022). The Past, Present, and Future of Non-Viral CAR T Cells. *Frontiers in Immunology*, *13*, 867013. https://doi.org/10.3389/FIMMU.2022.867013/BIBTEX

Morgan, R. A., & Boyerinas, B. (2016). Genetic Modification of T Cells. *Biomedicines 2016, Vol. 4, Page 9*, *4*(2), 9. https://doi.org/10.3390/BIOMEDICINES4020009

Morgan, R. A., Yang, J. C., Kitano, M., Dudley, M. E., Laurencot, C. M., & Rosenberg, S. A. (2010). Case Report of a Serious Adverse Event Following the Administration of T Cells Transduced With a Chimeric Antigen Receptor Recognizing ERBB2. *Molecular Therapy*, *18*(4), 843–851. https://doi.org/10.1038/MT.2010.24

Morin, S. O., Giroux, V., Favre, C., Bechah, Y., Auphan-Anezin, N., Roncagalli, R., Mège, J. L., Olive, D., Malissen, M., & Nunès, J. A. (2015). In the absence of its cytosolic domain, the CD28 molecule still contributes to T cell activation. *Cellular and Molecular Life Sciences*, *72*(14), DOI 10.1007/s00018. https://doi.org/10.1007/S00018-015-1873-7

Morita, D., Nishio, N., Saito, S., Tanaka, M., Kawashima, N., Okuno, Y., Suzuki, S., Matsuda, K., Maeda, Y., Wilson, M. H., Dotti, G., Rooney, C. M., Takahashi, Y., & Nakazawa, Y. (2018). Enhanced Expression of Anti-CD19 Chimeric Antigen Receptor in piggyBac Transposon-

Engineered T Cells. *Molecular Therapy - Methods & Clinical Development*, *8*, 131–140. https://doi.org/10.1016/J.OMTM.2017.12.003

Moroz, A., Eppolito, C., Li, Q., Tao, J., Clegg, C. H., & Shrikant, P. A. (2004). IL-21 enhances and sustains CD8+ T cell responses to achieve durable tumor immunity: comparative evaluation of IL-2, IL-15, and IL-21. *Journal of Immunology (Baltimore, Md. : 1950)*, *173*(2), 900–909. https://doi.org/10.4049/JIMMUNOL.173.2.900

Moscou, M. J., & Bogdanove, A. J. (2009). A simple cipher governs DNA recognition by TAL effectors. *Science*, *326*(5959), 1501. https://doi.org/10.1126/SCIENCE.1178817/SUPPL_FILE/PAPV3.PDF

Mumm, J. B., Emmerich, J., Zhang, X., Chan, I., Wu, L., Mauze, S., Blaisdell, S., Basham, B., Dai, J., Grein, J., Sheppard, C., Hong, K., Cutler, C., Turner, S., LaFace, D., Kleinschek, M., Judo, M., Ayanoglu, G., Langowski, J., … Oft, M. (2011). IL-10 Elicits IFNγ-Dependent Tumor Immune Surveillance. *Cancer Cell*, *20*(6), 781–796. https://doi.org/10.1016/J.CCR.2011.11.003

Nakamura, K., Yagyu, S., Hirota, S., Tomida, A., Kondo, M., Shigeura, T., Hasegawa, A., Tanaka, M., & Nakazawa, Y. (2021). Autologous antigen-presenting cells efficiently expand piggyBac transposon CAR-T cells with predominant memory phenotype. *Molecular Therapy. Methods & Clinical Development*, *21*, 315–324. https://doi.org/10.1016/J.OMTM.2021.03.011

Nakazawa, Y., Huye, L. E., Dotti, G., Foster, A. E., Vera, J. F., Manuri, P. R., June, C. H., Rooney, C. M., & Wilson, M. H. (2009). Optimization of the PiggyBac Transposon System for the Sustained Genetic Modification of Human T-Lymphocytes. *Journal of Immunotherapy (Hagerstown, Md. : 1997)*, *32*(8), 826. https://doi.org/10.1097/CJI.0B013E3181AD762B

Nakazawa, Y., Matsuda, K., Kurata, T., Sueki, A., Tanaka, M., Sakashita, K., Imai, C., Wilson, M. H., & Koike, K. (2016). Anti-proliferative effects of T cells expressing a ligand-based chimeric antigen receptor against CD116 on CD34+ cells of juvenile myelomonocytic leukemia. *Journal of Hematology and Oncology*, *9*(1), 1–11. https://doi.org/10.1186/S13045-016-0256-3/FIGURES/6

Narayan, V., Barber-Rotenberg, J. S., Jung, I. Y., Lacey, S. F., Rech, A. J., Davis, M. M., Hwang, W. T., Lal, P., Carpenter, E. L., Maude, S. L., Plesa, G., Vapiwala, N., Chew, A., Moniak, M., Sebro, R. A., Farwell, M. D., Marshall, A., Gilmore, J., Lledo, L., … Haas, N. B. (2022). PSMA-targeting TGFβ-insensitive armored CAR T cells in metastatic castration-resistant prostate cancer: a phase 1 trial. *Nature Medicine 2022 28:4*, *28*(4), 724–734. https://doi.org/10.1038/s41591-022-01726-1

Narayanan, P., Lapteva, N., Seethammagari, M., Levitt, J. M., Slawin, K. M., & Spencer, D. M. (2011). A composite MyD88/CD40 switch synergistically activates mouse and human dendritic cells for enhanced antitumor efficacy. *The Journal of Clinical Investigation*, *121*(4), 1524. https://doi.org/10.1172/JCI44327

Nawaz, W., Huang, B., Xu, S., Li, Y., Zhu, L., Yiqiao, H., Wu, Z., & Wu, X. (2021). AAV-mediated in vivo CAR gene therapy for targeting human T-cell leukemia. *Blood Cancer Journal*, *11*(6). https://doi.org/10.1038/S41408-021-00508-1

Negishi, S., Girsch, J. H., Siegler, E. L., Bezerra, E. D., Miyao, K., & Sakemura, R. L. (2023). Treatment strategies for relapse after CAR T-cell therapy in B cell lymphoma. *Frontiers in Pediatrics*, *11*, 1305657. https://doi.org/10.3389/FPED.2023.1305657/BIBTEX

Nehlsen, K., Broll, S., Bode, J., & Article Kristina Nehlsen, R. (2006). Replicating minicircles: Generation of nonviral episomes for the efficient modification of dividing cells. *Gene Therapy and Molecular Biology*, *10*, 233–244. https://www.researchgate.net/publication/287566115

Newrzela, S., Cornils, K., Li, Z., Baum, C., Brugman, M. H., Hartmann, M., Meyer, J., Hartmann, S., Hansmann, M. L., Fehse, B., & Von Laer, D. (2008). Resistance of mature T cells to oncogene transformation. *Blood*, *112*(6), 2278–2286. https://doi.org/10.1182/BLOOD-2007-12-128751

Nguyen, N. T., Huang, K., Zeng, H., Jing, J., Wang, R., Fang, S., Chen, J., Liu, X., Huang, Z., You, M. J., Rao, A., Huang, Y., Han, G., & Zhou, Y. (2021). Nano-optogenetic engineering of CAR T cells for precision immunotherapy with enhanced safety. *Nature Nanotechnology*, *16*(12), 1424–1434. https://doi.org/10.1038/S41565-021-00982-5

Ni, J., Wangensteen, K. J., Nelsen, D., Balciunas, D., Skuster, K. J., Urban, M. D., & Ekker, S. C. (2016). Active recombinant Tol2 transposase for gene transfer and gene discovery applications. *Mobile DNA*, *7*(1). https://doi.org/10.1186/S13100-016-0062-Z

Nish, S. A., Zens, K. D., Kratchmarov, R., Lin, W. H. W., Adams, W. C., Chen, Y. H., Yen, B., Rothman, N. J., Bhandoola, A., Xue, H. H., Farber, D. L., & Reiner, S. L. (2017). CD4+ T cell effector commitment coupled to self-renewal by asymmetric cell divisions. *The Journal of Experimental Medicine*, *214*(1), 39. https://doi.org/10.1084/JEM.20161046

Nishio, N., Hanajiri, R., Ishikawa, Y., Murata, M., Taniguchi, R., Hamada, M., Nishikawa, E., Kawashima, N., Narita, A., Muramatsu, H., & Takahashi, Y. (2021). A Phase I Study of CD19 Chimeric Antigen Receptor-T Cells Generated By the PiggyBac Transposon Vector for Acute Lymphoblastic Leukemia. *Blood*, *138*(Supplement 1), 3831–3831. https://doi.org/10.1182/BLOOD-2021-150469

Noh, K. E., Lee, J. H., Choi, S. Y., Jung, N. C., Nam, J. H., Oh, J. S., Song, J. Y., Seo, H. G., Wang, Y., Lee, H. S., & Lim, D. S. (2021). TGF-β/IL-7 Chimeric Switch Receptor-Expressing CAR-T Cells Inhibit Recurrence of CD19-Positive B Cell Lymphoma. *International Journal of Molecular Sciences*, *22*(16). https://doi.org/10.3390/IJMS22168706

Northrop, J. P., Ullman, K. S., & Crabtree, G. R. (1993). Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells (NF-AT) complex. *Journal of Biological Chemistry*, *268*(4), 2917–2923. https://doi.org/10.1016/S0021-9258(18)53861-8

Numbenjapon, T., Serrano, L. M., Chang, W. C., Forman, S. J., Jensen, M. C., & Cooper, L. J. N. (2007). Antigen-independent and antigen-dependent methods to numerically expand CD19-specific CD8+ T cells. *Experimental Hematology*, *35*(7), 1083–1090. https://doi.org/10.1016/J.EXPHEM.2007.04.007

O'Cearbhaill, R. E., Park, J. H., Halton, E. F., Diamonte, C. R., Mead, E., Lakhman, Y., Kane, P., Riviere, I. C., & Brentjens, R. J. (2020). A phase I clinical trial of autologous chimeric antigen receptor (CAR) T cells genetically engineered to secrete IL-12 and to target the MUC16ecto antigen in patients (pts) with MUC16ecto+ recurrent high-grade serous ovarian cancer (HGSOC). *Gynecologic Oncology*, *159*, 42. https://doi.org/10.1016/j.ygyno.2020.06.089

O'Driscoll, M., & Jeggo, P. A. (2006). The role of double-strand break repair — insights from human genetics. *Nature Reviews Genetics 2006 7:1*, *7*(1), 45–54. https://doi.org/10.1038/nrg1746

Ogasawara, K., Lymp, J., Mack, T., Dell'Aringa, J., Huang, C. pin, Smith, J., Peiser, L., & Kostic, A. (2022). In Vivo Cellular Expansion of Lisocabtagene Maraleucel and Association With Efficacy and Safety in Relapsed/Refractory Large B-Cell Lymphoma. *Clinical Pharmacology and Therapeutics*, *112*(1), 81. https://doi.org/10.1002/CPT.2561

Oh, D. Y., & Fong, L. (2021). Cytotoxic CD4+ T cells in cancer: Expanding the immune effector toolbox. *Immunity*, *54*(12), 2701–2711. https://doi.org/10.1016/J.IMMUNI.2021.11.015

Orlando, E. J., Han, X., Tribouley, C., Wood, P. A., Leary, R. J., Riester, M., Levine, J. E., Qayed, M., Grupp, S. A., Boyer, M., De Moerloose, B., Nemecek, E. R., Bittencourt, H., Hiramatsu, H., Buechner, J., Davies, S. M., Verneris, M. R., Nguyen, K., Brogdon, J. L., … Winckler, W. (2018). Genetic mechanisms of target antigen loss in CAR19 therapy of acute lymphoblastic leukemia. *Nature Medicine*, *24*(10), 1504–1506. https://doi.org/10.1038/S41591-018-0146-Z

O'Rourke, D. M., Nasrallah, M. P., Desai, A., Melenhorst, J. J., Mansfield, K., Morrissette, J. J. D., Martinez-Lage, M., Brem, S., Maloney, E., Shen, A., Isaacs, R., Mohan, S., Plesa, G., Lacey, S. F., Navenot, J. M., Zheng, Z., Levine, B. L., Okada, H., June, C. H., … Maus, M. V. (2017). A

single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Science Translational Medicine*, *9*(399). https://doi.org/10.1126/SCITRANSLMED.AAA0984

Otten, G. R., & Germain, R. N. (1991). Split Anergy in a CD8+ T Cell: Receptor-dependent Cytolysis in the Absence of Interleukin-2 Production. *Science*, *251*(4998), 1228–1231. https://doi.org/10.1126/SCIENCE.1900952

Parayath, N. N., Stephan, S. B., Koehne, A. L., Nelson, P. S., & Stephan, M. T. (2020). In vitro-transcribed antigen receptor mRNA nanocarriers for transient expression in circulating T cells in vivo. *Nature Communications*, *11*(1). https://doi.org/10.1038/S41467-020-19486-2

Parkhurst, M. R., Yang, J. C., Langan, R. C., Dudley, M. E., Nathan, D. A. N., Feldman, S. A., Davis, J. L., Morgan, R. A., Merino, M. J., Sherry, R. M., Hughes, M. S., Kammula, U. S., Phan, G. Q., Lim, R. M., Wank, S. A., Restifo, N. P., Robbins, P. F., Laurencot, C. M., & Rosenberg, S. A. (2011). T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Molecular Therapy*, *19*(3), 620–626. https://doi.org/10.1038/mt.2010.272

Paszkiewicz, P. J., Fräßle, S. P., Srivastava, S., Sommermeyer, D., Hudecek, M., Drexler, I., Sadelain, M., Liu, L., Jensen, M. C., Riddell, S. R., & Busch, D. H. (2016). Targeted antibody-mediated depletion of murine CD19 CAR T cells permanently reverses B cell aplasia. *The Journal of Clinical Investigation*, *126*(11), 4262. https://doi.org/10.1172/JCI84813

Patrinostro, X., Hermanson, D., & Silaika, S. (2022). Accelerating cell therapy discovery & development with non-viral gene engineering. *Cell and Gene Therapy Insights*, *08*(08), 1023–1032. https://doi.org/10.18609/CGTI.2022.152

Pegram, H. J., Lee, J. C., Hayman, E. G., Imperato, G. H., Tedder, T. F., Sadelain, M., & Brentjens, R. J. (2012). Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning. *Blood*, *119*(18), 4133–4141. https://doi.org/10.1182/BLOOD-2011-12-400044

Peluso, I., Fantini, M. C., Fina, D., Caruso, R., Boirivant, M., MacDonald, T. T., Pallone, F., & Monteleone, G. (2007). IL-21 Counteracts the Regulatory T Cell-Mediated Suppression of Human CD4+ T Lymphocytes. *The Journal of Immunology*, *178*(2), 732–739. https://doi.org/10.4049/JIMMUNOL.178.2.732

Perkins, M. R., Grande, S., Hamel, A., Horton, H. M., Garrett, T. E., Miller, S. M., Latimer, H. J., Horvath, C. J., Kuczewski, M., Friedman, K. M., & Morgan, R. A. (2015). Manufacturing an Enhanced CAR T Cell Product By Inhibition of the PI3K/Akt Pathway During T Cell Expansion Results in Improved In Vivo Efficacy of Anti-BCMA CAR T Cells. *Blood*, *126*(23), 1893. https://doi.org/10.1182/BLOOD.V126.23.1893.1893

Pfeiffer, A., Thalheimer, F. B., Hartmann, S., Frank, A. M., Bender, R. R., Danisch, S., Costa, C., Wels, W. S., Modlich, U., Stripecke, R., Verhoeyen, E., & Buchholz, C. J. (2018). In vivo generation of human CD19-CAR T cells results in B-cell depletion and signs of cytokine release syndrome. *EMBO Molecular Medicine*, *10*(11). https://doi.org/10.15252/EMMM.201809158

Philip, B., Kokalaki, E., Mekkaoui, L., Thomas, S., Straathof, K., Flutter, B., Marin, V., Marafioti, T., Chakraverty, R., Linch, D., Quezada, S. A., Peggs, K. S., & Pule, M. (2014). A highly compact epitope-based marker/suicide gene for easier and safer T-cell therapy. *Blood*, *124*(8), 1277–1287. https://doi.org/10.1182/BLOOD-2014-01-545020

Ping, Y., Li, F., Nan, S., Zhang, D., Shi, X., Shan, J., & Zhang, Y. (2020). Augmenting the Effectiveness of CAR-T Cells by Enhanced Self-Delivery of PD-1-Neutralizing scFv. *Frontiers in Cell and Developmental Biology*, *8*. https://doi.org/10.3389/FCELL.2020.00803/FULL

Poirot, L., Philip, B., Schiffer-Mannioui, C., Le Clerre, D., Chion-Sotinel, I., Derniame, S., Potrel, P., Bas, C., Lemaire, L., Galetto, R., Lebuhotel, C., Eyquem, J., Cheung, G. W. K., Duclert, A., Gouble, A., Arnould, S., Peggs, K., Pule, M., Scharenberg, A. M., & Smith, J. (2015). Multiplex

genome-edited T-cell manufacturing platform for "off-the-shelf" adoptive T-cell immunotherapies. *Cancer Research*, *75*(18), 3853–3864. https://doi.org/10.1158/0008-5472.CAN-14-3321/651705/AM/MULTIPLEX-GENOME-EDITED-T-CELL-MANUFACTURING

Pomeroy, E. J., Lahr, W. S., Chang, J. W., Krueger, J., Wick, B. J., Slipek, N. J., Skeate, J. G., Webber, B. R., & Moriarity, B. S. (2021). Non-Viral Engineering of CAR-NK and CAR-T cells using the Tc Buster Transposon System™. *BioRxiv*, 2021.08.02.454772. https://doi.org/10.1101/2021.08.02.454772

Poorebrahim, M., Sadeghi, S., Fakhr, E., Abazari, M. F., Poortahmasebi, V., Kheirollahi, A., Askari, H., Rajabzadeh, A., Rastegarpanah, M., Linē, A., & Cid-Arregui, A. (2019). Production of CAR T-cells by GMP-grade lentiviral vectors: latest advances and future prospects. *Critical Reviews in Clinical Laboratory Sciences*, *56*(6), 393–419. https://doi.org/10.1080/10408363.2019.1633512

Porter, D. L., Hwang, W. T., Frey, N. V., Lacey, S. F., Shaw, P. A., Loren, A. W., Bagg, A., Marcucci, K. T., Shen, A., Gonzalez, V., Ambrose, D., Grupp, S. A., Chew, A., Zheng, Z., Milone, M. C., Levine, B. L., Melenhorst, J. J., & June, C. H. (2015). Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. *Science Translational Medicine*, *7*(303), 303ra139. https://doi.org/10.1126/SCITRANSLMED.AAC5415

Potter, K. N., Faulkner, P., & Mackinnon, E. A. (1976). Strain selection during serial passage of Trichoplusia in nuclear polyhedrosis virus. *Journal of Virology*, *18*(3), 1040. https://doi.org/10.1128/JVI.18.3.1040-1050.1976

Pouw, N., Treffers-Westerlaken, E., Mondino, A., Lamers, C., & Debets, R. (2010). TCR gene-engineered T cell: Limited T cell activation and combined use of IL-15 and IL-21 ensure minimal differentiation and maximal antigen-specificity. *Molecular Immunology*, *47*(7–8), 1411–1420. https://doi.org/10.1016/J.MOLIMM.2010.02.022

Prommersberger, S., Monjezi, R., Shankar, R., Schmeer, M., Hudecek, M., Ivics, Z., & Schleef, M. (2022). Minicircles for CAR T Cell Production by Sleeping Beauty Transposition: A Technological Overview. *Methods in Molecular Biology*, *2521*, 25–39. https://doi.org/10.1007/978-1-0716-2441-8_2/FIGURES/3

Prommersberger, S., Reiser, M., Beckmann, J., Danhof, S., Amberger, M., Quade-Lyssy, P., Einsele, H., Hudecek, M., Bonig, H., & Ivics, Z. (2021). CARAMBA: a first-in-human clinical trial with SLAMF7 CAR-T cells prepared by virus-free Sleeping Beauty gene transfer to treat multiple myeloma. *Gene Therapy 2021 28:9*, *28*(9), 560–571. https://doi.org/10.1038/s41434-021-00254-w

Prosser, M. E., Brown, C. E., Shami, A. F., Forman, S. J., & Jensen, M. C. (2012). Tumor PD-L1 co-stimulates primary human CD8+ cytotoxic T cells modified to express a PD1:CD28 chimeric receptor. *Molecular Immunology*, *51*(3–4), 263–272. https://doi.org/10.1016/J.MOLIMM.2012.03.023

Ptáčková, P., Musil, J., Štach, M., Lesný, P., Němečková, Š., Král, V., Fábry, M., & Otáhal, P. (2018). A new approach to CAR T-cell gene engineering and cultivation using piggyBac transposon in the presence of IL-4, IL-7 and IL-21. *Cytotherapy*, *20*(4). https://doi.org/10.1016/j.jcyt.2017.10.001

Pulè, M. A., Straathof, K. C., Dotti, G., Heslop, H. E., Rooney, C. M., & Brenner, M. K. (2005). A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. *Molecular Therapy*, *12*(5), 933–941. https://doi.org/10.1016/J.YMTHE.2005.04.016

Qin, H., Ramakrishna, S., Nguyen, S., Fountaine, T. J., Ponduri, A., Stetler-Stevenson, M., Yuan, C. M., Haso, W., Shern, J. F., Shah, N. N., & Fry, T. J. (2018). Preclinical Development of Bivalent Chimeric Antigen Receptors Targeting Both CD19 and CD22. *Molecular Therapy - Oncolytics*, *11*, 127–137. https://doi.org/10.1016/J.OMTO.2018.10.006

Qiu, Y., Wan, C. L., Xu, M. Z., Zhou, H. X., Liu, M. J., Gong, W. J., Kang, L. Q., Sun, A. N., Yu, L., Wu, D. P., Qian, C. S., & Xue, S. L. (2023). Safety and efficacy of CD22 and CD19 CAR-T bridging auto-HSCT as consolidation therapy for AYA and adult B-ALL. *Blood Cancer Journal 2023 13:1*, *13*(1), 1–4. https://doi.org/10.1038/s41408-023-00837-3

Querques, I., Mades, A., Zuliani, C., Miskey, C., Alb, M., Grueso, E., Machwirth, M., Rausch, T., Einsele, H., Ivics, Z., Hudecek, M., & Barabas, O. (2019). A highly soluble Sleeping Beauty transposase improves control of gene insertion. *Nature Biotechnology*, *37*(12), 1502. https://doi.org/10.1038/S41587-019-0291-Z

Rafiq, S., Yeku, O. O., Jackson, H. J., Purdon, T. J., van Leeuwen, D. G., Drakes, D. J., Song, M., Miele, M. M., Li, Z., Wang, P., Yan, S., Xiang, J., Ma, X., Seshan, V. E., Hendrickson, R. C., Liu, C., & Brentjens, R. J. (2018). Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy in vivo. *Nature Biotechnology*, *36*(9), 847. https://doi.org/10.1038/NBT.4195

Rajan, T. S., Gugliandolo, A., Bramanti, P., & Mazzon, E. (2020). In Vitro-Transcribed mRNA Chimeric Antigen Receptor T Cell (IVT mRNA CAR T) Therapy in Hematologic and Solid Tumor Management: A Preclinical Update. *International Journal of Molecular Sciences 2020, Vol. 21, Page 6514*, *21*(18), 6514. https://doi.org/10.3390/IJMS21186514

Ramírez-Chacón, A., Betriu-Méndez, S., Bartoló-Ibars, A., González, A., Martí, M., & Juan, M. (2022). Ligand-based CAR-T cell: Different strategies to drive T cells in future new treatments. *Frontiers in Immunology*, *13*, 932559. https://doi.org/10.3389/FIMMU.2022.932559/BIBTEX

Ramos, C. A., Rouce, R., Robertson, C. S., Reyna, A., Narala, N., Vyas, G., Mehta, B., Zhang, H., Dakhova, O., Carrum, G., Kamble, R. T., Gee, A. P., Mei, Z., Wu, M. F., Liu, H., Grilley, B., Rooney, C. M., Heslop, H. E., Brenner, M. K., … Dotti, G. (2018). In Vivo Fate and Activity of Second- versus Third-Generation CD19-Specific CAR-T Cells in B Cell Non-Hodgkin's Lymphomas. *Molecular Therapy*, *26*(12), 2727–2737. https://doi.org/10.1016/J.YMTHE.2018.09.009

Ramsay, A. G., Clear, A. J., Fatah, R., & Gribben, J. G. (2012). Multiple inhibitory ligands induce impaired T-cell immunologic synapse function in chronic lymphocytic leukemia that can be blocked with lenalidomide: establishing a reversible immune evasion mechanism in human cancer. *Blood*, *120*(7), 1412–1421. https://doi.org/10.1182/BLOOD-2012-02-411678

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols 2013 8:11*, *8*(11), 2281–2308. https://doi.org/10.1038/nprot.2013.143

Rataj, F., Jacobi, S. J., Stoiber, S., Asang, F., Ogonek, J., Tokarew, N., Cadilha, B. L., van Puijenbroek, E., Heise, C., Duewell, P., Endres, S., Klein, C., & Kobold, S. (2018). High-affinity CD16-polymorphism and Fc-engineered antibodies enable activity of CD16-chimeric antigen receptor-modified T cells for cancer therapy. *British Journal of Cancer 2018 120:1*, *120*(1), 79–87. https://doi.org/10.1038/s41416-018-0341-1

Ren, J., Liu, X., Fang, C., Jiang, S., June, C. H., & Zhao, Y. (2017). Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *23*(9), 2255. https://doi.org/10.1158/1078-0432.CCR-16-1300

Ren, J., Zhang, X., Liu, X., Fang, C., Jiang, S., June, C. H., & Zhao, Y. (2017). A versatile system for rapid multiplex genome-edited CAR T cell generation. *Oncotarget*, *8*(10), 17002. https://doi.org/10.18632/ONCOTARGET.15218

Rennert, P., Su, L., Dufort, F., Birt, A., Sanford, T., Wu, L., Ambrose, C., & Lobb, R. (2019). A Novel CD19-Anti-CD20 Bridging Protein Prevents and Reverses CD19-Negative Relapse from CAR19 T Cell Treatment In Vivo. *Blood*, *134*(Supplement_1), 252–252. https://doi.org/10.1182/BLOOD-2019-130654

Robertson, M. J., Kirkwood, J. M., Logan, T. F., Koch, K. M., Kathman, S., Kirby, L. C., Bell, W. N., Weisenbach, J., Thurmond, L. M., & Dar, M. M. (2008). A Dose-Escalation Study of Recombinant Human Interleukin-18 Using Two Different Schedules of Administration in Patients with Cancer. *Clinical Cancer Research*, *14*(11), 3462–3469. https://doi.org/10.1158/1078-0432.CCR-07-4740

Rodgers, D. T., Mazagova, M., Hampton, E. N., Cao, Y., Ramadoss, N. S., Hardy, I. R., Schulman, A., Du, J., Wang, F., Singer, O., Ma, J., Nunez, V., Shen, J., Woods, A. K., Wright, T. M., Schultz, P. G., Kim, C. H., & Young, T. S. (2016). Switch-mediated activation and retargeting of CAR-T cells for B-cell malignancies. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(4), E459–E468. https://doi.org/10.1073/PNAS.1524155113/-/DCSUPPLEMENTAL

Romeo, C., & Seed, B. (1991). Cellular immunity to HIV activated by CD4 fused to T cell or Fc receptor polypeptides. *Cell*, *64*(5), 1037–1046. https://doi.org/10.1016/0092-8674(91)90327-U

Romero, P., Zippelius, A., Kurth, I., Pittet, M. J., Touvrey, C., Iancu, E. M., Corthesy, P., Devevre, E., Speiser, D. E., & Rufer, N. (2007). Four Functionally Distinct Populations of Human Effector-Memory CD8+ T Lymphocytes. *The Journal of Immunology*, *178*(7), 4112–4119. https://doi.org/10.4049/JIMMUNOL.178.7.4112

Rosenberg, S. A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R. A., Moen, R., Karson, E. M., Lotze, M. T., Yang, J. C., Topalian, S. L., Merino, M. J., Culver, K., Miller, A. D., Blaese, R. M., & Anderson, W. F. (1990). Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *The New England Journal of Medicine*, *323*(9), 570–578. https://doi.org/10.1056/NEJM199008303230904

Rosenberg, S. A., Yang, J. C., Sherry, R. M., Kammula, U. S., Hughes, M. S., Phan, G. Q., Citrin, D. E., Restifo, N. P., Robbins, P. F., Wunderlich, J. R., Morton, K. E., Laurencot, C. M., Steinberg, S. M., White, D. E., & Dudley, M. E. (2011). Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clinical Cancer Research*, *17*(13), 4550–4557. https://doi.org/10.1158/1078-0432.CCR-11-0116/84227/AM/DURABLE-COMPLETE-RESPONSES-IN-HEAVILY-PRETREATED

Rostovskaya, M., Fu, J., Obst, M., Baer, I., Weidlich, S., Wang, H., Smith, A. J. H., Anastassiadis, K., & Francis Stewart, A. (2012). Transposon-mediated BAC transgenesis in human ES cells. *Nucleic Acids Research*, *40*(19), e150. https://doi.org/10.1093/NAR/GKS643

Rouet, P., Smih, F., & Jasin, M. (1994). Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proceedings of the National Academy of Sciences*, *91*(13), 6064–6068. https://doi.org/10.1073/PNAS.91.13.6064

Roybal, K. T., Rupp, L. J., Morsut, L., Walker, W. J., McNally, K. A., Park, J. S., & Lim, W. A. (2016). Precision Tumor Recognition by T Cells With Combinatorial Antigen Sensing Circuits. *Cell*, *164*(4), 770. https://doi.org/10.1016/J.CELL.2016.01.011

Ruella, M., Barrett, D. M., Kenderian, S. S., Shestova, O., Hofmann, T. J., Perazzelli, J., Klichinsky, M., Aikawa, V., Nazimuddin, F., Kozlowski, M., Scholler, J., Lacey, S. F., Melenhorst, J. J., Morrissette, J. J. D., Christian, D. A., Hunter, C. A., Kalos, M., Porter, D. L., June, C. H., … Gill, S. (2016). Dual CD19 and CD123 targeting prevents antigen-loss relapses after CD19-directed immunotherapies. *The Journal of Clinical Investigation*, *126*(10), 3814–3826. https://doi.org/10.1172/JCI87366

Ruella, M., Barrett, D. M., Shestova, O., Perazzelli, J., Posey, A. D., Hong, S. J., Kozlowski, M., Lacey, S. F., Melenhorst, J. J., June, C. H., & Gill, S. I. (2020). A cellular antidote to specifically deplete anti-CD19 chimeric antigen receptor–positive cells. *Blood*, *135*(7), 505. https://doi.org/10.1182/BLOOD.2019001859

Rupp, L. J., Schumann, K., Roybal, K. T., Gate, R. E., Ye, C. J., Lim, W. A., & Marson, A. (2017). CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric

antigen receptor T cells. *Scientific Reports 2017 7:1*, *7*(1), 1–10. https://doi.org/10.1038/s41598-017-00462-8

Rurik, J. G., Tombácz, I., Yadegari, A., Méndez Fernández, P. O., Shewale, S. V., Li, L., Kimura, T., Soliman, O. Y., Papp, T. E., Tam, Y. K., Mui, B. L., Albelda, S. M., Puré, E., June, C. H., Aghajanian, H., Weissman, D., Parhiz, H., & Epstein, J. A. (2022). CAR T cells produced in vivo to treat cardiac injury. *Science*, *375*(6576), 91–96. https://doi.org/10.1126/SCIENCE.ABM0594/SUPPL_FILE/SCIENCE.ABM0594_MDAR_REPRODUCIBILITY_CHECKLIST.PDF

Sabatino, M., Hu, J., Sommariva, M., Gautam, S., Fellowes, V., Hocker, J. D., Dougherty, S., Qin, H., Klebanoff, C. A., Fry, T. J., Gress, R. E., Kochenderfer, J. N., Stroncek, D. F., Ji, Y., & Gattinoni, L. (2016). Generation of clinical-grade CD19-specific CAR-modified CD8+ memory stem cells for the treatment of human B-cell malignancies. *Blood*, *128*(4), 519. https://doi.org/10.1182/BLOOD-2015-11-683847

Saito, S., Nakazawa, Y., Sueki, A., Matsuda, K., Tanaka, M., Yanagisawa, R., Maeda, Y., Sato, Y., Okabe, S., Inukai, T., Sugita, K., Wilson, M. H., Rooney, C. M., & Koike, K. (2014). Anti-leukemic potency of piggyBac-mediated CD19-specific T cells against refractory Philadelphia chromosome–positive acute lymphoblastic leukemia. *Cytotherapy*, *16*(9), 1257. https://doi.org/10.1016/J.JCYT.2014.05.022

Sakemura, R., Terakura, S., Watanabe, K., Julamanee, J., Takagi, E., Miyao, K., Koyama, D., Goto, T., Hanajiri, R., Nishida, T., Murata, M., & Kiyoi, H. (2016). A Tet-On Inducible System for Controlling CD19-Chimeric Antigen Receptor Expression upon Drug Administration. *Cancer Immunology Research*, *4*(8), 658–668. https://doi.org/10.1158/2326-6066.CIR-16-0043

Sallusto, F., Geginat, J., & Lanzavecchia, A. (2004). Central memory and effector memory T cell subsets: Function, generation, and maintenance. *Annual Review of Immunology*, *22*(Volume 22, 2004), 745–763. https://doi.org/10.1146/ANNUREV.IMMUNOL.22.012703.104702/CITE/REFWORKS

Salter, A. I., Ivey, R. G., Kennedy, J. J., Voillet, V., Rajan, A., Alderman, E. J., Voytovich, U. J., Lin, C., Sommermeyer, D., Liu, L., Whiteaker, J. R., Gottardo, R., Paulovich, A. G., & Riddell, S. R. (2018). Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function. *Science Signaling*, *11*(544). https://doi.org/10.1126/SCISIGNAL.AAT6753

Salzer, B., Schueller, C. M., Zajc, C. U., Peters, T., Schoeber, M. A., Kovacic, B., Buri, M. C., Lobner, E., Dushek, O., Huppa, J. B., Obinger, C., Putz, E. M., Holter, W., Traxlmayr, M. W., & Lehner, M. (2020). Engineering AvidCARs for combinatorial antigen recognition and reversible control of CAR function. *Nature Communications*, *11*(1). https://doi.org/10.1038/S41467-020-17970-3

Sandoval-Villegas, N., Nurieva, W., Amberger, M., & Ivics, Z. (2021). Contemporary Transposon Tools: A Review and Guide through Mechanisms and Applications of Sleeping Beauty, piggyBac and Tol2 for Genome Engineering. *International Journal of Molecular Sciences*, *22*(10). https://doi.org/10.3390/IJMS22105084

Santegoets, S. J. A. M., Turksma, A. W., Suhoski, M. M., Stam, A. G. M., Albelda, S. M., Hooijberg, E., Scheper, R. J., van den Eertwegh, A. J. M., Gerritsen, W. R., Powell, D. J., June, C. H., & de Gruijl, T. D. (2013). IL-21 promotes the expansion of CD27+CD28+ tumor infiltrating lymphocytes with high cytotoxic potential and low collateral expansion of regulatory T cells. *Journal of Translational Medicine*, *11*(1), 37. https://doi.org/10.1186/1479-5876-11-37

Sarikonda, G., Mathieu, M., Natalia, M., Pahuja, A., Xue, Q., Pierog, P. L., Trampont, P. C., Decman, V., Reynolds, S., Hanafi, L. A., Sun, Y. S., Eck, S., Hedrick, M. N., Stewart, J. J., Tangri, S., Litwin, V., & Dakappagari, N. (2021). Best practices for the development, analytical validation and clinical implementation of flow cytometric methods for chimeric antigen receptor T cell

analyses. *Cytometry Part B: Clinical Cytometry*, *100*(1), 79–91. https://doi.org/10.1002/CYTO.B.21985

Sato, T., Neschadim, A., Konrad, M., Fowler, D. H., Lavie, A., & Medin, J. A. (2007). Engineered Human tmpk/AZT As a Novel Enzyme/Prodrug Axis for Suicide Gene Therapy. *Molecular Therapy*, *15*(5), 962–970. https://doi.org/10.1038/MT.SJ.6300122

Schluns, K. S., Kieper, W. C., Jameson, S. C., & Lefrançois, L. (2000). Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo. *Nature Immunology 2000 1:5*, *1*(5), 426–432. https://doi.org/10.1038/80868

Schneider, D., Xiong, Y., Wu, D., Hu, P., Alabanza, L., Steimle, B., Mahmud, H., Anthony-Gonda, K., Krueger, W., Zhu, Z., Dimitrov, D. S., Orentas, R. J., & Dropulic, B. (2021). Trispecific CD19-CD20-CD22-targeting duoCAR-T cells eliminate antigen-heterogeneous B cell tumors in preclinical models. *Science Translational Medicine*, *13*(586). https://doi.org/10.1126/SCITRANSLMED.ABC6401/SUPPL_FILE/ABC6401_SM.PDF

Schneider, D., Xiong, Y., Wu, D., Nölle, V., Schmitz, S., Haso, W., Kaiser, A., Dropulic, B., & Orentas, R. J. (2017). A tandem CD19/CD20 CAR lentiviral vector drives on-target and off-target antigen modulation in leukemia cell lines. *Journal for Immunotherapy of Cancer*, *5*(1). https://doi.org/10.1186/S40425-017-0246-1

Schnell, A., Bod, L., Madi, A., & Kuchroo, V. K. (2020). The yin and yang of co-inhibitory receptors: toward anti-tumor immunity without autoimmunity. *Cell Research 2020 30:4*, *30*(4), 285–299. https://doi.org/10.1038/s41422-020-0277-x

Scholler, J., Brady, T. L., Binder-Scholl, G., Hwang, W. T., Plesa, G., Hege, K. M., Vogel, A. N., Kalos, M., Riley, J. L., Deeks, S. G., Mitsuyasu, R. T., Bernstein, W. B., Aronson, N. E., Levine, B. L., Bushman, F. D., & June, C. H. (2012). Decade-long safety and function of retroviral-modified chimeric antigen receptor T cells. *Science Translational Medicine*, *4*(132). https://doi.org/10.1126/SCITRANSLMED.3003761

Schwartz, R. H. (1990). A Cell Culture Model for T Lymphocyte Clonal Anergy. *Science*, *248*(4961), 1349–1356. https://doi.org/10.1126/SCIENCE.2113314

Shenoy, A. R., Kirschnek, S., & Häcker, G. (2014). IL-15 regulates Bcl-2 family members Bim and Mcl-1 through JAK/STAT and PI3K/AKT pathways in T cells. *European Journal of Immunology*, *44*(8), 2500–2507. https://doi.org/10.1002/EJI.201344238

Shin, J. H., Park, H. B., Oh, Y. M., Lim, D. P., Lee, J. E., Seo, H. H., Lee, S. J., Eom, H. S., Kim, I. H., Lee, S. H., & Choi, K. (2012). Positive conversion of negative signaling of CTLA4 potentiates antitumor efficacy of adoptive T-cell therapy in murine tumor models. *Blood*, *119*(24), 5678–5687. https://doi.org/10.1182/BLOOD-2011-09-380519

Shum, T., Omer, B., Tashiro, H., Kruse, R. L., Wagner, D. L., Parikh, K., Yi, Z., Sauer, T., Liu, D., Parihar, R., Castillo, P., Liu, H., Brenner, M. K., Metelitsa, L. S., Gottschalk, S., & Rooney, C. M. (2017). Constitutive signaling from an engineered IL7 receptor promotes durable tumor elimination by tumor-redirected T cells. *Cancer Discovery*, *7*(11), 1238–1247. https://doi.org/10.1158/2159-8290.CD-17-0538/333264/AM/CONSTITUTIVE-SIGNALING-FROM-AN-ENGINEERED-IL-7

Siegel, A. M., Heimall, J., Freeman, A. F., Hsu, A. P., Brittain, E., Brenchley, J. M., Douek, D. C., Fahle, G. H., Cohen, J. I., Holland, S. M., & Milner, J. D. (2011). A Critical Role for STAT3 Transcription Factor Signaling in the Development and Maintenance of Human T Cell Memory. *Immunity*, *35*(5), 806–818. https://doi.org/10.1016/J.IMMUNI.2011.09.016

Singh, H., Figliola, M. J., Dawson, M. J., Huls, H., Olivares, S., Switzer, K., Mi, T., Maiti, S., Kebriaei, P., Lee, D. A., Champlin, R. E., & Cooper, L. J. N. (2011). Reprogramming CD19-specific T cells with IL-21 signaling can improve adoptive immunotherapy of B-lineage malignancies. *Cancer Research*, *71*(10), 3516. https://doi.org/10.1158/0008-5472.CAN-10-3843

Singh, H., Srour, S. A., Milton, D. R., McCarty, J., Dai, C., Gaballa, M. R., Ammari, M., Olivares, S., Huls, H., De Groot, E., Marin, D., Petropoulos, D., Olson, A. L., Anderlini, P., Im, J. S., Khouri, I., Hosing, C. M., Rezvani, K., Champlin, R. E., … Kebriaei, P. (2022). Sleeping beauty generated CD19 CAR T-Cell therapy for advanced B-Cell hematological malignancies. *Frontiers in Immunology*, *13*, 1032397. https://doi.org/10.3389/FIMMU.2022.1032397/BIBTEX

Slovin, S. F., Dorff, T. B., Falchook, G. S., Wei, X. X., Gao, X., McKay, R. R., Oh, D. Y., Wibmer, A. G., Spear, M. A., McCaigue, J., Shedlock, D. J., Dhar, M., Coronella, J., Martin, C. E., Ghodussi, M., Murphy, A., & Ostertag, E. M. (2022). Phase 1 study of P-PSMA-101 CAR-T cells in patients with metastatic castration-resistant prostate cancer (mCRPC). *Https://Doi.Org/10.1200/JCO.2022.40.6_suppl.098*, *40*(6_suppl), 98–98. https://doi.org/10.1200/JCO.2022.40.6_SUPPL.098

Smith, T. T., Stephan, S. B., Moffett, H. F., McKnight, L. E., Ji, W., Reiman, D., Bonagofski, E., Wohlfahrt, M. E., Pillai, S. P. S., & Stephan, M. T. (2017). In situ programming of leukaemia-specific T cells using synthetic DNA nanocarriers. *Nature Nanotechnology*, *12*(8), 813–822. https://doi.org/10.1038/NNANO.2017.57

Sommermeyer, D., Hudecek, M., Kosasih, P. L., Gogishvili, T., Maloney, D. G., Turtle, C. J., & Riddell, S. R. (2015). Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity in vivo. *Leukemia 2016 30:2*, *30*(2), 492–500. https://doi.org/10.1038/leu.2015.247

Song, D. G., Ye, Q., Poussin, M., Harms, G. M., Figini, M., & Powell, D. J. (2012). CD27 costimulation augments the survival and antitumor activity of redirected human T cells in vivo. *Blood*, *119*(3), 696–706. https://doi.org/10.1182/BLOOD-2011-03-344275

Sotillo, E., Barrett, D. M., Black, K. L., Bagashev, A., Oldridge, D., Wu, G., Sussman, R., Lanauze, C., Ruella, M., Gazzara, M. R., Martinez, N. M., Harrington, C. T., Chung, E. Y., Perazzelli, J., Hofmann, T. J., Maude, S. L., Raman, P., Barrera, A., Gill, S., … Thomas-Tikhonenko, A. (2015). Convergence of Acquired Mutations and Alternative Splicing of CD19 Enables Resistance to CART-19 Immunotherapy. *Cancer Discovery*, *5*(12), 1282. https://doi.org/10.1158/2159-8290.CD-15-1020

Srivastava, S., Salter, A. I., Liggitt, D., Yechan-Gunja, S., Sarvothama, M., Cooper, K., Smythe, K. S., Dudakov, J. A., Pierce, R. H., Rader, C., & Riddell, S. R. (2019). Logic-gated ROR1 chimeric antigen receptor expression rescues T cell-mediated toxicity to normal tissues and enables selective tumor targeting. *Cancer Cell*, *35*(3), 489. https://doi.org/10.1016/J.CCELL.2019.02.003

Štach, M., Ptáčková, P., Mucha, M., Musil, J., Klener, P., & Otáhal, P. (2020). Inducible secretion of IL-21 augments anti-tumor activity of piggyBac-manufactured chimeric antigen receptor T cells. *Cytotherapy*, *22*(12). https://doi.org/10.1016/j.jcyt.2020.08.005

Štach, M., Pytlík, R., Šmilauerová, K., Rychlá, J., Mucha, M., Musil, J., Koladiya, A., Nemec, M., Petráčková, M., Kaštánková, I., Pecherková, P., Šrámková, L., Polgárová, K., Trněný, M., Lesný, P., Vydra, J., & Otáhal, P. (2023). Characterization of the input material quality for the production of tisagenlecleucel by multiparameter flow cytometry and its relation to the clinical outcome. *Pathology and Oncology Research*, *29*. https://doi.org/10.3389/PORE.2023.1610914/FULL

Stamova, S., Koristka, S., Keil, J., Arndt, C., Feldmann, A., Michalk, I., Bartsch, H., Bippes, C. C., Schmitz, M., Cartellieri, M., & Bachmann, M. (2012). Cancer Immunotherapy by Retargeting of Immune Effector Cells via Recombinant Bispecific Antibody Constructs. *Antibodies 2012, Vol. 1, Pages 172-198*, *1*(2), 172–198. https://doi.org/10.3390/ANTIB1020172

Stasi, A. Di, Tey, S.-K., Dotti, G., Fujita, Y., Kennedy-Nasser, A., Martinez, C., Straathof, K., Liu, E., Durett, A. G., Grilley, B., Liu, H., Cruz, C. R., Savoldo, B., Gee, A. P., Schindler, J., Krance, R. A., Heslop, H. E., Spencer, D. M., Rooney, C. M., & Brenner, M. K. (2011). Inducible Apoptosis as a Safety Switch for Adoptive Cell Therapy. *The New England Journal of Medicine*, *365*(18), 1673. https://doi.org/10.1056/NEJMOA1106152

Stavrou, M., Philip, B., Traynor-White, C., Davis, C. G., Onuoha, S., Cordoba, S., Thomas, S., & Pule, M. (2018). A Rapamycin-Activated Caspase 9-Based Suicide Gene. *Molecular Therapy*, *26*(5), 1266–1276. https://doi.org/10.1016/J.YMTHE.2018.03.001

Stepanov, A. V., Kalinin, R. S., Shipunova, V. O., Zhang, D., Xie, J., Rubtsov, Y. P., Ukrainskaya, V. M., Schulga, A., Konovalova, E. V., Volkov, D. V., Yaroshevich, I. A., Moysenovich, A. M., Belogurov, A. A., Zhang, H., Telegin, G. B., Chernov, A. S., Maschan, M. A., Terekhov, S. S., Wu, P., … Altman, S. (2022). Switchable targeting of solid tumors by BsCAR T cells. *Proceedings of the National Academy of Sciences of the United States of America*, *119*(46), e2210562119. https://doi.org/10.1073/PNAS.2210562119/SUPPL_FILE/PNAS.2210562119.SAPP.PDF

Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C., & Doudna, J. A. (2014). DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature 2014 507:7490*, *507*(7490), 62–67. https://doi.org/10.1038/nature13011

Sterner, R. M., Cox, M. J., Sakemura, R., & Kenderian, S. S. (2019). Using CRISPR/Cas9 to Knock Out GM-CSF in CAR-T Cells. *JoVE (Journal of Visualized Experiments)*, *2019*(149), e59629. https://doi.org/10.3791/59629

Stock, S., Benmebarek, M. R., Kluever, A. K., Darowski, D., Jost, C., Stubenrauch, K. G., Benz, J., Freimoser-Grundschober, A., Moessner, E., Umana, P., Subklewe, M., Endres, S., Klein, C., & Kobold, S. (2022). Chimeric antigen receptor T cells engineered to recognize the P329G-mutated Fc part of effector-silenced tumor antigen-targeting human IgG1 antibodies enable modular targeting of solid tumors. *Journal for Immunotherapy of Cancer*, *10*(7). https://doi.org/10.1136/JITC-2022-005054

Stock, S., Klüver, A. K., Fertig, L., Menkhoff, V. D., Subklewe, M., Endres, S., & Kobold, S. (2023). Mechanisms and strategies for safe chimeric antigen receptor T-cell activity control. *International Journal of Cancer*, *153*(10), 1706–1725. https://doi.org/10.1002/IJC.34635

Stock, S., Schmitt, M., & Sellner, L. (2019). Optimizing Manufacturing Protocols of Chimeric Antigen Receptor T Cells for Improved Anticancer Immunotherapy. *International Journal of Molecular Sciences*, *20*(24). https://doi.org/10.3390/IJMS20246223

Straathof, K. C., Pulè, M. A., Yotnda, P., Dotti, G., Vanin, E. F., Brenner, M. K., Heslop, H. E., Spencer, D. M., & Rooney, C. M. (2005). An inducible caspase 9 safety switch for T-cell therapy. *Blood*, *105*(11), 4247–4254. https://doi.org/10.1182/BLOOD-2004-11-4564

Strengell, M., Matikainen, S., Sirén, J., Lehtonen, A., Foster, D., Julkunen, I., & Sareneva, T. (2003). IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *170*(11), 5464–5469. https://doi.org/10.4049/JIMMUNOL.170.11.5464

Strzalkowski, T., Cadilha, B., Dalloul, I., Manske, K., Endres, S., & Kobold, S. (2022). 09.01 Armoring anti-HER2 CAR-T cells with C-C-motive receptor 8 (CCR8) and a dominant negative TGF-b receptor (DNR) to enable efficacy in solid tumor models. *Journal for ImmunoTherapy of Cancer*, *10*(Suppl 1), A3.2-A4. https://doi.org/10.1136/JITC-2022-ITOC9.6

Suarez, E. R., Chang, D. K., Sun, J., Sui, J., Freeman, G. J., Signoretti, S., Zhu, Q., & Marasco, W. A. (2016). Chimeric antigen receptor T cells secreting anti-PD-L1 antibodies more effectively regress renal cell carcinoma in a humanized mouse model. *Oncotarget*, *7*(23), 34341. https://doi.org/10.18632/ONCOTARGET.9114

Suhoski, M. M., Golovina, T. N., Aqui, N. A., Tai, V. C., Varela-Rohena, A., Milone, M. C., Carroll, R. G., Riley, J. L., & June, C. H. (2007). Engineering Artificial Antigen-presenting Cells to Express a Diverse Array of Co-stimulatory Molecules. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *15*(5), 981. https://doi.org/10.1038/MT.SJ.6300134

Sun, Y., Su, Y., Wang, Y., Liu, N., Li, Y., Chen, J., Qiao, Z., Niu, J., Hu, J., Zhang, B., Ning, H., & Hu, L. (2021). CD19 CAR-T Cells With Membrane-Bound IL-15 for B-Cell Acute

Lymphoblastic Leukemia After Failure of CD19 and CD22 CAR-T Cells: Case Report. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/FIMMU.2021.728962/FULL

Suster, M. L., Sumiyama, K., & Kawakami, K. (2009). Transposon-mediated BAC transgenesis in zebrafish and mice. *BMC Genomics*, *10*, 477. https://doi.org/10.1186/1471-2164-10-477

Svoboda, J., Gerson, J. N., Landsburg, D. J., Chong, E. A., Barta, S. K., Dwivedy Nasta, S., Ruella, M., Hexner, E. O., Marshall, A., Leskowitz, R., Four, M., Shea, J., Matlawski, T., Cervini, A., Davis, M. M., Gohil, M., Scholler, J., Hasenmayer, D., Rojas Amortegui, J., … June, C. H. (2022). Interleukin-18 Secreting Autologous Anti-CD19 CAR T-Cells (huCART19-IL18) in Patients with Non-Hodgkin Lymphomas Relapsed or Refractory to Prior CAR T-Cell Therapy. *Blood*, *140*(Supplement 1), 4612–4614. https://doi.org/10.1182/BLOOD-2022-162393

Swan, S. L., Mehta, N., Ilich, E., Shen, S. H., Wilkinson, D. S., Anderson, A. R., Segura, T., Sanchez-Perez, L., Sampson, J. H., & Bellamkonda, R. V. (2023). IL7 and IL7 Flt3L co-expressing CAR T cells improve therapeutic efficacy in mouse EGFRvIII heterogeneous glioblastoma. *Frontiers in Immunology*, *14*, 1085547. https://doi.org/10.3389/FIMMU.2023.1085547/BIBTEX

Tamada, K., Geng, D., Sakoda, Y., Bansal, N., Srivastava, R., Li, Z., & Davila, E. (2012). Redirecting gene-modified T cells toward various cancer types using tagged antibodies. *Clinical Cancer Research*, *18*(23), 6436–6445. https://doi.org/10.1158/1078-0432.CCR-12-1449/273520/AM/REDIRECTING-GENE-MODIFIED-T-CELLS-TOWARD-VARIOUS

Tamang, D. L., Redelman, D., Alves, B. N., Vollger, L., Bethley, C., & Hudig, D. (2006). Induction of granzyme B and T cell cytotoxic capacity by IL-2 or IL-15 without antigens: Multiclonal responses that are extremely lytic if triggered and short-lived after cytokine withdrawal. *Cytokine*, *36*(3–4), 148–159. https://doi.org/10.1016/J.CYTO.2006.11.008

Tammana, S., Huang, X., Wong, M., Milone, M. C., Ma, L., Levine, B. L., June, C. H., Wagner, J. E., Blazar, B. R., & Zhou, X. (2010). 4-1BB and CD28 Signaling Plays a Synergistic Role in Redirecting Umbilical Cord Blood T Cells Against B-Cell Malignancies. *Human Gene Therapy*, *21*(1), 75. https://doi.org/10.1089/HUM.2009.122

Tang, N., Cheng, C., Zhang, X., Qiao, M., Li, N., Mu, W., Wei, X. F., Han, W., & Wang, H. (2020). TGF-β inhibition via CRISPR promotes the long-term efficacy of CAR T cells against solid tumors. *JCI Insight*, *5*(4). https://doi.org/10.1172/JCI.INSIGHT.133977

Tanikawa, T., Wilke, C. M., Kryczek, I., Chen, G. Y., Kao, J., Núñez, G., & Zou, W. (2012). Interleukin-10 ablation promotes tumor development, growth, and metastasis. *Cancer Research*, *72*(2), 420–429. https://doi.org/10.1158/0008-5472.CAN-10-4627/649753/AM/INTERLEUKIN-IL-10-ABLATION-PROMOTES-TUMOR

Tasian, S. K., Kenderian, S. S., Shen, F., Li, Y., Ruella, M., Fix, W. C., Kozlowski, M., Carroll, M., Aplenc, R., June, C. H., Grupp, S. A., & Gill, S. I. (2015). Efficient Termination of CD123-Redirected Chimeric Antigen Receptor T Cells for Acute Myeloid Leukemia to Mitigate Toxicity. *Blood*, *126*(23), 565. https://doi.org/10.1182/BLOOD.V126.23.565.565

Tasian, S. K., Kenderian, S. S., Shen, F., Ruella, M., Shestova, O., Kozlowski, M., Li, Y., Schrank-Hacker, A., Morrissette, J. J. D., Carroll, M., June, C. H., Grupp, S. A., & Gill, S. (2017). Optimized depletion of chimeric antigen receptor T cells in murine xenograft models of human acute myeloid leukemia. *Blood*, *129*(17), 2395–2407. https://doi.org/10.1182/BLOOD-2016-08-736041

Teoh, J., & Brown, L. F. (2022). Developing lisocabtagene maraleucel chimeric antigen receptor T-cell manufacturing for improved process, product quality and consistency across CD19+ hematologic indications. *Cytotherapy*, *24*(9), 962–973. https://doi.org/10.1016/J.JCYT.2022.03.013

Thomis, D. C., Marktel, S., Bonini, C., Traversari, C., Gilman, M., Bordignon, C., & Clackson, T. (2001). A Fas-based suicide switch in human T cells for the treatment of graft-versus-host disease. *Blood*, *97*(5), 1249–1257. https://doi.org/10.1182/BLOOD.V97.5.1249

Tian, Y., & Zajac, A. J. (2016). IL-21 and T Cell Differentiation: Consider the Context. *Trends in Immunology*, *37*(8), 557–568. https://doi.org/10.1016/J.IT.2016.06.001

Till, B. G., Jensen, M. C., Wang, J., Qian, X., Gopal, A. K., Maloney, D. G., Lindgren, C. G., Lin, Y., Pagel, J. M., Budde, L. E., Raubitschek, A., Forman, S. J., Greenberg, P. D., Riddell, S. R., & Press, O. W. (2012). CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. *Blood*, *119*(17), 3940–3950. https://doi.org/10.1182/BLOOD-2011-10-387969

Tiraby, M., Cazaux, C., Baron, M., Drocourt, D., Reynes, J.-P., & Tiraby, G. (1998). Concomitant expression of E. coli cytosine deaminase and uracil phosphoribosyltransferase improves the cytotoxicity of 5-fluorocytosine. *FEMS Microbiology Letters*, *167*(1), 41–49. https://doi.org/10.1111/J.1574-6968.1998.TB13205.X

Todorovic, Z., Todorovic, D., Markovic, V., Ladjevac, N., Zdravkovic, N., Djurdjevic, P., Arsenijevic, N., Milovanovic, M., Arsenijevic, A., & Milovanovic, J. (2022). CAR T Cell Therapy for Chronic Lymphocytic Leukemia: Successes and Shortcomings. *Current Oncology 2022, Vol. 29, Pages 3647-3657*, *29*(5), 3647–3657. https://doi.org/10.3390/CURRONCOL29050293

Toffalori, C., Zito, L., Gambacorta, V., Riba, M., Oliveira, G., Bucci, G., Barcella, M., Spinelli, O., Greco, R., Crucitti, L., Cieri, N., Noviello, M., Manfredi, F., Montaldo, E., Ostuni, R., Naldini, M. M., Gentner, B., Waterhouse, M., Zeiser, R., … Vago, L. (2019). Immune signature drives leukemia escape and relapse after hematopoietic cell transplantation. *Nature Medicine 2019 25:4*, *25*(4), 603–611. https://doi.org/10.1038/s41591-019-0400-z

Tokarew, N., Ogonek, J., Endres, S., von Bergwelt-Baildon, M., & Kobold, S. (2018). Teaching an old dog new tricks: next-generation CAR T cells. *British Journal of Cancer 2018 120:1*, *120*(1), 26–37. https://doi.org/10.1038/s41416-018-0325-1

Tokatlian, T., Asuelime, G. E., Mock, J. Y., Diandreth, B., Sharma, S., Warshaviak, D. T., Daris, M. E., Bolanos, K., Luna, B. L., Naradikian, M. S., Deshmukh, K., Hamburger, A. E., & Kamb, A. (2022). Mesothelin-specific CAR-T cell therapy that incorporates an HLA-gated safety mechanism selectively kills tumor cells. *Journal for ImmunoTherapy of Cancer*, *10*(1), e003826. https://doi.org/10.1136/JITC-2021-003826

Torikai, H., Reik, A., Liu, P. Q., Zhou, Y., Zhang, L., Maiti, S., Huls, H., Miller, J. C., Kebriaei, P., Rabinovitch, B., Lee, D. A., Champlin, R. E., Bonini, C., Naldini, L., Rebar, E. J., Gregory, P. D., Holmes, M. C., & Cooper, L. J. N. (2012). A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. *Blood*, *119*(24), 5697. https://doi.org/10.1182/BLOOD-2012-01-405365

Torikai, H., Reik, A., Soldner, F., Warren, E. H., Yuen, C., Zhou, Y., Crossland, D. L., Huls, H., Littman, N., Zhang, Z., Tykodi, S. S., Kebriaei, P., Lee, D. A., Miller, J. C., Rebar, E. J., Holmes, M. C., Jaenisch, R., Champlin, R. E., Gregory, P. D., & Cooper, L. J. N. (2013). Toward eliminating HLA class I expression to generate universal cells from allogeneic donors. *Blood*, *122*(8), 1341. https://doi.org/10.1182/BLOOD-2013-03-478255

Tsukahara, T., Iwase, N., Kawakami, K., Iwasaki, M., Yamamoto, C., Ohmine, K., Uchibori, R., Teruya, T., Ido, H., Saga, Y., Urabe, M., Mizukami, H., Kume, A., Nakamura, M., Brentjens, R., & Ozawa, K. (2015). The Tol2 transposon system mediates the genetic engineering of T-cells with CD19-specific chimeric antigen receptors for B-cell malignancies. *Gene Therapy*, *22*(2), 209. https://doi.org/10.1038/GT.2014.104

Urasaki, A., Morvan, G., & Kawakami, K. (2006). Functional Dissection of the Tol2 Transposable Element Identified the Minimal cis-Sequence and a Highly Repetitive Sequence in the Subterminal Region Essential for Transposition. *Genetics*, *174*(2), 639–649. https://doi.org/10.1534/GENETICS.106.060244

Urbanska, K., Lanitis, E., Poussin, M., Lynn, R. C., Gavin, B. P., Kelderman, S., Yu, J., Scholler, N., & Powell, D. J. (2012). A universal strategy for adoptive immunotherapy of cancer through use

of a novel T cell antigen receptor. *Cancer Research*, *72*(7), 1844. https://doi.org/10.1158/0008-5472.CAN-11-3890

Urbanska, K., Lynn, R. C., Stashwick, C., Thakur, A., Lum, L. G., & Powell, D. J. (2014). Targeted cancer immunotherapy via combination of designer bispecific antibody and novel gene-engineered T cells. *Journal of Translational Medicine*, *12*(1). https://doi.org/10.1186/S12967-014-0347-2

Van Bruggen, J. A. C., Martens, A. W. J., Fraietta, J. A., Hofland, T., Tonino, S. H., Eldering, E., Levin, M. D., Siska, P. J., Endstra, S., Rathmell, J. C., June, C. H., Porter, D. L., Melenhorst, J. J., Kater, A. P., & Van Der Windt, G. J. W. (2019). Chronic lymphocytic leukemia cells impair mitochondrial fitness in CD8+ T cells and impede CAR T-cell efficacy. *Blood*, *134*(1), 44–58. https://doi.org/10.1182/BLOOD.2018885863

Van Den Broek, T., Borghans, J. A. M., & Van Wijk, F. (2018). The full spectrum of human naive T cells. *Nature Reviews Immunology 2018 18:6*, *18*(6), 363–373. https://doi.org/10.1038/s41577-018-0001-y

Van Der Stegen, S. J. C., Hamieh, M., & Sadelain, M. (2015). The pharmacology of second-generation chimeric antigen receptors. *Nature Reviews. Drug Discovery*, *14*(7), 499. https://doi.org/10.1038/NRD4597

Vella, A., Teague, T. K., Ihle, J., Kappler, J., & Marrack, P. (1997). Interleukin 4 (IL-4) or IL-7 Prevents the Death of Resting T Cells: Stat6 Is Probably Not Required for the Effect of IL-4. *The Journal of Experimental Medicine*, *186*(2), 325. https://doi.org/10.1084/JEM.186.2.325

Vicari, A. P., Ait-Yahia, S., Chemin, K., Mueller, A., Zlotnik, A., & Caux, C. (2000). Antitumor Effects of the Mouse Chemokine 6Ckine/SLC Through Angiostatic and Immunological Mechanisms. *The Journal of Immunology*, *165*(4), 1992–2000. https://doi.org/10.4049/JIMMUNOL.165.4.1992

Vigdal, T. J., Kaufman, C. D., Izsvák, Z., Voytas, D. F., & Ivics, Z. (2002). Common physical properties of DNA affecting target site selection of Sleeping Beauty and other Tc1/mariner transposable elements. *Journal of Molecular Biology*, *323*(3), 441–452. https://doi.org/10.1016/S0022-2836(02)00991-9

Vlachonikola, E., Stamatopoulos, K., & Chatzidimitriou, A. (2021). T Cells in Chronic Lymphocytic Leukemia: A Two-Edged Sword. *Frontiers in Immunology*, *11*, 612244. https://doi.org/10.3389/FIMMU.2020.612244/BIBTEX

Vogler, I., Newrzela, S., Hartmann, S., Schneider, N., Von Laer, D., Koehl, U., & Grez, M. (2010). An Improved Bicistronic CD20/tCD34 Vector for Efficient Purification and In Vivo Depletion of Gene-Modified T Cells for Adoptive Immunotherapy. *Molecular Therapy*, *18*(7), 1330. https://doi.org/10.1038/MT.2010.83

Voigt, F., Wiedemann, L., Zuliani, C., Querques, I., Sebe, A., Mátés, L., Izsvák, Z., Ivics, Z., & Barabas, O. (2016). Sleeping Beauty transposase structure allows rational design of hyperactive variants for genetic engineering. *Nature Communications*, *7*. https://doi.org/10.1038/NCOMMS11126

Vormittag, P., Gunn, R., Ghorashian, S., & Veraitch, F. S. (2018). A guide to manufacturing CAR T cell therapies. *Current Opinion in Biotechnology*, *53*, 164–181. https://doi.org/10.1016/J.COPBIO.2018.01.025

Wagner, D. L., Koehl, U., Chmielewski, M., Scheid, C., & Stripecke, R. (2022). Review: Sustainable Clinical Development of CAR-T Cells – Switching From Viral Transduction Towards CRISPR-Cas Gene Editing. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/FIMMU.2022.865424

Waldmann, T. A. (2006). The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nature Reviews Immunology 2006 6:8*, *6*(8), 595–601. https://doi.org/10.1038/nri1901

Walker, R. E., Bechtel, C. M., Natarajan, V., Baseler, M., Hege, K. M., Metcalf, J. A., Stevens, R., Hazen, A., Blaese, R. M., Chen, C. C., Leitman, S. F., Palensky, J., Wittes, J., Davey, R. T., Falloon, J., Polis, M. A., Kovacs, J. A., Broad, D. F., Levine, B. L., … Clifford Lane, H. (2000). *Long-term in vivo survival of receptor-modified syngeneic T cells in patients with human immunodeficiency virus infection*. *96*, 467–474.

Wang, H., Wang, X., Ye, X., Ju, Y., Cao, N., Wang, S., & Cai, J. (2022). Nonviral mcDNA-mediated bispecific CAR T cells kill tumor cells in an experimental mouse model of hepatocellular carcinoma. *BMC Cancer*, *22*(1), 1–18. https://doi.org/10.1186/S12885-022-09861-1/FIGURES/7

Wang, M., Munoz, J., Goy, A., Locke, F. L., Jacobson, C. A., Hill, B. T., Timmerman, J. M., Holmes, H., Jaglowski, S., Flinn, I. W., McSweeney, P. A., Miklos, D. B., Pagel, J. M., Kersten, M. J., Milpied, N., Fung, H., Topp, M. S., Houot, R., Beitinjaneh, A., … Reagan, P. M. (2020). KTE-X19 CAR T-Cell Therapy in Relapsed or Refractory Mantle-Cell Lymphoma. *The New England Journal of Medicine*, *382*(14), 1331. https://doi.org/10.1056/NEJMOA1914347

Wang, T., Tang, Y., Cai, J., Wan, X., Hu, S., Lu, X., Xie, Z., Qiao, X., Jiang, H., Shao, J., Yang, F., Ren, H., Cao, Q., Qian, J., Zhang, J., An, K., Wang, J., Luo, C., Liang, H., … Pui, C. H. (2023). Coadministration of CD19- and CD22-Directed Chimeric Antigen Receptor T-Cell Therapy in Childhood B-Cell Acute Lymphoblastic Leukemia: A Single-Arm, Multicenter, Phase II Trial. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *41*(9), 1670–1683. https://doi.org/10.1200/JCO.22.01214

Wang, X., Chang, W. C., Wong, C. L. W., Colcher, D., Sherman, M., Ostberg, J. R., Forman, S. J., Riddell, S. R., & Jensen, M. C. (2011). A transgene-encoded cell surface polypeptide for selection, in vivo tracking, and ablation of engineered cells. *Blood*, *118*(5), 1255. https://doi.org/10.1182/BLOOD-2011-02-337360

Wang, Y., Jiang, H., Luo, H., Sun, Y., Shi, B., Sun, R., & Li, Z. (2019). An IL-4/21 Inverted Cytokine Receptor Improving CAR-T Cell Potency in Immunosuppressive Solid-Tumor Microenvironment. *Frontiers in Immunology*, *10*, 1691. https://doi.org/10.3389/FIMMU.2019.01691/BIBTEX

Wang, Y., Tong, C., Lu, Y., Wu, Z., Guo, Y., Liu, Y., Wei, J., Wang, C., Yang, Q., & Han, W. (2023). Characteristics of premanufacture CD8+ T cells determine CAR-T efficacy in patients with diffuse large B-cell lymphoma. *Signal Transduction and Targeted Therapy 2023 8:1*, *8*(1), 1–11. https://doi.org/10.1038/s41392-023-01659-2

Watanabe, N., Bajgain, P., Sukumaran, S., Ansari, S., Heslop, H. E., Rooney, C. M., Brenner, M. K., Leen, A. M., & Vera, J. F. (2016). Fine-tuning the CAR spacer improves T-cell potency. *Oncoimmunology*, *5*(12). https://doi.org/10.1080/2162402X.2016.1253656

Watts, T. H. (2005). TNF/TNFR family members in costimulation of T cell responses. *Annual Review of Immunology*, *23*, 23–68. https://doi.org/10.1146/ANNUREV.IMMUNOL.23.021704.115839

Webber, B. R., Lonetree, C. lin, Kluesner, M. G., Johnson, M. J., Pomeroy, E. J., Diers, M. D., Lahr, W. S., Draper, G. M., Slipek, N. J., Smeester, B. S., Lovendahl, K. N., McElroy, A. N., Gordon, W. R., Osborn, M. J., & Moriarity, B. S. (2019). Highly efficient multiplex human T cell engineering without double-strand breaks using Cas9 base editors. *Nature Communications 2019 10:1*, *10*(1), 1–10. https://doi.org/10.1038/s41467-019-13007-6

Wei, W., Chen, Z. N., & Wang, K. (2023). CRISPR/Cas9: A Powerful Strategy to Improve CAR-T Cell Persistence. *International Journal of Molecular Sciences*, *24*(15). https://doi.org/10.3390/IJMS241512317

Wen, W., Song, S., Han, Y., Chen, H., Liu, X., & Qian, Q. (2020). An efficient Screening System in Yeast to Select a Hyperactive piggyBac Transposase for Mammalian Applications. *International Journal of Molecular Sciences*, *21*(9). https://doi.org/10.3390/IJMS21093064

Wilber, A., Frandsen, J. L., Geurts, J. L., Largaespada, D. A., Hackett, P. B., & McIvor, R. S. (2006). RNA as a source of transposase for Sleeping Beauty-mediated gene insertion and expression in

somatic cells and tissues. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *13*(3), 625–630. https://doi.org/10.1016/J.YMTHE.2005.10.014

Williams, J. Z., Allen, G. M., Shah, D., Sterin, I. S., Kim, K. H., Garcia, V. P., Shavey, G. E., Yu, W., Puig-Saus, C., Tsoi, J., Ribas, A., Roybal, K. T., & Lim, W. A. (2020). Precise T cell recognition programs designed by transcriptionally linking multiple receptors. *Science (New York, N.Y.)*, *370*(6520), 1099. https://doi.org/10.1126/SCIENCE.ABC6270

Willingham, S. B., Volkmer, J. P., Gentles, A. J., Sahoo, D., Dalerba, P., Mitra, S. S., Wang, J., Contreras-Trujillo, H., Martin, R., Cohen, J. D., Lovelace, P., Scheeren, F. A., Chao, M. P., Weiskopf, K., Tang, C., Volkmer, A. K., Naik, T. J., Storm, T. A., Mosley, A. R., … Weissman, I. L. (2012). The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(17), 6662–6667. https://doi.org/10.1073/PNAS.1121623109/-/DCSUPPLEMENTAL

Wilson, M. H., Coates, C. J., & George, A. L. (2007). PiggyBac Transposon-mediated Gene Transfer in Human Cells. *Molecular Therapy*, *15*(1), 139–145. https://doi.org/10.1038/SJ.MT.6300028

Wu, C. Y., Roybal, K. T., Puchner, E. M., Onuffer, J., & Lim, W. A. (2015). Remote control of therapeutic T cells through a small molecule-gated chimeric receptor. *Science (New York, N.Y.)*, *350*(6258), aab4077. https://doi.org/10.1126/SCIENCE.AAB4077

Wu, X., Li, Y., Crise, B., & Burgess, S. M. (2003). Transcription start regions in the human genome are favored targets for MLV integration. *Science*, *300*(5626), 1749–1751. https://doi.org/10.1126/SCIENCE.1083413/SUPPL_FILE/WU.SOM.PDF

Wu, X., Shi, B., Zhang, J., Shi, Z., Di, S., Fan, M., Gao, H., Wang, H., Gu, J., Jiang, H., & Li, Z. (2017). A Fusion Receptor as a Safety Switch, Detection, and Purification Biomarker for Adoptive Transferred T Cells. *Molecular Therapy*, *25*(10), 2270–2279. https://doi.org/10.1016/J.YMTHE.2017.06.026

Wu, Y., Liu, Y., Huang, Z., Wang, X., Jin, Z., Li, J., Limsakul, P., Zhu, L., Allen, M., Pan, Y., Bussell, R., Jacobson, A., Liu, T., Chien, S., & Wang, Y. (2021). Control of the activity of CAR-T cells within tumours via focused ultrasound. *Nature Biomedical Engineering*, *5*(11), 1336. https://doi.org/10.1038/S41551-021-00779-W

Wynn, T. A. (2015). Type 2 cytokines: mechanisms and therapeutic strategies. *Nature Reviews. Immunology*, *15*(5), 271–282. https://doi.org/10.1038/NRI3831

Xia, A., Zhang, Y., Xu, J., Yin, T., & Lu, X. J. (2019). T Cell Dysfunction in Cancer Immunity and Immunotherapy. *Frontiers in Immunology*, *10*, 1719. https://doi.org/10.3389/FIMMU.2019.01719

Xiao, X., Huang, S., Chen, S., Wang, Y., Sun, Q., Xu, X., & Li, Y. (2021). Mechanisms of cytokine release syndrome and neurotoxicity of CAR T-cell therapy and associated prevention and management strategies. *Journal of Experimental & Clinical Cancer Research 2021 40:1*, *40*(1), 1–23. https://doi.org/10.1186/S13046-021-02148-6

Xie, Y. J., Dougan, M., Ingram, J. R., Pishesha, N., Fang, T., Momin, N., & Ploegh, H. L. (2020). Improved Antitumor Efficacy of Chimeric Antigen Receptor T Cells that Secrete Single-Domain Antibody Fragments. *Cancer Immunology Research*, *8*(4), 518. https://doi.org/10.1158/2326-6066.CIR-19-0734

Xiong, X., Xi, J., Liu, Q., Wang, C., Jiang, Z., Yue, S. Y., Shi, L., & Rong, Y. (2021). Co-expression of IL-7 and PH20 promote anti-GPC3 CAR-T tumour suppressor activity in vivo and in vitro. *Liver International*, *41*(5), 1033–1043. https://doi.org/10.1111/LIV.14771

Xu, Y., Zhang, M., Ramos, C. A., Durett, A., Liu, E., Dakhova, O., Liu, H., Creighton, C. J., Gee, A. P., Heslop, H. E., Rooney, C. M., Savoldo, B., & Dotti, G. (2014). Closely related T-memory stem cells correlate with in vivo expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15. *Blood*, *123*(24), 3750. https://doi.org/10.1182/BLOOD-2014-01-552174

Yamamoto, T. N., Lee, P. H., Vodnala, S. K., Gurusamy, D., Kishton, R. J., Yu, Z., Eidizadeh, A., Eil, R., Fioravanti, J., Gattinoni, L., Kochenderfer, J. N., Fry, T. J., Aksoy, B. A., Hammerbacher, J. E., Cruz, A. C., Siegel, R. M., Restifo, N. P., & Klebanoff, C. A. (2019). T cells genetically engineered to overcome death signaling enhance adoptive cancer immunotherapy. *The Journal of Clinical Investigation*, *129*(4), 1551. https://doi.org/10.1172/JCI121491

Yang, Z., Li, L., Turkoz, A., Chen, P., Harari-Steinfeld, R., Bobbin, M., Stefanson, O., Choi, H., Pietrobon, V., Alphson, B., Goswami, A., Balan, V., Kearney, A., Patel, D., Yang, J., Inel, D., Vinod, V., Cesano, A., Wang, B., … Marincola, F. M. (2021). Contextual reprogramming of CAR-T cells for treatment of HER2+ cancers. *Journal of Translational Medicine*, *19*(1), 1–18. https://doi.org/10.1186/S12967-021-03132-6/FIGURES/7

Yang, Z., Pietrobon, V., Bobbin, M., Stefanson, O., Yang, J., Goswami, A., Alphson, B., Choi, H., Magallanes, K., Cai, Q., Barrett, D., Wang, B., Qi, L. S., & Marincola, F. M. (2023). Nanoscale, antigen encounter-dependent, IL-12 delivery by CAR T cells plus PD-L1 blockade for cancer treatment. *Journal of Translational Medicine*, *21*(1), 1–15. https://doi.org/10.1186/S12967-023-04014-9/FIGURES/6

Yant, S. R., Wu, X., Huang, Y., Garrison, B., Burgess, S. M., & Kay, M. A. (2005). High-Resolution Genome-Wide Mapping of Transposon Integration in Mammals. *Molecular and Cellular Biology*, *25*(6), 2085. https://doi.org/10.1128/MCB.25.6.2085-2094.2005

Yasuda, K., Nakanishi, K., & Tsutsui, H. (2019). Interleukin-18 in Health and Disease. *International Journal of Molecular Sciences*, *20*(3). https://doi.org/10.3390/IJMS20030649

Ye, X., Wu, Y., Zhang, H., Shi, L., Zhou, Y., Guo, S., Dong, J., & Cai, J. (2023). Rapid generation of CD19 CAR-T cells by minicircle DNA enables anti-tumor activity and prevents fatal CAR-B leukemia. *Cancer Letters*, *568*, 216278. https://doi.org/10.1016/J.CANLET.2023.216278

Yeku, O. O., Purdon, T. J., Koneru, M., Spriggs, D., & Brentjens, R. J. (2017). Armored CAR T cells enhance antitumor efficacy and overcome the tumor microenvironment. *Scientific Reports 2017 7:1*, *7*(1), 1–14. https://doi.org/10.1038/s41598-017-10940-8

Yin, Y., Boesteanu, A. C., Binder, Z. A., Xu, C., Reid, R. A., Rodriguez, J. L., Cook, D. R., Thokala, R., Blouch, K., McGettigan-Croce, B., Zhang, L., Konradt, C., Cogdill, A. P., Panjwani, M. K., Jiang, S., Migliorini, D., Dahmane, N., Posey, A. D., June, C. H., … Johnson, L. A. (2018). Checkpoint Blockade Reverses Anergy in IL-13Rα2 Humanized scFv-Based CAR T Cells to Treat Murine and Canine Gliomas. *Molecular Therapy Oncolytics*, *11*, 20. https://doi.org/10.1016/J.OMTO.2018.08.002

Yuan, X., Wu, H., Xu, H., Xiong, H., Chu, Q., Yu, S., Wu, G. S., & Wu, K. (2015). Notch signaling: An emerging therapeutic target for cancer treatment. *Cancer Letters*, *369*(1), 20–27. https://doi.org/10.1016/J.CANLET.2015.07.048

Yusa, K., Zhou, L., Li, M. A., Bradley, A., & Craig, N. L. (2011). A hyperactive piggyBac transposase for mammalian applications. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(4), 1531–1536. https://doi.org/10.1073/PNAS.1008322108/-/DCSUPPLEMENTAL

Zah, E., Lin, M. Y., Anne, S. B., Jensen, M. C., & Chen, Y. Y. (2016). T cells expressing CD19/CD20 bi-specific chimeric antigen receptors prevent antigen escape by malignant B cells. *Cancer Immunology Research*, *4*(6), 498. https://doi.org/10.1158/2326-6066.CIR-15-0231

Zakeri, B., Fierer, J. O., Celik, E., Chittock, E. C., Schwarz-Linek, U., Moy, V. T., & Howarth, M. (2012). Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(12), E690. https://doi.org/10.1073/PNAS.1115485109/-/DCSUPPLEMENTAL

Zander, R., & Cui, W. (2023). Exhausted CD8+ T cells face a developmental fork in the road. *Trends in Immunology*, *44*(4), 276. https://doi.org/10.1016/J.IT.2023.02.006

Zannikou, M., Duffy, J. T., Levine, R. N., Seblani, M., Liu, Q., Presser, A., Arrieta, V. A., Chen, C. J., Sonabend, A. M., Horbinski, C. M., Lee-Chang, C., Miska, J., Lesniak, M. S., Gottschalk, S., & Balyasnikova, I. V. (2023). IL15 modification enables CAR T cells to act as a dual targeting agent against tumor cells and myeloid-derived suppressor cells in GBM. *Journal for ImmunoTherapy of Cancer*, *11*(2), e006239. https://doi.org/10.1136/JITC-2022-006239

Zeng, R., Spolski, R., Finkelstein, S. E., Oh, S. K., Kovanen, P. E., Hinrichs, C. S., Pise-Masison, C. A., Radonovich, M. F., Brady, J. N., Restifo, N. P., Berzofsky, J. A., & Leonard, W. J. (2005). Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. *The Journal of Experimental Medicine*, *201*(1), 139. https://doi.org/10.1084/JEM.20041057

Zhang, C., Liu, J., Zhong, J. F., & Zhang, X. (2017). Engineering CAR-T cells. *Biomarker Research*, *5*(1). https://doi.org/10.1186/S40364-017-0102-Y

Zhang, L., Feldman, S. A., Zheng, Z., Chinnasamy, N., Xu, H., Nahvi, A. V., Dudley, M. E., Rosenberg, S. A., & Morgan, R. A. (2012). Evaluation of γ-retroviral vectors that mediate the inducible expression of IL-12 for clinical application. *Journal of Immunotherapy*, *35*(5), 430–439. https://doi.org/10.1097/CJI.0B013E31825898E8

Zhang, L., Morgan, R. A., Beane, J. D., Zheng, Z., Dudley, M. E., Kassim, S. H., Nahvi, A. V., Ngo, L. T., Sherry, R. M., Phan, G. Q., Hughes, M. S., Kammula, U. S., Feldman, S. A., Toomey, M. A., Kerkar, S. P., Restifo, N. P., Yang, J. C., & Rosenberg, S. A. (2015). Tumor-infiltrating lymphocytes genetically engineered with an inducible gene encoding interleukin-12 for the immunotherapy of metastatic melanoma. *Clinical Cancer Research*, *21*(10), 2278–2288. https://doi.org/10.1158/1078-0432.CCR-14-2085

Zhang, R. Y., Wei, D., Liu, Z. K., Yong, Y. Le, Wei, W., Zhang, Z. Y., Lv, J. J., Zhang, Z., Chen, Z. N., & Bian, H. (2019). Doxycycline Inducible Chimeric Antigen Receptor T Cells Targeting CD147 for Hepatocellular Carcinoma Therapy. *Frontiers in Cell and Developmental Biology*, *7*. https://doi.org/10.3389/FCELL.2019.00233

Zhang, Y., Wang, P., Wang, T., Fang, Y., Ding, Y., & Qian, Q. (2021). Chimeric antigen receptor T cells engineered to secrete CD40 agonist antibodies enhance antitumor efficacy. *Journal of Translational Medicine*, *19*(1), 1–10. https://doi.org/10.1186/S12967-021-02750-4/FIGURES/4

Zhang, Y., Zhang, X., Cheng, C., Mu, W., Liu, X., Li, N., Wei, X., Liu, X., Xia, C., & Wang, H. (2017). CRISPR-Cas9 mediated LAG-3 disruption in CAR-T cells. *Frontiers of Medicine*, *11*(4), 554–562. https://doi.org/10.1007/S11684-017-0543-6/METRICS

Zhang, Y., Zhang, Z., Ding, Y., Fang, Y., Wang, P., Chu, W., Jin, Z., Yang, X., Wang, J., Lou, J., & Qian, Q. (2021). Phase I clinical trial of EGFR-specific CAR-T cells generated by the piggyBac transposon system in advanced relapsed/refractory non-small cell lung cancer patients. *Journal of Cancer Research and Clinical Oncology*, *147*(12), 3725–3734. https://doi.org/10.1007/S00432-021-03613-7/FIGURES/5

Zhang, Y., Zhuang, Q., Wang, F., Zhang, C., Xu, C., Gu, A., Zhong, W. H., Hu, Y., & Zhong, X. (2022). Co-expression IL-15 receptor alpha with IL-15 reduces toxicity via limiting IL-15 systemic exposure during CAR-T immunotherapy. *Journal of Translational Medicine*, *20*(1), 1–13. https://doi.org/10.1186/S12967-022-03626-X/FIGURES/4

Zhao, Y., Chen, J., Andreatta, M., Feng, B., Xie, Y. Q., Wenes, M., Wang, Y., Gao, M., Hu, X., Romero, P., Carmona, S., Sun, J., Guo, Y., & Tang, L. (2024). IL-10-expressing CAR T cells resist dysfunction and mediate durable clearance of solid tumors and metastases. *Nature Biotechnology 2024*, 1–12. https://doi.org/10.1038/s41587-023-02060-8

Zhao, Y., Moon, E., Carpenito, C., Paulos, C. M., Liu, X., Brennan, A. L., Chew, A., Carroll, R. G., Scholler, J., Levine, B. L., Albelda, S. M., & June, C. H. (2010). Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. *Cancer Research*, *70*(22), 9053–9061. https://doi.org/10.1158/0008-5472.CAN-10-2880

Zhao, Z., Condomines, M., van der Stegen, S. J. C., Perna, F., Kloss, C. C., Gunset, G., Plotkin, J., & Sadelain, M. (2015). Structural Design of Engineered Costimulation Determines Tumor Rejection Kinetics and Persistence of CAR T Cells. *Cancer Cell*, *28*(4), 415–428. https://doi.org/10.1016/j.ccell.2015.09.004

Zhao, Z., Li, Y., Liu, W., & Li, X. (2020). Engineered IL-7 Receptor Enhances the Therapeutic Effect of AXL-CAR-T Cells on Triple-Negative Breast Cancer. *BioMed Research International*, *2020*. https://doi.org/10.1155/2020/4795171

Zhou, J., Jin, L., Wang, F., Zhang, Y., Liu, B., & Zhao, T. (2019). Chimeric antigen receptor T (CAR-T) cells expanded with IL-7/IL-15 mediate superior antitumor effects. *Protein & Cell*, *10*(10), 764–769. https://doi.org/10.1007/S13238-019-0643-Y

Zhou, J. T., Liu, J. H., Song, T. T., Ma, B., Amidula, N., & Bai, C. (2020). EGLIF-CAR-T Cells Secreting PD-1 Blocking Antibodies Significantly Mediate the Elimination of Gastric Cancer. *Cancer Management and Research*, *12*, 8893. https://doi.org/10.2147/CMAR.S260915

Zhou, Z. (joy), Han, Y., Pan, H.-B., Sang, C.-J., Shi, D.-L., Feng, C., Xiao, H., Zhuang, Q.-C., Wang, P.-Y., & Fan, X.-H. (Frank). (2021). Tri-Specific CD19xCD20xCD22 VHH CAR-T Cells (LCAR-AIO) Eradicate Antigen-Heterogeneous B Cell Tumors, Enhance Expansion, and Prolong Persistence in Preclinical In Vivo Models. *Blood*, *138*(Supplement 1), 1700–1700. https://doi.org/10.1182/BLOOD-2021-150650

Zhu, W., Zhang, Z., Chen, J., Chen, X., Huang, L., Zhang, X., Huang, X., Ma, N., Xu, W., Yi, X., Lu, X., Fu, X., Li, S., Mo, G., Wang, Y., Yuan, G., Zang, M., Li, Q., Jiang, X., … Hou, J. (2024). A novel engineered IL-21 receptor arms T-cell receptor-engineered T cells (TCR-T cells) against hepatocellular carcinoma. *Signal Transduction and Targeted Therapy 2024 9:1*, *9*(1), 1–16. https://doi.org/10.1038/s41392-024-01792-6

8. INTERNET SOURCES

https://www.cancer.gov/about-cancer/treatment/types/immunotherapy/t-cell-transfer-therapy [Online] [Cited: 2023-10-02]

https://blog.addgene.org/plasmids-101-using-transposons-in-the-lab [Online] [Cited: 2023-11-17]

https://www.addgene.org/guides/retrovirus/ [Online] [Cited: 2023-11-17]

https://www.addgene.org/guides/lentivirus/ [Online] [Cited: 2024-01-10]

https://www.thermofisher.com/order/catalog/product/11132D [Online] [Cited: 2024-02-11]

https://www.miltenyibiotec.com/US-en/products/t-cell-transact-human.html#130-111-160 [Online] [Cited: 2024-02-11]

https://www.bio-techne.com/p/cell-culture/cloudz-human-t-cell-activation-kit_cld001 [Online] [Cited: 2024-02-11]

https://www.wilsonwolf.com/product-and-order-info/ [Online] [Cited: 2024-01-12]

https://acmerevival.com/wp-content/uploads/2021/03/GEWaveBioreactorSystem210EHBrochure.pdf [Online] [Cited: 2024-01-12]

https://www.medicalexpo.com/prod/terumo-bct/product-75244-850081.html [Online] [Cited: 2024-01-12]

https://www.miltenyibiotec.com/DE-en/products/clinimacs-prodigy.html [Online] [Cited: 2024-01-12]

https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/yescarta [Online] [Cited: 2024-04-15]

https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/tecartus [Online] [Cited: 2024-04-15]

https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/kymriah [Online] [Cited: 2024-04-15]

https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/breyanzi-lisocabtagene-maraleucel [Online] [Cited: 2024-04-15]

https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/breyanzi-lisocabtagene-maraleucel [Online] [Cited: 2024-04-15]

https://www.fda.gov/vaccines-blood-biologics/abecma-idecabtagene-vicleucel [Online] [Cited: 2024-04-15]

https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/carvykti [Online] [Cited: 2024-04-15]