Developing Chimeric Antigen Receptor T cells for the Treatment of Acute Myeloid Leukaemia

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Statement of originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Daochen Tong

Ethical clearance

All the research in this thesis was approved by the Western Sydney Local Health District Human Research Ethics Committee (2019/ETH02263: In-vitro Enhancement of T-cells for the Immunotherapy of Malignancy).

Abstract

Acute myeloid leukaemia (AML) is the most common acute leukaemia in adults with a high incidence of relapse following conventional induction therapy and allogeneic haematopoietic stem cell transplant. In the event of relapse after allogeneic stem cell transplant, only a few therapeutic options remain, and many of those with refractory or relapsed leukaemia prior to transplant are unable to achieve remission with standard therapies, leading to a dismal prognosis. Chimeric antigen receptor (CAR) T cell therapy has exploded onto the medical scene with its remarkable outcomes in relapsed/refractory B-cell leukaemia and lymphoma and multiple myeloma. However, replicating this success in AML has been challenging chiefly due to heterogeneity of leukaemia associated and lineage specific antigen expression in AML within and between patients, leading to immune escape and treatment failure.

CD123 is a leading target for CAR T cell development for AML. It is frequently overexpressed in bulk tumour and leukaemic stem cells, with relatively lower expression on myeloid cells. Others in the Westmead Cell Therapies Laboratory have shown that CAR T cells generated using the *PiggyBac* transposon/transposase transfection system targeting CD123 with the extracellular antigen recognition domain derived from its natural ligand IL-3 (CARIL3) are effective *in vitro*. However, the development of CAR T derived lymphomas in patients treated with *PiggyBac* transduced CAR T cells prompted us to look at alternative gene modification systems for clinical translation. In this thesis, I explored 2 main themes, namely (i) to improve the genetic safety aspect of CARIL3 therapy; and (ii) to increase the efficacy of the anti-AML CAR T therapy developed.

In Chapter 3 of this thesis, I describe the pre-clinical development of alternative gene modification systems for CARIL3 using lentiviral and *PiggyBat* transposon/transposase

systems with a focus on maintaining efficacy while exploring differences in gene integration patterns.

On the theme of improving efficacy, Chapter 4 of the thesis looked at TIM-3 as another potential antigen target for CAR T therapy against AML. CAR T cells against TIM-3 were developed with the ultimate aim of combining with CARIL3 and reducing the risk of antigen escape which underpins a significant proportion of treatment failure of CAR T therapy in AML.TIM-3 is canonically studied in T cells in the context of cell activation and exhaustion. Expression of TIM-3 is usually low on myeloid precursors. However, TIM-3 overexpression is described in a proportion of cases in AML including in the leukaemic stem cell compartment, making it an attractive target. The issue of fratricide is crucial to overcome due to universal expression of TIM-3 on T cells, which is increased upon activation. I explored *in vitro* function of TIM-3 knockout by CRISPR/Cas9 on CAR T cells expressing anti-TIM-3 CARs.

In Chapter 5 of the thesis, I explore whether the knockout of TIM-3 could also serve to improve efficacy of CARIL3, on the basis that TIM-3 is a well-studied T cell exhaustion marker. This chapter also further explores the theme of genetic safety, as it looked at using CRISPR-Cas9 directed knock-out and knock-in strategies into the TIM-3 locus to further minimise the risk of random genetic integration, which is particularly relevant as it has recently emerged that other transduction systems including lentiviral CAR T can still result in CAR T derived lymphomas.

While further work is required to enable safe and efficacious CAR T cells against AML in the clinic, the work in this thesis presents several proof-of-concept strategies taking significant steps towards realising this goal.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

~	approximately
+	positive
aAPC	artificial antigen presenting cell
ACT	adoptive T cell therapy
ADC	antibody-drug conjugates
ADCC	antibody-directed cellular cytotoxicity
AdV	adenovirus
AF647	Alexa fluor 647
AGRF	Australian Genome Research Facility
aGvHD	acute graft versus host disease
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
AML1	acute myeloid leukemia-1 transcription factor, also known as RUNX1, CBFA2, or PEBP2αB
ANOVA	analysis of variance
APC	antigen presenting cell
axi-cel	axicabtagene ciloleucel
B-ALL	B-cell acute lymphoblastic leukaemia
BCMA	B-cell maturation antigen
BiTE	bispecific T cell engager
BLESS	breaks labelling, enrichment on streptavidin and next-generation sequencing
BLISS	breaks labelling in situ and sequencing
BPDCN	blastic plasmacytoid dendritic cell neoplasm
°C	degree Celsius
calcein AM	calcein acetoxymethyl
CAR	chimeric antigen receptor
CARIL3dm	double mutant IL-3 based CAR (K116W, E22R substitutions)
CARIL3sm	single mutant IL-3 based CAR (K116W substitution)
CARIL3wt	wild-type IL-3 based CAR
Cas9	CRISPR-associated protein 9
CBFB	core binding factor beta subunit
CD	cluster of differentiation
CDC	complement dependent cytotoxicity
cDMEM	complete Dulbecco’s Modified Eagle Medium
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1
cGvHD	chronic graft versus host disease
cilta-cel	ciltacabtagene autoleucel
cIMDM	complete Iscove’s modified Dulbecco’s media
CLEC12A	C-type lectin domain family 12 member A
CLL-1	C-type lectin-like molecule 1
CMV	cytomegalovirus
CNV	copy number variation
CPI	checkpoint inhibitor

CR	complete remission
CRi	complete remission with incomplete hematologic recovery
CRISPR	clustered regularly interspaced short palindromic repeats
cRPMI	complete Roswell Park Memorial Institute medium-1640
crRNA	CRISPR RNA
CRS	cytokine release syndrome
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
Cy	cyclophosphamide
DART	dual-affinity retargeting
DC	dendritic cells
DCAL2	dendritic-cell-associated C-type lectin 2
ddPRC	Droplet Digital PCR
DFS	disease free survival
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco’s phosphate buffered saline
DSB	double stranded break
dUTP	deoxyuridine triphosphate
E:T	effector to target ratio
EBV	Epstein-Barr virus
EDTA	ethylene diamine tetra-acetic acid (disodium)
EF1α	Elongation factor 1 alpha
eGFP	enhanced green fluorescent protein
EGFR	epithelial growth factor receptor
ETO	eight-twenty-one corepressor, also known as MTG8
Fab	fragment antigen-binding
FACS	fluorescence activated cell sorting
FAM	fluorescein amidites
FBS	foetal bovine serum
Fc	fragment crystallisable region
FDA	Food and Drug Administration of USA
FLT3	FMS-like tyrosine kinase
FLT3-ITD	FMS-like tyrosine kinase-3 internal tandem duplication
Flu	fludarabine
FMO	fluorescence minus one
fwd	forward
g	acceleration relative to gravity (9.8 m/s^2)
gDNA	genomic DNA
gen	Generation
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	Good Manufacturing Practice
GO	gemtuzumab ozogamicin
GUIDE-Seq	Genome-wide, Unbiased Identification of DSBs Enabled by Sequencing

GvHD	graft versus host disease
GvL	graft versus leukaemia
HA	homology arm
HAVCR2	hepatitis A virus cellular receptor 2
HBS	HEPES-buffered saline
HCC	hepatocellular carcinoma
HDR	homology directed repair
HEK293	human embryonic kidney 293 cell line
HEK293T	human embryonic kidney 293 cell line with constitutive expression of SV40 T-antigen
HEPES	2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid
HEX	hexachlorofluorescein
HLA	human leukocyte antigen
HMGB1	high-mobility group protein B1
HOMER	Hypergeometric Optimization of Motif EnRichment
HSC	haematopoietic stem cell
HSCT	haematopoietic stem cell transplant
hTERT	human telomerase reverse transcriptase
ICAHT	immune effector cell–associated haematotoxicity
ICANS	immune effector cell-associated neurotoxicity syndrome
ICD	intracellular domain
ICOS	inducible T cell co-stimulation
ICPI	immune-checkpoint inhibitors
IDH1	isocitrate dehydrogenase 1
IFNγ	interferon gamma
Ig	immunoglobulin
IgG (H+L)	heavy and light chains of immunoglobulin gamma
IL	interleukin
IL1RAP	interleukin 1 receptor accessory protein
IL-3Rα	interleukin-3 receptor alpha
IL3Rβ	interleukin 3 receptor β subunit, common β subunit
IMDM	Iscove’s modified Dulbecco’s media
IR’d	irradiated
ITAM	immunoreceptor tyrosine-based activation motifs
ITIM	immunoglobulin and tyrosine-based inhibitory motif
IV	intravenous
IVIG	Intravenous immunoglobulin G
junc	junction
KD	knock-down
KI	knock-in
KO	knock-out
KRAS	Kirsten Rat Sarcoma viral oncogene homolog
LAA	leukaemia associated antigen
LAG-3	lymphocyte activation gene-3
LAIP	leukaemia associated immunophenotypes
LB	Luria Bertani

LFS	leukaemia free survival
LFT	liver function tests
LSC	leukaemic stem cells
LTR	lentiviral terminal repeat
LV	lentiviral
MAGE-A3	melanoma-associated antigen-A3
MART-1	melanoma-associated antigen recognized by T-cells
MDS	myelodysplastic syndrome
MHC	major histocompatibility complexes
MICL	myeloid inhibitory C-type lectin-like receptor
min	minutes
MM	multiple myeloma
MOI	multiplicity of infection
MRD	measurable/minimal residue disease
mRNA	messenger ribonucleic acid
ms, sec	milliseconds, seconds
MYH11	smooth muscle myosin heavy chain 11
NCCN	National Comprehensive Cancer Network
neg	negative
NFAT	nuclear factor of activated T cell
NHEJ	non-homologous end joining
NK	natural killer
NPM1	nucleophosmin
NSCLC	non-small cell lung cancer
NYESO-1	New York oesophageal squamous cell carcinoma 1
OR	odds ratio
OS	overall survival
OX40	TNF receptor superfamily, member 4 (TNFRSF4)
P2A	self-cleaving peptide derived from porcine teschovirus-1 2A
PAM	protospacer adjacent motif
PASTE	programmable addition via site-specific targeting elements
PB, PBac	*PiggyBac*
PBat	*PiggyBat*
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PD-1	programmed cell death protein 1
PE	phycoerythrin
pg, ng, mg, g	picogram, nanogram, milligram, gram
Ph	Philadelphia chromosome
pmol	picomoles
PML	promyelocytic leukemia
PR	Partial remission
PRAME	preferentially expressed antigen in melanoma
PtdSer, PS	phosphatidylserine
R/R	relapsed/refractory
RACE	rapid amplification of cDNA ends

RARA	retinoic acid receptor alpha
RCL	replication competent lentivirus
rev	reverse
RFS	relapse free survival
RHAMM	receptor for hyaluronan-mediated motility
RIC	reduced intensity conditioning
RNA	ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute medium-1640
RPP30	ribonuclease P protein subunit p30
rRNA	ribosomal RNA
RT PCR	reverse transcription polymerase chain reaction
scFv	single chain variable fragment
SF3B1	splicing factor 3b subunit 1
sg37	sgRNA CRISPR699737_SGM
sg49	sgRNA CRISPR699749_SGM
sgRNA	synthetic gRNA
siRNA	small interfering RNA
SOC	super-optimal broth with catabolite repression
ssODN	single stranded donor oligonucleotide
T25, T75, T150	25cm^2, 75cm^2, 150cm^2 rectangular canted-neck cell culture flasks
T2A	self-cleaving peptide derived from thosea asigna virus 2A
TAA	tumour associated antigen
TALEN	transcription activator-like effector nucleases
TBI	total body irradiation
TCR	T cell receptors
TE	Tris EDTA buffer
tEGFR	truncated epithelial growth factor receptor
tEGFRz	truncated epithelial growth factor receptor-based CAR
TEN	Tris EDTA sodium chloride buffer
TET2	Tet Methylcytosine dioxygenase 2
Thio	thiotepa
TIGIT	T-cell immunoreceptor with immunoglobulin and ITIM domains
TIL	tumour-infiltrating lymphocytes
TIM-3	T cell immunoglobulin and mucin-domain-containing-3
TIR	terminal inverted repeat
tisa-cel	tisagenlecleucel
TLR	toll-like receptor
TM	transmembrane
TME	tumour microenvironment
TNFα	tumour necrosis factor alpha
TP53	tumour protein p53
Tregs	regulatory T cells
UniCAR, UCAR	universal CAR
WHO	World Health Organisation
WT1	Wilms’ tumour 1
z or ζ	Zeta

βc	common beta subunit
ΔICD	intracellular domain deletion
μL, mL, L	microlitre, millilitre, litre
μM, mM, M	micromolar, millimolar, molar

List of Presentations

CD123-targeting CAR T-cells for the treatment of AML

Tong D, Lee K, & Micklethwaite K.

Poster presentation at Westmead Hospital Week 2021

Oral presentation at The Cancer Research Network Postgraduate & ECR Cancer Research Symposium 2021

Putative TIM-3 directed CAR T-cells may be expanded with concomitant TIM-3 KO

Tong D, Lee K, & Micklethwaite K.

Oral presentation at ISCT ANZ Regional Meeting 2022

TIM-3 targeting Chimeric Antigen Receptor T-cells for treating Acute Myeloid Leukemia

Tong D, Lee K, & Micklethwaite K.

Poster presentation at Westmead Hospital Week 2023

Poster presentation at New South Wales Cancer Conference 2023

TIM-3 targeting CAR T-cells for treating acute myeloid leukaemia may be generated with simultaneous knock-out or knock-in to the endogenous TIM-3 locus to prevent fratricide

Tong D, Lee K, & Micklethwaite K.

Oral presentation at ISCT ANZ Regional Meeting 2024

Chapter 1. Introduction

1.1 AML

1.1.1 Overview

Acute myeloid leukaemia (AML) is the most common acute leukaemia in adults with a dismal 5-year survival rate of 25.9%. AML is driven by genetically diverse mutations dictating prognosis and optimal therapy. However, standard treatment for AML has remained limited and largely unchanged for the majority of patients for more than 50 years, consisting of induction chemotherapy followed by consolidation with either further chemotherapy or allogeneic haematopoietic stem cell transplant (HSCT). For patients unfit for intensive chemotherapy, the combination of venetoclax and azacitidine produced superior median overall survival compared to azacitidine alone (14.7 months vs 9.6 months) (DiNardo *et al.*, 2020), leading to establishment of this combinations as the standard of care treatment. However, this is not considered curative.

Although first-line induction chemotherapy achieves high remission rates in AML patients, relapse is very common. For many patients, allogeneic HSCT remains the best chance of cure, and this has been postulated to be due to T cell mediated graft versus leukaemia (GvL) effect in eradicating minimal residual disease (MRD) and leukaemic stem cells (LSCs). The role of the immune system in mediating cure post-transplant is supported by the inverse correlation of relapse with development of chronic graft versus host disease (GvHD). Unmanipulated donor lymphocyte infusions (DLIs) are sometimes utilised following allogeneic HSCT to eradicate persistent MRD or early morphological relapse, providing evidence for the central role of the immune system in transplant's efficacy. However, unmanipulated T cells are ineffective in frank relapse and can cause lethal GvHD, highlighting the need for a more targeted immunotherapy.

Chimeric antigen receptor (CAR) T cells have been successfully implemented to target tumour antigens, demonstrating outstanding efficacy in B cell leukaemia, lymphoma, and multiple myeloma. However, the application of CAR T cells in AML has yet to achieve significant clinical success, due to a lack of easily targetable AML antigens, tumour heterogeneity and immune escape, and myeloid-specific toxicities.

This introduction aims to summarise relevant literature and build a case for the need of novel therapies such as advanced CAR T cell therapy for the treatment of AML.

1.1.2 AML epidemiology and survival outcomes

Acute myeloid leukaemia (AML) is a blood cancer caused by uncontrolled proliferation of undifferentiated myeloid progenitor cells in the bone marrow, where it adversely affects normal blood cell production. It is the second most common type of leukaemia and the commonest aggressive leukaemia in adults, with the lowest survival rate (AIHW, 2024, CRUK, 2020, Deschler and Lubbert, 2006, Siegel *et al.*, 2020). In Australia, about 4.5 / 100,000 people are diagnosed each year, with a median age of diagnosis of about 70 years (AIHW, 2024, Gangatharan *et al.*, 2013).

The prognosis for AML is dismal, having a 5-year overall survival (OS) of 23 – 25%. Despite the 5-year OS improving in the last 5 decades, it remains unacceptably low for elderly patients > 70 years old (<11%) compared to younger patients (>49%) (AIHW, 2024, Beckmann *et al.*, 2022).

1.1.3 Genetic basis of AML

AML is a genetically heterogenous disease with many variations between individual cases. The World Health Organisation (WHO) classification of AML subtypes is based on commonly observed driver mutations which are related to their biological and prognostic properties. Most of these are chromosomal translocations which are easily detected by traditional karyotype and well characterised, while others are more recently discovered point mutations (Arber *et al.*, 2016). Common mutations are used to stratify patients into favourable, intermediate and adverse risk groups according to the European LeukemiaNet Standardized System to inform treatment decisions (Döhner *et al.*, 2022). About 21% of cases remain unclassifiable according to the 2022 World Health Organisation Classification, being of normal karyotype and lacking a commonly occurring mutation, emphasising the heterogeneity of AML (Yun, 2025).

1.1.4 Leukaemic stem cells in AML relapse

It is now recognised that in a significant proportion of cases, relapse is due to persistence of a subpopulation of leukaemic cells with relative chemotherapy resistance (Ishikawa *et al.*, 2007), to which some have ascribed stem cell like properties, calling them leukaemic stem cells (LSCs) (Röllig *et al.*, 2011).

The immunophenotype of LSCs is typically CD34^{+}, CD38^{neg}, CD71^{neg}, HLA-DR^{neg}, CD90^{neg}, CD117^{neg} and CD123^{+} (Jordan, 2002), however most studies uses the first two markers, namely CD34^{+}CD38^{neg}, for identification of LSCs. Animal studies have shown that leukaemia cells which possess a stem cell CD34^{+}CD38^{neg} phenotype are sufficient to recapitulate the entire leukaemic population with similar morphology to the original AML. In contrast CD34^{neg} and CD34^{+}CD38^{+} fractions are not capable of leukaemogenesis, (Bonnet and Dick, 1997, Lapidot *et al.*, 1994). However, AML leukaemia initiating cells have also been found in the CD33^{+}CD34^{neg} fraction (Taussig *et al.*, 2010). Moreover, there is a contrary view that the lack of leukaemic initiating capacity by the CD34^{+}CD38^{+} fraction is due to the detrimental effect of anti-CD38 antibodies used in cell sorting (Taussig *et al.*, 2008). Irrespective of whether leukaemic stem cells are true stem cells, the identification and characterisation of specific populations responsible for relapse provides an opportunity for "cure" by specifically targeting these cells with novel treatments such as immunotherapy.

CD123 over-expression on CD34^{+}CD38^{neg} LSCs was established more than 20 years ago, while its expression is not detected on normal bone marrow haematopoietic stem cells (HSCs) (Jordan *et al.*, 2000). The same group also found that these CD34^{+}CD123^{+} cells can initiate and maintain AML *in vivo*, a hallmark of LSCs, and provides a strong rationale for testing CD123-targeted therapies in AML (Jordan *et al.*, 2000).

T cell immunoglobulin and mucin-domain-containing-3 (TIM-3) is a relatively new AML marker, and its high gene and protein expression levels have both been associated with poorer

OS and disease free survival (DFS). LSC markers were also found upregulated in the TIM-3-high group, indicating the potential in targeting TIM-3 as a LSC marker (Wu *et al.*, 2023). Most importantly, the TIM-3^{+} population contained all functional LSCs, where only the TIM-3^{+} fraction, but not the TIM-3^{neg} cells, reconstituted human AML in mice model (Kikushige *et al.*, 2010). This suggests that targeting TIM-3 may be a valid strategy to eliminate all LSCs in patients to prevent relapse.

1.1.5 Current treatments for AML

Current treatment for AML is guided by patient fitness and disease cytogenetic molecular factors which prognosticate patient relapse risks. The standard of care is generally categorised into chemotherapy, hypomethylating agents, targeted agents, HSCT and immunotherapy.

1.1.5.1 Cytotoxic and hypomethylating agents

First-line intensive induction chemotherapy for AML has not changed substantially for over 50 years, consisting of induction with cytarabine and an anthracycline (particularly idarubicin or daunorubicin), abbreviated as "7+3" (Döhner *et al.*, 2017) followed by consolidation with high dose cytarabine in younger patients who can tolerate intensive chemotherapy. This protocol induces complete remission (CR) rates of between 50-80%, though CR rates are lower in the higher risk group (Fernandez *et al.*, 2009). Despite the satisfactory CR rates achieved, relapse is common (50-70%) and long-term survival post relapse is abysmal, especially for patients who are ineligible to proceed to allogeneic HSCT, having median OS between only 1.5 – 11.9 month (Nath *et al.*, 2019). For older patients unfit for chemotherapy, hypomethylating agents such as azacitidine alone can extend overall survival by only a modest 3.8 months compared to conventional therapies (Dombret *et al.*, 2015).

Many drugs and formulations are in development or being repurposed for AML to improve outcomes especially for higher risk groups. CPX351, a liposomal encapsulation of cytarabine and daunorubicin in a fixed 5:1 ratio, is a Food and Drug Administration (FDA) approved drug for the treatment of subset of AML patients with myelodysplasia-related changes and therapy-related AML which moderately improved median OS from 5.95 to 9.56 months compared to conventional chemotherapy (Lancet *et al.*, 2018). Other drugs such as cladribine, clofarabine, actinomycin D and statins are still being trialled, but have yielded minimal patient outcome improvements (Gionfriddo *et al.*, 2021, Holowiecki *et al.*, 2004, Lancet *et al.*, 2018, Löwenberg *et al.*, 2017, Pluta *et al.*, 2017, Shadman *et al.*, 2015).

1.1.5.2 Targeted therapies

In light of recurring mutations discovered in AML, targeted therapies have emerged in recent years. While their use as monotherapies results in only modest efficacies (DiNardo and Lachowiez, 2019), their combination with chemotherapy induction or consolidation has improved patient outcomes leading to approval of several agents for routine clinical use. For example, FMS-like tyrosine kinase 3 (FLT3) inhibitors midostaurin and gilteritinib have been approved for newly diagnosed and relapsed/refractory (R/R) AML patients with FLT3 mutations respectively (Wander *et al.*, 2014). The FLT3 inhibitor gilterinib has shown superiority to intensive chemotherapy for relapsed refractory patients with FLT3 mutation, but the miniscule improvement in median overall survival (9.3 months vs. 5.6 months) highlights the ongoing need for novel therapies. (Almatani *et al.*, 2021, Perl Alexander *et al.*, 2019). In patients ineligible for standard chemotherapy, venetoclax, a BCL-2 inhibitor, combined with azacitidine gave an improvement in OS of about 5 months compared with azacitidine alone (DiNardo *et al.*, 2020). Inhibitors of mutated cytosolic isocitrate dehydrogenase 1 (IDH1), ivosidenib and enasidenib, have also been approved for R/R AML with IDH mutations. However, outcomes are still dismal for this group of patients treated with these two agents, with CR rate, 2-year OS rate and median OS of 21%, 15% and 8.21 months, respectively (Chen *et al.*, 2023b). Considering the abysmal survival rates for R/R AML patients using even the latest standard-of-care treatments listed above, novel therapies are urgently warranted.

1.1.5.3 Allogeneic haematopoietic stem cell transplant

For most transplant-eligible intermediate and high-risk AML patients, allogeneic haematopoietic stem cell transplant (HSCT) remains the best post remission consolidation therapy with the highest probability of cure and where the reduction in relapse rate outweighs the risk of non-relapse mortality and morbidity (Burnett *et al.*, 2002, Cornelissen and Blaise, 2016, Daver *et al.*, 2021b). With both haploidentical and matched sibling transplants, a high probability of 2-year leukaemia free survival (LFS) is achieved in intermediate-risk (56% and 70% in haploidentical and matched sibling donors) and high-risk AML patients (61% and 55% in haploidentical and matched sibling donors) (Salvatore *et al.*, 2018). The benefit from HSCT is not only due to the high dose conditioning therapy that could be administered to patients, but also due to the immunologically mediated graft versus leukaemia (GvL) effect. The central role of T lymphocytes in the GvL effect was inferred initially by the higher rates of relapse observed in T cell depleted allogeneic transplants (Marmont *et al.*, 1991) and was supported by reports of eradication of MRD following DLI (Biederstädt and Rezvani, 2023). This recognition of the role of the immune system in transplant has led to the development of reduced intensity conditioning (RIC) allowing for similar cure rates with lower toxicity in older, less fit patients (Alatrash *et al.*, 2019, Ali *et al.*, 2020a, Alyea *et al.*, 2006) as well as use of DLI for remission consolidation (Gooptu *et al.*, 2024).

Unfortunately, allogeneic HSCT and DLI are hampered by graft versus host disease (GvHD) where donor T cells damage normal host tissue, leading to significant mortality and morbidity (Nivison-Smith *et al.*, 2016). Allogeneic HSCT has a wide range of complications in addition to GvHD, including chemotherapy related toxicity, sepsis, organ failure and opportunistic viral and fungal infections (Nivison-Smith *et al.*, 2016, Takami, 2018). Furthermore, relapse can occur even when severe GvHD is present due to insufficient GvL effect as both GvHD and GvL are caused by alloantigen and minor histocompatibility antigens on recipient cells, but due to the tissue location of the reactive donor T cells, can elicit detrimental or beneficial effects (Baron *et al.*, 2023, Teshima and Hashimoto, 2023). Therefore, while some AML cases can be effectively treated with DLIs, the specificity and activity of unmanipulated allogeneic T cells are sub-optimal and improvements to this crude type of immunotherapy is required.

1.1.5.4 Immunotherapies

Immunotherapy aims to utilise or augment the activities of the immune system to eradicate cancerous cells or infections. They function via antigen specific delivery of toxins, recruiting effector molecules and cells, removing immune inhibition or conferring new antigen recognition capabilities to cells. Antibody based immunotherapy and adoptive T cell therapies are examples of options that harness the anti-leukaemic effect of the immune system.

1.1.5.5 Antibody based immunotherapy

Naked monoclonal antibodies are the most common type of antibody-based treatment in treating cancer. Naked antibodies can boost the immune response by binding and thus marking cancer cells for complement dependent cytotoxicity (CDC) and antibody-directed cellular cytotoxicity (ADCC). However, for AML, monoclonal antibodies such as anti-CD33 and anti-CD123 antibodies have had little observable anti-leukaemic activities as single agents or in combination with chemotherapy in R/R cases, which may be attributed to impaired ADCC function, loss of ligands required for ADCC on cancer cells, and down-regulation of CD33 expression (Feldman *et al.*, 2003, Feldman *et al.*, 2005, Montesinos *et al.*, 2021, Zahavi and Weiner, 2020).

Antibody-drug conjugates (ADCs) are biological drugs made up of a therapeutic chemical attached to an antibody by a permanent or labile linker. This strategy couples the precision of the antibody targeting ability with the cytocidal activity of the therapeutic drug which is usually too toxic to be administered systemically, thus reducing off-target toxicities in patients (Birrer *et al.*, 2019). Gemtuzumab ozogamicin (GO) is a humanised anti-CD33 antibody conjugated with calicheamicin, a cytotoxic DNA damage drug. GO was approved by the FDA in 2000 for AML induction therapy in combination with chemotherapy but was retracted in 2010 due to bone marrow toxicities. In a meta-analysis of five randomised controlled trials consisting of 3325 patients established that although the addition of low dose GO (3mg/m^2) to induction therapy did not increase CR rates, it did reduce risk of relapse (OR = 0.81) and improve 5-year OS (OR = 0.90) especially for those with favourable risk cytogenetics (Hills *et al.*, 2014). This led to the FDA approval of GO being reinstated in 2018. Other CD33, CD123 and C-type lectin-like molecule 1 (CLL-1) targeting ADCs are also in various stages of early clinical development. Although some are showing promising results, some trials have been prematurely terminated due to concerning high death rates in the ADC cohorts (Yu and Liu, 2019), emphasising the need for alternate immunotherapies for treating AML.

Tagraxofusp, an IL-3 ligand-drug conjugate targeting CD123, approved for blastic plasmacytoid dendritic cell neoplasm (BPDCN) has shown improvements in survival for previously untreated high-risk AML patient in combination with azacitidine and venetoclax in early trials (Lane *et al.*, 2024). This supports the use of CD123 as a potential AML target, though further trials would be needed in R/R AML patients.

1.2 Adoptive T cell therapies

Adoptive T cell therapy (ACT) is a specialised subtype of immunotherapy in which T cells specific to infections or cancers are administered with the goal of improving immune functionality of the patient. The Westmead Cell Therapies Laboratory focuses on the research and development of adoptive T cell therapies against viruses and haematological malignancies (Bishop *et al.*, 2021, Bishop *et al.*, 2018, Clancy *et al.*, 2013, Jiang *et al.*, 2023, Lee *et al.*, 2020, Ramanayake *et al.*, 2015).

Tumour specific T cells may be pre-existing and directly isolated from blood or tumours and expanded *in vitro*, or non-specific T cells can be genetically modified with receptors to recognise tumour antigens (Table 1.1).

All T cell immunotherapies, including chimeric antigen receptors leverage physiological T cell activation to produce a therapeutic benefit. Rational design of T cell therapies requires an understanding of this physiology as well as potential AML antigen targets, these will be explored in the following section.

Table 1.1 **Comparison of major adoptive T cell therapies.** HLA, human leukocyte antigen; TCR, T cell receptors. Adapted from (Biernacki et al., 2019)

	T cell therapy	Target antigen	Advantage	Disadvantage
Non-genetically modified T cell therapy	Tumour infiltrating lymphocytes	Neo-antigens	Polyclonal (reduce potential for escape through antigen loss) Targets from intracellular and cell surface proteins	T cell specificity generally unknown Cannot be generated for all patients Not well established for hematologic malignancies Patient specific
	Allogeneic cytotoxic T lymphocytes	Alloantigens Minor histocompatibility antigens	Monoclonal or polyclonal Targets from intracellular and cell surface proteins T cell specificity may be single or multiple	Cannot be generated for all patients Patient specific
Genetically modified T cell therapy	Transgenic TCR T cells	Neo antigens Virus antigens in virus driven tumours Tumour associated antigens (cancer testis antigens) Minor histocompatibility antigens	Targets derived from intracellular and cell surface proteins Defined specificity	HLA restriction Tumour escape through antigen loss (altered processing of peptides, HLA downregulation) Unexpected off-target side-effects Mispairing with endogenous TCR α and β chains
	CAR T cells	Lineage specific antigens	No HLA-restriction Defined specificity	Cell surface targets only Tumour escape through antigen loss (downregulation or loss of target protein)

1.2.2 Physiological T cell activation

Physiological T cell activation is initiated by engagement of the T cell receptor with a specific short antigen peptide epitope presented in the context of major histocompatibility complexes (MHC) on the surface of a diseased or professional antigen presenting cell (APC) (Figure 1.1).

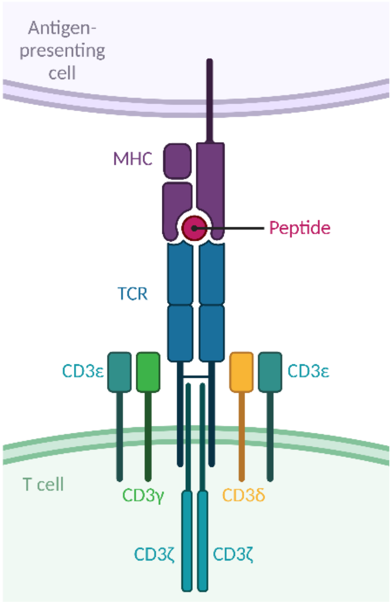


Figure 1.1 Diagram of TCR/CD3 complex engagement of peptide/MHC class I molecule.. MHC, major histocompatibility complex; TCR, T cell receptor. Created with BioRender.

Antigens are processed by an APC into peptides and loaded onto MHC class I or class II molecules through a number of pathways involving endosomes, endoplasmic reticulum and the Golgi complex, and eventually presented on the cell surface (Harryvan *et al.*, 2022, Pishesha *et al.*, 2022).

T cell receptors (TCR) are heterodimers consisting of an α and a β chains in most T cells. These each contain an immunoglobulin (Ig)-like extracellular variable (V) and constant (C) domain, a membrane-proximal connecting peptide (CP), a single transmembrane (TM) region, and a short cytoplasmic tail. T cell activation is initiated by binding of the variable regions of the TCR to a specific antigen peptide bound to the MHC molecule (Figure 1.2). This interaction

(Signal 1) is stabilised by co-receptors (CD4 with MHC class II and CD8 with MHC class I). Once the TCR is engaged, activation is mediated by an associated CD3 complex, with CD3ζ effecting downstream secondary messengers, leading to its cytotoxic activity and cytokine release. However, full T cell activation requires not only engagement of the peptide/MHC complex but also additional co-stimulatory signals (Smith-Garvin *et al.*, 2009). Without co-stimulatory signals, T cells will either become anergic or die by programmed cell death.

The co-stimulatory signal (Signal 2) is also necessary for sustained T cell activation. Common co-stimulatory T cell receptors include CD28 which binds to B7.1 (CD80) or B7.2 (CD86) on activated APCs. Other co-stimulatory receptors include 4-1BB (CD137), OX40 receptor (CD134) and inducible T cell co-stimulation (ICOS) receptor. To prevent excessive T cell mediated damage to healthy tissue, negative feedback is provided by co-inhibitory receptors/immune checkpoint receptors such as TIM-3, programmed cell death protein 1 (PD-1), lymphocyte activation gene-3 (LAG-3), and cytotoxic T lymphocyte antigen 4 (CTLA-4) (Thangavelu et al., 2010). However, these inhibitory receptors can be exploited by tumours for immune evasion by suppressing activation of naturally occurring tumour specific T cells and promoting their exhaustion. Exhausted or dysfunctional T cells are characterised by high expression of co-inhibitory receptors, low proliferation, impaired cytokine producing capacities and increased apoptosis (Schnell *et al.*, 2020). Immune checkpoint blockade such as anti-PD-1, anti-PD-L1 and anti-CTLA-4 antibodies have demonstrated clear clinical efficacies against solid tumours (He and Xu, 2020). TIM-3, LAG-3 and T cell immunoreceptor with immunoglobulin and tyrosine-based inhibitory motif (ITIM) domain (TIGIT) represent the next immune checkpoints which show great promise as cancer treatment targets (Cai *et al.*, 2023). For example, LAG-3 or TIM-3 inhibition both showed increasing cytokine production and reduced tumour in combination with PD-1 inhibition (Burova *et al.*, 2019, Kikushige *et al.*, 2010, Ngiow *et al.*, 2011, Zettl *et al.*, 2022, Zhou *et al.*, 2011), thus disruption of these co-inhibitory receptors may also improve the efficacy of ACT.

T cell activation also leads to expression of cytokines (Signal 3), such as interleukin (IL)-2, which is crucial for autocrine stimulation of clonal expansion, effector function, and formation of memory phenotype T cells. Naïve CD4 and CD8 T cells normally differentiate into effector cells to eliminate the pathogen or tumour, while some T cells form memory cells to confer immune memory, with faster and increased activity upon future re-exposure to the antigen (Golubovskaya and Wu, 2016).

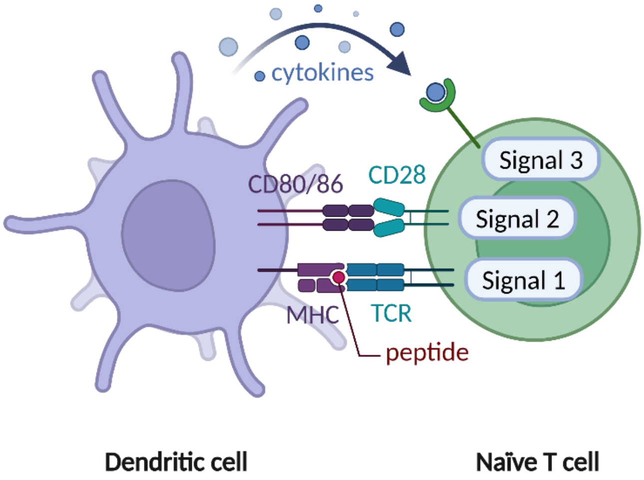
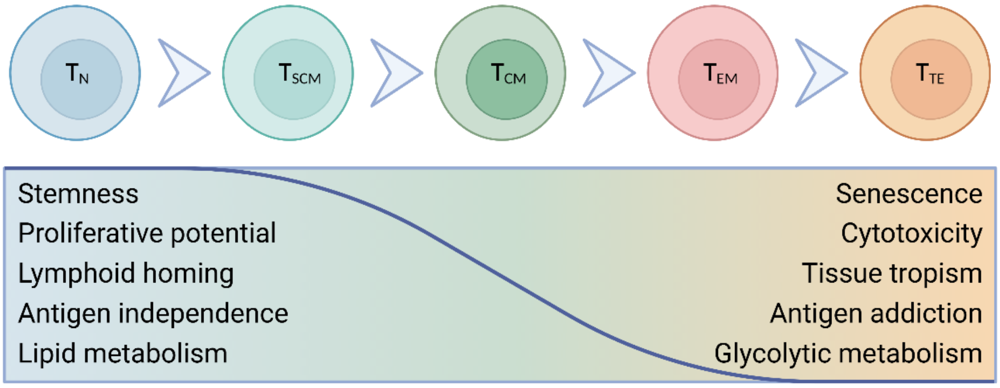


Figure 1.2 The three signals required for T cell activation. Signal 1 is antigen recognition of peptide-MHC complex by TCR. Signal 2 is co-stimulatory molecule binding such as CD28 by one of the B7 ligands (CD80 or CD86). Signal 3 is release of cytokines, essential for T cell expansion and differentiation. Created with BioRender.

1.2.3 T cell memory phenotype

At birth, the T cell repertoire consists of up to 10^{15} naïve clones ready to respond to specific peptides through their unique TCR V regions. Upon encountering their target antigen for the first time, naïve T (T_N) cells undergo expansion and differentiation into various memory subsets which can be identified by their differing phenotypes and functions. This leads to a progressive narrowing of the TCR repertoire over an individual's life due to outgrowth of T cells specific for common infections. Phenotypic, gene expression and functional studies suggest that memory T cells follow a linear progression along a spectrum (Figure 1.3), where less differentiated cells give rise to more differentiated daughter cells that are less proliferative, but more cytotoxic (Gattinoni *et al.*, 2017). Following encounter with an antigen, the quiescent T cells develop into effectors. After clearance of the antigen, the effector T cells that survive return to a quiescent memory state, ready to activate if the same antigen is encountered again. Multiple studies have provided support that higher proportions of naïve and central memory CD8 T cells are associated with improved outcome in cell therapy due to greater self-renewal potential, anti-tumour activity and persistence (López-Cantillo *et al.*, 2022).



	T_N	T_{SCM}	T_{CM}	T_{EM}	T_{TE} or T_{EMRA}
CD62L	+	+	+	–	–
CCR7	+	+	+	–	–
CD45RA	+	+	–	–	+
CD45RO	–	–	+	+	–
CD28	+	+	+	+/–	–
CD95	–	+	+	+	+
CXCR3	–	+	+	–	–

Figure 1.3 **Memory phenotypes of T cells.** Linear progression of memory T cell differentiation. T_N, naïve; T_{SCM}; memory stem cell; T_{CM}, central memory; T_{EM}, effector memory; T_{TE}, terminal effector. Adapted from (Gattinoni *et al.*, 2017, Mahnke *et al.*, 2013). Illustrated with BioRender.com.

1.2.4 Selecting AML antigens

The choice of target antigen is the first crucial step in successful immunotherapies. The National Cancer Institute has devised a list of criteria for antigen selection in T cell therapies weighted by their relative importance (Canichella et al., 2023, Cheever et al., 2009) (Table 1.2).

Table 1.2 Factors in antigen selection for T cell therapies.
Adapted from (Canichella *et al.*, 2023, Cheever *et al.*, 2009)

Criteria	Relevance / interpretation
Therapeutic function	Efficacy demonstrated in clinical trials targeting the antigen
Immunogenicity	T cell response can be elicited (relevant for *ex vivo* expanded CTL/TILs)
Oncogenicity	The antigen has been implicated in development, maintenance and/or progression of AML
Specificity	The antigen is present only on leukaemic cells, or only on immune privileged sites, to avoid on-target, off-tumour side effects
Expression level and % positive cells	High levels of expression in most leukaemic blasts
Expression on cancer stem cell	Evidence of expression on leukaemic stem cells, to prevent relapse.
Stable expression	Not downregulated with treatment or course of disease.
Coverage of patient cohort	High % of patients that have antigen-positive cancer across different subtypes
Number of epitopes	Longer antigens with multiple epitopes, has potential to bind most MHC molecules
Cellular location	Expressed on cell surface, with no/little circulating antigen

In the list above, immunogenicity of target antigens is not relevant in the context of CAR T cells, but it is necessary for the target antigens to be significantly expressed on the cell surface.

A wide range of antigens found in AML have been characterised using transcriptomic and flow cytometric techniques, comparing AML and healthy haematopoietic cells in order to discover factors suitable for determining prognosis and treatment (Koehnke *et al.*, 2017, Perna *et al.*, 2017), but no single antigen fulfills all the criteria of an ideal target. These AML antigens may be grouped into neo-antigens, tumour/leukaemia associated antigens and lineage specific antigens which are described in more details below.

1.2.4.2 AML neo-antigens

Leukemia specific neo-antigens resulting from somatic mutations would be ideal antigens as they are only present in AML cells and not normal tissue (Xie *et al.*, 2023b, Zhang *et al.*, 2021). AML neo-antigens may arise from gene fusions (e.g. CBFB-MHY11, PML-RARA), frameshift insertion/deletions (e.g. in NPM1, FLT3-ITD) and non-synonymous point mutations (e.g. in IDH1, KRAS, TP53) and splicing variants (e.g. SF3B1) (Koehnke *et al.*, 2017, Zhou *et al.*, 2022). Due to their intracellular expression, many well-described leukemia specific neo-antigens such as AML1-ETO, PML-RARA fusion proteins and mutated NPM1, are not amendable to CAR T cell therapy (Anguille et al., 2012). Moreover, due to the vast number of possible mutations and the resultant epitopes generated, it is a laborious, time-consuming and highly technical process to isolate and identify the TCR sequences which are reactive to the immunogenic epitopes from pools of neo-antigen specific T cells, requiring specialised instrumentations and data searching algorithms such as MS HLA-ligand profiling, exon sequencing and RNA-sequencing (Zhou *et al.*, 2022).

1.2.4.3 Tumour associated antigens / Leukaemia associated antigens

Tumour/leukaemia associated antigens (TAAs/LAAs) are antigens present on normal tissues, but overexpressed on AML blasts (Table 1.3). Commonly studied LAAs include preferentially expressed antigen in melanoma (PRAME), Wilms' tumour 1 (WT1), survivin, melanoma-associated antigen-A3 (MAGE-A3) and mesothelin (Bleakley et al., 2016, Cheever et al., 2009, Goswami et al., 2014) with promising pre-clinical data in solid tumours such as medulloblastoma (Orlando et al., 2018). However, like AML neo-antigens, these antigens are not usually readily targetable by CAR T cell therapy as they are intracellular and recognised in an HLA-dependent context.

1.2.4.4 Lineage specific antigens

AML CAR T cell research have been mainly focused on lineage-specific antigens which are antigens normally present on haematopoietic cells, but overexpressed on AML (Table 1.4). Commonly targeted lineage-specific antigens include CD33, CD123 and C-type lectin domain family 12 member A (CLEC12A/CLL-1), with over 60 clinical trials against these 3 targets as of June 2023 (Pérez-Amill *et al.*, 2023).

Instead of targeting a single antigen, it is also possible to target multiple antigens. Leukaemia associated immunophenotypes (LAIPs) are aberrant combinations of surface antigens detected by flow cytometry, used to identify individualised measurable/minimal residue disease (MRD). Usually, LAIPs are used for prognostic purposes (Dix *et al.*, 2020, Feller *et al.*, 2013, Sui *et al.*, 2019). However, strategies such as dual targeting and split CARs are being created to target common LAIPs, to combat antigen escape and off-target toxicities, respectively (Atilla *et al.*, 2022, Petrov *et al.*, 2018).

Table 1.3 **Leukemia-associated target antigens in AML.** Antigen expression: - negative, (-) infrequent, (+) low, + positive, ++ highly expressed; HSC, haematopoietic stem cells; HCC, hepatocellular carcinoma; hTERT, human telomerase reverse transcriptase, MAGE-A3, melanoma- associated antigen-A3; MM, multiple myeloma; PRAME, preferentially expressed antigen in melanoma; RHAMM, receptor for hyaluronan-mediated motility; TIM-3, T cell immunoglobulin and mucin-domain containing-3; WT1, Wilms' tumour 1. Adapted from (Daver *et al.*, 2021a).

Target antigen	Expression	Physiological function	Expression on bulk AML cells	Expression on LSCs	Expression on HSCs	Non-haematopoietic expression	Spontaneous immune response
Lewis Y (CD174)	Surface	Unknown	+	Not assessed	(-)	Epithelial cells	
Mucin-1	Surface	Mucosal protection	+ (Myelomonocytic/ monocytic AMLs)	+	(-)	Epithelial cells	
CD44v6	Surface	Cell-cell interactions/ cell-matrix interactions	+ (64–72%)	Probable	-	Keratinocytes	
CD244/2B4	Surface	Activating/inhibitory receptor of NK cells	++	++	++	Gut, kidney, liver, pancreas, parathyroid	
CD96	Surface	Immune cell adhesion	+	+	(-)	-	
TIM-3	Surface	Co-inhibitory receptor of immune cells	+	+	-	Bladder?	
CD70	Surface	Ligand of CD27 involved in immune cell homeostasis	+ (>95%)	+	-	Medullary thymic epithelial cells	
WT1	Intracellular	Transcription factor	+ (73–100%)	+	+	Kidney, spleen, heart, lung, prostate	CD8+ T cell responses observed in AML

Target antigen	Expression	Physiological function	Expression on bulk AML cells	Expression on LSCs	Expression on HSCs	Non-haematopoietic expression	Spontaneous immune response
PRAME	Intracellular	Cancer testis antigen	+ (41–55%)	+	Minimal	Testis	CD8+ T cell responses observed in AML
RHAMM	Intracellular	Cell–matrix interactions	+	Questioned	+	Colon	CD8+ T cell responses observed in AML
Survivin	Intracellular	Anti-apoptotic protein (relevance in embryogenesis)	+	+	+	Endothelial cells	T cell responses observed in breast cancer, melanoma, and CLL
hTERT	Intracellular	Subunit of the telomerase complex	+	Questioned	(+)	Keratinocytes, testis, endometrium, placenta	CD8+ T cell responses rarely observed in HCC or MM

Table 1.4 Lineage-restricted antigens in AML. Antigen expression: - negative, (-) infrequent, (+) low, + positive, ++ highly expressed. CLEC12A, C-type lectin domain family 12 member A; CLL-1, C-type lectin-like molecule-1; FLT3, fms-like tyrosine kinase 3; HSC, haematopoietic stem cells; IL1RAP, interleukin 1 receptor accessory protein. Adapted from (Daver *et al.*, 2021a).

Target antigen	Expression	Physiological function	Expression on bulk AML cells	Expression on LSCs	Expression on HSCs	Non-haematopoietic expression	Spontaneous immune response
CD33	Surface	Sialic-acid dependent cytoadhesion molecule	>99% (higher expression with normal karyotype or e.g., NPM1+)	+	+	Kupffer cells (liver), microglial cells (CNS)	
CD123	Surface	Interleukin 3 receptor	45-80% (higher expression in FLT3-ITD mutated AML)	++	(+)	Bronchus/ gastrointestinal tissue	
CLEC12A/CLL-1	Surface	Immunological homeostasis	78–92% (lower expression in adverse risk cytogenetics)	+	-	None reported	
CD117	Surface	Mast/stem cell growth factor receptor	78–90%	(-)	+	Epithelial cells (e.g., in skin, breast tissue), Cajal cells, melanocytes	
CD135/FLT3	Surface	Cytokine receptor	54-90%	++	(+)	CNS, intestine, testis (no surface expression)	
Folate receptor β	Surface	Folate uptake	~70%	Not assessed	(+)	None reported	
IL1RAP	Surface	IL1 receptor accessory protein	~80%	+	(-)	Oesophagus	
PR1/ proteinase-3- derived epitope peptide	Intracellular	Neutrophilic serine proteases	+	+	+	-	CD8+ T cell responses observed in AML

1.2.5 Immunotherapies that activate pre-existing T cells

T cells in the patient may be redirected or activated using specialised molecules and cells to eradicate tumour cells. Examples include immune checkpoint inhibitors, cancer vaccination and T cell recruiting antibodies, which are briefly discussed below.

1.2.5.1 Immune checkpoint inhibitors

Lymphocytes which migrate into tumours are termed tumour-infiltrating lymphocytes (TILs). Although TILs possess potent anti-tumour activities, their responses are suppressed by the tumour and the tumour microenvironment (TME). Immune-checkpoint inhibitors (ICPIs) are monoclonal antibodies that target immunoinhibitory receptors in order to restore immune responses against cancer cells (Makkouk and Weiner, 2015). ICPIs such as anti-PD-1, and anti-CTLA antibodies have been especially successful in in solid tumours such as melanoma due to their high somatic mutational burden and associated neo-antigen load (Büttner *et al.*, 2019, Carlino *et al.*, 2021, Chabanon *et al.*, 2016). Many trials are underway investigating ICPIs as single agents or in combination with hypomethylating agents or chemotherapy (Daver *et al.*, 2021a). However, AML is an immunologically “cold tumour”, having relatively few activated TILs in the bone marrow patients, and is more resistant to immune checkpoint blockade (Maleki Vareki, 2018).

1.2.5.2 Cancer vaccination

Cancer vaccination can potentially boost the patient immune system to recognise tumour antigens which have been tolerated in an immunosuppressive TME. Different methods have been trialled, including direct vaccination of tumour peptide (Jochen *et al.*, 2010), infusion of antigen presenting dendritic cells (DCs) loaded with leukaemia associated antigens (Anguille *et al.*, 2017, Lichtenegger *et al.*, 2020) and fusion of dendritic cells with AML blasts. For example, TLR7/8-matured DCs transferred with WT1 and PRAME RNA induced antibody responses in 3/7 initially seronegative AML patients in CR at high risk of relapse (Lichtenegger *et al.*, 2020). A molecular remission in 30% of patients was obtained using DCs electroporated with WT1 messenger RNA (mRNA) (Anguille *et al.*, 2017). However, while various studies have demonstrated enhancement of assays of vaccine-specific immune responses, these have not led to significant clinical disease control and may be more suited in the setting of consolidation of an established complete remission rather than active disease (Anguille *et al.*, 2017, Di Stasi *et al.*, 2015, Lichtenegger *et al.*, 2020, Lin and Li, 2013, Rosenblatt *et al.*, 2016).

1.2.5.3 T cell recruiting bispecific antibodies

A broad range of bispecific antibodies have been developed, such as bispecific T cell engagers (BiTEs), dual-affinity retargeting (DART) molecules, tandem diabodies, and others (Daver *et al.*, 2021a), mainly due to the lack of efficacy from naked antibodies.

BiTEs are made of two linked single chain variable fragments (scFvs), one which targets CD3 and the other targets a TAA. The CD19 targeting BiTE blinatumomab was FDA approved after showing 43% CR in Ph-negative R/R B-ALL in a multi-centre trial (Curran and O'Brien, 2020). A major logistical disadvantage of initially developed BiTEs is their short half-lives which requires continuous intravenous (IV) infusions (Daver *et al.*, 2021a). This has been addressed with newer bispecific T-cell recruiting antibodies with longer half-lives such as glofitamab (Hutchings *et al.*, 2021). Early phase I clinical trials of different CD33 x CD3 BiTEs in R/R AML have induced some low remission rates with varying levels of manageable adverse reactions depending on BiTE design (Ravandi *et al.*, 2020, Subklewe *et al.*, 2019, Westervelt *et al.*, 2019).

DART molecules work similarly to BiTEs, but have heavy and light chain variable domains on separate polypeptides stabilised by C-terminal disulfide bridges. Flotetuzumab, a CD123 x CD3 DART induced a remission rate of 26.7% in R/R AML patients as a single agent, with manageable side effects (Uy *et al.*, 2021). This provides support for the notion that adoptive T cell therapies targeting CD123 may be a promising approach in AML.

1.2.6 Non-genetically modified T cell therapies

1.2.6.1 Alloantigen and minor histocompatibility antigen specific T cells

In HSCT, donor T cells may recognise non-donor self-antigens on recipient cells due to genomic polymorphisms such as (a) complexes of allelic variants of HLA molecules presenting peptides in HLA-mismatched HSCT, (b) processed peptide epitopes from mismatched HLA molecules presented by shared HLA molecules, and (c) minor histocompatibility antigens which are peptides derived from normal self-proteins that differ between donor and recipient due to genetic polymorphisms outside the HLA loci (Biernacki *et al.*, 2020, Bleakley and Riddell, 2004). Although both alloantigen and minor histocompatibility antigen specific T cells mediates GvL effect, they are also involved in the pathogenesis of GvHD when the antigens are presented on healthy non-haematopoietic tissues. However, current trials of anti-minor histocompatibility antigen specific T cells have not shown any clinical responses in relapsed patients, possibly due to the low cell numbers and exhaustion from long culture times (Meij *et al.*, 2012, Roex *et al.*, 2020). Moreover, alloantigen and minor histocompatibility antigens are recognised in a matched-HLA context, which would limit the availability of eligible donors for patients with unusual HLA types (Falkenburg *et al.*, 2003).

1.2.6.2 Tumour infiltrating lymphocytes

Neo-antigens may be presented by the tumour cells via MHC class I molecules which can be recognised by tumour infiltrating lymphocytes (TILs) and lead to tumour elimination. TILs can be extracted and expanded *in vitro* to overcome immune suppression and have demonstrated remarkable efficacy in melanoma (Rosenberg et al., 1988). TILs have been successfully isolated from AML patients' bone marrow, expanded, and exhibited cytotoxicity against autologous AML blast cells *in vitro* (Teo et al., 2019). However, TILs may only be detected and isolated from a fraction of AML patients (Wei et al., 2019), likely due to the overall low mutational burden compared to solid organ tumours such as melanoma (Büttner et al., 2019), which significantly reduces the number of neo-antigens available to prime the patient's own anti-tumour response.

1.2.6.3 Tumour associated antigen-specific cytotoxic T lymphocytes

In contrast to TIL, T cells specific for TAAs found in the peripheral blood of healthy donors or patients are termed TAA-specific cytotoxic T lymphocytes (CTLs). These can be selectively activated and expanded for CTL therapy via dendritic cells pulsed with tumour antigens. In the case of AML, a variety of TAAs have been targeted, such as PRAME, WT1, survivin and New York oesophageal squamous cell carcinoma 1 (NYESO-1) (Lee *et al.*, 2020, Lulla *et al.*, 2021, Weber *et al.*, 2013). A selection of AML CTL clinical trials is summarised in Table 1.5 below. In an early clinical trial, autologous lymphocytes expanded *ex vivo* using five TAAs proved to be safe and demonstrated some efficacy in achieving MRD negativity in 2/5 MRD$^+$ AML patients at high risk of relapse, however 1/4 MRD^{neg} patients relapsed (Xue *et al.*, 2019). CTLs also caused less GvHD with similar efficacy to DLI at maintaining remission, however only displayed limited efficacy against active relapse (Lulla *et al.*, 2021). The INTACT clinical trial conducted by the Westmead Cell Therapies Laboratory prophylactically infused WT1 and/or PRAME specific T cells with multi-pathogen specific (CMV, EBV, AdV and Aspergillus) T cells after HSCT of AML or high risk MDS patients in order to prevent relapse and infection. Out of 10 patients, seven remained in CR, ranging between 1 and 3.5 years post-HSCT (Jiang *et al.*, 2023). Therefore, the current evidence indicates that CTL may be more suitable as prophylactic or adjuvant therapy against relapse than treating active disease.

Table 1.5 Selected clinical trials of non-genetically modified adoptive T cells in AML. aGvHD, acute graft versus host disease; APC, antigen presenting cell; cGvHD, chronic graft versus host disease; CR, complete response; DC, dendritic cells; LFT, liver function tests; MAGE-A3, melanoma-associated antigen-A3; NYESO-1, New York oesophageal squamous cell carcinoma 1; PR, partial response; PRAME, preferentially expressed antigen in melanoma; RHAMM, Receptor for hyaluronan-mediated motility; WT1, Wilms' tumour 1. Adapted from (Lulla et al., 2019)

Target(s)/ HLA	Cell source and APC	Prior chemo	Cell dose	Patient numbers	Outcome	Toxicity Grade ≥3	Reference
WT1, PRAME, multi-pathogen	Allogeneic donor / peptide pulsed mononuclear cell	myeloablative or non-myeloablative allogeneic transplantation	2×10^7 cells/m^2	6 AML, 4 MDS	7 of 10 remained in CR	2 aGvHD 2 cGvHD	(Jiang *et al.*, 2023)
WT1 (HLA-A2)	Allogeneic donor / peptide pulsed DC	None	Up to 10^{10} /m^2	11 in relapse or CR	4 of 11 CR	None reported	(Chapuis et al., 2013)
WT1 (all HLAs)	Allogeneic donor / WT1 transduced DC	None	4×10^7	10 in CR post-transplant	5 of 10 alive at 8 years, 3 died of relapses, 2 died from treatment related mortality	None reported	(Kim et al., 2019)
WT1, PRAME, survivin, NYESO-1 (all HLAs)	Allogeneic donor / peptide pulsed DC	None	Up to 10×10^7/m^2	17 in CR, 8 in relapse	11/17 CR at 1.9 years in CR group, 2/7 responders in relapse group (1 CR, 1 PR)	None reported	(Lulla et al., 2021)
WT1, PRAME, survivin, NYESO-1, MAGE-A3 (all HLAs)	Autologous donor / peptide-pulsed DC	Decitabine or none	Up to three times, 1–5×10^7 cells/m^2 each time	9 (8 CR, 1 relapse)	5 / 9 became MRD^{neg}	None reported	(Xue *et al.*, 2019)
WT1, PRAME, survivin (all HLAs)	Allogeneic donor / peptide pulsed DC	None	Up to 4×10^7/m^2	Up to 90 (CR or relapse)	Still recruiting	Still recruiting	NCT02203903

Target(s)/ HLA	Cell source and APC	Prior chemo	Cell dose	Patient numbers	Outcome	Toxicity Grade ≥3	Reference
WT1, PRAME, NYESO-1, RHAMM, proteinase 3, HA-1 (HLA*02:01)	Allogeneic HA-1 negative HLA-A*0201 positive donor / not cultured	10 myeloablative, 17 non-myeloablative	Median 2.3×10^6 (0.1 – 25.5×10^6), includes other viral specific T cell products	24	13 infused TAA-specific T cells, none detected in peripheral blood.	No severe GvHD	(Roex *et al.*, 2020)
HA-1 (HLA-A*02:01)	Allogeneic HA-1 negative HLA-A*0201 positive donor / peptide pulsed DC	Ara-C and daunorubicin in one patient, unknown for another 2 patients	10–170×10^6 HA-1-specific T cells	3	No clinical response	No toxicity	(Meij *et al.*, 2012)

1.2.7 Genetically modified T cell therapies

1.2.7.1 Transgenic TCR T cells

One way to direct non-specific T cells against tumour cells is by genetic modification to express tumour antigen specific TCR.

Transgenic TCR T cells require the introduction of transgenes encoding the TCR α and β chains targeting tumour antigens. The TCR sequences are usually derived from single cell clones of tumour-specific T cells by mRNA extraction and reverse transcription polymerase chain reaction (RT PCR) using universal primer sets which recognise all TCR genes (Moysey *et al.*, 2004) or 5' rapid amplification of cDNA ends (5'-RACE) (Wälchli *et al.*, 2011). High throughput phage display may also be used to screen large TCR repertoires for high affinity TCRs against specific TAAs (Li *et al.*, 2005).

One intrinsic shortcoming of using TCR is that TCRs are HLA-restricted, so can only be used in patients with a corresponding HLA type, while CAR T cells are HLA independent. In addition, transgenic TCR carries the risk of mispairing of α and β chains due to the presence of both endogenous and transgenic TCR α and β chains in the same cell, which can lead to reduced activity against intended TAA and risk of autoimmune toxicity (Shao *et al.*, 2010, Wei *et al.*, 2022). Several strategies exist to increase preferential pairing between transgenic α and β chains such as introducing cysteine di-sulphide bridges (Govers *et al.*, 2010), introducing interacting amino acids (Voss *et al.*, 2008), or using murine TCR chains (Cohen *et al.*, 2006). A complimentary strategy is to knock out (KO) or knock down (KD) endogenous TCR, using molecular techniques including small interfering RNA (siRNA) (Casey *et al.*, 2020), zinc finger nucleases (Provasi *et al.*, 2012), transcription activator-like effector nucleases (TALEN) (Berdien *et al.*, 2014) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 (Knipping *et al.*, 2017). However, these solutions add another layer of complexity to the design and testing of transgenic TCRs.

Transgenic TCR T cell therapy had most notable clinical success in melanoma, pancreatic cancer, synovial cell sarcoma and multiple myeloma (Chodon *et al.*, 2014, Leidner *et al.*, 2022, Rapoport *et al.*, 2015, Robbins *et al.*, 2015, Robbins *et al.*, 2011). However, unexpected cross-reactivity with other antigens is also a concern such as TCR against the melanoma associated antigen MART 1 which targeted cardiac tissue, resulting in patient deaths (Linette *et al.*, 2013). Currently, only two reports demonstrated transgenic TCR T cells are efficacious in AML patients overexpressing HLA-A*02:01-and HLA-A*24:02 restricted WT1 (Chapuis *et al.*, 2019, Tawara *et al.*, 2017). Other ongoing trials are listed in Table 1.6 below.

The other main category of gene-modified T cells, CAR T cells in the context of AML is discussed in detail in the following section.

Table 1.6 Selected clinical trials of transgenic TCR T cells in AML. Bu, busulfan; CR, complete remission; Cy, cyclophosphamide; Flu, Fludarabine; PRAME, preferentially expressed antigen in melanoma; siRNA, small interfering RNA; TBI, total body irradiation; Thio, thiotepa; WT1, Wilms' tumour 1. Adapted from (Campillo-Davo *et al.*, 2021, Lulla *et al.*, 2019)

Target/ HLA	Cell source / transduction	Prior chemo	Cell dose	Patient numbers	Outcome	Toxicity Grade ≥3	Reference
WT1 (HLA-A2)	Allogeneic donor EBV-specific CTL / lentiviral	Flu/Cy and IL-2	Up to 10^{10}/m²	11 in CR, 11 in relapse arm	11/11 remaining in CR at 1y, no durable response in relapse arm	None	(Chapuis *et al.*, 2016)
WT1 (HLA-A2)	Allogeneic donor EBV-specific CTL / lentiviral	Various: Flu/Bu, Flu/TBI, Bu/Cy, Flu/TBI, Thio,	At least 10^{10}/m²	12 in CR	12/12 patients remaining in CR at 44 months	2/12 cytokine release syndrome, 12/12 lymphopenia 2/12 thrombocytopenia 2/12 neutropenia 7/12 anaemia	(Chapuis *et al.*, 2019)
WT1 (HLA-A2)	Autologous T cell / retroviral	Standard conditioning; 10^6 units/m² IL-2	Cohort 1: $\leqslant 2 \times 10^7$ T cells/kg Cohort 2: $\leqslant 1 \times 10^8$ T cells/kg	Cohort 1: 3 Cohort 2: 4	Cohort 1: 1/3 CR; 2/3 no response Cohort 2: 3/4 CR; 1/4 no response	Cohort 1: 1/3 febrile neutropenia	NCT01621724 UCL, UK
WT1 (HLA-A24)	Autologous T cell / retroviral with siRNA for native TCR	None	Up to 5×10^9	8 in relapse	Transient responses only	None	(Tawara *et al.*, 2017)
PRAME (HLA-A2)	Autologous T cell / has iCaspase switch	Not shown	Up to 5×10^6/kg	28 recruited	Not yet reported	Not yet reported	NCT02743611
HA-1 (HLA-A2)	Allogeneic / Lentiviral with iCaspase switch	Fludarabine	Up to 30×10^6/kg	9	2/9 CR sustained with bridging chemotherapy,	No cytokine release syndrome, immune effector cell-associated	(Krakow *et al.*, 2024)

Target/ HLA	Cell source / transduction	Prior chemo	Cell dose	Patient numbers	Outcome	Toxicity Grade ≥3	Reference
					2/9 reduction in marrow blasts for > 30 days	neurotoxicity syndrome or dose-limiting toxicity. 2/9 Grade 1 or 2 GvHD, 3/9 self-resolving fever	

1.3 CAR T cells

1.3.1 Chimeric antigen receptors

Chimeric antigen receptors (CARs) are artificially engineered receptors which specifically direct effector cells (such as T cells and natural killer (NK) cells) to a target antigen to elicit effector function. In the most basic form, CARs comprise of three components: an extracellular antigen recognition domain joined to a transmembrane domain and an intracellular signalling domain (Figure 1.4). The majority of antigen recognition domains are comprised of a single chain variable fragment (scFv) derived from the variable light and variable heavy chains of a monoclonal antibody that binds to the antigen of interest. Other recognition domains are possible as well, such as cytokines, ligands and nanobodies (He *et al.*, 2020, Wang *et al.*, 2018b). The commonality between these different antigen recognition domains is that they are almost always designed to bind extracellular antigens regardless of MHC type. This unique feature of CARs also has the drawback of being only able to recognise extracellular, but not intracellular antigens, though this paradigm is shifting with the advent of peptide-centric CARs (Yarmarkovich *et al.*, 2023). The hinge region is also crucial, affecting the cytokine release and cytotoxic activities of the CAR T cell (Laborda *et al.*, 2017).

CAR T cells have been hailed as a monumental success story in immunotherapy since the first publicised CAR T cell infusion of 6-year-old Emily Whitehead (Awasthi *et al.*, 2023). Since then, large international clinical trials have demonstrated that CAR T cells are able to induce long-lasting remissions in a significant proportion of R/R leukaemia and lymphoma patients with otherwise dismal prognosis (Table 1.7) which led to the eventual FDA approval of six CAR T cell products for B-cell haematological malignancies as of 2024 (Table 1.8). What is most encouraging is that real world experiences of approved CAR T cells have reflected comparable outcomes to the early clinical trials, even in patients with co-morbidities which would have excluded them from previous clinical studies (Jacobson *et al.*, 2024, Yassine *et al.*, 2020).

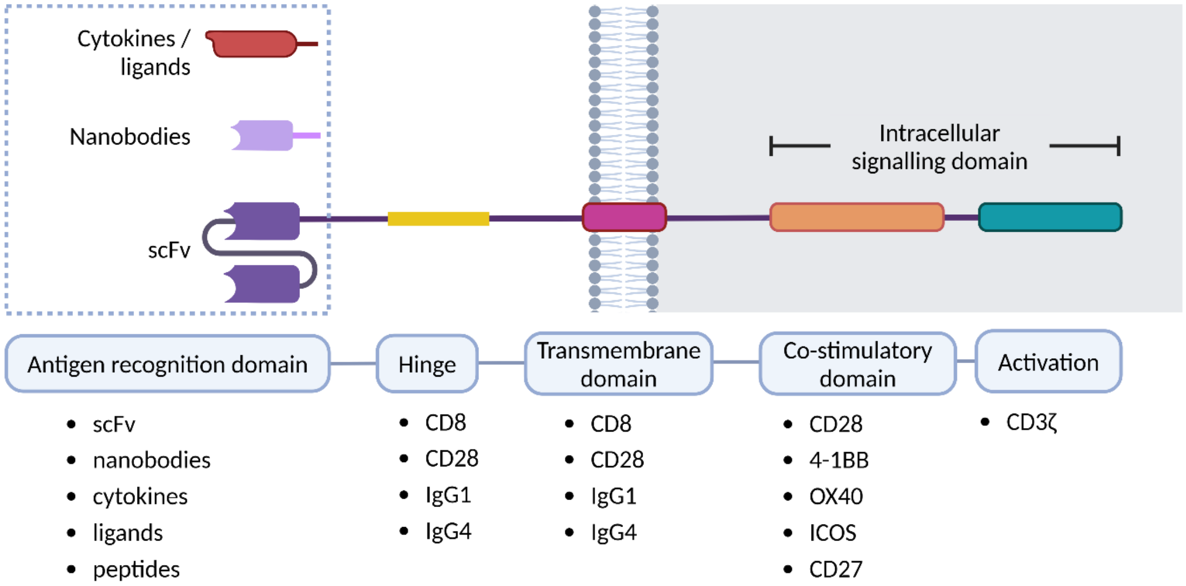


Figure 1.4 Schematic of basic CAR design with different options available for each domain. Adapted from (Rafiq *et al.*, 2020). ICOS, inducible T cell co-stimulator; scFv, short chain variable fragment. Created with BioRender.com.

Table 1.7 Select FDA approved CAR T cell product efficacies and toxicity. AE, adverse events; CR, complete response; CRS, cytokine-release syndrome; DLBCL, diffuse large B-cell lymphoma; HGG, hypogammaglobulinaemia; ICANS, immune effector cell–associated neurotoxicity syndrome; NHL, non-Hodgkin lymphoma; ORR, objective response rate; OS, overall survival

Product name	Indication; Pivotal study	Efficacy outcome	Safety outcome	Reference
Yescarta® (axicabtagene ciloleucel)	Refractory DLBCL; ZUMA-1	Patients: N = 101 ORR: 83% CR: 58% Median OS: 25.8 months	Patients: N = 108 CRS, grade ≥ 3: 11% ICANS, grade ≥ 3: 32% Received tocilizumab: 45%	(Neelapu *et al.*, 2017) (Locke *et al.*, 2019)
Yescarta® (axicabtagene ciloleucel)	Relapsed/refractory Follicular lymphoma; ZUMA-5	Patients: N = 84 ORR: 94% CR: 80% Median OS: not reached	Patients: N = 84 CRS, grade ≥ 3: 6% ICANS, grade ≥ 3: 15% Treatment-related AE, grade 5: 1 patient (CRS) Neutropenia Grade ≥ 3: 33% Anaemia, Grade ≥ 3: 23%	(Jacobson *et al.*, 2020)
Tecartus® (brexucabtagene autoleucal)	Relapsed/refractory mantle-cell lymphoma; ZUMA-2	Patients: N = 68 ORR: 91% CR: 68% Median PFS: 25.8 months Median OS: 46.6 months	Patients: N = 68 CRS, grade ≥ 3: 15% ICANS, grade ≥ 3: 31% Treatment-related AE, grade 5: 2 patients Received tocilizumab: 59% Neutropenia Grade ≥ 3: 85% Thrombocytopenia, Grade ≥ 3: 53% Anaemia, Grade ≥ 3: 53% Infection Grade ≥ 3: 34%	(Wang *et al.*, 2020a) (Wang *et al.*, 2020b) (Wang *et al.*, 2023)
Breyanzi® (lisocabtagene maraleucel)	Relapsed/refractory large B-cell lymphomas; TRANSCEND NHL 001	Patients: N = 256 ORR: 73% CR: 53% Median OS: 21.1 months	Patients: N = 269 CRS, grade ≥ 3: 2% ICANS, grade ≥ 3: 10% Treatment-related AE, grade 5: 0 Received tocilizumab: 18% Neutropaenia, Grade ≥ 3: 60% Thrombocytopenia, Grade ≥ 3: 27% Anaemia, Grade ≥ 3: 37%	(Abramson *et al.*, 2020)

Product name	**Indication; Pivotal study**	**Efficacy outcome**	**Safety outcome**	**Reference**
Kymriah® (tisagenlecleucel)	Relapsed/refractory DLBCL; JULIET	Patients: N = 93 ORR: 52% CR: 40%	Patients: N = 111 CRS, grade ≥ 3: 22% ICANS, grade ≥ 3: 12% Treatment-related AE, grade 5: 0 Received tocilizumab: 14% Neutropaenia, Grade ≥ 3: 14% Infections, Grade ≥ 3: 20%	(Schuster *et al.*, 2019)
Kymriah® (tisagenlecleucel)	Relapsed/refractory B-cell acute lymphoblastic leukemia in adults <25years old; ELIANA	Patients: N = 75 MRD neg CR= 81% 12 month PFS: 50% 12 month OS: 76%	Patients: N = 75 CRS, grade ≥ 3: 46% ICANS, grade ≥ 3: 13% Neutropaenia, Grade ≥ 3: 35%	(Maude *et al.*, 2018)
Abecma® (idecabtagene vicleucel)	Relapsed/refractory multiple myeloma; KarMMa-2	Patients: N = 31 ORR: 87.1% CR: 74.2% 24 month PFS: 83.1% 24 month OS: 100%	Patients: N = 31 CRS, Grade ≥ 3: 12.9% ICANS, Grade ≥ 3: 6.5% Treatment-related AE, grade 5: 0	(Dhodapkar *et al.*, 2022)
Abecma® (idecabtagene vicleucel)	Relapsed/refractory multiple myeloma; KarMMa-3	N = 254 ORR: 71% CR: 39% Median PFS: 13.3 month	N = 225 CRS, Grade ≥ 3: 5% Neurotoxicity, Grade ≥ 3: 3% Neutropenia: 7.1% HGG: 6.7%	(Rodriguez-Otero *et al.*, 2023) (Baz *et al.*, 2023)
Carvykti® (ciltacabtagene autoleucel)	Relapsed/refractory multiple myeloma; CARTITUDE-1	N = 97 ORR: 97.9% CR: 82.5% 27 month PFS: 54.9% 27 month OS: 70.4%	N = 97 CRS, Grade ≥ 3: 4.1% Neurotoxicity, Grade ≥ 3: 12.3% Treatment related AE, grade 5: 6.2% Neutropenia, Grade ≥ 3: 94.8% Anaemia, Grade ≥ 3 68.0%	(Martin *et al.*, 2023)

Table 1.8 FDA approved CAR T cell products. R/R, relapsed/refractory; B-ALL, B-cell acute lymphoblastic leukaemia. Adapted from (Chen *et al.*, 2023c)

Product name and year approved	Target antigen	Indication(s)	Pivotal studies	References
Kymriah® (tisagenlecleucel) - Approved 2017	CD19	Children and young adults with R/R B-ALL Adults with R/R B-cell non-Hodgkin lymphoma Adults with R/R follicular lymphoma	ELIANA JULIET	(Laetsch *et al.*, 2019) (Schuster *et al.*, 2019)
Yescarta® (axicabtagene ciloleucel) - Approved 2017	CD19	Adults with R/R B-cell non-Hodgkin lymphoma Adults with R/R follicular lymphoma	ZUMA-1 ZUMA-5	(Neelapu *et al.*, 2017) (Jacobson *et al.*, 2021)
Tecartus® (brexucabtagene autoleucal) - Approved 2020	CD19	Adults with R/R mantle cell lymphoma Adults with R/R B-ALL	ZUMA-2 ZUMA-3	(Wang *et al.*, 2020a) (Shah *et al.*, 2021)
Breyanzi® (lisocabtagene maraleucel) - Approved 2021	CD19	Adults with R/R B-cell non-Hodgkin lymphoma	TRANSCEND	(Abramson *et al.*, 2020)
Abecma® (idecabtagene vicleucel) - Approved 2021	BCMA	Adults with R/R multiple myeloma	KarMMa	(Munshi *et al.*, 2021)
Carvykti® (ciltacabtagene autoleucel) - Approved 2022	BCMA	Adults with R/R multiple myeloma	CARTITUDE-1	(Berdeja *et al.*, 2021)

1.3.2 Advancement in CAR structures

Altering and adding different domains to the CAR structure has been an area of intense research since its inception in 1993 (Eshhar *et al.*, 1993). CAR designs have come a long way since the first generation which elicited *in vitro* activity but lacked *in vivo* efficacy (Brocker and Karjalainen, 1995, Till *et al.*, 2008). Second generation CARs incorporate a co-stimulation domain with significantly enhanced CAR T cell anti-tumour activity and persistence *in vivo* (Brentjens *et al.*, 2007, Finney *et al.*, 1998, Kowolik *et al.*, 2006, Milone *et al.*, 2009, Savoldo *et al.*, 2011, Song *et al.*, 2011) (Figure 1.5). Most clinical trials and all commercially available CARs use a second-generation design.

Third generation CARs contain two co-stimulatory domains, the most common being CD28 and 4-1BB, which are associated with rapid onset of T cell activity and increased persistence, respectively (Baroni *et al.*, 2020, Li *et al.*, 2018c, Salter *et al.*, 2018). Compared to second-gen CARs, third-gen anti-CD19 CAR T cells consistently had better expansion and persistence in trial patients (Ramos *et al.*, 2018). However, *in vivo* studies show that third-generation CAR T cells could have inferior activity due to exhaustion and activation-induced cell death (Künkele *et al.*, 2015).

Fourth generation CARs are not as well defined, but include further modifications such as the expression of cytokines, receptors, antibodies, enzymes and transcriptional factors to overcome roadblocks in T cell activity and tumour microenvironment (Chmielewski and Abken, 2015, Tang *et al.*, 2023). Fourth generation CARs are still mostly in the preclinical development stage due to the complexity of immune responses initiated.

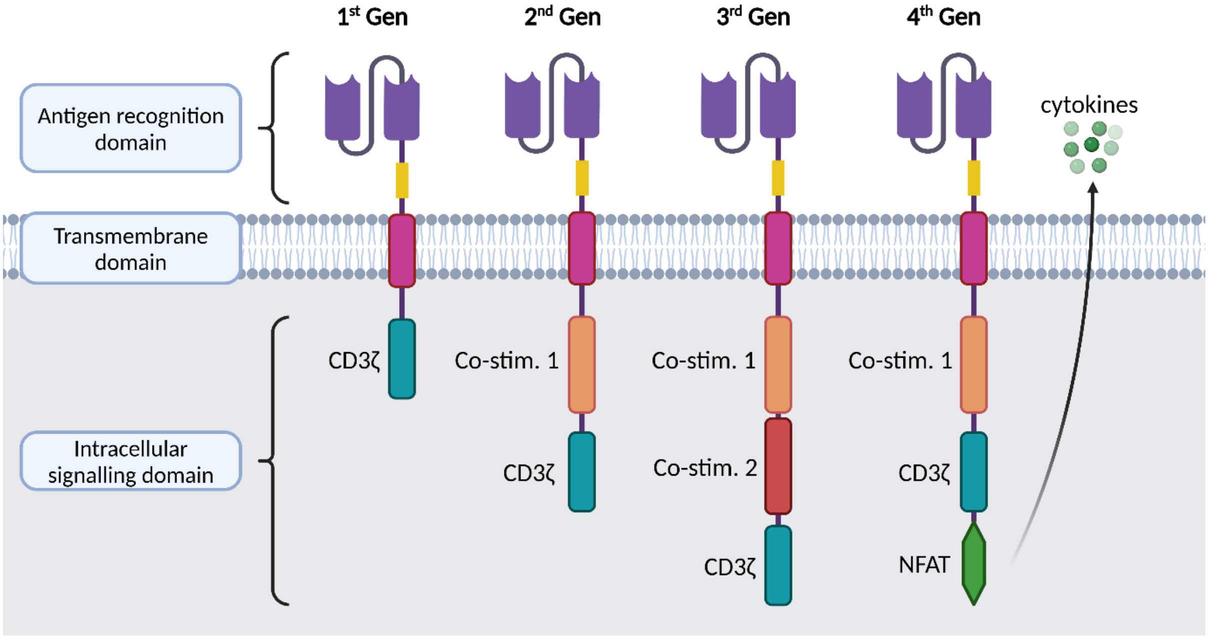


Figure 1.5 Different generations of CARs. First generation CAR only contains a CD3ζ activation module intracellularly. Second and third generation CARs has one and two co-stimulatory domains respectively. An example of fourth generation CAR T cells is those engineered with nuclear factor of activated T cell (NFAT) responsible for expression of a transgenic product such as cytokines. Adapted from (Elahi *et al.*, 2018). Created with BioRender.com.

1.3.3 CAR T cells' unique toxicity challenges

Despite the remarkable efficacy of CAR T cells, being living drugs, they present a unique toxicity profile. Cytokine release syndromes (CRS), immune effector cell-associated neurotoxicity syndromes (ICANS), and cytopenias / immune effector cell–associated haematotoxicity (ICAHT) are some of the common challenges encountered by patients (Ali *et al.*, 2020b) (Table 1.7).

CRS is a supraphysiological inflammatory state triggered by cytokines and chemokines released by CAR T cells and host immune cells. Symptoms range from fever, fatigue, myalgia, but can rapidly progress to hypotension, tachycardia, respiratory distress and even shock and organ dysfunction (Brudno and Kochenderfer, 2016). Fortunately, effective therapies for CRS such as tocilizumab, an anti-IL-6 monoclonal antibody and corticosteroids, are readily available and have become standard-of-care for moderate to severe CRS (Le et al., 2018), without affecting the expansion, persistence and response rates of CAR T cells (Gardner *et al.*, 2019, Liu *et al.*, 2020b).

While the pathophysiology of ICANS is less well understood, evidence suggests that CAR T cells promote an inflammatory response from myeloid cells, resulting in systemic inflammation and activation of endothelial cells, systemic capillary leak and subsequent dysfunction of the blood-brain-barrier exposing cerebrospinal fluid to cytokines (Gust *et al.*, 2017). Expressive aphasia and changes in handwriting are specific and early signs of ICANS, which can deteriorate to loss of consciousness, coma and fatal cerebral oedema. Management is dependent on grading of ICANS, ranging from supportive care to lympho-toxic doses of corticosteroids.

Late ICAHT (cytopenia beyond 30 days) is observed in approximately 30% of patients treated in seminal clinical trials with axicabtagene ciloleucel (axi-cel) or tisagenlecleucel (tisa-cel) (Lee *et al.*, 2015, Maude *et al.*, 2018, Neelapu *et al.*, 2017, Schuster *et al.*, 2019), but may be up to 60% in recent real-world reports (Zenner *et al.*, 2024) which is reflected in the supportive transfusions of packed red blood cells and platelets (approximately 63-66% of patients and 42-52% of patients respectively), and granulocyte colony-stimulating factor administered (62-88% of patients) (Jain *et al.*, 2020, Logue *et al.*, 2022).

Due to the expression of CD19 on B cells, B cell aplasia and hypogammaglobulinemia are expected on-target, off-tumour toxicities after anti-CD19 CAR T cell therapy, which exposes patients to risk of infections (Doan and Pulsipher, 2018). Intravenous immunoglobulin G (IVIG) supplementation during B cell aplasia is routinely undertaken for paediatric patients and may be considered for adults, though CD19^{neg} memory plasma cells capable of secreting antibodies have been described (Bhoj *et al.*, 2016, Halliley *et al.*, 2015, Hill *et al.*, 2019). More recently, BCMA-targeting CAR T cell ciltacabtagene autoleucel (cilta-cel) has been implicated in the development of grade ≥3 parkinsonism (Van Oekelen *et al.*, 2021), but whether it was true on-target off-tumour toxicity is debatable (Marella *et al.*, 2022). On-target off tumour toxicity remains a crucial point to consider in terms of CAR T cell safety, which is explored in more detail below, specifically in relation to AML-specific CAR T cells.

1.4 CAR T cells for AML

One of the major contributing factors to the success of commercial CAR T cells is due to the near-exclusive expression of CD19 and BCMA on malignant cells. The major on-target off-tumour side effects are due to eradication of B cells and subsequent hypogammaglobulinemia which may be rectified by immunoglobulin infusions. Mimicking the enormous success of CD19 and BCMA targeting CAR T cell therapy in AML is proving to be challenging due to the lack of a single targetable antigen on AML. Myelosuppression would be a likely consequence that can prove to be deadly if any overexpressed TAA is targeted due to on-target off-tumour toxicity.

Currently only a few small clinical trials have been published for AML CAR T cells, despite much pre-clinical research against different targets, with generally excellent *in vitro* and *in vivo* efficacies (Canichella *et al.*, 2023). Of the few small early clinical trials reported, no serious side effects were observed, and some trials even demonstrated promising results of a few MRD negative complete remissions achieved (Table 1.9). One major common trend of the trials' designs is the dependence on allogeneic haematopoietic stem cell rescue post CAR T cell infusion due to myelotoxicities. Nonetheless, ongoing efforts are evident in the voluminous early clinical trials that are open to recruitment globally, mainly in China and USA (Table 1.10). The most difficult issue so far is the selection of an appropriate antigen that satisfies the ideal criteria for a CAR target (Table 1.2). Although a myriad of potential antigens exists for AML (Table 1.3 and Table 1.4), the following section highlights the most commonly used ones in AML CAR T therapy as well as ones relevant to this thesis.

Table 1.9 Reported clinical trials of CAR T cells against AML. CR, complete remission; CRi, CR with incomplete count recovery; Cy, cyclophosphamide; Flu, fludarabine; ICANS, Immune effector cell-associated neurotoxicity syndrome. Adapted from (Atilla and Benabdellah, 2023, Canichella *et al.*, 2023).

Study	Target	CAR T Cell/Lymphodepletion	Number of Patients	Response	Safety	Reference
Phase I	CLL-1	1 – 2 × 10^6/kg / Cy+Flu	10	7/10 CR/CRi	10/10 CRS: 4 low grade, 6 high grade No CAR T cell-related encephalopathy syndrome 10/10 pancytopenia: severe 2 death due to chronic agranulocytosis	(Jin *et al.*, 2022)
Phase I/II	CLL-1	0.35 – 1 × 10^6/kg anti-CLL-1-CD8-4-1BB / Cy+Flu	8	4/8 morphological leukemia-free, MRD$^-$ 1 morphological leukemia-free, MRD$^+$, 1 CRi MRD$^+$, 1 PR, 1 SD	CRS: 5 grade 1, 3 grade 2	(Zhang *et al.*, 2022)
Phase I	CLL-1	0.5 – 2 × 10^6/kg / Cy+Flu	30	12 MRD$^-$ CR/CRi 10 MRD$^+$ CR/CRi	30/30 CRS: 18 grade 0-2, 11 grade 3, 1 grade 4 One patient grade 4 neurotoxicity 30/30 cytopenia	(Zhang *et al.*, 2023)
Phase I	CLL-1 or CD33	1 – 3 × 10^6/kg anti-CLL-1 anti-CD33 compound CAR (separated by P2A peptide) / Cy+Flu	9	7/9 MRD^{neg}	8/9 CRS: 6 grade 1-2, 2 grade 3 4/9 neurotoxicity: 1 grade 1, 2 grade 3 9/9 grade 4 pancytopenia	(Liu *et al.*, 2018a) (Liu *et al.*, 2020a)
Phase I	CD33	0.3 × 10^6/kg anti- CD33-4-1BB	3	0/3 response	2 CRS; 1 ICANS. One patient had grade 3 tumour lysis syndrome-acute kidney injury, mucositis. Another patient experienced grade 2 intermittent orthostatic hypotension, increased bilirubin, ALT and AST	(Tambaro *et al.*, 2021)
Case report	CD33	1.12 × 10^9 anti-CD33-4-1BB	1	Disease progression at week 9	Grade 4 chills and a high fever, pancytopenia	(Wang *et al.*, 2015)

Study	Target	CAR T Cell/Lymphodepletion	Number of Patients	Response	Safety	Reference
Case report	CD123	1.8 × 10^6/kg apoptosis-inducible 4th gen 123-scFv-CD28-CD137-CD27-CD3ζ-iCasp9 / Cy	1	Partial remission: reduction of bone marrow blast 59% to 45%	Severe CRS	(Luo *et al.*, 2015)
Phase I	CD123	Dose escalation 50 × 10^6 – 200 × 10^6 anti-CD123-IgG4-CD28 / Cy+Flu	6	4/6 CR 2 reduced blasts	CRS: 4/6 grade 1, 1/6 grade 2 1 adenoviral pneumonia requiring intubation; and 1 grade 3 rash due to drug hypersensitivity	(Budde *et al.*, 2017)
Case report	CD123	100 – 250 × 10^6 CD28 universal CAR using CD123 targeting module	3	2/3 CRi 1/3 PR	2/3 CRS: grade 1 0 neurotoxicity 3/3 myelosuppression recovered after withdrawal of CD123 targeting module	(Wermke *et al.*, 2021)
Phase I	CD38	6.1–10 × 10^6/kg CD28-4-1BB/ Cy+Flu	6	4/6 CR with full donor chimerism (3/6 CR, 1/6 CRi)	5/6 mild CRS (Grade 1-2), 1/6 grade 3 CRS: hepatotoxicity with elevated serum transaminase and bilirubin levels 0 ICANS 6/6 neutropenia, thrombocytopenia	(Cui *et al.*, 2021)
Phase I	LeY	Anti-LeY-CD28 / Flu-Cy	4	1/4 with active leukemia had temporary reduction in peripheral blood blast cells. 1/4 achieved a cytogenetic remission, 2/4 had SD	One patient had transient grade 2 neutropenia	(Ritchie *et al.*, 2013)
Phase I	NKG2D ligands	1 × 10^6 –3 × 10^7 viable T cells CD3ζ-NKG2D	7	0/7 response	Not available	(Baumeister *et al.*, 2019)

Table 1.10 Current AML CAR T cell clinical trials open to recruitment. CLL-1, C-type lectin-like molecule 1; FLT3, FMS-like tyrosine kinase 3; R/R, relapsed refractory; UniCAR, universal CAR. Adapted from (Canichella *et al.*, 2023)

Target Antigen	Clinical Trial ID	Phase	Disease Status	Intervention	Institution
CD33	NCT05672147	Phase I	AML R/R	CD33 CAR T	City of Hope Medical Center
	NCT05248685	Phase I	AML R/R	CD33/CLL-1 CAR T	Beijing Boren Hospital
	NCT04835519	Phase I/II	AML R/R	CD33 CAR T	Beijing Boren Hospital
	NCT04010877	Phase I/II	AML R/R	UCARTCD33 CLL-1, CD33, CD38 and CD123	Shenzhen Geno-Immune Medical Institute
	NCT03971799	Phase I/II	AML R/R	CD33 CAR T	Center for International Blood and Marrow Transplant Research
	NCT05105152	Phase I	AML R/R	CD33 CAR T	Seattle Children's Hospital
CD123	NCT04265963	Phase I	AML R/R	CD123 CAR T	Chongqing Precision Biotech Co., Ltd.
	NCT04272125	Phase I	AML R/R	CD123 CAR T	Chongqing Precision Biotech Co., Ltd.
	NCT04010877	Phase I	AML R/R	CAR T CLL-1, CD33 and/or CD123	Shenzhen Geno-Immune Medical Institute
	NCT05457010	Phase I	AML R/R	SPRX002 and ARC T	Arcellx, Inc.
	NCT04230265	Phase I	AML R/R and BPDCN	UniCAR02-T	AvenCell Europe GmbH
	NCT06125652	Phase I/II	AML R/R	anti Tim3/CD123	Xuzhou Medical University
CLL-1	NCT04923919	Early Phase 1	AML R/R	CLL-1 CAR T	920th Hospital of Joint Logistics Support Force of People's Liberation Army of China
	NCT05248685	Phase 1	AML R/R	Dual CD33/CLL-1 CAR T	Beijing Boren Hospital
	NCT04219163	Phase 1	AML R/R	CLL-1 CAR T	Baylor College of Medicine
	NCT04884984	Phase 1/2	AML R/R	CLL-1 CAR T	The First Affiliated Hospital of Soochow University
	NCT04010877	Phase 1/2	AML R/R	CLL-1 CAR T	Shenzhen Geno-Immune Medical Institute
	NCT06017258	Phase 1	AML R/R	CD371-YSNVZ-IL18 CAR T	Memorial Sloan Kettering Cancer Center (Responsible Party)
	NCT04789408	Phase 1	AML R/R	CLL-1 CAR T	Kite, A Gilead Company

Target Antigen	Clinical Trial ID	Phase	Disease Status	Intervention	Institution
	NCT06110208	Early Phase 1	AML R/R	CLL-1 and CD38 dual-target CAR T injection	920th Hospital of Joint Logistics Support Force of People's Liberation Army of China
CD70	NCT04662294	Early Phase 1	Non-Hodgkin's Lymphoma Multiple Myeloma AML R/R	CD70 CAR T	Zhejiang University
FLT3	NCT05023707	Phase 1/2	R/R AML	Anti-Flt3 CAR T	The First Affiliated Hospital of Soochow University
	NCT05445011	Phase 1	R/R AML	Anti-Flt3 CAR T	Wuhan Union Hospital, China
CD7	NCT04762485	Phase 1/2	R/R AML R/R T ALL	Anti-CD7 CAR T	The First Affiliated Hospital of Soochow University
	NCT04033302	Phase 1/2	R/R AML R/R T ALL	Anti-CD7 CAR T	Shenzhen Geno-Immune Medical Institute
	NCT05907603	Early Phase 1	R/R AML R/R T ALL	RD13-02 cell infusion	Kai Lin Xu, MD

1.4.2 Anti-CD33 CAR T cells

CD33 is a myeloid lineage antigen normally present on myeloid progenitors and mature myelocytes (Haubner *et al.*, 2019), but overexpressed in AML patients. CD33 overexpression is demonstrated on blasts of 65-90% AML patients in many large studies collating hundreds of patient samples (Baroni *et al.*, 2020, Ehninger *et al.*, 2014, Kaleem *et al.*, 2003). However, the median expression of CD33 on LSCs is on par with normal haematopoietic stem cells, raising concerns of myelosuppression and/or LSC escape (Haubner *et al.*, 2019), which was confirmed by *in vitro* and *in vivo* models. Although anti-CD33 CAR T cells are effective pre-clinically, most researchers found impairment of haematopoietic reconstitution of human CD34$^+$ HSC (Dutour *et al.*, 2012, Kenderian *et al.*, 2015a, Li *et al.*, 2018c, Pizzitola *et al.*, 2014, Rafiq *et al.*, 2016). In clinical trials, one case report demonstrated a partial reduction of blast cells 14 days after CAR T cell, while no response was demonstrated in another trial with 3 infused patients (Tambaro *et al.*, 2021, Wang *et al.*, 2015). Other clinical trials involving CD33 CARs are still actively recruiting.

1.4.3 Anti-CLEC12A/CLL-1 CAR T cells

Another commonly researched lineage-specific AML target is C-type lectin domain family 12 (CLEC12A), which is also known by many other names – CLL-1, CD371, dendritic-cell-associated C-type lectin 2 (DCAL2), myeloid inhibitory C-type lectin-like receptor (MICL) and KLRL1. It is a type II transmembrane glycoprotein (Marshall *et al.*, 2004), but its natural human ligand remains a mystery, while its physiological functions are also poorly understood. CLEC12A is normally present on most mature myeloid cells, but absent on normal haematopoietic stem (CD34$^+$CD38^{neg}) and progenitor (CD34$^+$CD33^{neg}) cells, erythrocytes, NK, B, T cells and plasmacytoid dendritic cells (Morsink *et al.*, 2019), with little evidence to suggest that it is found on other tissue types (Bakker *et al.*, 2004, Chen *et al.*, 2006, Haubner *et al.*, 2019).

CLEC12A is detected on 77.5-92% of AML cases at diagnosis (Bakker *et al.*, 2004, Haubner *et al.*, 2019, Wang *et al.*, 2018a), with its expression retained at relapse in a about 71% of patients (Haubner *et al.*, 2019, Larsen *et al.*, 2012), which implies that CLEC12A is a stable target for immunotherapy. CLEC12A is also implicated in relapse as higher expression in the LSC (CD34^{+}CD38^{neg}) population is associated with faster relapse (van Rhenen *et al.*, 2007b). However, CLEC12A expression on LSCs is extremely variable, being present on 33-45% diagnostic and 20% relapse samples respectively (Haubner *et al.*, 2019, van Rhenen *et al.*, 2007a, van Rhenen *et al.*, 2007b). Hence targeting CLEC12A alone may not be enough to eradicate all LSCs to prevent relapse.

Several preclinical studies have published encouraging results of anti-CLEC12A CAR T cells, demonstrating excellent *in vitro* and *in vivo* activities against cell lines and primary patient samples. These studies also showed that normal HSCs were spared in cytotoxicity and colony forming assays as predicted by expression patterns (De Togni *et al.*, 2018, Laborda *et al.*, 2017, Tashiro *et al.*, 2017, Wang *et al.*, 2018a). One group observed modest *in vivo* activity with anti-CLEC12A CAR T cells as monotherapy, but when combined with cytarabine, it induced remarkable 100% survival at up to 200 days (Kenderian *et al.*, 2016). Research efforts have focused on optimising CAR designs by changing intracellular domains and the hinge regions, with CD28 intracellular signalling domain with a short hinge or CD8 endodomain producing the greatest anti-tumour activity *in vitro*, and improved persistence *in vivo* (Atilla et al., 2019). PD-1 silencing also enhanced *in vitro* activity of CLEC12A CAR T cells (Lin *et al.*, 2021).

Several clinical trials have shown promising results, with most patients achieving CR/CRi in those studies. However, consolidation with HSCT was needed to rescue from myelosuppression due to on-target off-tumour toxicity as in other AML CAR T cell trials (Jin *et al.*, 2022, Zhang *et al.*, 2022, Zhang *et al.*, 2023).

1.4.4 Anti-CD123 CAR T

CD123 or interleukin-3 receptor alpha, (IL-3Rα) is the alpha subunit of the IL-3 receptor, which associates with the common beta subunit (βc) to bind IL-3 and regulate growth and differentiation of multipotent and lineage-committed progenitor cells. It is absent on mature lymphoid cells, at low levels on HSCs, and present on granulocytes, monocytes, (Sato *et al.*, 1993), endothelial cells (Sun *et al.*, 2019) and wide range of other organs such as the respiratory tract, gastrointestinal tract, breast and cervix (Perna *et al.*, 2017). Studies have found it to be overexpressed in 45-80% AML samples of different subtypes (Baroni *et al.*, 2020, Ehninger *et al.*, 2014, Testa *et al.*, 2002). Its expression is also retained between diagnosis and relapse (Haubner *et al.*, 2019), making it a viable option for both first-line and consolidation treatments. Although CD123 is lower in expression level than CD33, it is less heterogeneous (Baroni *et al.*, 2020, Ehninger *et al.*, 2014), which is also evident in its similar expression on both bulk AML cells and LSCs, while CD33 is higher on bulk AML cells than LCSs. Furthermore, CD123 has a greater relative difference in expression on AML than healthy HSCs compared to CD33 (Haubner *et al.*, 2019). Thus, in theory, its more restricted expression on bulk AML and LSCs implies that targeting CD123 would be more effective for eradicating LSCs, reducing relapse rates, while also being less toxic to HSCs and myeloid cells.

Studies demonstrate that CD123 also plays a biological role in AML development. Binding of IL-3R by IL-3 alone, or with granulocyte colony stimulating factor (G-CSF) stimulates AML proliferation (Pébusque *et al.*, 1989). Overexpression of CD123 on AML is associated with enhanced blast proliferation (Testa *et al.*, 2002) and an independent predictor of worse OS (Lamble *et al.*, 2019). Pre-clinical and clinical data of CD123 antibody-drug conjugates and

DART molecules further supports the therapeutic function of targeting CD123 in AML (Campagne et al., 2018, Kovtun et al., 2018, Li et al., 2018b). Therefore, CD123 satisfies multiple parameters of an ideal target antigen (Table 1.2) in its expression patterns, oncogenicity and therapeutic function.

Anti-CD123 CAR T cells have been very successful in preclinical studies. Multiple reports demonstrate cytokine production and specific lysis against AML cell lines and primary AML samples (Baroni *et al.*, 2020, Gill *et al.*, 2014, Kenderian *et al.*, 2015a, Magnani *et al.*, 2016, Mardiros *et al.*, 2013, Pizzitola *et al.*, 2014). Furthermore, the anti-CD123 CAR T cells also exhibited *in vivo* immunologic memory as they expanded upon antigen re-challenge following primary challenge (Gill *et al.*, 2014). Moreover, T cells from both healthy and MDS/AML patient were able to be transduced with CD123 CARs to kill cancer cells in tumour xenograft models (Baroni *et al.*, 2020, Mardiros *et al.*, 2013, Stevens *et al.*, 2019). Therefore, anti-CD123 CAR T cells are clearly efficacious in the preclinical setting.

Evidence for anti-CD123 CAR T cell's potential myeloid toxicity has been more encouraging than anti-CD33 CAR. Although most have reported impaired clonogenic potentials as well as *in vivo* myelopoiesis of CD34$^+$ cells (Baroni *et al.*, 2020, Gill *et al.*, 2014, Kenderian *et al.*, 2015a, Laborda *et al.*, 2017), a direct comparison with CD33 CAR demonstrated that CD123 CAR T cells did not exhibit significant *in vitro* reduction in colony formation (Tettamanti *et al.*, 2013), *in vivo* myelosuppression, nor impaired secondary engraftment of CD34$^+$ HSCs (Pizzitola *et al.*, 2014). Therefore, CD123 may be a safer target than CD33 for CAR T cell development.

CD123 has the most clinical evidence reported for AML CAR T cells. The first case was in a relapsed AML patient in 2015 (Luo *et al.*, 2015) where a fourth generation CAR T cell reduced BM blast from 59% to 45%. Severe CRS was experienced by the patient, but controlled with tocilizumab. Encouragingly, no myeloid toxicity was observed. A phase I dose escalation trial of 6 R/R AML and one BPDCN patient highlighted the potency of a second generation CD123-directed CAR inducing 2 CRs and 4 blast reductions (Budde *et al.*, 2017). Furthermore, no treatment related cytopenia was observed, while CRS and neurotoxicity were lower than grade 3 and reversible. As a result of the trial, this CAR T product MB-102 was granted FDA orphan drug designation for AML. A novel universal CAR (uniCAR) construct using soluble CD123-specific targeting modules to recruit CAR T cells has also been tested in three AML patients (Wermke et al., 2021). Two patients achieved CR with incomplete hematologic recovery (CRi) while the third had a partial remission. Additionally, adverse events were mild, and myelosuppression was immediately reversed on withdrawal of the targeting module. Though small, these clinical trials highlight the promising safety and efficacy of CD123-targeting CAR T cells, warranting further testing.

Current CD123-targeting CARs have, to the best of my knowledge, used scFvs as the recognition domain. However, our laboratory was the first to utilise IL-3, the natural ligand of CD123/IL-3Rα to recognise the CD123 antigen on AML cells (Lee, 2021).

Using natural ligands have theoretical advantages over scFv-based CARs. Because scFv are synthetic peptides consisting of variable regions of an antibody's light and heavy chains taken out of their natural context of an antibody, factors intrinsic to the scFv could destabilise its structure, such as solvent exposure of hydrophobic residues at the variable-constant interface and loss of stabilising interactions in the constant region of the antibody. Hydrophobic amino acid residues that are normally found between variable and constant regions of an antibody are exposed to solvent, when can lead to protein unfolding (Nieba *et al.*, 1997). These unstable scFv designs are usually identified and discarded or modified due to lack of efficacy in preclinical studies, adding to the time to develop functional CARs. Multiple scFvs could also aggregate through domain swapping, where V_H region of one scFv incorrectly associate with V_L region of another scFv, which leads to tonic signalling, exhaustion and lack of efficacy

(Branella and Spencer, 2021, Long *et al.*, 2015). Moreover, natural ligands are usually shorter than scFvs, which would increase the gene delivery efficiency via transfection or viral transduction.

Out of the 133 amino acids of the IL-3 mature peptide, amino acids 15-125 is sufficient for ligand function (Klein et al., 2001). Furthermore, certain point mutations affect binding affinities to α and βc subunits of the IL-3R. The K116W substitution greatly increases binding affinity of IL-3 to CD123 (Lopez et al., 1992) while E22R substitution disrupts recruitment of βc which subsequently abrogates downstream signalling of IL3-R (Barry et al., 1994). The Westmead Cell Therapies Laboratory has constructed CARs with wild-type (wt) IL-3, single mutant (sm) IL-3 (with K116W substitution), and double mutant (dm) IL-3 (with K116W, E22R dual substitutions) binding domains, which would theoretically have higher binding capacity without signal induction in CD123 bearing tumour cells, potentially making the dm IL-3 CARs (CARIL3dm) more effective and safer for use in humans.

1.4.5 Anti-TIM-3 CAR T cells

T cell immunoglobulin and mucin domain 3 (TIM-3) is traditionally studied as an immune checkpoint receptor on T cells. Thus far, four ligands of TIM-3 have been discovered – galectin-9, phosphatidylserine (PtdSer), high-mobility group protein B1 (HMGB1), and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), all of which have been implicated in tumour progression of different cancers (Acharya *et al.*, 2020, Shin and Ribas, 2015, Zhou *et al.*, 2011), presumably as a result of exhausted and dysfunctional T cells. Higher TIM-3 levels on marrow resident T cells of AML patients are associated with induction failure (Dama et al., 2019). TIM-3 expression levels on peripheral blood $CD4^+$ and $CD8^+$ cells of AML patients are also correlated with high-risk groups of FLT3-ITD mutation and National Comprehensive Cancer Network (NCCN) high risk stratification respectively (Li *et al.*, 2014).

Moreover, in solid tumours such as non-small cell lung cancer (NSCLC) and hepatocellular carcinoma, higher TIM-3 expression on T cells is also a negative prognostic marker of patient survival (Gao *et al.*, 2012, Li *et al.*, 2012).

TIM-3 as a target on AML is a relatively new concept (Kikushige *et al.*, 2010). It is overexpressed in both AML bulk cells and LSCs, but not on normal CD34$^+$ HSC cells (Haubner *et al.*, 2019, Jan *et al.*, 2011, Kikushige and Miyamoto, 2013). A TIM-3/galectin-9 autocrine loop has also been demonstrated to be necessary for LSC renewal *in vivo* (Kikushige *et al.*, 2015). TIM-3 is also implicated in AML pathogenesis, able to initiate AML in mice, while TIM-3 negative cells could not (Jan *et al.*, 2011), suggesting that targeting TIM-3 could possibly eradicate all LSCs to prevent relapse. Moreover, anti-TIM-3 antibodies could also block AML reconstitution and reduce AML burden, without affecting normal CD34$^+$ engraftment (Kikushige *et al.*, 2010). These results together suggest that TIM-3 satisfies key selection criteria of desirable target antigens in terms of therapeutic function, oncogenicity, expression level on both bulk and LSCs.

The possibility of using TIM-3 as a target for CAR T cells has often been discounted due to the issue of fratricide, where CAR T cells against TIM-3 are likely to lyse and destroy other CAR T cells which also express TIM-3 following activation. However, the advent of genetic tools available to knock out TIM-3 expression, enables the exploration of TIM-3 as a potential target for CAR T cells.

There is limited literature on TIM-3 directed CAR T cells. One group constructed a second-generation anti-TIM-3 CAR T cell which possessed *in vitro* cytotoxicity against TIM-3 cell lines and the TIM-3 positive population within primary AML samples. However, specific cytotoxicity was quite low ($\leq$50% for most cell lines) and slow (50 – 100 hrs for maximal cytolysis). The CAR T cells also possessed *in vivo* activity as demonstrated by elevated plasma cytokine concentrations and tumour suppression in xenograft models (Lee *et al.*, 2021).

Another group developed a bispecific split CAR T cell targeting both CD13 and TIM-3 which reduced CD13$^+$ TIM-3$^+$ tumour burden and prolonged survival *in vivo*. However, some toxicity against normal HSCs was still observed (He *et al.*, 2020).

If the issue of fratricide can be resolved, TIM-3 targeting CAR T cells could be a useful addition to the arsenal of CAR T cells in AML, which could potentially be used in concert with other CARs to prevent immune escape, as explored further in this chapter.

1.5 Barriers to clinical implementation of AML CAR T cell therapy

Besides the selection of an appropriate target antigen, additional challenges exist in the implementation of AML CAR T cells, some of which are unique to AML as discussed below.

1.5.1 AML immune evasion

When endogenous immune response or GvL is elicited by tumour associated antigens (TAAs) or neo-antigens, AML is able to evade the immune mediated destruction using several mechanisms arising from the bone marrow tumour microenvironment (TME) or intrinsic leukaemic cell properties (Khaldoyanidi *et al.*, 2021, Tettamanti *et al.*, 2022). These need to be considered to design any effective immunotherapy.

The immediate niche where tumour cells reside is termed the TME. It plays substantial roles in contributing to immune evasion using biochemical and immunological methods. For example, depletion of arginine and tryptophan by myeloid suppressor cells in the TME may contribute to T-cell dysfunction and increased immunosuppressive regulatory T cells (Tregs) (Folgiero *et al.*, 2014, Mussai *et al.*, 2013). Tregs have been shown to aid immune escape via secretion of inhibitory cytokines (Szczepanski *et al.*, 2009).

AML also has intrinsic abilities to avoid immune detection, including downregulation of MHC class I molecules to avoid immune surveillance (Masuda *et al.*, 2007), upregulating CD47 "self"-signal to shield against phagocytosis (Jaiswal *et al.*, 2009) and induce peripheral tolerance (Zhang et al., 2013). Overexpression of immunoinhibitory ligands such as programmed death ligand-1 (PD-L1), galectin-9 (a TIM-3 ligand) by AML could also contribute to immune evasion by immune checkpoint inhibition of T cells and NK cells (Brodská *et al.*, 2016, Gonçalves Silva *et al.*, 2017, Kandel *et al.*, 2021). Moreover, studies have shown an upregulation of a whole host of immunoinhibitory receptors such as PD-1, CTLA-4, TIM-3 and LAG-3 on T cells of patients with AML, (Williams et al., 2019) indicating T cell dysfunction in the AML bone marrow. It has also been hypothesised that TIM-3 on AML plays a role in the maintenance of the LSC population while suppressing anti-tumour immunity by inducing expansion of myeloid-derived suppressor cells (MDSCs) and differentiation into tumour-associated macrophages (Gao *et al.*, 2014).

Additional agents such as immune checkpoint inhibitors can be co-administered to make AML more susceptible to endogenous or CAR T cell mediated killing (Daver *et al.*, 2021a, Kenderian *et al.*, 2015b). The hypomethylating agent decitabine also appears to enhance CD123 CAR T cell activity by promoting naïve, early memory and non-exhausted gene expressions (You *et al.*, 2020). The knock-out of co-inhibitory receptors, in particular TIM-3, to enhance CAR activity (Kamiya *et al.*, 2018, Ren *et al.*, 2017) has been explored by others (Lee *et al.*, 2021) and is assessed in this thesis.

While antigen loss of CD33 or CD123 following CAR T therapy has not been demonstrated in *in vivo* studies (Pizzitola *et al.*, 2014), compound CARs where the T cell express multiple CARs against different antigens can potentially target a larger proportion of leukaemic cells and prevent immune escape. Pre-clinical data of compound CAR T cells targeting CD33 or CD123, CD33 or CLEC12A, and CD123 or CLEC12A have demonstrated potent cytotoxicity *in vitro* and *in vivo* against dual positive cell lines as well as single antigen expressing cell lines (Atilla *et al.*, 2022, Petrov *et al.*, 2018). A first in-human trial of CD33 and CLEC12A targeting CAR induced remarkable MRD-negative remission rates for 7 out of 9 R/R AML patients who were heavily pre-treated with bone marrow blasts of 1.6 – 87.8% at time of CAR T infusion (Liu *et al.*, 2020a).

1.5.2 On-target off-tumour toxicity

On-target off-tumour toxicity refers to the unwanted injury of healthy tissues that express the target antigen. A few fatal examples have been observed in CAR T cells for other cancers, such as liver toxicity from carbonic anhydrase IX CAR in renal cell carcinoma (Lamers *et al.*, 2013) and pulmonary toxicity in anti-HER2 CAR T therapy (Morgan *et al.*, 2010), which emphasises the need for careful target choice.

Different CAR designs may be able to mitigate these toxicities. Several groups have modulated the affinity of the antigen binding domain of CD123-directed CAR T cells to target AML blasts preferentially (Arcangeli *et al.*, 2017, Thokala *et al.*, 2016). In this thesis, point mutations are introduced into the IL-3 ligand-based CAR to alter its affinity to increase its safety.

Other groups attempt to minimise on-target off-tumour toxicities by targeting leukemia associated immunophenotypes (LAIPs), which are combinations of antigens that are unique to AML, but absent on normal tissue (Leick and Maus, 2019). This is mainly achieved using Boolean logic AND-gate where the CAR T cells are only activated in the presence of two target antigens, some of which are shown in Figure 1.6 (Bucher and Feucht, 2023, Klein *et al.*, 2001, Kloss *et al.*, 2013, Lanitis *et al.*, 2013, Roybal *et al.*, 2016, Wilkie *et al.*, 2012).

However, a focus on specificity of CARs to avoid on-target, off-tumour toxicity comes at a cost of reduced coverage when only a subset of leukaemic blasts express the specific combination of antigens targeted by AND-gate CARs. This could lead to tumour antigen escape and relapse from antigen negative/single antigen positive clones. Due to the pervasive issue of immune escape and refractory disease in patients treated with CAR T cells in AML in trials thus far, work in this thesis does not pursue the AND-gate approach to reduce toxicity.

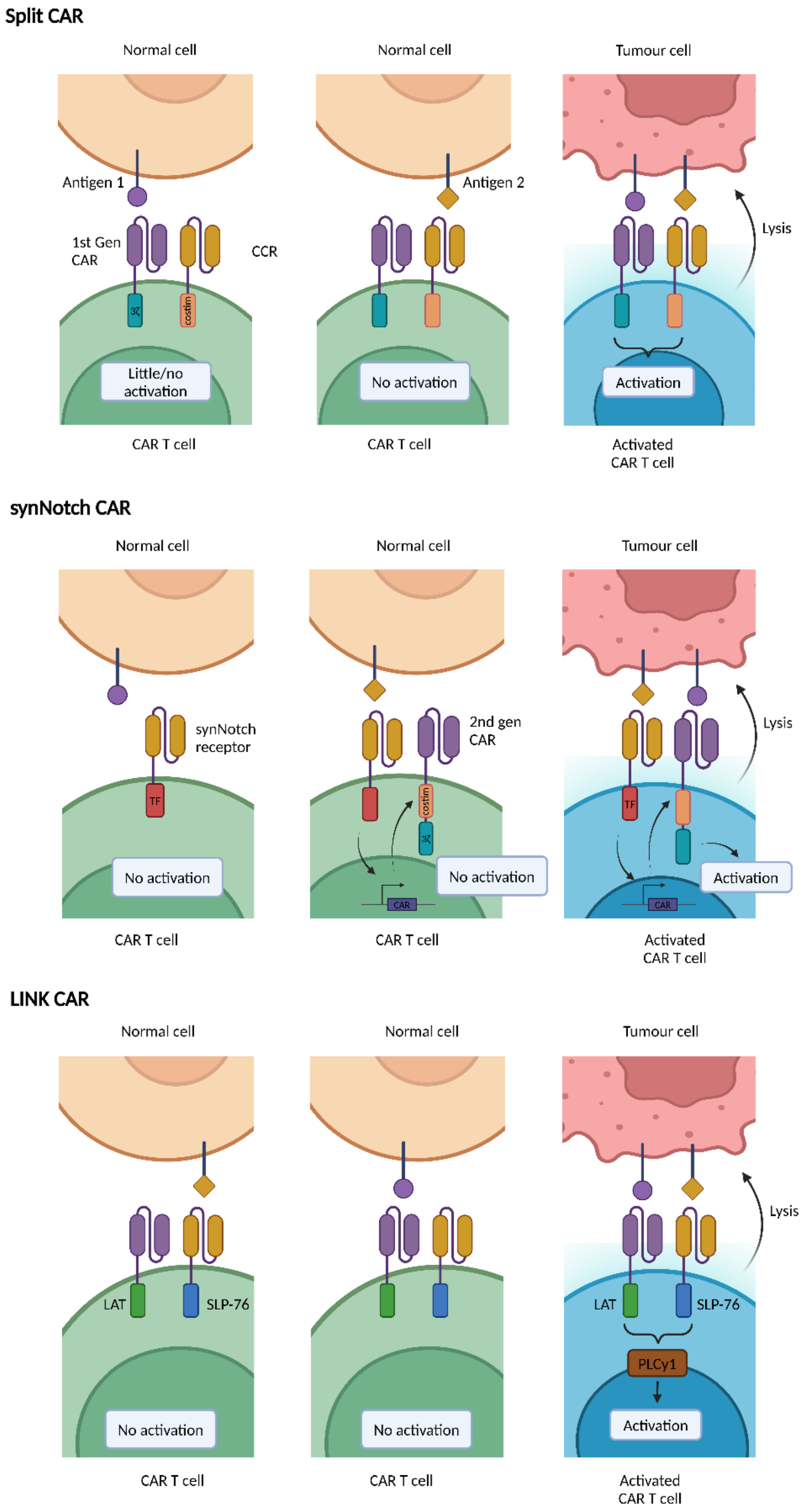


Figure 1.6 Examples of different molecular approaches of AND-gate CARs Costim, costimulatory domain; CCR, chimeric co-stimulatory receptor; LINK, logic-gated intracellular network; synNotch, synthetic Notch. Adapted from (Bucher and Feucht, 2023, Savanur *et al.*, 2021). Created in BioRender.com

1.5.2.2 Myelosuppression

Most AML targets investigated for CAR T cell therapy are lineage-restricted antigens that are also present on myeloid cells (e.g. CD33 and CD123), of which ablation would lead to on-target off-tumour toxicity of life-threatening myelosuppression. A few approaches have been explored to overcome this.

In most early phase clinical trials, patients achieving CR underwent allogeneic HSCT as a rescue of myelosuppression. Alemtuzumab which binds endogenous CD52 on T cells has also been demonstrated to be capable of depleting CAR T cells *in vivo*, and has been employed by some groups to deplete CAR T cells prior to subsequent HSCT (Petrov *et al.*, 2018, Tasian *et al.*, 2017). Co-expressing transgenic proteins such as truncated epithelial growth factor receptor (tEGFR) and then eliminating unwanted CAR T cells using corresponding antibodies such as cetuximab has also been tested (Wang *et al.*, 2011). This method has been successfully demonstrated in both CD33 (Rafiq *et al.*, 2016) and CD123-targeting (Mardiros *et al.*, 2013, Stevens *et al.*, 2019) CARs.

Another possible approach is to transiently express the CAR using mRNA. Anti-CD33 RNA-CAR T cells were most active 24 hours post electroporation which suppressed disseminated AML *in vivo* and prolonged survival of lymphodepleted mice (Kenderian *et al.*, 2015a).

Several groups have also explored gene KO of CD33 from normal HSPCs by CRISPR/Cas9, which are resistant to CD33-targeted killing, effectively making CD33 a leukemia-specific antigen. Remarkably, the CD33-KO HSPCs were completely functional and able to reconstitute all haematopoietic compartments in mice and rhesus macaque models. More importantly, CAR T cells specifically eradicated AML cells in mice models, while allowing engraftment of human CD34$^+$ cells (Borot *et al.*, 2019, Kim *et al.*, 2018).

These results highlight the possibilities of using safety switches or rescue strategies for CARs targeting lineage-restricted antigens alone.

1.5.2.3 Fratricide and T cell toxicities by CAR T cells

Another example of on-target off-tumour effect is the presence of target antigen on T cells in general or activated CAR T cells, which is predicted to cause fratricide between the CAR T cells. TIM-3, an upregulated inhibitory receptor on activated T cells, has been shown to lead to about 25% fratricide between TIM-3-targeting CAR T cells (Lee *et al.*, 2021).

However, CRISPR/Cas9 KO has proven to be a useful tool in circumventing fratricide. It has enabled the manufacture of CD7-targeting CAR T cells in T-ALL that would otherwise be unamenable to CAR T cell therapy due to fratricide (Cooper *et al.*, 2018, Gomes-Silva *et al.*, 2019, Xie *et al.*, 2023a). Furthermore, TIM-3 KO has been shown to not have any direct effect on CD19-specific CAR T cells' function *in vitro*, while increasing persistence (Schairer *et al.*, 2022). Therefore TIM-3 KO may have the added benefit of reducing T cell exhaustion as well as preventing fratricide which will be both explored in this thesis.

1.5.3 New malignancies arising from genetically modified T cells

Recently, the safety of genetically modified T cells has come into question. In a clinical trial, malignant transformation of CAR T cell into lymphomas have been reported after *PiggyBac* CAR T infusion in spite of similar CAR integration profiles to viral vectors which is the gold standard accepted for commercial manufacture (Bishop *et al.*, 2021). This highlights the need for a better understanding of contributing factors to CAR T cell genotoxicity, which may include high CAR copy number, CAR overexpression with basal activation and prolonged stimulation through CAR or endogenous TCRs.

The University of Pennsylvania also reported a case of T cell lymphoma which was most likely CAR negative 3-month post infusion of anti-CD19 CAR T cells. The same group also collated and calculate the projected 5-year cumulative incidence of secondary malignancies to be about 17%, which would be comparable to chemotherapy and/or radiation treatment and less than for stem-cell transplant patients, highlighting the relatively low risk of secondary malignancies post CAR T infusion (Ghilardi *et al.*, 2024).

Lentiviral vectors preferentially integrate within transcribed genes, but insertional mutagenesis, whether beneficial or detrimental cannot be entirely excluded (Fraietta *et al.*, 2018). Recently, there have been 3 cases of patients treated with the LV generated CAR T cell product cilta-cel who developed a CAR$^+$ T cell lymphoma (Harrison *et al.*, 2023, Ozdemirli *et al.*, 2024, Perica *et al.*, 2025). Although the exact cause is unknown, the contribution of insertional mutagenesis cannot be excluded. The FDA have also issued a statement of investigating into the serious risk of T cell derived malignancies, including CAR T cell lymphomas among patients receiving approved CAR T cells after case reports of 12 T cell lymphomas (Prasad, 2024).

Thus, the potential for genotoxicity remains a major incentive for finding safer approaches. Both transposon and lentiviral gene modification systems rely on random insertions of the transgene into the genome, carrying with it inherent dangers of insertional mutagenesis, therefore this thesis looked for more precise sequence-directed ways such as the CRISPR/Cas9 system for introducing the CAR transgene into T cells. These three gene modification systems are discussed in more detail below.

1.6 Molecular genetic techniques for generating CAR T cells

Gene modification to generate CAR T cells could be achieve by introducing DNA vectors (viral transgenes, plasmids, transposons), mRNA, antisense oligonucleotides (e.g. siRNA) and gene editing nucleases (e.g. CRISPR/Cas9, TALEN) (Stewart *et al.*, 2016) into T cells with gene delivery systems such as viral vectors, or by mechanical (e.g. electroporation) or carrier based (e.g. liposomes) technologies (Stewart *et al.*, 2016).

1.6.1 Transposon/transposase system

The transposon/transposase gene delivery systems such as *Sleeping Beauty*, *PiggyBac* and *PiggyBat* offer simpler, cheaper alternatives to viral transduction systems. DNA transposons are mobile genetic elements which may be excised from and inserted into genomes by transposases (Figure 1.7). The *PiggyBac* family of transposons are found across species, including *PiggyBac* from the cabbage looper moth (*Trichoplusia ni*) (Elick *et al.*, 1996, Fraser *et al.*, 1996), *PiggyBat* from the little brown bat (*Myotis lucifugus*) (Mitra *et al.*, 2013) and *PiggyBac* transposable element derived 5 (PGBD5) from humans (Smit and Riggs, 1996).

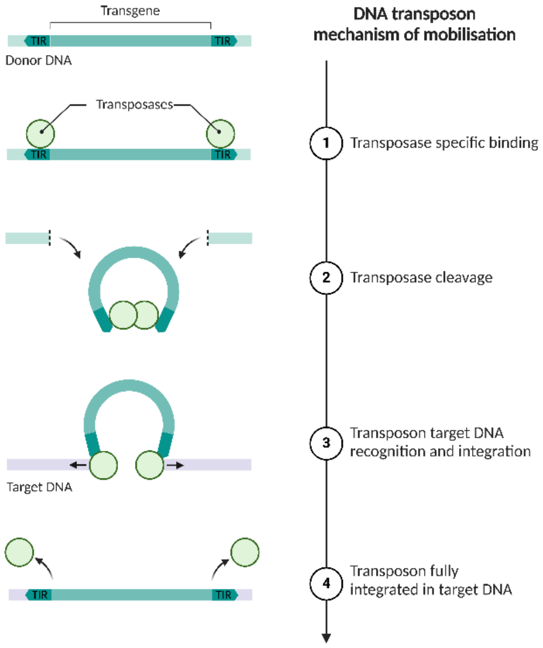


Figure 1.7 Diagram of transposon/transposase mobilisation. TIR, terminal inverted repeat. Based on (Sandoval-Villegas *et al.*, 2021). Created with BioRedner.com.

Transposon/transposase systems have already been successfully employed in manufacturing CAR T cells against CD19 (Bishop *et al.*, 2020, Bishop *et al.*, 2018, Magnani *et al.*, 2016, Ramanayake *et al.*, 2015), CD123 (Magnani *et al.*, 2016) and CD33 (Rotiroti *et al.*, 2020). Moreover, in contrast to γ-retroviral and lentiviral vectors, *PiggyBac* has the least integration frequency into or within 50kb of transcriptional start site of proto-oncogenes (Galvan *et al.*, 2009). *PiggyBac* and *PiggyBat* transposases have also demonstrated lack of cross-species reactivity against the human transposon PGBD5, which reduces the chances of inadvertent genome edits in human cells. However, genotoxicity is still a concern especially after the report of two CAR$^+$ lymphomas occurred in two patients after *PiggyBac* CAR T cells were infused (Bishop *et al.*, 2021, Micklethwaite *et al.*, 2021). It has been hypothesised that the cycle of transposition may continue in the cells, causing more double stranded breaks and likelihood of chromosomal rearrangements (Bushman, 2023).

Another transposon, the *PiggyBat* transposon has been described in 2014 (Campos-Sánchez *et al.*, 2014), but has not been used widely for CAR T cell generation. The Westmead Cell Therapies Laboratory has demonstrated that CD19 CAR T cells generated using the *PiggyBat* system has more consistent and lower integration number than the *PiggyBac* counterpart, possibly making *PiggyBat* CAR T cells a more consistent product (Sutrave *et al.*, 2019). Therefore, *PiggyBat* transposon system presents an unexplored, avenue to produce CAR T cells quickly and conveniently for proof of principle preclinical testing.

1.6.2 Lentiviral transduction system

Currently viral vectors are the gold standard of introducing transgenes into cells, which is evidenced by the fact that all FDA-approved CAR T cells are produced using viral vectors. Lentiviral (LV) vectors are derived from the *retroviridae* family, most commonly the human immunodeficiency virus type 1 (HIV-1) which normally delivers their retroviral RNA genome into host cells, which is reverse transcribed to DNA and preferentially integrated into transcriptionally active regions (Schröder *et al.*, 2002, Wang *et al.*, 2009). LV vectors exploits their natural ability of genome integration for persistent alteration of the cell's function and is applicable for both dividing and non-dividing cells (Milone and O'Doherty, 2018). Lentiviral vector systems have been developed over at least three generations to improve their safety profile. The third-generation lentiviral vector system splits HIV-1 genes into packaging, envelope and transfer plasmids, removal of viral accessory proteins from the packaging plasmid associated with disease progression and pathogenicity, and replacement of the 5' LTR sequence with a strong promoter, enabling the removal of the *tat* gene involved in enhancing transcription. The Rev element was also moved onto a new regulatory plasmid, which further decreased the possibility of replication competent lentivirus (RCL) formation (Figure 1.8).

Lentiviral vectors containing the transgene cassette are produced in cell lines transfected with all four plasmids (Figure 1.9).

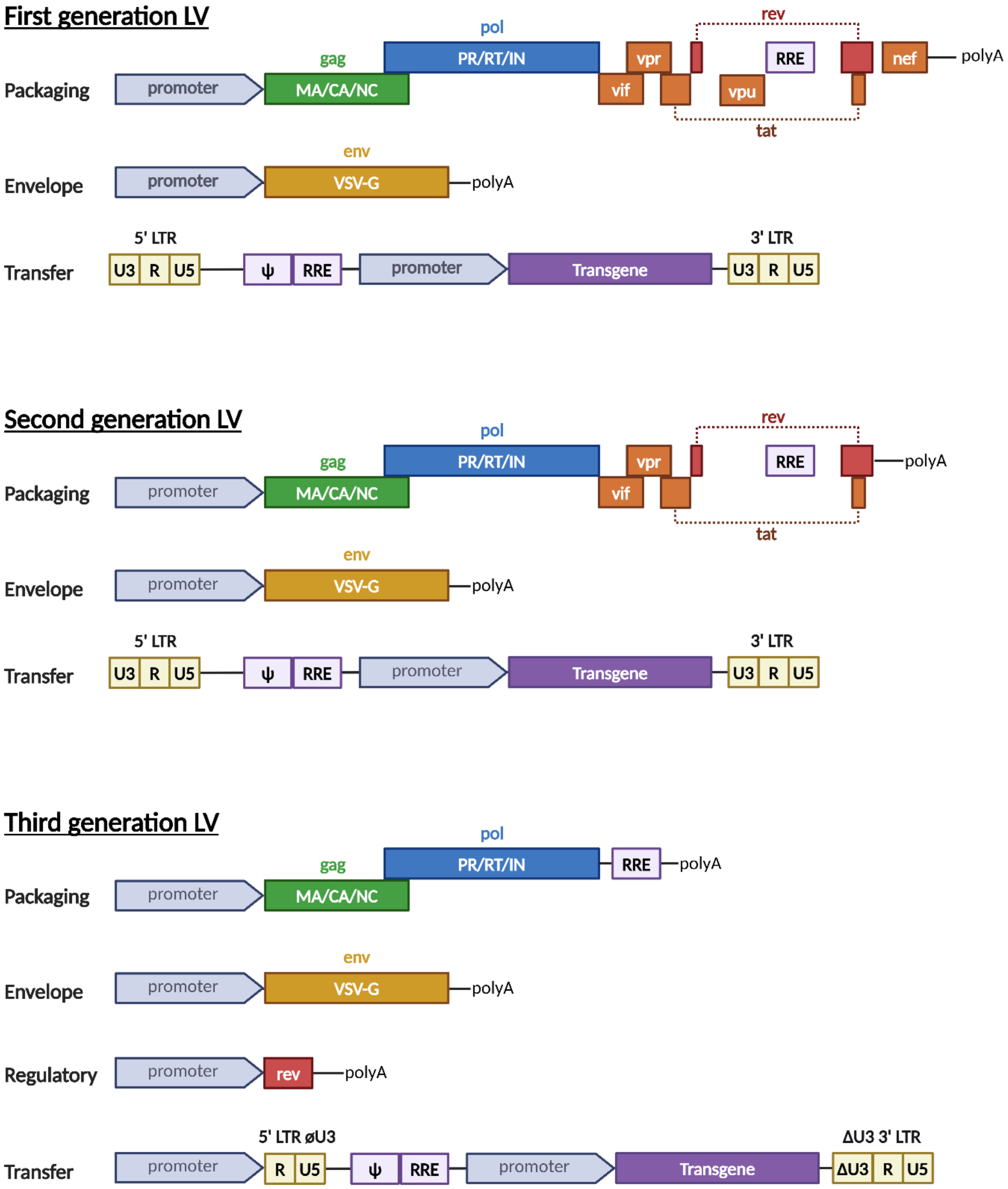


Figure 1.8 The three generations of LV vectors Each generation consists of plasmid constructs necessary for the production of LV vectors. Adapted from (Labbe *et al.*, 2021). Created with BioRender.com.

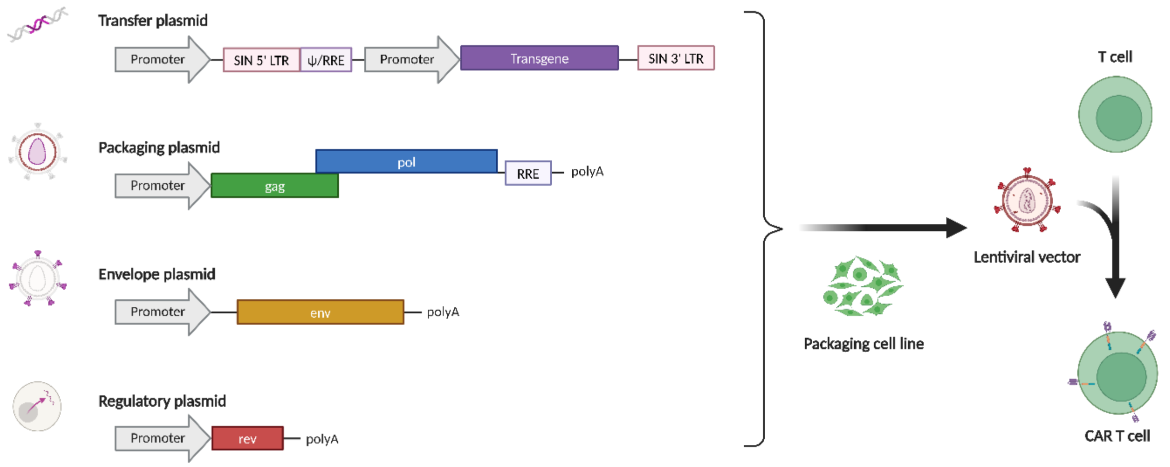


Figure 1.9 Schematic of third generation LV vector. SIN, self-inactivating; LTR, long terminal repeat; RRE, Rev response element; Ψ, retroviral psi packaging element; gag, group specific antigen; pol, polymerase; env, envelope; polyA, poly-adenine tail. Based on (Labbe *et al.*, 2021), created with BioRender.com

Although well characterised and utilised, viral vectors are associated with drawbacks in terms of safety concerns, production challenges and limited flexibility.

Current literature shows that lentiviral vectors carry a low risk of viral reactivation and insertional oncogenesis. From long-term follow up of 308 patients, 375 manufactured T cell products and 17 vector lots over 10 years, no replication competent retrovirus/lentivirus event was detected, which estimates to be one positive event every 52.8 years of patient follow-up (Marcucci *et al.*, 2018). The same study also demonstrated rapid decline in gene modified cells, with the median time to fall below the FDA recommended 1% vector sequence threshold at less than or equal to 1.4 month. No T cell transformation was observed even in cases of lentivirus mediated transgene insertion into tumour suppressor gene loci such as Tet Methylcytosine dioxygenase 2 (TET2) (Fraietta *et al.*, 2018, Shah *et al.*, 2019).

Although the potential for recombination events leading to generation of replication competent viruses is low, as an additional safety concern, the FDA demands extensive and expensive testing for replication competent viruses during vector production and at release of CAR T cell products (Levine *et al.*, 2017, Li *et al.*, 2019). In addition, patients are recommended to be followed up long-term for the presence of replication competent retroviruses.

Lentiviruses have limited cargo capacity of up to 10 kb (Powell *et al.*, 2015). LV vectors are also limited to introducing only DNA into cells whereas other non-viral delivery methods such as electroporation and nano-needling are more flexible, suitable for delivering a variety of effector molecules including DNA, RNA and enzymes (Stewart *et al.*, 2016). With increasingly complex T cell engineering, such as 4th generation "armoured CARs" expressing suicide genes, cytokines, BiTEs, antibodies and/or additional receptors, larger transgene payload capacity and delivery methods that can accommodate other effector molecules such as siRNA and protein complexes will be needed.

1.6.3 CRISPR/Cas9 system

A wide range of genome editing tools are now available to researchers, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and meganucleases. However, CRISPR/Cas9 based systems outshines these other enzymes by virtue of its simplicity and versatility and is the ideal tool for targeted 'knock-out' and 'knock-in' of genes.

Since its first discovery as a mechanism of adaptive immunity in bacteria against bacteriophages (Barrangou *et al.*, 2007), it has been extensively studied and utilised for genome editing by thousands of laboratories around the world. Currently, the most commonly used type II CRISPR/Cas9 system involves two components: a single guide RNA (sgRNA) and the Cas9 (CRISPR-associated protein 9). The gRNA contains a sequence of about 18-20 nucleotides which is complementary to the target gene of interest which directs the Cas9 endonuclease to cut the genomic DNA at the protospacer adjacent motif (PAM) site, which is a specific sequence of DNA of between 2-5 nucleotides (the exact sequence of which depends on the bacterial species of the Cas9). The cut DNA is repaired by the cell's endogenous pathways: the error-prone non-homologous end joining (NHEJ) which often leads to small insertion/deletion (indel) of DNA, or homology directed repair (HDR) which uses a homologous piece of DNA as a repair template (Figure 1.10). The former NHEJ is employed to knock out specific gene loci by introducing frame-shift mutations and/or early termination codons, whilst the latter is used in gene/cell therapy to repair faulty genes in genetic disorders or introduce transgenes such as CARs for novel functions (Redman *et al.*, 2016, Tao *et al.*, 2024).

Because conventional CRISPR/Cas9 relies on double stranded DNA breakage, major chromosomal abnormalities such as chromosome loss or translocations could result (Tsuchida *et al.*, 2023). As a counter-measure, newer derivatives of CRISPR/Cas9 have been developed for safer gene knock-out and knock-in, such as base editing and prime editing without DNA breakage, Cas9 nickases to reduce off-target cuts and catalytically dead Cas9 for regulating specific gene expressions by recruiting transcriptional activators and repressors (Dimitri *et al.*, 2022). However, recent evidence reveals that base and prime editing in human haematopoietic stem cells were still fraught with DNA double-stranded breaks, deletions and translocations, albeit at lower frequencies than Cas9 (Fiumara *et al.*, 2024).

CRISPR/Cas9 has already being investigated to address various genetic diseases including sickle cell disease, β-thalassemia, cystic fibrosis and muscular dystrophy (Pandey *et al.*, 2017). Thus, it has naturally extended into the CAR T cell research space as deletion of inhibitory molecules and precise insertion of CAR presents alluring opportunities in improving the effectiveness and safety of CAR T cells.

While lentiviral vectors are generally safe, the risk of malignant transformation is still a legitimate concern due to insertional mutagenesis of tumour suppressor genes or oncogenes (Schlimgen *et al.*, 2016). Furthermore, the semi-random insertion patterns lead to variable CAR expression levels and inconsistent product qualities. Although placing the CAR transgene under the control of a constitutive promoter leads to high CAR expression, constant elevated CAR expression and interaction with other CARs can lead to tonic signalling, T cell exhaustion and poor function. To address these safety and efficacy issues, CRISPR/Cas9 allows targeted knock-in and placement of the CAR such that expression is under the control of an endogenous promoter. As an example CAR19 inserted into the T cell receptor α constant (TRAC) locus promoted basal expression which prevented tonic signalling and delayed T cell differentiation, exhaustion, with the added benefit of producing a universal CAR T product (Eyquem *et al.*, 2017).

CRISPR/Cas9 KO has also allowed the manufacture of CAR T cells against T cell malignancies that are otherwise unamenable to CAR T cell therapy due to fratricide such as in CD7-targeting CAR T cells in T-ALL (Cooper *et al.*, 2018, Xie *et al.*, 2023a). This thesis explores the use of CRISPR/Cas9 gene editing for both KO and knock-in (KI) at the TIM-3 locus.

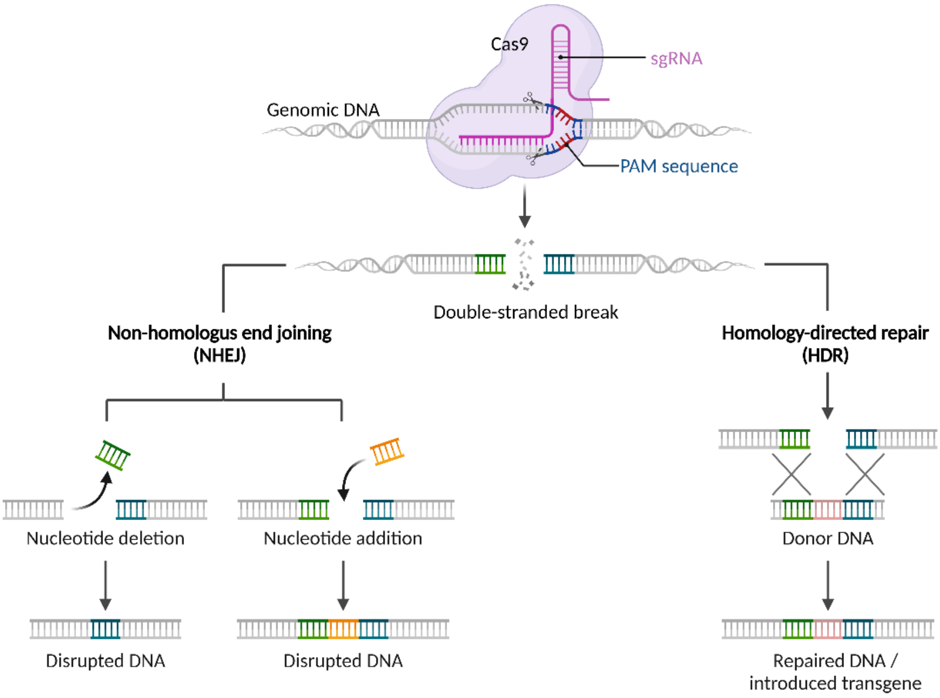


Figure 1.10 Schematic of CRISPR/Cas9 genome editing technology. The two pathways of repair after DNA double-stranded break are shown. Based on (Ran *et al.*, 2013b). PAM, protospacer adjacent motif; sgRNA, single guide RNA. Created with BioRender.

1.7 Hypothesis and Aims

In view of the small but favourable clinical trial results for CD123-directed CARs in AML, the overarching aim of this thesis is to bring CD123-directed CARIL3 T cells closer to a clinical trial. The *PiggyBac* system was associated with CAR T cell lymphomas in a recent clinical trial. Therefore, this thesis explores the feasibility of alternative gene modification including lentiviral, *PiggyBat* and CRISPR/Cas9 KI for the production of the anti-CD123 CAR. TIM-3 was chosen as the target locus for CAR KI, as simultaneous disruption of the TIM-3 inhibitory receptor and introduction of the CAR could potentially improve efficacy through elimination of a common immune checkpoint. Furthermore, the TIM-3 directed CAR can be tested in the context of KO and KI at the TIM-3 locus, avoiding possible fratricide between CAR T cells.

The aims of this thesis are to:

1) Determine if CARIL3 T cells can be manufactured in the *PiggyBat* transposon and lentiviral systems and retain efficacy *in vitro*.
2) Test CRISPR/Cas9 KO and KI at the TIM-3 locus, while concurrently expressing TIM-3-directed CAR as a step toward KI CARIL3 T cells.
3) Demonstrate the *in vitro* efficacy of CARIL3 T cells in the context of CRISPR/Cas9 KI at the TIM-3 locus.

Chapter 2. General materials and methods

2.1 General reagents and materials

2.1.1 Cell culture media

Dulbecco's Phosphate Buffered Saline (DPBS): 0.14 M NaCl, 2.68 mM KCl, 1.47 mM KH_2HPO_4, 8.06 mM Na_2HPO_4, pH 7.4 (BE17-516F, Lonza, Basel, Switzerland, Thermo Fisher, MA, USA).

Complete AIM-V (cAIM-V): CTS AIM V SFM (0870112DK, Gibco-BRL, Waltham, MA, USA), 10% (v/v) heat inactivated foetal bovine serum (FBS) (S-FBS-AU-015, Serana, Bunbury, Australia).

Complete Roswell Park Memorial Institute medium (cRPMI): RPMI-1640 (BE12-167F, Lonza), 10% (v/v) heat inactivated FBS (Serana), 2mM L-glutamine (G7513-100ML, Sigma-Aldrich, St. Louis, MO, USA; BE17-605E, Lonza).

Complete Dulbecco's Modified Eagle Medium (cDMEM): DMEM (BE17-516F, Lonza), 10% (v/v) FBS (Serana), 2mM L-glutamine (Sigma-Aldrich; Lonza)

Complete Iscove's modified Dulbecco's media (cIMDM): IMDM (BE12-726F, Lonza), 20% (v/v) FBS (Serana), 4mM L-glutamine (Sigma-Aldrich).

2X freezing medium: 40% (v/v) DPBS (Lonza), 40% (v/v) FBS (Serana) and 20% (v/v) di-methyl sulfoxide (DMSO) (WAK-DMSO-10, WAK-Chemie Medical GmbH, Steinbach, Germany).

TrypLE Express Enzyme (1X), no phenol red (12604021, Gibco): 0.14 M NaCl, 2.67 mM KCl, 1.47 mM KH_2HPO_4, 8.06 mM Na_2HPO_4, 1.1mM EDTA, recombinant protease (proprietary concentration).

2.1.2 Buffers

FACS (Fluorescence activated cell sorting) PBA buffer: PBS with 1% v/v bovine serum albumin (BSA) (Gibco, CA, USA) and 0.05% w/v sodium azide (0639-250G, Amresco).

Complete CliniMACS (Magnetic assisted cell sorting) buffer: CliniMACS (PBS with 1mM EDTA adjusted to pH 7.2) (Miltenyi Biotec, Bergisch Gladbach, Germany) with 0.5% v/v human albumin (Albumex20, CSL Behring, Parkville, Victoria, Australia)

2X HEPES-buffered saline (HBS) made in-house: 280 mM NaCl, 50 mM HEPES adjusted to pH 7.1, and autoclaved at 121 °C for 20 mins.

10% EDTA made in-house: 1 mM Tris, 0.1mM EDTA, adjusted to pH 8.0 and autoclaved at 121 °C for 20 mins.

2.1.3 Chemicals

2M $CaCl_2$ made in house by dissolving $CaCl_2$ in MilliQ water and autoclaved at 121 °C for 20 mins.

0.15 M Na_2HPO_4 made in house, adjusted to pH 7.10 and autoclaved at 121 °C for 20 mins.

2.1.4 Cell lines

The following cell lines were kindly provided by A/Prof Linda Bendall (The University of Sydney, Australia): Nalm-6 (DSMZ ACC128), K562 (ATCC CCL-243), KG-1 (ATCC CCL-246), TF-1 (ATCC CRL-2003), Raji (ATCC CCL-86), HEK-293 (ATCC CRL-1573).

2.1.5 Bacterial strains

NEB stable competent E.coli (C3040I, NEB biosciences, Ipswich, MA, USA). Genotype: F' proA+B+ $lacI^q$ Δ(lacZ)M15 zzf::Tn10 (Tet^R)/ Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- Φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (Str^R) rph spoT1 Δ(mrr-hsdRMS-mcrBC).

2.1.6 Bacterial culture reagents

Luria Bertani (LB) broth: made in-house with 10 g tryptone, 5 g yeast extract, 10 g NaCl; suspended in 1L of deionised water and autoclaved at 121 °C for 20 mins.

10-beta/Stable Outgrowth Medium (B9035S, NEB biosciences).

Ampicillin stocks (100 mg/mL) and kanamycin stocks (50 mg/mL) were made in house by dissolving powder in MilliQ water and sterilised through a 0.22 μm syringe filter and stored in 100 μL aliquots in -30°C.

2.1.7 PCR reagents

Polymerase chain reactions (PCRs) were performed using Q5 High-Fidelity 2X Master Mix (M0492S, NEB biosciences, MA, USA) which contains thermostable Q5 DNA polymerase, $MgCl_2$ at 4 mM and each dNTP with a proprietary buffer.

2.1.8 Restriction enzymes

All restriction enzymes and reaction buffers used were purchased from NEB biosciences (Table 2.1).

Table 2.1 List of NEB restriction enzymes used

Restriction enzyme	Catalogue number
BanI	R0118S
BspEI	R0540S, R0540L
BstBI	R0519S, R0519L
ClaI	R0197S, R0197L
EcoRI-HF	R3101T, R3101M
PmlI	R0532S, R0532L
PshAI	R0593S, R0593L
SalI-HF	R3138T, R3138M
SpeI-HF	R3133M
XbaI	R0145T, R0145M

2.1.9 DNA purification kits

QIAquick gel extraction kit (28704, QIAGEN, Germantown, USA) was used to extract DNA from agarose gels. QIAquick PCR Purification Kit (28104, QIAGEN) was used to purify PCR product from PCR reagents.

2.1.10 Plasmid DNA extraction kits

QIAprep Spin Miniprep Kit (27106, QIAGEN) and EndoFree Plasmid Maxi Kit (12362, QIAGEN) were used according to manufacturer's instructions:

2.1.11 Genomic TIM-3 knockout reagents

Invitrogen TrueGuide synthetic gRNA (sgRNA) (A35533) and Cas9 reagents (A36499) were purchased from ThermoFisher Scientific. Pre-designed sgRNAs targeting exon 2 of TIM-3 (HAVCR2) gene (CRISPR699749_SGM (sg49) and CRISPR699737_SGM (sg37)) were selected from the ThermoFisher Scientific catalogue (Figure 2.1).

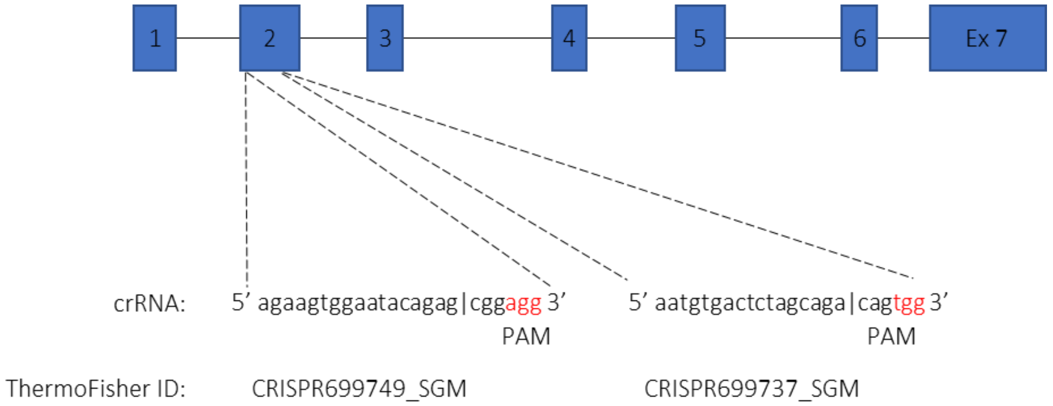


Figure 2.1 Schematic of CRISPR target sites on the TIM-3 (HAVCR2) locus. crRNA, CRISPR RNA; PAM (red), protospacer adjacent motif; |, predicted cleavage site.

2.2 Isolation and enumeration of peripheral blood mononuclear cells

2.2.1 Donor

Venesection blood from therapeutic phlebotomies were donated by haemochromatosis patients who were otherwise healthy. They provided informed consent in accordance with the Declaration of Helsinki following ethics approval from the Western Sydney Local Health District Human Research Ethics Committee.

2.2.2 Ficoll density separation

Heparinised whole blood was aliquoted into 50 mL V-bottom tubes and centrifuged at 480x *g* (2400 rpm) (Spintron, VIC, Australia) for 20 minutes at room temperature. The plasma was discarded, and buffy coat was collected in new 50 mL V-bottom tubes and diluted with equal-volume of warmed PBS before being underlaid with 10 mL of Ficoll-Paque (17144003, GE health care, Chicago IL, USA). These tubes were centrifuged at 400x *g* (2000 rpm) for 20 minutes with no brake at room temperature and mononuclear cell (MNC) layer isolated into new 50mL V-bottom tubes. The MNCs were washed twice with warmed PBS, each time pelleted by centrifuging at 225x *g* (1500 rpm) for 5 minutes at room temperature. The pooled cell pellet was red cell lysed with Pharm Lyse (555899, BD biosciences, San Jose, CA, USA) at room temperature for 5-10 minutes before being washed twice with warmed DPBS and resuspended for cell enumeration.

2.2.3 Enumeration

Viable cells were enumerated manually by trypan blue (T8154-100ML, Sigma-Aldrich) exclusion with light microscopy using a haemocytometer (Neubauer improved, Camlab, Cambridge UK).

2.2.4 Cryopreservation and thawing of peripheral blood mononuclear cells and cell lines

Peripheral blood mononuclear cells (PBMCs) and cell lines were cryopreserved by mixing at 1:1 ratio with freezing medium (40% PBS, 40% heat inactivated FBS and 20% DMSO), at concentrations of 50 x 10^6/mL and 2-50 x 10^6/mL respectively.

Cryopreserved PBMCs and cell lines were thawed by placing cryovials in a 37°C water bath for 2-3 minutes until just thawed. Thawed cells were then transferred into a V-bottom tube and washed with 9x volumes of warmed PBS before being resuspended in fresh media for subsequent use.

2.3 Maintenance of cell lines

2.3.1 Routine maintenance of cell lines

Cell lines were maintained in vented 25cm² (T25), 75cm² (T75) or 150cm² (T150) rectangular canted-neck cell culture flasks (Corning, Corning, NY, USA) at 37°C in a 5% CO_2 incubator at cell concentrations recommended by ATCC or DSMZ and passaged 2-3 times per week. The cell lines were passaged for a maximum of 25 times or 3 months in culture to reduce phenotypic or genotypic drift. The purity and expression of relevant target antigens of the cell lines used were confirmed by flow cytometry prior to their experimental use.

The culture media used for each cell line are listed in Table 2.2.

Table 2.2 Culture media for cell lines

Cell line	Culture media
Nalm6, Raji, K562, Jurkat	Complete RPMI
TF-1	Complete RPMI with 50U/mL GM-CSF
KG-1	Complete IMDM
HEK293	Complete DMEM

2.3.2 Dissociation of adherent cell lines from culture flask

Culture media was aspirated and discarded, and cell monolayer was released from culture flask using 5 mL TrypLE Express Enzyme per 75cm² flask area and incubated at 37°C until the cells were completely dissociated. Enzyme action was terminated by equal volume of complete media and cell suspension was transferred into a V-bottom flask for centrifugation at 300 x *g* for 5 minutes at room temperature. Supernatant was discarded and cell pellet resuspended in fresh complete media.

2.4 Molecular techniques for plasmid engineering

2.4.1 Templates for plasmid engineering

The *PiggyBat* transposase plasmid (pVAX1 PBat) was previously created in the lab by cloning the *PiggyBat* transposase sequence (Mitra *et al.*, 2013) into the kanamycin-resistant plasmid pVAX1 (V260-20, Life Technologies, Carlsbad, CA, USA) backbone (Sutrave, 2021). *PiggyBat* transposon plasmids used in the thesis consist of the pVAX1 backbone, terminal inverted repeats (TIRs), EF1α promoter, SV40 polyadenylation sequence and the CAR constructs.

Lentiviral transduction was undertaken using third generation lentiviral system consisting of packaging plasmid (pMDLg.pRRE), regulatory plasmid (pRSV-Rev), envelope plasmid (pMD2.VSV-g) and transfer plasmid carrying the CAR constructs (pRRLsin.cPPT.EF1α.CAR.Wpre).

Further details regarding individual CAR constructs are elaborated in Chapters 3, 4 and 5.

2.4.2 General approach for transposon plasmid engineering

Plasmid constructs were synthesised *de novo* (GenScript, Piscataway, United States) or isolated from existing plasmids through restriction enzyme digestion or PCR followed by cloning into transfer plasmids by ligation with T4 DNA Ligase (M0202T, NEB Biosciences). The schematic of this process is shown in Figure 2.2. Further details regarding methods of molecular cloning of expression plasmids are listed in Chapters 3, 4 and 5 of the thesis.

Plasmids were transformed into competent *E. coli* by heat shock and selected on agar plates with antibiotics (100 μg/mL ampicillin or 50 μg/mL kanamycin) corresponding to the resistance profile of the plasmid. Plasmid DNA from clones were screened using restriction enzyme digestion and ligation junctions sequence verified by Sanger sequencing.

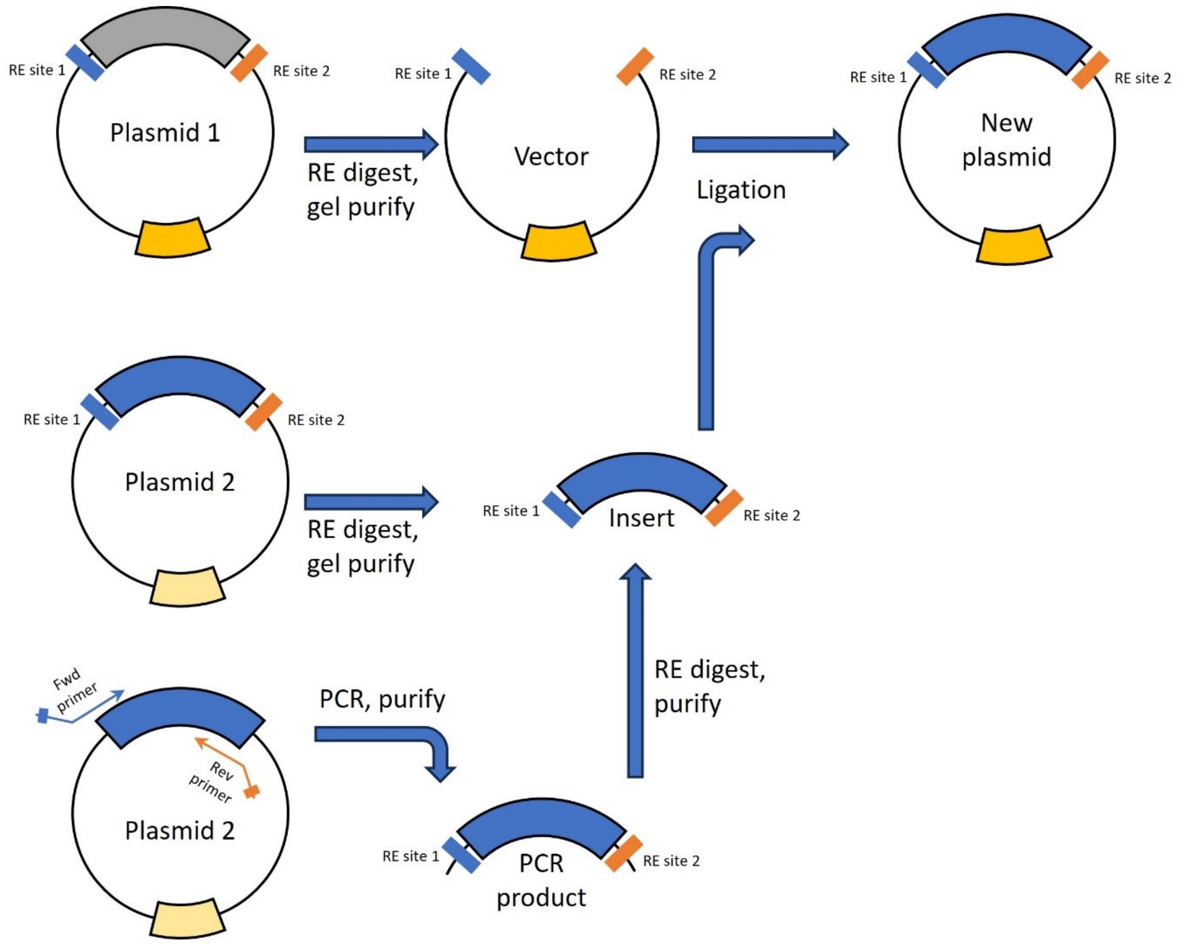


Figure 2.2 **General plasmid cloning method.** Vector plasmid is digested with restriction enzymes and purified. Insert plasmid is either derived by digesting a second plasmid or PCR products amplified with 5' extension primers containing compatible restriction sites. Cut insert and vector fragments were ligated using T4 ligase. RE, restriction enzyme.

2.4.3 Polymerase chain reaction

PCRs were performed using the MiniAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) with reaction mix setup as outlined in Table 2.3 and cycling conditions as shown in Table 2.4. PCR products were resolved on a 1-2% agarose gel and purified using QIAquick PCR Purification Kit (QIAGEN) and QIAquick gel extraction kit (QIAGEN) for pure and non-pure PCR products respectively.

Table 2.3 PCR setup volumes

Reagent	Volume (µL)
2x Q5 high fidelity master-mix	25
DNA	Genomic DNA 1 ng–1 µg Plasmid 1 pg–10 ng
Forward primer, 10 µM	2.5
Reverse primer, 10 µM	2.5
Nuclease free water	To 50 µL

Table 2.4 PCR cycling conditions

Temperature	Temperature	Time
Initial denaturation	98°C	180 sec
30 Cycles	98°C	5 sec
	Annealing temperature (Tm)	10 sec
	72°C	30 sec per kb product size
Final extension	72°C	2 min
Hold	4 °C	∞

2.4.3.2 KI confirmation PCR

Genomic DNA was extracted from cultured TIM-3^{KI} CAR T cells using QIAamp Blood Mini Kits according to the manufacturer's protocol (51104, Qiagen, Germany). The extracted gDNA were used as template for PCRs with primers targeting the sg37 and sg49 KI junctions of TIM-3 (Figure 2.3, Table 2.5). The PCR products were resolved on a 2% agarose gel electrophoresis. Target and non-specific bands were excised, and the DNA were extracted using the QIAquick gel extraction kit. The purified PCR products were quantified and any samples with low concentrations were concentrated in an Eppendorf 5301 Vacufuge Concentrator at 60°C. The purified PCR products were sent to Australian Genome Research Facility (AGRF) for Sanger sequencing (section 2.4.9).

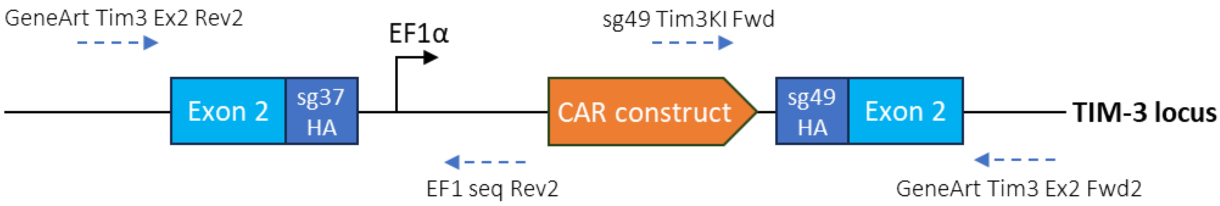


Figure 2.3 **Schematic of CAR knock-in confirmation PCR.** Two primer pairs were used to amplify the sg37 and sg49 KI junctions separately. "GeneArt Tim3 Ex2 Rev2" pairs with "EF1 seq Rev2", "sg49 Tim3KI Fwd" pairs with "GeneArt Tim3 Ex2 Fwd2". EF1α, EF1α promoter; HA, homology arm; TIM-3, T cell immunoglobulin and mucin-domain-containing-3.

Table 2.5 **Primer sets for KI confirmation PCR**

Name	**Sequence** (5' → 3')	**Target**	**Expected size (bp)**
GeneArt Tim3 Ex2 Rev2	GATGGCATGCAAATGTCCACTC	sg37 KI site	714
EF1 seq Rev2	TGGATCCGAGGTAGCTTGAG		
sg49 Tim3KI Fwd	ACGTAAACGGCCACAAGTTC	sg49 KI site	1020
GeneArt Tim3 Ex2 Fwd2	GCATTGTCACAGCGAATCATCC		

2.4.4 Restriction enzyme digest of plasmid DNA

Plasmid DNA was digested using a 2- to 10-fold excess of enzyme over DNA (Table 2.6). For increased amounts of DNA, the mix was scaled up appropriately.

Table 2.6 **Restriction enzyme digestion setup volumes**

Reagent	**Cloning volume (µL)**	**Miniprep screening volume (µL)**
10X restriction buffer	5	2
DNA	(5-8 µg)	1-2 µL (about 1 µg)
Restriction enzyme	1 per enzyme	1 per enzyme
Nuclease free water	Make volume to 50	Make volume to 20

The reaction mixes were incubated at the manufacturer recommended temperature for 2 hours. DNA fragments were resolved on a 1% agarose gel and fragments of interest were extracted using QIAquick gel extraction kit per manufacturer's instructions.

2.4.5 DNA ligation

Ligation mixes were prepared according to the following table (Table 2.7). The reactions were performed at room temperature for 10 minutes for sticky-end ligations, or 2 hours if one of the ligation sites was a blunt-end ligation.

Table 2.7 DNA ligation setup volumes

Reagent	Volume / Amount
Nuclease free water	Make volume to 20 µL
T4 ligase buffer	1 µL
Vector DNA fragment	0.1 µg
Insert DNA fragment	3x molar equivalent of vector
T4 DNA ligase	1 µL

2.4.6 Bacterial transformation

One to 10 ng of plasmid DNA or 2 µL of ligation mix was added to 10 µL of competent *E. coli* and placed on ice for 30 minutes after mixing by gentle flicking. The mixture was placed on a heat block at 42°C for exactly 30 seconds before being placed back on ice for 5 minutes. 10-beta/Stable Outgrowth Medium (300µL) was added, and the mixture was shaken at 225 rpm for 60 minutes at 30°C. Thirty µL of the mixture was then spread onto an appropriate antibiotic selection agar plate. The remaining volume was pelleted using a Biofuge pico at 13,000 rpm for 60 seconds to remove excess media and spread on another antibiotic selection agar plate. Both plates were incubated overnight at 37°C.

2.4.7 Miniprep and diagnostic digest by restriction enzyme

Bacteria colonies were picked and inoculated into 5 mL of LB medium containing the appropriate antibiotic in a 50 mL V-bottom tubes and shaken at 225 rpm overnight at 37°C. DNA was extracted from 4 mL of each bacterial culture using the QIAprep spin miniprep kit per manufacturer's instructions. Plasmid DNA obtained from each colony was digested with restriction enzymes as described above in section 2.4.4 and resolved on 1% agarose gels.

2.4.8 Maxiprep

One mL from each bacterial starter culture was inoculated into 150 mL of LB medium containing the appropriate antibiotic in 2 L conical flasks and shaken at 225 rpm overnight at 37°C.

The EndoFree Plasmid Maxi Kit (QIAGEN, Germantown, USA) was used to extract plasmid DNA according to the manufacturer's instructions.

2.4.9 Sanger sequencing

Reaction mixtures prepared as shown in Table 2.8 and Table 2.9 were sent to AGRF for Sanger sequencing to verify plasmid sequences and PCR products respectively. Sequencing traces were aligned with predicted sequences using SnapGene v5.3 (GSL Biotech, San Diego, USA).

Table 2.8 Sanger sequencing setup for plasmids

Reagent	**Volume / Amount**
Column purified plasmid DNA	600 – 1500 ng
Sequencing primer (10 µM)	1 µL
30% (v/v) DMSO	2 µL
Nuclease free water	Make volume to 12 µL

Table 2.9 Sanger sequencing setup for PCR products

Reagent	**Volume / Amount**
PCR purified or gel-extracted PCR product	3 – 8 ng (100 – 200 bp) 6 – 12 ng (200 – 400 bp) 12 – 18 ng (400 – 600 bp)
Sequencing primer (10 µM)	1 µL
30% (v/v) DMSO	2 µL
Nuclease free water	Make volume to 12 µL

2.4.10 Glycerol stock

Following overnight incubation for Maxiprep as described in section 2.4.8, 700µL of the bacterial Maxiprep cultures were added to 300 µL of 50% (v/v) glycerol diluted in nuclease free water, vortexed and frozen at -80°C.

To revive competent *E coli* from glycerol stocks, the vials were partially thaw on ice and 5 – 20 µL volumes were inoculated into 5 mL of LB medium containing the appropriate antibiotics in 50 mL V-bottom tubes and shaken at 225 rpm overnight at 37°C.

2.5 Generation and enrichment of antigen overexpressing cell lines

2.5.1 K562-cetuximab

An artificial antigen presenting cell (aAPC) comprised of K562 overexpressing cetuximab was generated by others in the group (Sutrave, 2021). Briefly, a DNA construct encoding Fab of cetuximab with signal peptide, CD8α stalk, CD8 transmembrane and the first 10 amino acids of the intracellular domains of CD28 linked to the fluorescent protein mCherry by a Gly-Gly-Gly-Gly-Ser linker was synthesised after codon optimisation. The cetuximab construct was cloned into the *PiggyBac* transposon vector to make pVAX1.PB.cetuximab.mCherry and electroporated into K562 cell lines, followed by screening for high expressing clones.

2.5.2 Electroporation

Cell lines were washed twice with PBS and resuspended in electroporation buffer R (HEK293 at 25x10^6/mL, K562 at 10x10^6/mL). Electroporation cuvette was filled with 3mL buffer E. Plasmids containing the antigen transposon and Super *PiggyBac* transposase (pVAX1.SPBase) sequences were each added at 50 µg/mL.

Electroporation was performed using the 100µL tips from Neon Transfection System (Thermo Fisher) with cell line dependent settings (Table 2.10).

Table 2.10 Electroporation settings

HEK293	K562
1100 V	1450 V
20 ms	10 ms
2 pulses	3 pulses

Electroporated cells were immediately transferred to 5 mL pre-warmed media in 24 well tissue culture plates (0.2x10^6 cells/mL for K562, 0.5x10^6/mL for HEK293). 3-4 days later, cultured cells were either bulk sorted and seeded by limiting dilution or single cell sorted into 96-well plates.

2.5.3 Fluorescence-activated cell sorting

Cells overexpressing selection marker mCherry or antigen of interest (e.g. TIM-3) were either bulk sorted into 1 mL FBS in 15 mL centrifuge tubes or single cell sorted into 96-well plates containing 100 µL culture media each well using the BD Influx or Aria III flow cytometers. Single cell sorted cells were cultured and expanded into 24-well plates over time and screened using mCherry marker or staining for antigen of interest against untransfected cells as negative controls. The highest expressing single clone was further expanded and frozen as primary and secondary stocks.

Bulk sorted cells were limiting diluted as follows.

2.5.4 Limiting dilution

Bulk sorted cells were diluted to 0.67 cells/µL and plated into 96-well plates with appropriate complete media and monitored for colony expansion. Suspension cell lines were resuspended, and adherent cell lines were dissociated from bottom of plate and screened for the appropriate selection marker or antigen of interest using the Canto II or LSRFortessa (BD). The highest expressing single clone was expanded and frozen as primary and secondary stocks.

2.6 Generation and expansion of CAR T cells

2.6.1 Functional expansion by co-expression of separate CAR

The *PiggyBat* CAR constructs in this thesis were multi-cistronic, which contains in cis, the CAR-of-interest and a second generation truncated epithelial growth factor receptor CAR (tEGFRz) separated by 2A peptides. The tEGFRz CAR was developed by others in the group, comprising of an extracellular tEGFR linked to a CD28 transmembrane and intracellular domain with CD3ζ (Sutrave, 2021, Sutrave *et al.*, 2022). The tEGFRz CAR does not have any natural ligands, but allowed the functional expansion of the CAR T cells upon engagement with the Fab derived from EGFR-specific monoclonal antibody cetuximab expressed on genetically modified K562 cells. This multi-cistronic system enabled the activation, expansion and enrichment of the CAR T cell by exposure to the K562-cetuximab artificial “antigen” presenting cell, without interfering with the CAR of interest (Figure 2.4). Furthermore, the detection of tEGFR extracellular domain using cetuximab also acted as a surrogate marker for CAR expression.

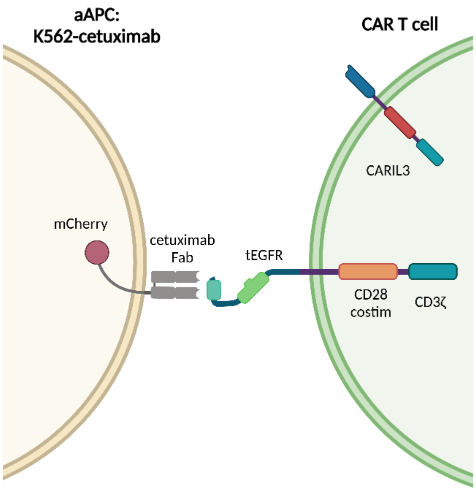


Figure 2.4 Schematic of tEGFRz interaction with cetuximab Fab. CARIL3 and tEGFRz were co-expressed on the same CAR T cell, where engagement of tEGFR with cetuximab Fab presented by K562 leads to activation and expansion of CAR T cell, irrespective of CARIL3. aAPC, artificial antigen presenting cell.

2.6.2 Immunomagnetic column depletion of CD3 negative cells in PBMCs

T cells derived from healthy donor PBMCs were selected using the Pan T Cell Isolation Kit (130-096-535, Miltenyi Biotec) according to the manufacturer's instructions. Briefly, the thawed PBMCs were washed and resuspended in complete CliniMACS buffer and magnetically labelled with cocktails of biotin-conjugated monoclonal antibodies against CD14, CD15, CD16, CD19, CD34, CD36, CD56, CD123, and CD235a (GlycophorinA) followed by microbeads conjugated to biotin and anti-CD61 antibody. Unlabelled T cells were isolated with LS columns using MidiMACS or QuadroMACS Separators, washed twice with complete CliniMACS and rested for 18-24 hours at around 2 x 10^6 cells/mL in complete AIM-V.

2.6.3 Electroporation of T cells

Electroporation with the Neon Transfection System is used to introduce the transposase and transgene for generation of *PiggyBat* CAR T cells, as well as CRISPR mediated knock-out and knock-in modifications.

Plasmids containing PBat transposase (1.0 µg / 10^6 cells) and PBat CAR transposon (equimolar to PBat transposase) were diluted in 50 µL buffer T (MPK10025, Thermo Fisher Scientific, MA, USA). The T cells isolated as detailed in section 2.6.2 were pelleted and resuspended in buffer T to 100x10^6/mL and 5x10^6 cells were added to the plasmids mixture, making volume up to 125 µL with buffer T (MPK10025, Thermo Fisher Scientific, MA, USA). Electroporation cuvette was filled with 3 mL buffer E2.

Electroporation was performed using 100 µL tips of the Neon Transfection System (MPK5000, Thermo Fisher) with a single pulse at 2400 V for 20 ms. Electroporated cells were immediately transferred to 2 mL pre-warmed complete AIM-V media in a 24 well tissue culture plate at 2 x 10^6 cells/mL.

2.6.3.1 CRISPR knockout of TIM-3

The knockout of TIM-3 in CAR T cells was mediated with CRISPR/Cas9. The Cas9 enzyme and CRISPR guide RNAs were introduced by electroporation simultaneously with *PiggyBat* transposase and CAR transposon (Figure 4.4).

Cas9 enzyme (75 pmol i.e. 12.5 μg) and the two TIM-3 sgRNA (CRISPR699737_SGM and CRISPR699749_SGM, 75 pmol each) were incubated in 50 μL of buffer T for about 15 minutes at room temperature. Following incubation, PBat transposase (5 μg) and CAR transposon (equimolar at 8.3 μg for CARTIM3a or CARTIM3b, 8 μg for CARIL3dm, and 6.8 μg for tEGFRz) plasmids were mixed with 5x10^6 T cells and made up to 125 μL total volume with Buffer T immediately prior to electroporation using the conditions described above.

2.6.3.2 CRISPR knock-in to TIM-3 locus

The use of CRISPR Cas9 for knock-in (KI) to the TIM-3 locus described in Chapter 4 and 5 of the thesis required preparation of KI homology directed repair (HDR) templates prior to electroporation, which will be elaborated in the methods section of Chapter 4.

Additionally, CRISPR knock-in CAR-T cells required additional pre-activation with TransAct prior to electroporation. Selected T cells were activated immediately following immunomagnetic T cell isolation with 1/100 TransAct for 24 hours in complete AIM-V supplemented with IL-7 (200 IU/mL) and IL-15 (200 IU/mL), then half-exchanged with fresh media supplemented with IL-7 and IL-15 without TransAct for another overnight culture. The Cas9 enzyme, two TIM-3 sgRNA (CRISPR699737_SGM and CRISPR699749_SGM, 75 pmol each) were co-electroporated with dsDNA CAR HDR templates (3.0 μg for CARIL3dm, 3.2 μg for CARTim3b and 2.2 μg for tEGFRz) with the same settings as described above (2400V - voltage, 20ms - pulse width, 1 - pulse number).

2.6.4 Culture and expansion of CAR T cells

All *PiggyBat* CAR-T cell cultures described in the thesis apart from TIM-3 knock-in CAR T cells were cultured and expanded with a standardised protocol. on days 1, 8 and 15 post-electroporation, the viable cells were enumerated by Trypan Blue exclusion. Small aliquots of cells were taken for flow cytometry and the remaining cells were pelleted with 0.2x irradiated (IR'd) K562-cetuximab with or without 2x irradiated PBMC and resuspended in complete AIM-V at $0.5x10^6$/mL live T cells. Cultures were supplemented with IL-15 at 200 IU/mL every 2-3 days and media added once colour changed to orange/yellow.

For TIM-3 knock-in, the T cells were expanded with 0.2x irradiated K562-cetuximab following the same culture protocol as described above. However, at day 15, live cells were enumerated by Trypan Blue exclusion and sorted for $tEGFR^+$ cells using fluorescence-activated cell sorting (FACS). Sorted cells were pelleted and cultured with 4x irradiated PBMC from the same donor with complete AIM-V ($0.5x10^6$ CAR T cell/mL) and rested overnight. 1/500 (v/v) TransAct was added the next day and T cells cultured for another 9 or 10 days with media exchange and IL-15 supplement every 2-3 days.

2.7 Flow cytometry

2.7.1 General cell washing technique

Primary PBMC, T cells and cell lines were washed by adding buffer and pelleted at 427x g for 5 min at room temperature (Heraeus Multifuge X3R or Multifuge X4 Pro, Waltham, MA, USA). Supernatant is tipped off and pellet loosened by dragging across a rack and resuspended in appropriate buffer.

2.7.2 Staining

Cultured cells were enumerated by trypan blue exclusion and 0.1 – 1 x 10^6 live cells were washed twice with PBS. Cocktails of fluorochrome-conjugated antibodies at previously determined saturating concentrations were made up to 100 µL with FACS buffer and added to cells, vortexed briefly before being incubated at 4°C in the dark for at least 20 minutes. Cells were then washed with FACS buffer and resuspended in 200 – 300µL of FACS buffer for acquisition.

For experiments with unlabelled antibodies (e.g. cetuximab), cells were incubated with unlabelled antibody for 20 minutes at room temperature in the dark, then washed with FACS buffer and incubated with a cocktail of fluorochrome-conjugated antibodies and appropriate secondary antibody, before second wash with FACS buffer prior to acquisition.

2.7.3 Monoclonal antibodies and fluorescent reagents for flow cytometry

Flow cytometry reagents used are as listed below in Table 2.11

Table 2.11 Flow cytometry stains

Target	Clone	Isotype	Fluorochrome	Supplier	Catalogue number
CD3	SK7	Mouse IgG1, κ	BV510	BD Biosciences	563109
CD3	UCHT1	Mouse IgG1, κ	Alexa-Fluor 647	BD Biosciences	557706
CD3	UCHT1	Mouse IgG1, κ	BV605	BD Biosciences	563219
CD4	SK3	Mouse IgG1, κ	PerCP	BD Biosciences	347324
CD4	RPA-T4	Mouse IgG1, κ	BUV395	BD Biosciences	564724
CD8	RPA-T8	Mouse IgG1, κ	PacBlue	BD Biosciences	558207
CD25	2A3	Mouse IgG1, κ	APC	BD Biosciences	340939
CD69	L78	Mouse IgG1, κ	PE	BD Biosciences	341652
CD62L	DREG-56	Mouse IgG1, κ	PE-Cy7	BD Biosciences	565535
CD45RA	HI100	Mouse IgG2b, κ	BV510	BD Biosciences	563031
PD1	EH12.1	Mouse IgG1, κ	BV711	BD Biosciences	564017
LAG3	T47-530	Mouse IgG1, κ	PE	BD Biosciences	565616
Tim-3	F38-2E2	Mouse IgG1, κ	APC-Cy7	Biolegend	345026
TNF-α	MAb1	Mouse IgG1, κ	PE-Cy7	BD Biosciences	557647
Interferon-γ	B27	Mouse IgG1, κ	PE	BD Biosciences	559327
CD107a	H4A3	Mouse IgG1, κ	BV786	BD Biosciences	563869
CD107b	H4B4	Mouse IgG1, κ	BV786	BD Biosciences	565304
IL-3	BVD8-3G11	Rat IgG1	Unconjugated	BD Bioscience	554672
CD123	9F5	Mouse IgG1, κ	BV510	BD Biosciences	563072
Mouse IgG1, κ isotype	X40	Mouse IgG1, κ	BV510	BD Biosciences	562946
Galectin-9	9M1-3	Mouse IgG1, κ	PE	BioLegend	348905
Mouse IgG1, κ isotype	MOPC-21	Mouse IgG1, κ	PE	BD Biosciences	554680
CEACAM-1	103	Rabbit IgG	Unconjugated	Invitrogen	MA5-29142
Rabbit isotype	N/A	Rabbit IgG	Unconjugated	Invitrogen	02-6102
Annexin V	N/A	N/A	PE	BD Biosciences	556421
Viability dyes					
7-AAD	N/A	N/A	N/A	BD Biosciences	555816
Fluorogold	N/A	N/A	N/A	Molecular Probes	H22845
Zombie NIR	N/A	N/A	N/A	Biolegend	423106
Secondary reagents					
Human IgG (H+L)	Polyclonal	Goat AffiniPure F(ab')2	Alexa-Fluor 647	Jackson Immunoresearch	109-606-088
Rat Ig	Poly1271	Goat Ig	PE	BD Biosciences	550767
Rabbit IgG	Poly4064	Donkey polyclonal Ig	PE	BioLegend	406421

2.7.4 Data acquisition

Compensation was performed as per manufacturer's instructions to account for spectral overlap of fluorophores and autofluorescence using unstained control and single fluorochrome-conjugated antibody-stained control cells. At least 30,000 CD3 events were collected for each analysis tube.

2.7.5 Analysis

FACSDiva version 6.1.3 (BD biosciences) software was used for acquisition of data. Forward and side scatter properties were used to distinguish live and dead cells. Fluorescence minus one (FMO) controls or isotype controls were used to set gate positions. Post-acquisition analysis was performed with either FACSDiva or FlowJo version 10 for Windows (Becton, Dickinson and Company, Ashland, OR).

2.7.6 Fluorescence-activated cell sorting

The CAR T cells were washed once with DPBS (427*g* for 5 mins) and incubated with cetuximab for 10 minutes at room temperature in the dark with reaction volumes as shown in Table 2.12.

Table 2.12 Primary antibody cocktail for CAR sorting

Primary antibody	**Volume / 10^6 viable cells (µL)**
Cetuximab (1mg/mL)	0.2
DPBS	100
Total	100.2

Following incubation, the cells were washed with DPBS/10% FBS and (427*g* for 5 mins) and resuspended in cocktails of secondary stains (Table 2.13) for at least 20 minutes on ice in the dark.

Table 2.13 Secondary antibody cocktail for CAR sorting

Primary antibody	**Volume / 10^6 viable cells (µL)**
Goat anti-human IgG (H+L) AF647 (1.5mg/mL)	0.1
7-AAD	5
DPBS/10% FBS	95
Total	100.1

After the secondary stain, the cells were washed with DPBS/10% FBS and resuspended in with DPBS/10% FBS.

FACS was performed on either the BD Aria or Influx and sorted cells were collected into ~1mL 100% FBS in 15mL centrifuge tubes and kept on ice for further culturing or gDNA extraction.

2.8 Functional assessments

2.8.1 Intracellular cytokine flow cytometry

Cultured CAR T cells (effector cells) were incubated with target antigen-expressing cell line (target cells) at an effector to target (E:T) ratio of 1:1 for 5 hours in the presence of 1 μg/mL anti-CD28 (clone L293, 340975, BD Biosciences) and 1 μg/mL CD49d (clone L25, 340976, BD Biosciences) antibodies. Monensin/GolgiStop™ (2 μM) (554724, BD Biosciences) and brefeldin A/GolgiPlug™ (1 μg/mL) (555029, BD Biosciences) were added for the last 4 hours, followed by labelling with viability dye Zombie NIR and fluorochrome-conjugated antibodies against surface antigens (CD3, CD4, CD8). Cell fixation and permeabilisation was performed using the Cytofix/Cytoperm Kit (554714, BD Biosciences) according to the manufacturer's instructions, followed by intracellular staining with anti-IFN-γ PE and anti-TNF-α PE-Cy7. T cells stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL ionomycin (Sigma-Aldrich) were used as positive controls and unstimulated cultured CAR T cells were used as negative controls.

2.8.2 Calcein-AM release cytotoxicity assay

Cultured CAR T cells were incubated with K562 cells at a 1:1 ratio in complete AIM-V for 1 hour at 37°C to adsorb any natural killer (NK) cells. Target cells were labelled with 25 μg/mL calcein-AM (C3100MP, Sigma Aldrich) for 30 min at 37°C. Labelled target cells were co-cultured in triplicate with decreasing numbers of effector cells in complete RPMI without phenol red (BE12-918F, Lonza) to give E:T ratios ranging from 40:1 to 1.25:1. The spontaneous (spont) and the maximal (max) calcein release were determined by incubating target cells with complete RPMI alone or 2% (v/v) Triton X-100 (0694-1L, Amresco), respectively.

After incubating for 4 hours at 37°C, the cell were pelleted at 300x *g* for 3 minutes, and 300μL supernatant was transferred into a 96 well black flat bottom Optiplate (6005270, Perkin Elmer, Shelton, CT, USA) and the fluorescence (F) due to released calcein AM was measured with a SpectraMax iD5 Plate Reader (Molecular Devices, LLC., San Jose, CA), using excitation and emission wavelength of 485 and 538 nm, respectively. The percentage of specific target cell lysis at each E:T ratio (test) was calculated with the formula:

$$\%\ \text{specific lysis} = \frac{\mathrm{F_{test}} - \mathrm{F_{spont}}}{\mathrm{F_{max}} - \mathrm{F_{spont}}} \times 100\%$$

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 (GraphPad, La Jolla, CA, USA). For comparison between two groups, two-tailed Student's t-test was used. Paired t-test was used to compare between two different experimental conditions of the same culture (e.g. intracellular cytokine flow cytometry). Comparison of continuous variables between multiple groups were performed with ANOVA with Bonferroni's or Tukey's post-hoc multiple comparisons test. P-value < 0.05 was considered significant.

Chapter 3. *PiggyBat* vs. lentiviral modified CAR T cells using natural ligand IL-3 to target CD123

3.1 Introduction

CD123 is an attractive target in acute myeloid leukaemia for CAR T cell therapy. The use of natural ligands as CAR antigen binding domains has been found to reduce the risk of constitutive activation of CAR T cells from cross-linking of single chain variable fragments (scFvs) which could lead to autonomous proliferation and exhaustion (Branella and Spencer, 2021).

The Westmead Cell Therapies Laboratory have previously developed a number of second generation CD123-targeting CAR constructs, expressing the natural ligand of CD123, human IL-3, as the recognition domain using the *PiggyBac* transposase-transposon gene modification system (Lee, 2021). Point mutations were introduced into the human IL-3 derived recognition domain to improve binding efficacy and reduce downstream activation following binding to CD123 on target cells. Significant *in vitro* efficacy in the form of cytokine release and cytotoxicity were demonstrated against CD123 expressing cell lines.

One of the goals of the Westmead Cell Therapies Laboratory is the production of clinically functional products for use in AML patients. However, the development of T cell lymphoma arising from *PiggyBac* transposon-modified anti-CD19 CAR T cells has raised some concerns regarding its safety in human use (Bishop *et al.*, 2021). A high transgene copy number may have contributed to the development of these CAR T cell derived lymphomas (Micklethwaite *et al.*, 2021). Therefore, this chapter explored whether effective ligand based anti-CD123 CAR T cells can be generated using two alternative vector systems, namely *PiggyBat* transposon and lentivirus.

In comparison to *PiggyBac*, the recently characterised *PiggyBat* transposon has the advantage of more consistent integration copy numbers across a wide range of transfected plasmid concentrations, as well as lower inter-donor variability (Sutrave *et al.*, 2022). Other aspects of the *PiggyBat* system were provided in Chapter 1 Introduction.

All current FDA-approved and most clinical trial CAR T cells are manufactured using either γ-retroviral or lentiviral vectors as they have been well characterised and provide stable CAR expression in transduced T cell. A more detailed description was provided in Chapter 1. These systems have advantages and disadvantages compared with the transposon systems, as summarised in Table 3.1.

This chapter establishes the feasibility of manufacture of functional CD123-specific CAR T cell products utilising these alternative gene modification systems. Transgene copy number consistent with FDA recommendations (< 5/cell) (Chen *et al.*, 2020) and integration profile of the CARs were evaluated to determine the risk of gene toxicity. It also highlights already known challenges with lentiviral vector systems, particularly the effect of transgene cassette size on vector manufacturing efficiency.

Table 3.1 Comparison of viral and transposon vectors
Adapted from (Zhang *et al.*, 2017)

Vector	γ-retrovirus	Lentivirus	Transposon
Delivered molecule	RNA	RNA	DNA
Packaging capacity	9-12 kb	10-14 kb	Not determined (*PiggyBat*) Up to 200 kb (*PiggyBac*)
Genome integration	Preferentially near transcription start sites and in transcriptional regulatory regions	Preferentially in transcriptionally active regions	Mainly intergenic and intronic (*PiggyBac, PiggyBat*) Random (*Sleeping Beauty*)
Gene expression	Stable	Stable	Stable
Applicable cell types	Dividing cells only	Broad	Broad

3.2 Materials and methods

3.2.1 Generation of CD123 overexpressing cell lines

K562 and HEK293 cell lines overexpressing CD123 were generated using *PiggyBac* transposon system as described in Chapter 2 with the following construct. DNA encoding the following peptide sequence (in that order) were synthesised (Genscript) after codon optimisation for human expression: mCherry (UniProt: X5DSL3), Gly-Ser-Gly linker, P2A self-cleaving peptide (GenBank: WEX32095.1), CD123 (UniProt: P26951), Gly-Ser-Gly linker, T2A self-cleaving peptide (GenBank: UER86417.1), CD131 (UniProt: P32927). The synthesised insert (Figure 3.1) was cloned into existing *PiggyBac* transposon vector under the control of the constitutively active EF1α promoter to generate pVAX1.PB.mCherry.IL3α.β plasmid.

The plasmids were electroporated into K562 and HEK293 cell lines followed by culturing, and screening for highest expressing clones as described in Chapter 2. CD123 and CD131 expressions were also confirmed using flow cytometry.

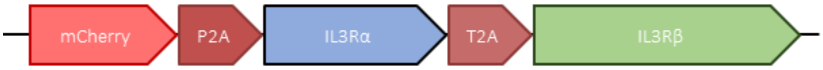


Figure 3.1 **CD123 overexpression construct** Consists of mCherry selection marker, CD123 (IL3Rα), CD131 (IL3Rβ/βc) separated by self-cleaving peptides P2A and T2A

3.2.2 The chimeric antigen receptor constructs

A wild type CARIL3 construct (CARIL3wt) consisted of truncated human IL-3 (UniProt P08700, amino acid residues 15 – 125 of mature human IL-3) linked to CD8α stalk, CD28 transmembrane domain, 4-1BB costimulatory intracellular domain (ICD), CD3ζ domain, P2A peptide, truncated EGFR, CD28 transmembrane and CD28 costimulatory ICD and CD3ζ, T2A peptide and eGFP (Figure 3.2 a.i).

Single mutant and double mutant CARIL3 constructs were identical to CARIL3wt, except for K116W and K116W/E22R single and dual amino acid substitutions respectively in the human IL-3 derived antigen recognition domain (Figure 3.2 b.i and c.i) (Barry *et al.*, 1994, Klein *et al.*, 2001, Lopez *et al.*, 1992). The CARIL3.tEGFRz.GFP constructs were 3501 bp in length.

Control tEGFRz as described in Chapter 2, consisted of truncated EGFR, CD28 transmembrane and CD28 costimulatory ICD and CD3ζ, T2A peptide and eGFP (Figure 3.2 d.i). The tEGFRz.GFP construct was 2316 bp.

All CAR inserts were synthesised previously by others in the group as *PiggyBac* transposon pVAX1 plasmids.

Intracellular deletion constructs (deletion of CD28 costimulatory domain, CD3ζ, T2A and eGFP at C-terminal end of tEGFR) (Figure 3.2 a.ii, b.ii, c.ii and d.ii) were created using 5'extension PCR and discussed below in 3.2.3.2. This reduced the transgene size for the purpose of increasing lentiviral titre.

Figure 3.2 **Schematic diagram of CARIL3 DNA constructs.** Full length and intracellular deletion forms (tEGFR without C-terminal signalling domain) are shown. (a) (i) wildtype CARIL3 (CARIL3wt) and (ii) intracellular deletion form (CARIL3wt.ΔICD). (b) single mutant CARIL3 (CARIL3sm) and (ii) intracellular deletion form (CARIL3sm.ΔICD). (c) double mutant CARIL3 (CARIL3dm) and (ii) intracellular deletion form (CARIL3dm.ΔICD). (d) tEGFR28ζ control CAR (tEGFRz) and (ii) intracellular deletion form (tEGFR.ΔICD). Point mutations are indicated by green and yellow stars. tEGFR, truncated epithelial growth factor receptor; TM, transmembrane; ICD, intracellular domain; eGFP, enhanced GFP; P2A, T2A, self-cleaving peptides; ΔICD, intracellular domain deletion.

3.2.3 Generation of CARIL3 plasmids

3.2.3.1 *PiggyBat* transposon plasmids

CAR constructs encoding CARIL3wt, CARIL3sm, CARIL3dm and tEGFRz were cloned from *PiggyBac* plasmids into *PiggyBat* vector plasmid using EcoRI and SalI restriction sites (Figure 3.3). The final pVAX1.PBat.CARIL3 and tEGFRz plasmids were 7566 bp and 6381 bp respectively.

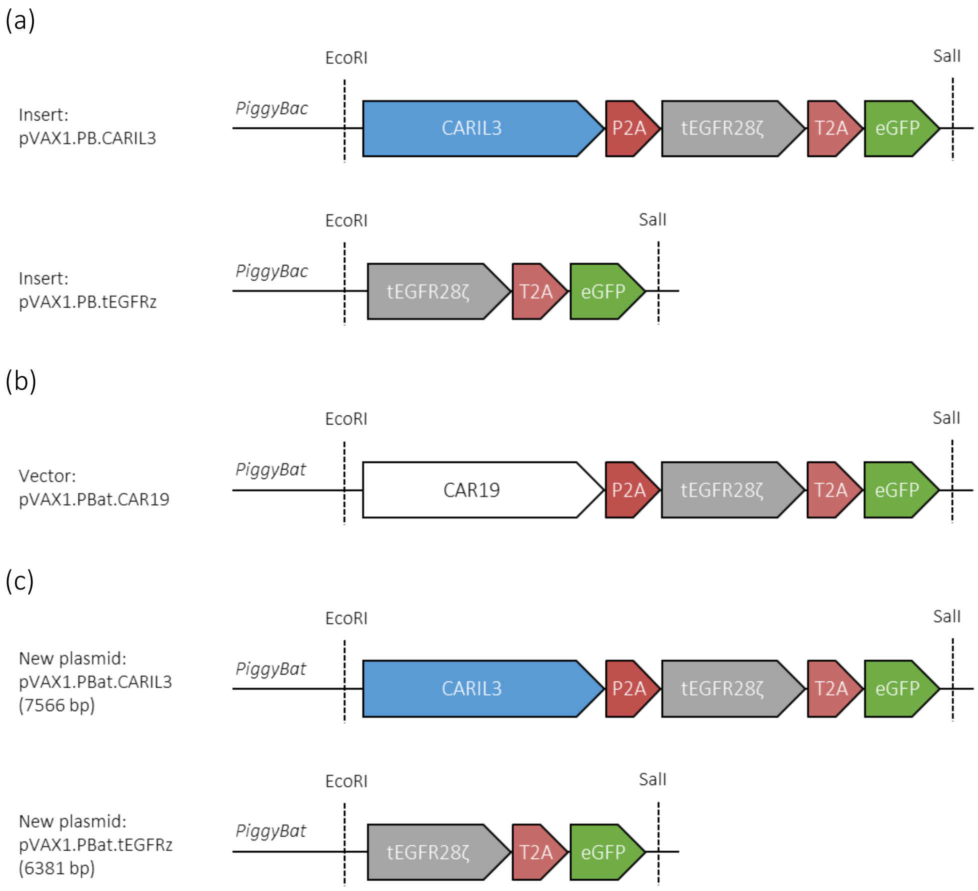


Figure 3.3 Schematic of pVAX1.PBat.CARIL3 cloning. (a) CARIL3wt, sm, dm (represented by CARIL3 blue arrow) and tEGFRz inserts were derived from *PiggyBac* plasmids using EcoRI/SalI double digest. (b) *PiggyBat* vector backbone was derived from a previously available pVAX1.PBat.CAR19. (c) Final pVAX1.PBat plasmids were created by ligating (a) and (b).

3.2.3.2 Lentiviral transfer plasmids

A third-generation self-inactivating lentiviral system was used to ensure safety by minimising likelihood of replication competent viruses. It consisted of one transfer plasmid transgene (pRRLsin.EF1a.transgene.Wpre, or pRRLsin.CMV.transgene.Wpre), one envelope plasmid (pMD2.VSV-G) and two packaging plasmids (pRSV-Rev, pMDLg.pRRE), generously supplied by A/Prof Grant Logan, Children's Medical Research Institute, Sydney.

CAR inserts were first cloned into a transfer plasmid with CMV promoter (Figure 3.4). SpeI and XbaI created compatible sticky ends for ligation. The CMV promoter was then replaced with the human EF1α promoter to be more relevant for translation into clinical practice (Figure 3.5). PmlI and PshAI created compatible blunt ends for ligation. Finally, to increase the viral titre (Sweeney and Vink, 2021), the transgene cargo size was reduced by deleting the intracellular domains of the tEGFRz (CD28 co-stimulatory domain, CD3ζ) and eGFP (Figure 3.6).

The CARIL3, tEGFRz, CARIL3.ΔICD, and tEGFR.ΔICD constructs length were 3501 bp, 2316 bp, 2316 bp and 1131 bp in length respectively (or 7390 bp, 6205 bp, 6208 bp and 5023 bp between the truncated 5' LTR and 3' LTR (ΔU3)) and were used for all subsequent lentiviral CAR cultures.

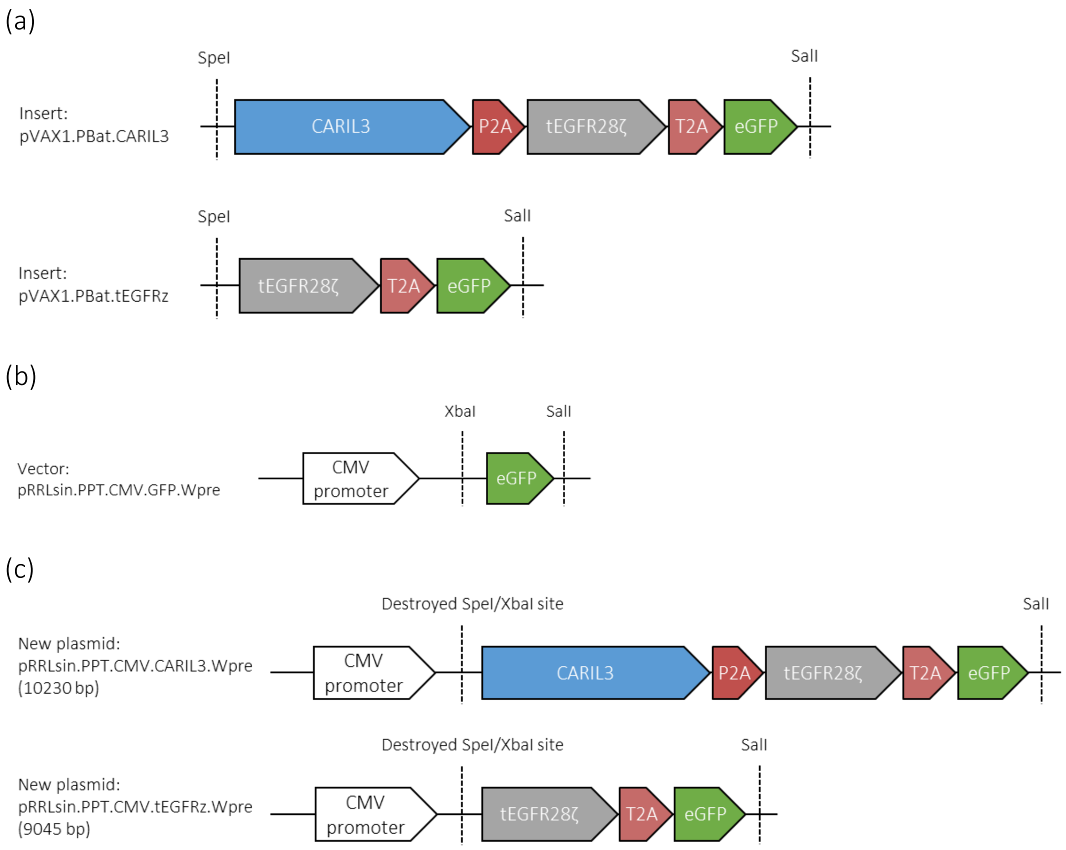


Figure 3.4 Schematic of CMV promoter transfer plasmid cloning. (a) Inserts were derived from *PiggyBat* plasmids carrying the CARIL3 transgenes and control tEGFRz plasmids by SpeI/SalI double digest. (b) The vector was obtained by XbaI/SalI double digesting the available transfer plasmid. (c) Ligated new CMV transfer plasmids showing destroyed SpeI/XbaI and preserved SalI sites.

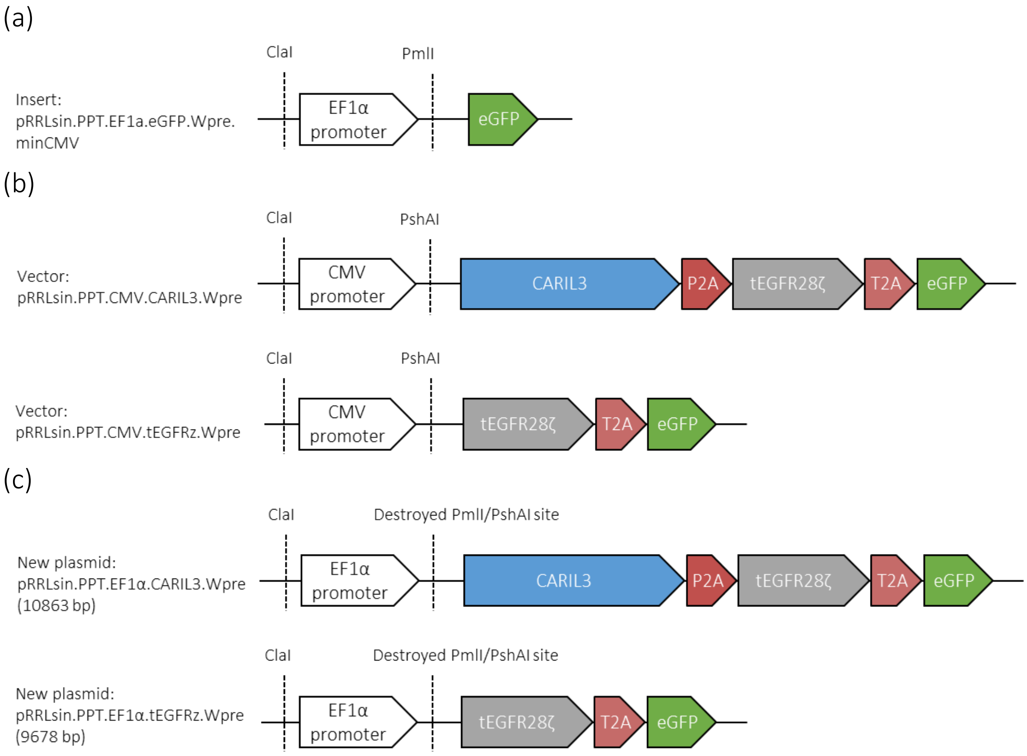


Figure 3.5 **Schematic of EF1α promoter cloning.** (a) Insert was derived from an available plasmid carrying the EF1α promoter using ClaI/PmlI double digest. (b) The vectors were derived from the CMV transfer plasmids by ClaI/PshAI double digest. (c) Ligated new EF1α transfer plasmids showing preserved ClaI and destroyed PmlI/PshAI sites.

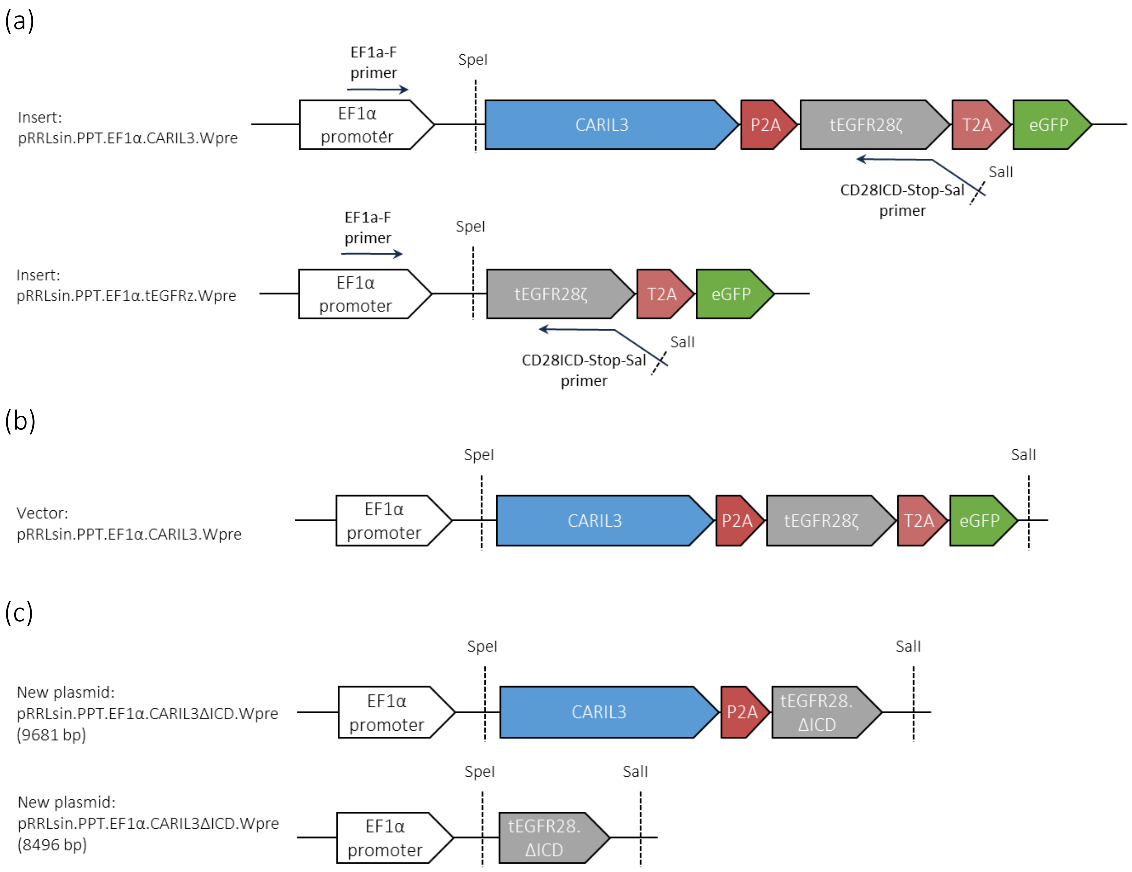


Figure 3.6 **Schematic of tEGFRz ICD and eGFP deletion.** (a) Insert was derived by PCR of the region of interest within the EF1α.CAR construct with 5' extension in the reverse primer to introduce the SalI digestion site followed by double digesting with SpeI/SalI. (b) The vector was derived from the same EF1α transfer plasmids by SpeI/SalI double digest. (c) Ligated new plasmids with truncation of the CD28 costimulatory and signalling domain of tEGFRz (ΔICD).

3.2.4 Lentiviral stock preparation

3.2.4.1 DNA transfection by calcium phosphate precipitation

HEK293T cells were grown in complete DMEM in T150 flasks to approximately 70% confluency. On the day of transfection, the media was replaced with 20 mL and incubated for 3.5 – 4 hrs. Solutions A and B were prepared as the following tables (Table 3.2).

Table 3.2 Calcium phosphate precipitation mixes

Solution A	
2x HEPES-buffered saline pH 7.0	1 mL
0.15 M Na_2HPO_4	10 µL

Solution B	
DNA	40 µg (equal-molar ratio of 4 lentiviral plasmids)
10% TE	Make up to 1 mL
2M $CaCl_2$	125 µL added last

Solution B was added dropwise to solution A, while lightly vortexing solution A. The calcium phosphate plasmid mixture was incubated at room temperature for 20-30 min, then added to a HEK293T flask for overnight incubation. Following the incubation period, the media was changed with 20 mL fresh complete DMEM and allowed to incubate for another 28 – 32 hrs.

3.2.4.2 Viral supernatant harvest and concentration

The lentivirus containing culture medium was harvested and filtered through cellulose acetate 0.45 µm syringe filters (SLHV033RS, Merck) into a sterile open-top round-bottom or conical shaped ultracentrifuge tube (C14292 or C14307, Beckman Coulter, CA, USA). The tubes were balanced to the nearest 0.1g and spun at 72,000 × *g* at 16°C for 90 min to pellet the viral particles. The supernatant was removed by suction and the tubes placed on ice. The viral pellet was fully resuspended in about 1/100 volume (~200 µL) of DPBS by pipetting up and down at least 20 times and incubated on ice for 1 hour, before freezing in 10 µL aliquots in PCR tube strips at -80°C.

3.2.4.3 Determining lentiviral vector titre

HEK293T cells were seeded into six-well tissue culture plates at 1x10^5/well. Increasing lentivirus volumes (1, 2, 3, 5, 10 μL) were added to in HEK293T in the presence of 8 μg/mL polybrene (TR-1003-G, Sigma-Aldrich, MO, USA). Cells were incubated overnight at 37°C in a humidified 5% CO_2 incubator. The next morning, the media was exchanged and cultured further for 2 more days. The HEK293T were harvested and transduced cells were detected by flow cytometry using GFP marker or cetuximab primary antibody with goat-anti human IgG (H+L) AF647 secondary antibody. Viral titre was calculated using the equation:

$$\frac{\text{Transducing units}}{\text{mL viral stock}} = \frac{\text{\% positive cells} \times \text{number of cells at transduction}}{\text{mL lentivirus added}}$$

3.2.5 Generation and expansion of CAR T cells

3.2.5.1 *PiggyBat* transfection

PiggyBat (PBat) CARIL3 T cells were generated and expanded as described in chapter 2.

3.2.5.2 Lentiviral transduction

It has been reported that lentiviral transduction efficiency is dramatically improved in actively dividing T cells (Ghassemi *et al.*, 2022). Hence, on day -2, selected T cells were activated with TransAct, a colloidal polymeric nanomatrix conjugated to humanised CD3 and CD28 agonists (130-111-160, Miltenyi Biotec) at a ratio of 1/100 total volume. The T cells were seeded at 1x10^6/cm^2 in complete AIM-V supplemented with IL-7 (200 IU/mL) (130-095-367, Miltenyi Biotec, Germany) and IL-15 (200 IU/mL) (130-095-760, Miltenyi Biotec, Germany) in flat-bottom tissue culture plates and incubated in a 37°C, 5% CO_2 incubator. On day -1, half of the supernatant was exchanged with fresh complete AIM-V supplemented with IL-7 and IL-15.

On day 0, activated T cells were pelleted at 427x *g* for 5 minutes and resuspended to 1.47x10^6 cells/mL in IL-7, IL-15 supplemented cAIM-V media. For each well of the 12-well plate, 441x10^3 (300μL) activated T cells were transduced with lentiviral vectors at multiplicity of infection (MOI) of 0.68 (200µL) in the presence of 8µg/mL Polybrene (TR-1003-G, Merck) in 500µL cAIM-V supplemented with IL-7 (200 IU/mL) and IL-15 (200 IU/mL). Cells were incubated in a 37ºC, 5% CO_2 incubator for 6 hours, then topped up with 500µL IL-7/IL-15 supplemented cAIM-V. On day +1, 3mL of cAIM-V/IL-7/IL-15 was added. Cultures were split 1 in 2 with IL-7 and IL-15 supplemented cAIM-V media every 2-3 days when media has turned orange/yellow, until the end of culture at 12 days post-transduction.

3.2.6 Phenotyping flow cytometry

Flow cytometry was performed every 7 days starting day 1 or 8 after electroporation or transduction as described in Chapter 2 to track proportion of $tEGFR^+$ CAR T cells.

For direct detection of CARIL3(wt/sm/dm), 1 µL (5 µg/mL final) primary Rat anti-human IL3 (554672, BD) and 20 µL (40 µg/mL final) secondary Goat anti-rat Ig PE (550767, BD) secondary antibody were used per 100,000 cells in 100 µL staining volume.

A more comprehensive immunophenotyping panel of CD4/CD8, memory phenotype and inhibitory receptors was performed at end of culture as described in Chapter 2.

3.2.7 Intracellular cytokine flow cytometry

Intracellular cytokine flow cytometry was undertaken at the end of cultures as described in Chapter 2. TNFα and IFNγ were assessed by co-culturing cultured CAR T cells at 1:1 ratio with target cell lines with or without CD123 expression.

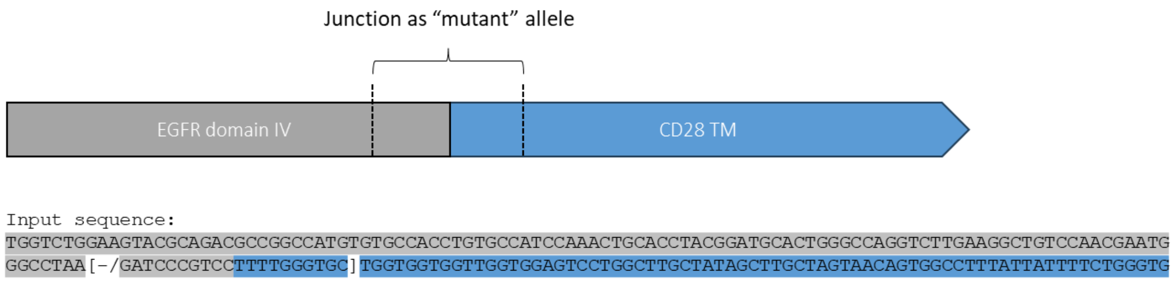
3.2.8 Calcein AM release assay

Cytotoxicity of CAR T cells at end of cultures was determined by calcein AM release assay at a range of effector:target ratios (E:T) using the protocol described in Chapter 2.

3.2.9 Copy number analysis

CAR T cells (3-15x10^6) were sorted by FACS for CAR$^+$ cells. Genomic DNA was extracted from 200,000 – 1.x10^6 sorted CAR$^+$ cells using QIAamp DNA Mini or Blood Mini Kits according to the manufacturer's protocol (51304 or 51104, Qiagen, Germany) and stored at -30°C.

CAR transgene copy number per cell was assessed by droplet digital PCR (ddPCR). Truncated EGFRz-specific primer/probe set (Unique Assay ID: dMDM5674380291) was designed in the BioRad ddPCR Mutant Detection Assay portal. DNA sequence input is shown in Figure 3.7 with the "mutant" allele being a stretch of the EGFR domain IV-CD28 transmembrane (TM) junction which does not normally occur in nature and represents only transgenes. The specification of the junction also forced the program to design primer pairs to be on both sides of the junction, with the probe specific to the junction itself. Two primer/probe sets are generated by the program with the mutant primer/probe set assigned to the FAM fluorophore. Predesigned RPP30-HEX primer/probe set (Unique Assay ID: dHsaCP1000485) was used as the house-keeping gene and its copy number is assumed to be 2 for calculations.



```
Input sequence:
TGGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCTGTGCCATCCAAACTGCACCTACGGATGCACTGGGCCAGGTCTTGAAGGCTGTCCAACGAATG
GGCCTAA[-/GATCCCGTCCTTTTGGGTGC]TGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTTGCTAGTAACAGTGGCCTTTATTATTTTCTGGGTG
```

Figure 3.7 Schematic and input sequence for ddPCR primer/probe design portal. Sequence in square brackets represent wild-type/mutant alleles. EGFR domain IV-CD28 TM junction (GATCCCGTCCTTTTGGGTGC) labelled as "mutant" allele and no DNA (-) as wild-type allele.

Table 3.3 Master mix of a single ddPCR reaction

Reagents	µL
10x NEB CutSmart buffer	0.18
HindIII-HF restriction enzyme (R3104S, NEB, MA, USA)	0.2
AccuGENE Molecular Biology Water (BE51200, Lonza, Switzerland)	1.62
ddPCR Supermix for Probes (no dUTP) (1863025, BioRad, CA, USA)	10
BioRad ddPCR EGFRIV-CD28TM junc – FAM (Unique Assay ID: dMDM5674380291)	1
BioRad ddPCR RPP30 assay (reference gene)-HEX (Unique Assay ID: dHsaCP1000485)	1
Total Volume	14

Master mixes were made according to Table 3.3 and aliquoted into PCR tubes. Genomic DNA (gDNA) was diluted to 3.75 ng/µL with molecular-grade water and 30 ng gDNA was added to each PCR tube. Tubes were capped and briefly vortexed and centrifuged. 20 µL of DNA/master mix was added to sample-row wells of DG8 Cartridges (1864008, BioRad, CA, USA) using a multichannel pipette. 70 µL of Droplet Generating Oil for Probes (1863005, BioRad, CA, USA) was added to the oil wells of the DG8 Cartridge. The cartridge was covered with the DG8 Gasket (1863009, BioRad, CA, USA) and inserted into a QX200 Droplet Generator (1864002, BioRad, CA, USA). Once droplet generation was completed, 40 µL of droplets were transferred to Twin-Tec 96-well semi-skirted plates (951020303, Eppendorf, Germany) and sealed with PCR Plate Heat Seal pierceable foil (1814040, BioRad, CA, USA) in a PX1 PCR plate sealer (1814000, BioRad, CA, USA). PCR cycling was performed using the MiniAmp Thermal Cycler for single temperature annealing/extension step (Applied Biosystems, MA, USA) or the VeritiPro Thermal Cycler for gradient annealing/extension step (Applied Biosystems, MA, USA) as per table below.

Table 3.4 PCR conditions for ddPCR

Step	Temperature	Time
Enzyme activation	95°C	10 min
40 Cycles	94°C	30 sec
	58°C optimised (or gradient temperature 50-60 °C at 2°C increments for optimisation)	1 min
Final extension	98°C	10 min
Hold	4°C	∞

After PCR, the plate was read in the BioRad QX200 Droplet Reader (1864003, BioRad, CA, USA) under the CNV2 program in QuantaSoft Software (BioRad, CA, USA).

Analysis was performed in the QuantaSoft Software, with positive threshold set at least 2.5x of negative events' median fluorescent intensity. Copy number per cell was calculated as follows:

$$\text{CAR copy number per cell} = \frac{\text{copies of CAR transgene junction}}{\text{copies of RPP30}} \times 2$$

Average results from 3 donors were compared between lentiviral and *PiggyBat* CARs by 2-tailed unpaired t-test.

3.2.10 Integration site analysis

MuA mediated integration site recovery was used for integration site analysis as previously described (Brady *et al.*, 2011) (Figure 3.8). The procedure is described in detail below.

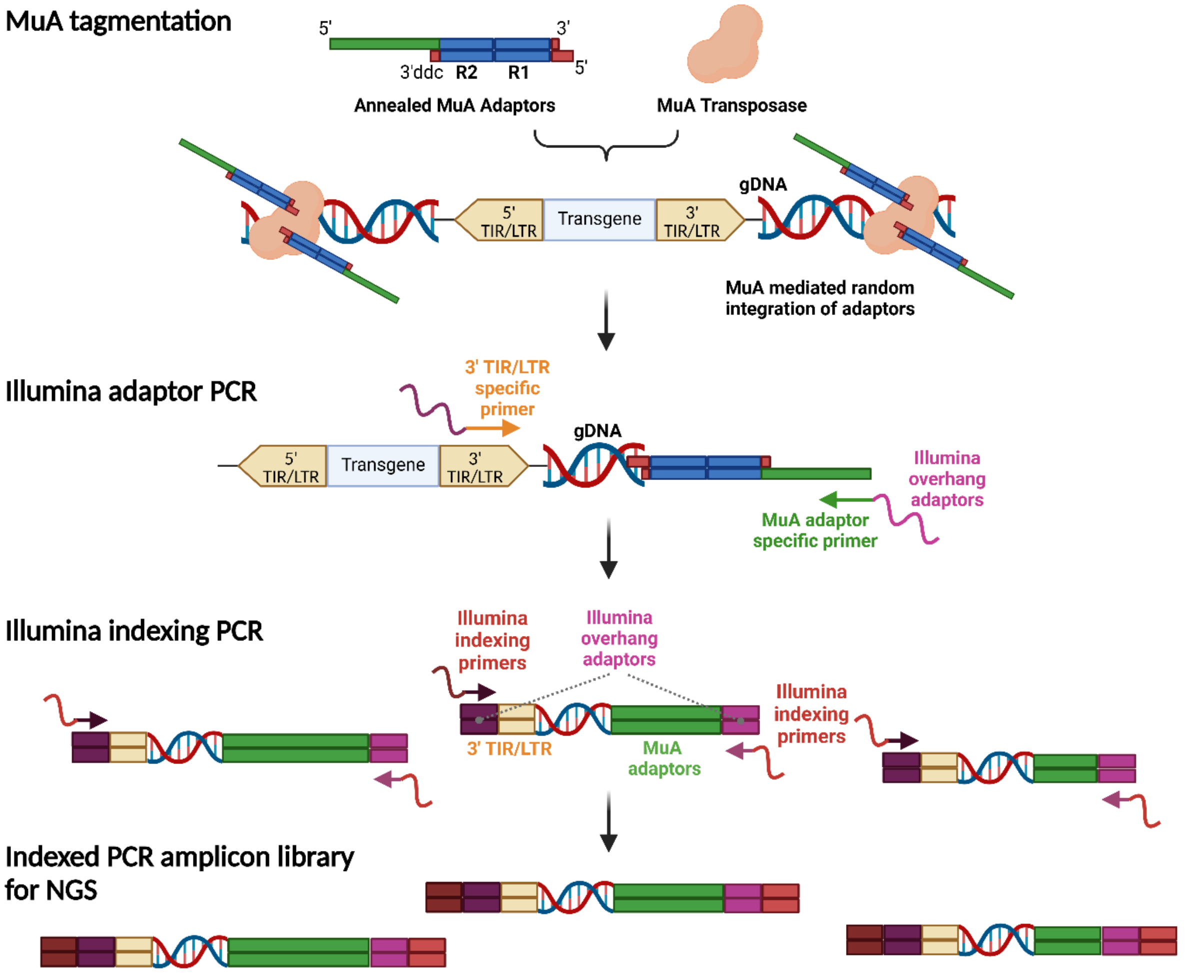


Figure 3.8 Schematic of integration site analysis by MuA transposase. gDNA, genomic DNA; TIR, terminal inverted repeat; LTR, lentiviral terminal repeat. Created using BioRender.com.

3.2.10.2 Mu (+) / (-) oligonucleotide annealing

Synthetic Mu (+) and Mu (-) oligonucleotides (100 µM) (Table 3.10) (Sigma-Aldrich, MO, USA) were diluted 1/10 with TEN buffer (10 mM Tris-HCI, pH 8, 1 mM EDTA, 50 mM NaCl) and equal volumes annealed by heating to 90°C for 1 minute, then cooling by 0.5°C per min down to 25°C or lower in a thermal cycler, then stored in -30°C freezer.

3.2.10.3 Lentiviral CAR T cell gDNA digestion and purification

Due to the lentiviral terminal repeat (LTR) primer binding to both 5’ LTR and 3’ LTR, to prevent amplification of the viral “internal fragment” (i.e. viral sequence from the 5’ LTR), the lentiviral CAR T cell gDNA (up to 400 ng) was first digested overnight with 40 U BanI (R0118S, NEB, MA, USA) in 100 µL. BanI cuts close to the 3’ end of the 5’ LTR, but not to the 3’ LTR. The digested LV CAR T cell gDNAs were purified using AMPure XP Bead-Based Reagent (A63882, Beckman Coulter, CA, USA) at 1.8x volume.

3.2.10.4 MuA tagmentation

Both PBat and purified digested LV CAR T cell gDNA was quantified using BR or HS Qubit dsDNA Quantification Assay Kits (Q32853 or Q32854, ThermoFisher, MA, USA) according to the manufacturer’s instructions and incubated with MuA transposase (F750, ThermoFisher, MA, USA) and annealed oligonucleotides at 30°C for 4 hours (Table 3.5) and stored at 4°C.

Table 3.5 MuA tagmentation mix

Component	**Volume (µL)**
5X Reaction Buffer	2.5
Annealed Mu Adapters (10 µM)	2.0
DNA (up to 250 ng in 6 µL)	Up to 6 µL
MuA (220 ng/µL)	2.0
Molecular biology water	To total 12.5

3.2.10.5 Illumina adaptor PCR

Tagmented gDNA were amplified using PBat 3’ TIR or lentiviral LTR-specific forward primer and MuA specific reverse primer (Table 3.6, Table 3.7, primer sequences in Table 3.10). PCR amplicons consist of DNA sequences spanning the intersection of 3’ PBat TIR/LV LTR and adjacent gDNA corresponding to the genomic integration site. The PCR amplicon library was purified using AMPure XP Bead-Based Reagent at 1.6x volume and quantified using BR Qubit dsDNA Quantification Assay Kit and stored at -30°C.

Table 3.6 Adaptor PCR master mix

Reagents	Volume (µL)
2x Q5 HF buffer	25
TIR or LTR adaptor fwd primer (10 µM)	2.5
MuA adaptor rev primer (10µM)	2.5
Template (Mu transposase reaction mix)	5.0
Molecular biology water	15
Total	50

Table 3.7 Adaptor PCR conditions

Step	Temperature	Time
Enzyme activation	95°C	3 min
39 Cycles	95°C	15 sec
	62°C	30 sec
	72°C	2 min
Final extension	72°C	5 min
Hold	4°C	∞

3.2.10.6 Illumina indexing PCR

Purified amplicon libraries were indexed using Nextera XT Index Kit (24 indexes, 96 samples) (FC-131-1001, Illumina, CA, USA) according to manufacturer’s instructions. Indices S502 and N701 – N706 were used to index the 6 samples (Table 3.8, Table 3.9, primer sequences in Table 3.10). Indexed PCR products were purified using AMPure XP Bead-Based Reagent (PBat PCR products at 1.8x volume; lentiviral PCR products at 1.8x volume, then 0.7x volume to deplete the peak at ~200bp). Purified indexed PCR products quality and quantities were assessed using D1000 ScreenTape assay (5067-5583 and 5067-5582, Agilent Technologies, CA, USA) according to the manufacturer’s instructions.

Table 3.8 Index PCR mix

Reagents	**Volume (µL)**
2x Q5 HF buffer	25
Index 1 primer (N7xx, orange cap)	5
Index 2 primer (S5xx, white cap)	5
Purified adapted PCR product	5
Molecular biology water	10
Total	50

Table 3.9 Index PCR conditions

Step	**Temperature**	**Time**
Enzyme activation	95°C	3 min
39 Cycles	95°C	30 sec
	55°C	30 sec
	72°C	30 sec
Final extension	72°C	5 min
Hold	4°C	∞

3.2.10.7 Illumina Next Generation Sequencing

Next generation sequencing of purified PCR amplicon libraries was performed on the Illumina MiSeq Reagent Kit v2 (pair-end reads, 500 cycles, MS-102-2003, Illumina, CA, USA) (Australian Genome Research Facility). Reads were trimmed of TIR or LTR and MuA sequences and mapped to chromosomal loci using Ub-ISAP bioinformatic algorithm (Kamboj *et al.*, 2017) and annotated to hg19 human genome as reference using HOMER (Heinz *et al.*, 2010). The frequency of integrations was expressed as a log fold change over expected for random insertion.

Table 3.10 Primer sequences for integration site analysis

Primer	**Primer sequence (5’ → 3’)**
Mu (+) strand	GTAATACGACTCACTATAGGGCTCCGCTTAAGGGACTGTTTTCGCATTTATCGTGAAACGCTTTCGCGTTTTTCGTGCGCCGCTTCA
Mu (-) strand	TCGGATGAAGCGGCGCACGAAAAACGCGAAAGCGTTTCACGATAAATGCGAAAACA[3ddC]
PBat TIR adaptor Fwd primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTTGGCGGGAAATTCACC
LTR adaptor Fwd primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTGAGTGCTTCAAGTAGTG
MuA Rev primer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTAATACGACTCACTATAGGGC

3.3 Results

3.3.1 CD123 specific CAR T cells could be successfully manufactured with the *PiggyBat* gene modification system

All three CARIL3 T cells (n = 4 donors) expanded in culture with weekly stimulation using irradiated autologous PBMC at a ratio of 4:1 PBMCs to cultured cells. CARIL3sm T cells showed significantly higher expansion compared to CARIL3wt and CARIL3dm at day 15 of culture (mean expansions: CARIL3sm – 34-fold; CARIL3wt – 18-fold; CARIL3dm – 16-fold; $p = 0.0031$, repeated measures ANOVA) (Figure 3.9a). Mean CAR expression was also highest in the CARIL3sm T cell culture (CARIL3sm – 85.6%; CARIL3dm – 73.9%; CARIL3wt – 53.3%; $p = 0.0246$) (Figure 3.9b).

Each culture contained both $CD4^+$ and $CD8^+$ CAR T cells, with no significant difference in the CD4:CD8 proportions between the CARIL3 constructs (mean ranges: $CD4^+$ CAR: 37.5% - 41.9%; $CD8^+$ CAR: 58.1% - 62.4%; $p = 0.9102$) (Figure 3.9c). Most CAR T cells were of either naïve/stem cell memory ($CD62L^+CD45RA^+$) (mean range: 38.6 - 48.2%) or central memory phenotype ($CD62L^+CD45RA^{neg}$) (mean range: 27.0 – 28.8%). A slightly lower proportion of effector memory cells ($CD62L^{neg}/CD45RA^{neg}$) was noted in CARIL3dm cultures (mean: 4.9%) compared to CARIL3wt and CARIL3dm (mean 12.3%, 11.1% respectively) (Figure 3.9d).

The expression of the co-inhibitory receptors (PD1, LAG3, TIM-3) was not significantly different between the three CARIL3 constructs. (mean ranges: $PD1^+$, 51.2 – 54.1%; $LAG3^+$, 84.6 – 87.3%; $TIM\text{-}3^+$, 85 – 85.5%) (Figure 3.9e).

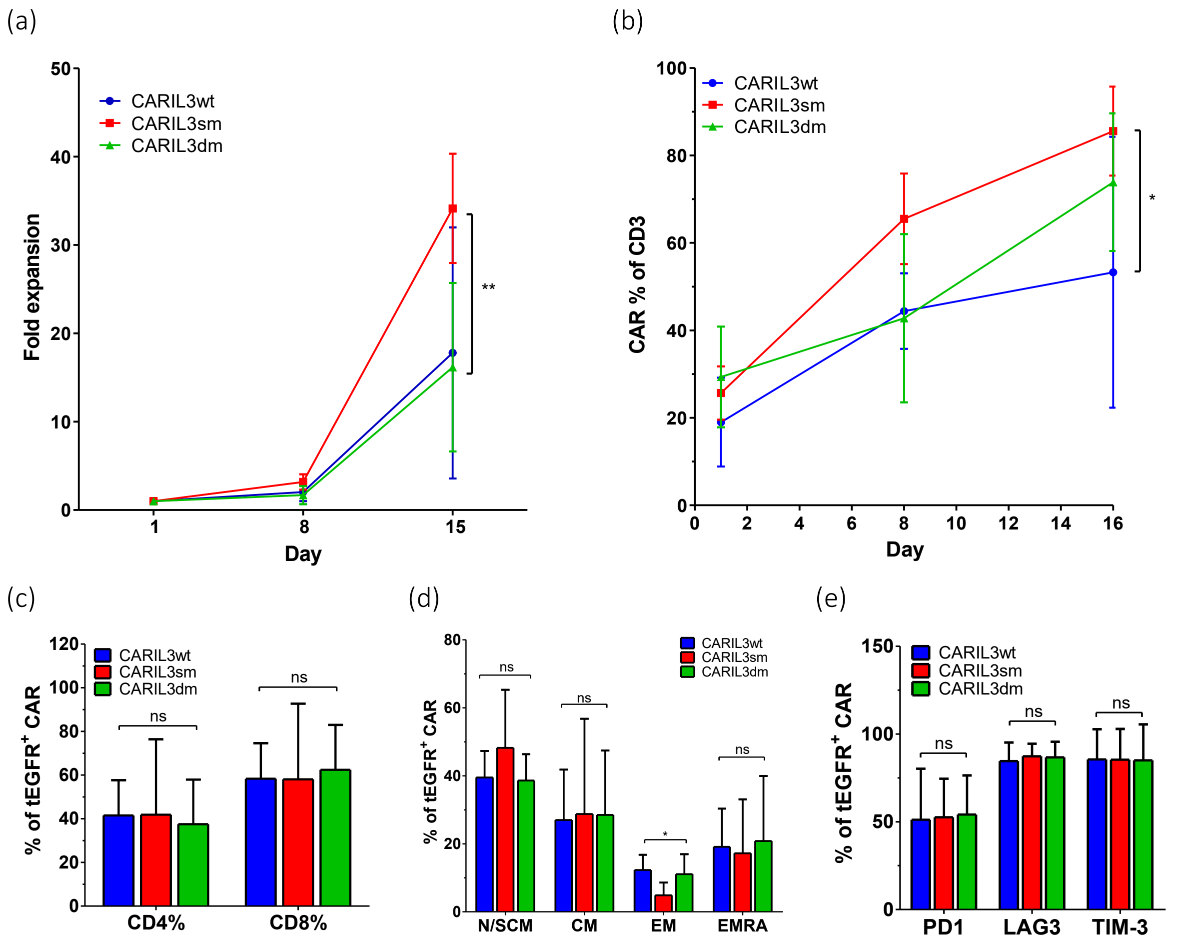


Figure 3.9 ***PiggyBat*** **CARIL3 CAR T cells expansion, enrichment and phenotypes after 15 days of culture.** (a) Cell number fold-expansion in all 3 CAR T cell constructs over 15 days following electroporation relative to day 1. (b) CAR expression was determined by tEGFRz CAR surrogate expression. (c) CD4/CD8 distribution, (d) memory phenotype, and (e) expression of co-inhibitory receptors on CAR T cells as determined by flow cytometry at the end of culture (day 15). CAR expression was measured via tEGFRz surrogate CAR. Pooled data (n = 4 donors per construct) represented as mean ± SD. P-values calculated using repeated measures ANOVA. *: <0.05, **: <0.01, ns: not significant. N/SCM: naïve/stem cell memory (CD62L^{+}CD45RA^{+}); CM: central memory (CD62L^{+}CD45RA^{neg}); EM: effector memory (CD62L^{neg}/CD45RA^{neg}); EMRA, effector memory RA (CD62L^{neg}CD45RA^{+}); PD1: Programmed cell death protein 1; Lag3: Lymphocyte-activation gene 3; Tim3: T cell immunoglobulin and mucin domain-containing protein 3.

3.3.2 *PiggyBat* CARIL3 T cells showed specific functional activity *in vitro*

The *PiggyBat* CARIL3 T cell cultures were tested for functional specificity by flow cytometry and calcein-AM cytotoxicity assay at the end of the 15-day culture. Flow cytometry was used to assess intracellular cytokine production of tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ) following 5 hours of co-culture with two CD123 negative cell lines (HEK293, Raji) and two leukaemic cell lines with low levels of CD123 expression (TF-1, KG-1) (Figure 3.10a). Media only control was used to determine basal activation.

CARIL3sm and CARIL3dm cultures expressed significantly higher TNFα following co-culture with CD123-low KG-1 cell lines, compared with media only ("no target") control (CARIL3sm 13.3% vs 0.2%, CARIL3dm 5.9% vs 0.4%). Production of TNFα following co-culture with CD123-low TF-1 was variable for single and double mutant CARIL3 T cells and did not reach statistical significance (mean range: 2.4 – 5.9%). In contrast, CARIL3wt T cells showed a minor, but significant increase in TNFα expression against CD123-low TF-1 compared to media only control (mean: 3.6% vs 0.3%), but not against CD123-low KG-1 (mean: 2.1% vs 0.3%). There was minimal non-specific TNFα expression by all three CARIL3, following co-culture with CD123^{neg} cell lines HEK293 and Raji cell lines (mean range: 0.2 – 1.3%) (Figure 3.10b).

Interferon gamma (IFNγ) production following co-culture with CD123 positive and negative cell lines followed a similar trend as TNFα, with CARIL3sm and CARIL3dm T cells producing significantly higher TNFα against CD123-low KG-1 compared to media only controls (CARIL3sm: 10.6% vs 0.3%, CARIL3dm: 5.1% vs 0.5%), but not CD123-low TF-1 (mean range: 2.4 – 4.4%) or CD123^{neg} cell lines HEK293 and Raji (mean range: 0.1 – 1.2%). Once again, significantly higher proportion of CARIL3wt T cells produced IFNγ against CD123-low TF-1 compared to media only control (mean: 2.6% vs 0.3%), but no difference was detected against CD123-low KG-1 (1.6%) or CD123^{neg} cell lines HEK293 and Raji (mean range 0.3 – 0.8%) (Figure 3.10c).

Cytotoxicity by calcein-AM with CD123^{neg} HEK293 as targets showed no significant killing by any of the CARIL3 T cell cultures (mean range at 40:1 E:T ratio: -3.0 to 3.3%) (Figure 3.10d.i). Dose-dependent target cell lysis against CD123-low KG-1 was observed in all cultures expressing the three variants of CARIL3 constructs without significant differences (mean lysis at 40:1 E:T ratio: CARIL3wt: 35.9%, CARIL3sm: 52.5%, CARIL3dm: 49.2%; $p > 0.05$, one-way ANOVA) (Figure 3.10d.ii). Each CARIL3 was significantly higher in specific cytotoxicity against KG-1 than HEK-293 targets ($p < 0.05$, two tailed paired t-test). (Figure 3.10d.iii).

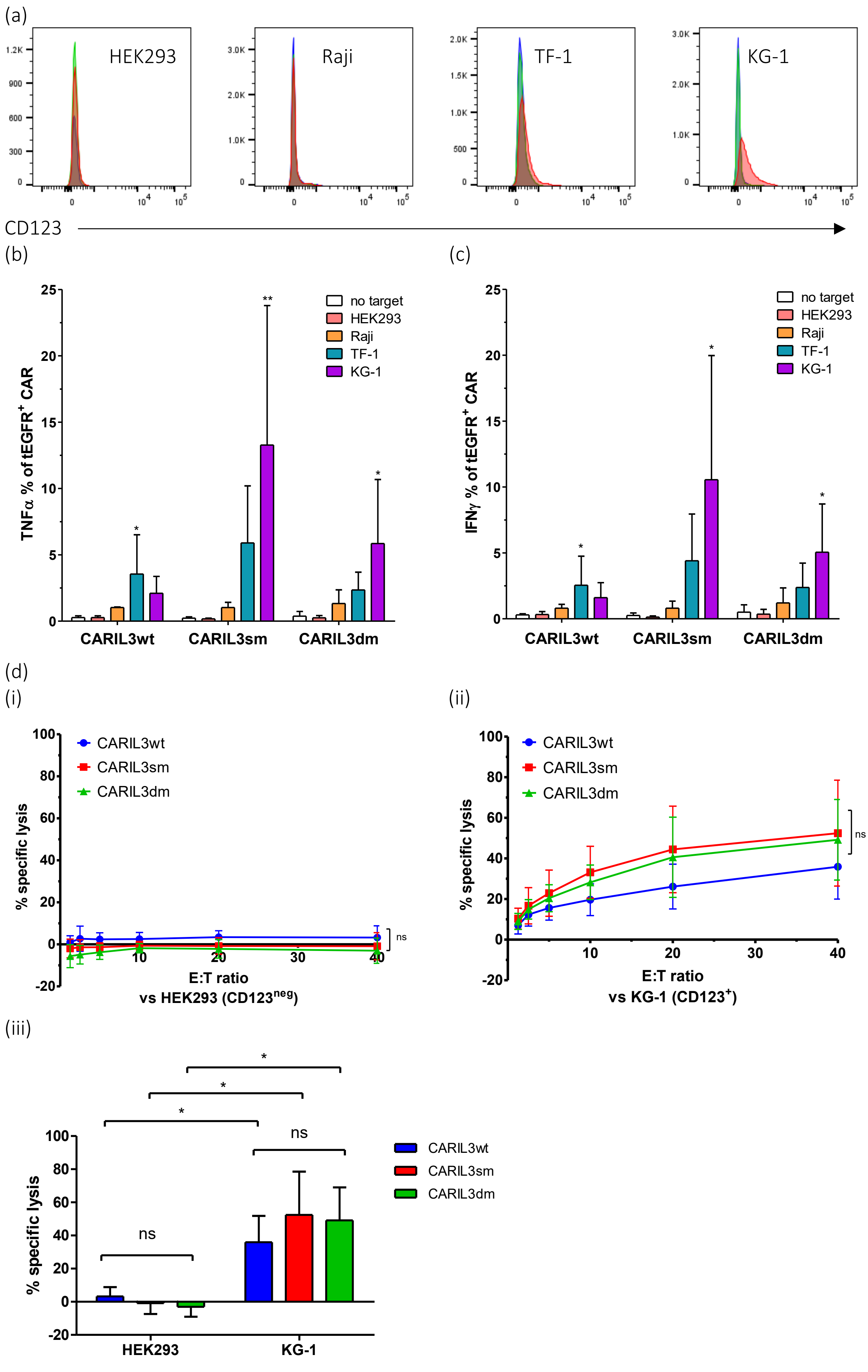

(a)
HEK293
Raji
TF-1
KG-1
CD123
(b)
TNFα % of tEGFR+ CAR
no target
HEK293
Raji
TF-1
KG-1
CARIL3wt
CARIL3sm
CARIL3dm
(c)
IFNγ % of tEGFR+ CAR
no target
HEK293
Raji
TF-1
KG-1
CARIL3wt
CARIL3sm
CARIL3dm
(d)
(i)
% specific lysis
CARIL3wt
CARIL3sm
CARIL3dm
ns
E:T ratio
vs HEK293 (CD123neg)
(ii)
% specific lysis
CARIL3wt
CARIL3sm
CARIL3dm
ns
E:T ratio
vs KG-1 (CD123+)
(iii)
% specific lysis
ns
ns
*
*
*
HEK293
KG-1
CARIL3wt
CARIL3sm
CARIL3dm

Figure 3.10 Functional assessment of *PiggyBat* CARIL3 CAR T cells *in vitro* against CD123^{+} cell lines. (a) Cell lines used as targets: HEK293 (CD123^{neg}), Raji (CD123^{neg}), TF-1 (CD123-low) and KG-1 (CD123-low). CD123 stained cells in red, overlayed with isotype control in green and unstained control in blue. Intracellular flow cytometry demonstrating proportions of CARIL3 CAR T cells producing (b) TNFα and (c) IFNγ in response to CD123-low cells lines (TF-1, KG-1). CAR expression was measured via tEGFRz surrogate CAR. (n = 4 donors; pooled data represented as mean ± SD; *: $p<0.05$, **: $p<0.01$; one-way ANOVA with Bonferroni's multiple comparison test against no target media only control). (d) Cytotoxicity of CARIL3 T cells was assessed against (i) CD123^{neg} HEK293 and (ii) CD123-low KG-1 using calcein AM assay by co-culturing at a range of effector to target (E:T) ratios. (iii) Summary data at 40:1 E:T ratio. (n = 4, pooled data represented as mean ± SD; *: $p<0.05$, two-tailed paired t-test was used for between targets; ns: not significant; one-way ANOVA was used for between CARs with Bonferroni's multiple comparison test).

3.3.3 Production of high titre lentiviral vectors required shortening the transgene construct

Lentiviral stocks preparation and titration was performed as described in section 3.2.4. The effect of different length transgene inserts on viral titre (functional viral transduction units) was explored. Specifically, the viral titre of full length CARIL3sm and tEGFRz were compared with shorter versions CARIL3sm.ΔICD, tEGFR.ΔICD made by deleting domains downstream of the CD28 transmembrane domain of tEGFRz (Figure 3.6). A significant negative correlation was observed between CAR transgene length and viral titre ($p = 0.0105$) (Figure 3.11). Therefore, to maximise the titre and subsequent multiplicity of infection (MOI) during T cell transduction, all following lentiviral transduction experiments were conducted using the intracellular deletion versions of the CARIL3 and tEGFRz constructs (i.e. CARIL3wt.ΔICD, CARIL3sm.ΔICD, CARIL3dm.ΔICD and tEGFR.ΔICD).

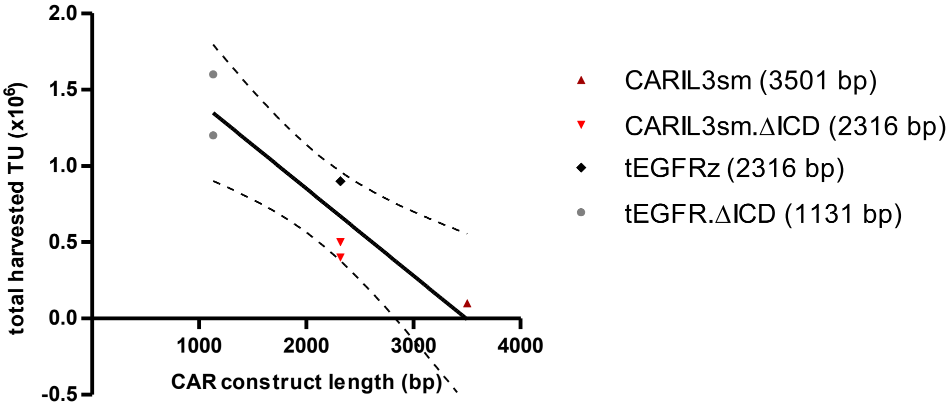


Figure 3.11 Total harvested lentiviral titre correlation with CAR construct length. Solid line represents Pearson's linear regression line, dashed lines represent 95% confidence intervals. CARIL3sm, single mutant (K116W) IL-3 CAR; tEGFR, truncated epithelial growth factor receptor; tEGFRz, truncated epithelial growth factor receptor CAR; ΔICD, intracellular domain deletion; TU, transduction units. Diagrams for full length and ΔICD CAR constructs are shown in Figure 3.2.

3.3.4 Lentiviral CAR T cells were expanded when stimulated with TransAct

After 2 days of TransAct incubation, T cell activation was confirmed by flow cytometry of early and late activation markers, CD69 and CD25 respectively (Figure 3.12a). TransAct activated T cell and CAR T cells expanded well after 12 days, with no significant difference between the un-transduced T cells (mock) and CAR T constructs (mean fold expansion range: 317.1 – 577.8, p = 0.7088) (Figure 3.12c).

To detect the human IL-3 recognition domain of CARIL3 directly, an unconjugated rat anti-human IL3 primary antibody (clone BVD8-3G11) and a goat anti-rat Ig PE secondary antibody were used in conjunction with routine tEGFR staining (cetuximab/goat anti-human IgG AF647). A strong concordance was found by flow cytometry between the direct detection of the truncated human IL-3 and tEGFR (Figure 3.12b). Therefore, tEGFR staining was used as a surrogate marker for CAR expression in the multi-cistronic system.

CAR expression was significantly higher for CARIL3sm.ΔICD and CARIL3dm.ΔICD T cells compared with CARIL3wt.ΔICD and tEGFR.ΔICD T cells. Un-transduced T cells had no expression of CARs (mean: sm – 72.9%, dm – 61.0%, wt, - 23.8%, tEGFR – 12.6%; P < 0.0004) (Figure 3.12d).

The LV CAR T cell cultures were CD4 dominant in which CARIL3sm.ΔICD CAR T cells were significantly higher in CD4 than tEGFR.ΔICD CAR T cell cultures (Mean ranges: CD4 CAR: 74.0 – 92%; CD8 CAR: 6.3 – 22.7%) (Figure 3.12e). TransAct expanded CAR T cells were mostly comprised of naïve/stem cell memory (mean range: 49.8 – 59.3%) and central memory (mean range: 29.0 – 34.3%) phenotypes. There was no statistical difference between the four CAR cultures in terms of distribution of memory phenotypes (Figure 3.12f).

Almost all LV CAR T cells were PD1, LAG3 positive by the end of culture (mean ranges: PD1: 93.3 – 97.5%, LAG3: 86.1 – 98.6%). CAR T cells expressing the non-functional control tEGFR.ΔICD had a lower level of TIM-3 (mean expression 10%), but no differences were detected between the cultures expressing the three CARIL3s constructs (mean range: 34.1% to 55.6%) (Figure 3.12g).

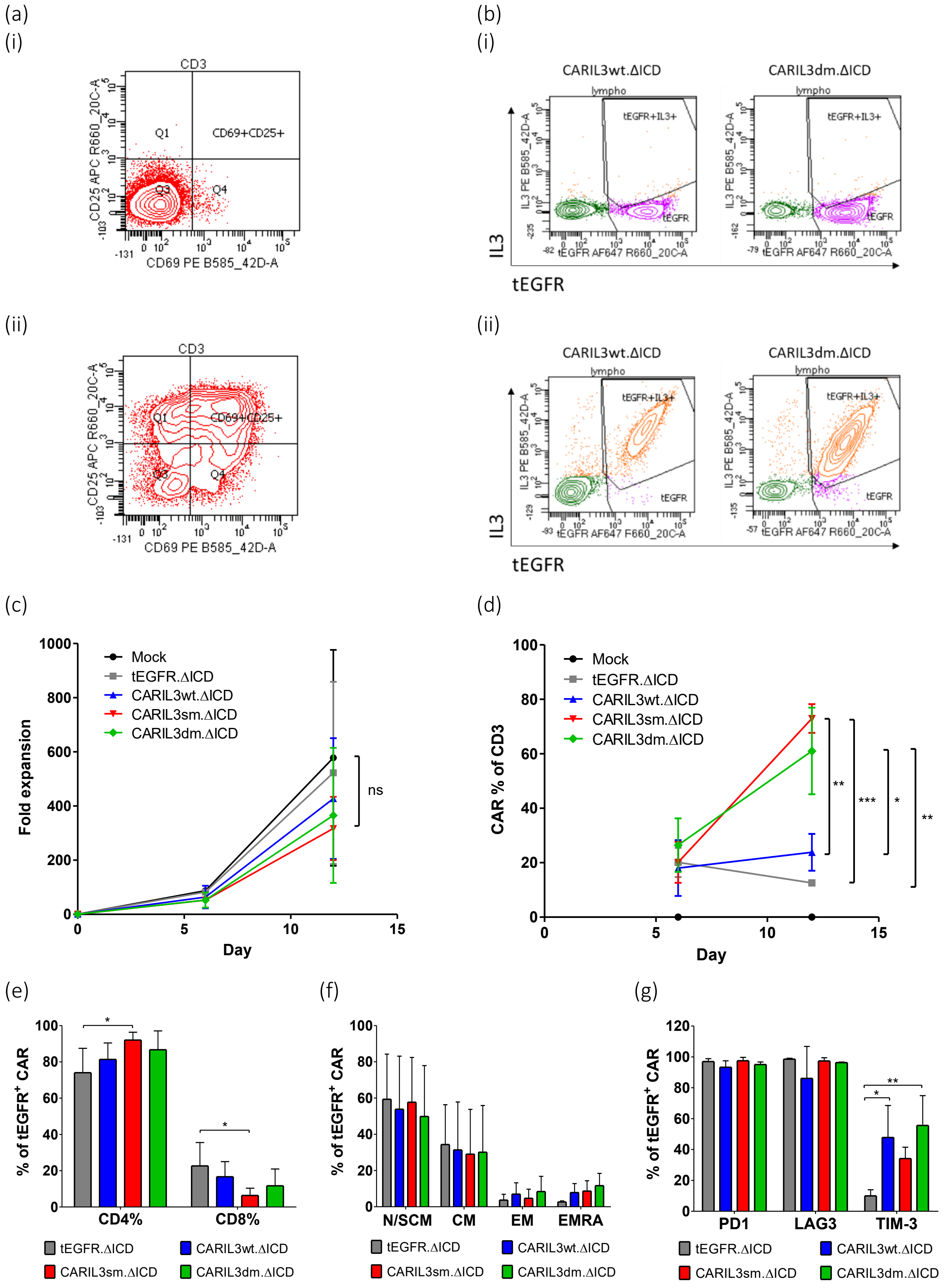

(a)
(i)
CD3
Q1
CD69+CD25+
Q3
Q4
CD25 APC R660_20C-A
CD69 PE B585_42D-A
(ii)
(b)
(i)
CARIL3wt.ΔICD
CARIL3dm.ΔICD
lympho
tEGFR+IL3+
tEGFR
IL3 PE B585_42D-A
tEGFR AF647 R660_20C-A
IL3
tEGFR
(ii)
(c)
Mock
tEGFR.ΔICD
CARIL3wt.ΔICD
CARIL3sm.ΔICD
CARIL3dm.ΔICD
Fold expansion
Day
ns
0
200
400
600
800
1000
0
5
10
15
(d)
CAR % of CD3
Day
**
***
*
**
0
20
40
60
80
100
(e)
% of tEGFR+ CAR
CD4%
CD8%
*
*
(f)
N/SCM
CM
EM
EMRA
(g)
PD1
LAG3
TIM-3
120
🟫 tEGFR.ΔICD
🟦 CARIL3wt.ΔICD
🟥 CARIL3sm.ΔICD
🟩 CARIL3dm.ΔICD

Figure 3.12 Expansion and enrichment of TransAct activated lentiviral CARIL3 CAR T cells after 12 days of culture. (a) Expression of activation markers CD69 and CD25 in T cells following 2 days of culture (i) without TransAct, and (ii) with TransAct exposure. (b) Representative contour plots of correlation between direct antibody detection of the truncated IL-3 recognition domain of CARIL3 and detection of tEGFR surrogate tag with (i) top row showing control staining without primary anti-IL3 antibody, and (ii) bottom row when anti-IL-3 primary antibody is added. (c) Numerical expansion of cells following 12 days in culture. (d) Lentiviral CARIL3 CAR expression was measured by tEGFR surrogate tag. (e) CD4:CD8 distribution, (f) memory immunophenotype and (g) expression of co-inhibitory receptors PD-1, TIM-3 and LAG3 on CAR T cells were assessed by flow cytometry at the end of culture. CAR expression was measured via tEGFR surrogate tag. Pooled data (n = 3 donors) represented as mean ± SD. P-values calculated using repeated measures ANOVA with Bonferroni's multiple comparison test. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: not significant. N/SCM: naïve/stem cell memory (CD62L^{+}CD45RA^{+}); CM: central memory (CD62L^{+}CD45RA^{neg}); EM: effector memory (CD62L^{neg}/CD45RA^{neg}); EMRA, effector memory RA (CD62L^{neg}CD45RA^{+}); PD1: Programmed cell death protein 1; LAG3: Lymphocyte-activation gene 3; TIM-3: T cell immunoglobulin and mucin domain-containing protein 3.

3.3.5 Lentiviral CARIL3 T cells showed variable *in vitro* activities against CD123-low KG-1 cell line

Lentiviral CAR T cells were tested for *in vitro* cytokine production and cytotoxicity against the CD123^{neg} HEK293 and CD123^{+} KG-1 cell lines at the end of the 12-day cultures (Figure 3.13a). The proportion of TNFα producing CAR T cells was significantly increased in CARIL3sm.ΔICD expressing culture following 5 hours co-culture with CD123-low KG-1 compared to media only (no target) control (mean 8.5% vs 0.7%, $p < 0.01$ repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test). The other two version of the LV CARIL3.ΔICD (wt and dm) were not significantly different to media only control (mean: wt: 3.0% vs 1.6%; dm: 4.2% vs 1.2%). Lentiviral CARIL3 T cells' TNFα release was not significantly increased against CD123^{neg} HEK293 cells (mean range: 0.7 – 1.8%) (Figure 3.13a).

Overall, very little IFNγ releases were observed across all three LV CAR constructs against both target cell lines and media only controls (mean range: 0.2% – 0.7%) (Figure 3.13b).

At a 40:1 E:T ratio, all three CARIL3.ΔICD T cells showed significant specific cytotoxicity against CD123-low KG-1 compared to CD123^{neg} HEK293 targets (mean: wt 43.2% vs 2.3%, $p < 0.05$; sm: 25.1% vs -4.2%, $p < 0.05$; dm: 49.3% vs -2.8%, $p < 0.01$, two-tailed paired t-test). CARIL3dm.ΔICD T cells were also significantly higher in cytotoxicity than CARIL3sm.ΔICD against CD123-low KG-1 cells (49.3% vs 25.1%, $p < 0.01$, repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test). (Figure 3.13c).

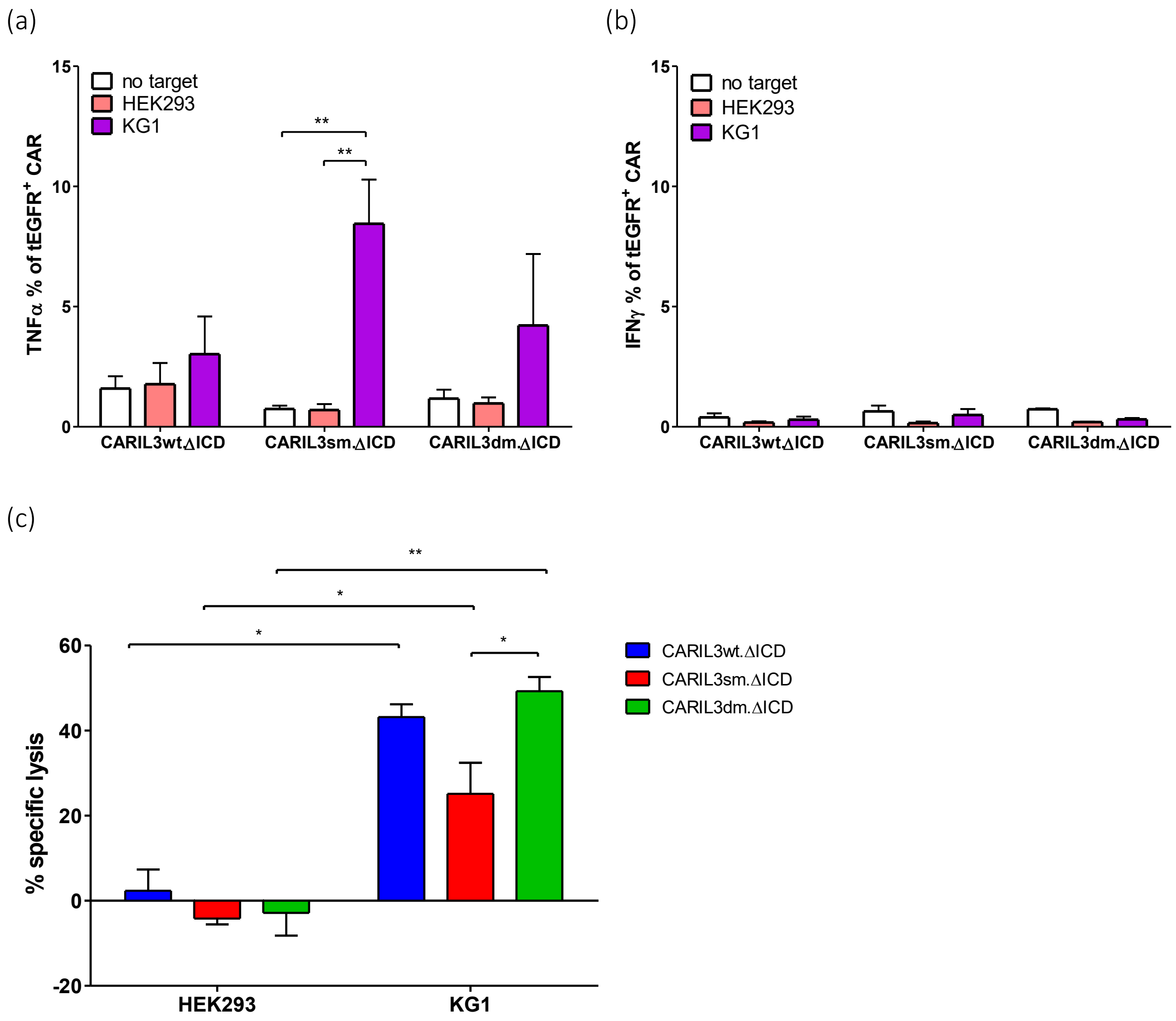


Figure 3.13 Specific activities of lentiviral CARIL3 T cells against CD123 low-expressing KG-1. (a) TNFα and (b) IFNγ production by CARIL3.ΔICD T cells against HEK293 ($CD123^{neg}$) and KG-1 (CD123-low) after co-culturing for 5 hours were assayed by intracellular flow cytometry. CAR expression was measured via tEGFR tag. (n = 3 donors, p-values calculated by repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test, *: $p<0.05$, **: $p<0.01$). (b) Specific cytotoxicity was assessed by Calcein AM assay after co-culture of 4 hours at effector : target (E:T) ratio of 40:1 (n = 3 donors; *: $p<0.05$, **: $p<0.01$, two-tailed paired t-test was used between targets; repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test was used between CARs).

3.3.6 Lentiviral CARIL3 T cells were functional *in vitro* against CD123 overexpressing cell lines

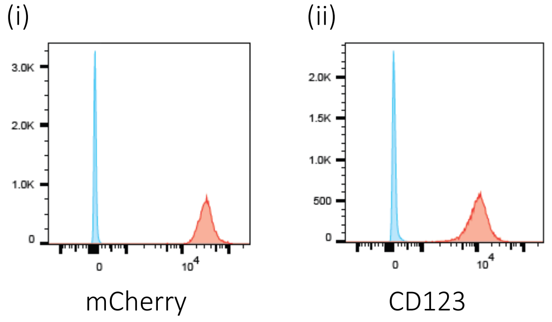
To discern efficacy of CARIL3 more clearly, CD123 overexpressing cell lines were created from CD123^{neg} cell lines HEK293 and K562 as targets (Figure 3.14a).

All three lentiviral CARIL3.ΔICD T cells displayed significantly more TNFα production against CD123-overexpressing cell lines compared to CD123^{neg} parental cell lines after 5 hours co-incubation (mean ranges: HEK293-IL3R vs HEK293: 14.2 – 27.9% vs 0.2 – 0.8%, K562-IL3R vs K562: 19.9 – 23.8% vs 1.8 – 4.6%). Control tEGFR.ΔICD had no significant increase in TNFα producing cells in any conditions above media only background (mean range: 0.1 – 0.3%) (Figure 3.14b).

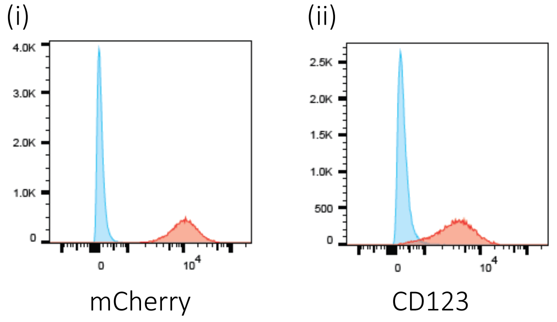
The proportion of cells with increased IFNγ production in each condition mirrored that of TNFα but did not reach statistical significance for the CARIL3.ΔICD cultures. (mean range: HEK293-IL3R vs HEK293: 1.9 – 5.2% vs 0.1 – 0.3%, K562-IL3R vs K562: 3.2 – 3.7% vs 0.3 – 0.5%). Control tEGFR.ΔICD again had minimal IFNγ production in all conditions (mean ranges 0.2% - 0.9%) (Figure 3.14c).

Specific cytotoxicity of the CAR T cells was assessed through calcein AM assays by co-incubating with target cells. CARIL3wt.ΔICD, CARIL3sm.ΔICD, and CARIL3dm.ΔICD T cells achieved mean lysis of 37.4%, 35.6% and 47.1% respectively at 20:1 E:T ratio against HEK293-IL3R which were significantly above tEGFR.ΔICD T cells (mean -16.6%). Similarly, CARIL3wt.ΔICD, CARIL3sm.ΔICD and CARIL3dm.ΔICD's lysis of K562-IL3R reached 23.0%, 50.1% and 60.9% respectively, though only the latter two were significantly higher than the tEGFR.ΔICD control (-7.0%) at 20:1 E:T ratio. No significant lysis against CD123^{neg} cell lines (HEK293 and K562) were observed at 20:1 E:T ratios (mean ranges: HEK293: -10.9 to 2.9%, K562: -8.5 to 4.2%) (Figure 3.14e).

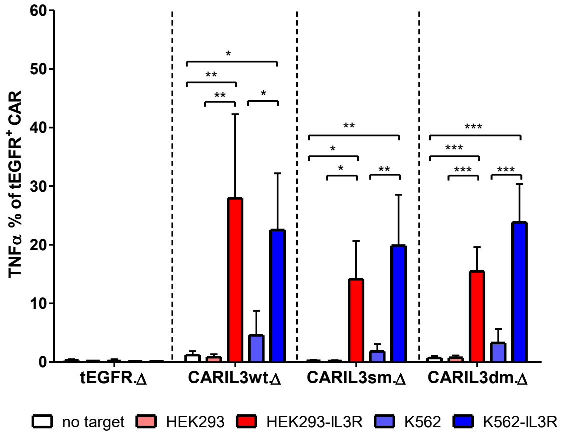
(a) HEK293 and HEK293-IL3R



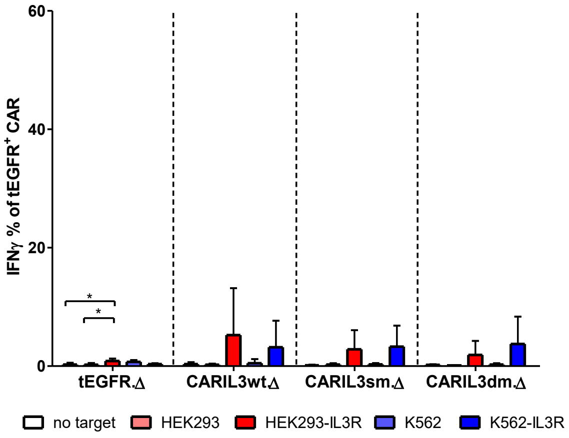
(b) K562 and K562-IL3R



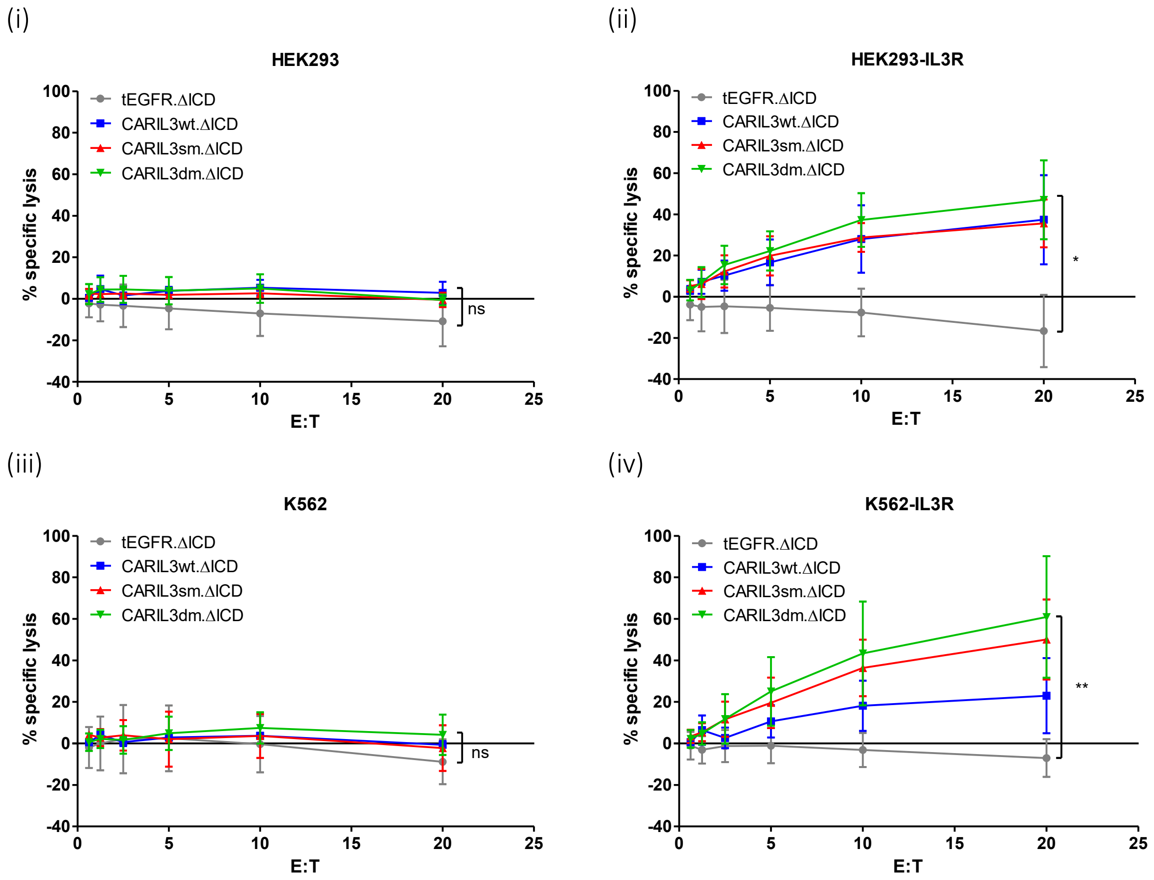
(c)



(d)



(e)



(e)
(v)

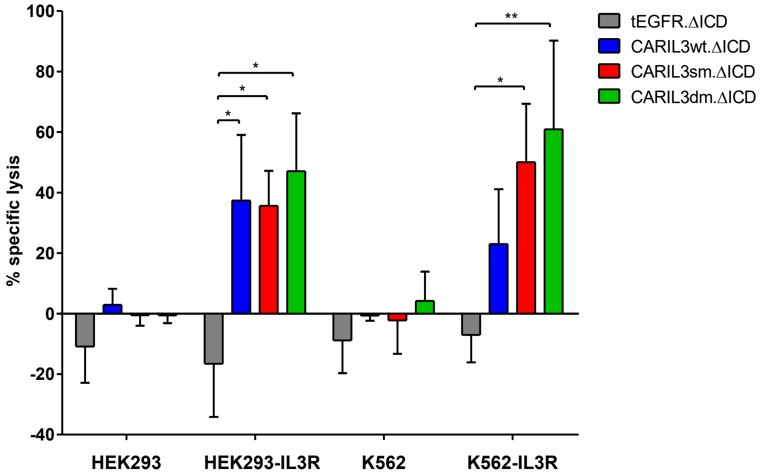


Figure 3.14 Specific activities of lentiviral CARIL3 T cells against CD123 overexpressing cell lines. (a-b) CD123 was overexpressed in CD123^{neg} cell lines (a) HEK293 and (b) K562 according to section 3.2.1. Flow cytometry confirmed the overexpression of (i) co-expressed marker mCherry and (ii) CD123. Overexpressing cell lines (in red) are overlayed with parental cell lines (in cyan). At the end of 12-day culture, CARIL3 CAR T cells were co-incubated with target cell lines HEK293 and K562 with or without CD123 overexpression. The proportions of (c) TNFα- and (d) IFNγ-producing CAR T cells were assessed using intracellular flow cytometry following co-incubation with target cells. CAR expression was measured via tEGFR surrogate tag (n = 3 donors; data represented as mean ± SD; Repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test performed with data, *: $p < 0.05$, **: $p < 0.01$, **: $p < 0.001$). (e) (i-iv) Dose-dependent specific cytotoxicity of lentiviral CARIL3.ΔICD T cells against CD123 overexpressing cell lines were assessed by calcein AM assay. Data at 20:1 E:T ratio was summarised in (e) (v) (n = 3 donors; data represented as mean ± SD; and P-values calculated by repeated measures ANOVA with post-hoc Tukey's multiple comparison test, ns: non-significant, *: $p < 0.05$, **: $p < 0.01$).

3.3.7 Lentiviral and *PiggyBat* gene delivery had similar integration patterns but different copy numbers and CAR expression levels

Transgene copy number analysis was performed using droplet digital PCR on genomic DNA (gDNA) extracted from CAR$^+$ sorted cells at end of CAR T cell cultures. Lentiviral CARIL3.ΔICD T cells consistently had lower CAR copy numbers per cell compared to *PiggyBat* CARIL3 T cells for all three constructs (mean ranges: 1.2 – 1.4 vs 2.8 – 3.0) (Figure 3.15a).

Integration site analysis via MuA integration site recovery was conducted by targeted next generation sequencing of amplicons spanning the junction of lentiviral terminal repeats (LTRs) or *PiggyBat* terminal inverted repeats (TIRs) and adjacent genomic DNA sequences. The total number of recovered unique integration sites were not significantly different between LV and *PiggyBat* CARIL3dm T cells (mean ± standard deviation: LV vs *PiggyBat* 1316 ± 615 vs 26695 ± 18862, $p = 0.1449$). The two different gene delivery systems also demonstrated similar integration patterns, mainly within intron and intergenic loci (Figure 3.15b). Relative to random integration patterns, *PiggyBat* have a large enrichment within rRNA and a slight enrichment in promoter regions relative to LV, while LV was not enriched in any regions compared to *PiggyBat* (Figure 3.15c).

CAR expression levels were calculated using staining index of tEGFR$^+$ population relative to no primary (no cetuximab) stained population. Lentiviral CAR T cells had higher expression levels than *PiggyBat* CAR T cells (mean staining index range: LV: 77.3 – 135.7; *PiggyBat*: 8.2 – 43.2), though due to substantial difference in culturing conditions and flow cytometer settings, they were not statistically compared. (Figure 3.15d)

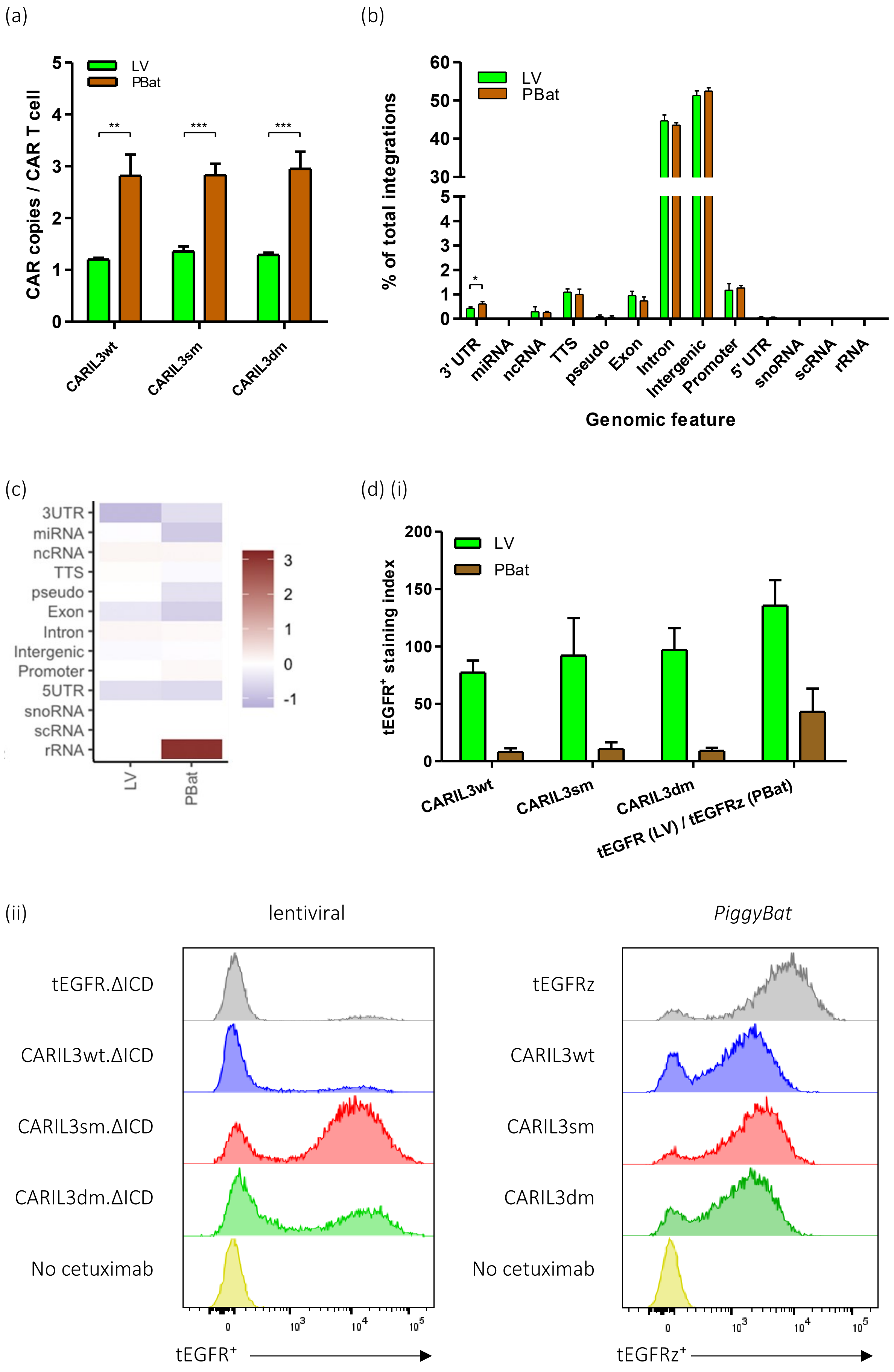

(a)
CAR copies / CAR T cell
0
1
2
3
4
5
LV
PBat
**
***
***
CARIL3wt
CARIL3sm
CARIL3dm
(b)
% of total integrations
0
1
2
3
4
5
30
40
50
60
LV
PBat
*
3' UTR
miRNA
ncRNA
TTS
pseudo
Exon
Intron
Intergenic
Promoter
5' UTR
snoRNA
scRNA
rRNA
Genomic feature
(c)
3UTR
miRNA
ncRNA
TTS
pseudo
Exon
Intron
Intergenic
Promoter
5UTR
snoRNA
scRNA
rRNA
LV
PBat
3
2
1
0
-1
(d) (i)
tEGFR$^+$ staining index
0
50
100
150
200
LV
PBat
CARIL3wt
CARIL3sm
CARIL3dm
tEGFR (LV) / tEGFRz (PBat)
(ii)
lentiviral
PiggyBat
tEGFR.ΔICD
CARIL3wt.ΔICD
CARIL3sm.ΔICD
CARIL3dm.ΔICD
No cetuximab
tEGFRz
CARIL3wt
CARIL3sm
CARIL3dm
No cetuximab
0
10^3
10^4
10^5
tEGFR$^+$
tEGFRz$^+$

Figure 3.15 *PiggyBat* and lentiviral CAR T cells' transgene integration copy numbers, integration patterns and expression levels. (a) CAR transgene copy-numbers of sorted CAR$^+$ T cells were assayed by droplet digital PCR at end of cultures. (b) Distribution of unique integration sites of CARIL3dm T cells were assayed by MuA integration recovery and amplicon library was sequenced by Illumina MiSeq kit v2 (n = 3 donors, data represented as mean ± SD). (c) Heat map of mean $\log_2$ ratio of observed / expected random integrations into different genomic features. 3'UTR, three prime untranslated region; 5'UTR, five prime untranslated region; miRNA, micro RNA; ncRNA, non-coding RNA; pseudo, pseudogene; rRNA, ribosomal RNA; scRNA, small conditional RNA; snoRNA, small nucleolar RNA; TTS, transcription termination site. (d) CAR expression levels as measured by tEGFR$^+$ expression using flow cytometry at end of cultures (n = 3 for day 12 lentiviral CAR, n = 4 for day 16 *PiggyBat*). Staining index of CAR population = (tEGFR$^+$ MFI – no cetuximab MFI) / (2 * robust SD of no cetuximab population). Figure (i) is summary of representative data in (ii).

3.4 Discussion

In this Chapter, second generation 4-1BB CARs using wildtype human IL-3 and 2 mutant forms as the extracellular recognition domain demonstrated *in vitro* activities against CD123-low cell lines, regardless of *PiggyBat* or lentiviral manufacturing methods, however there is significant room for optimisation, particularly with respect to the lentiviral vector mediated CAR T cell production.

In the *PiggyBat* system, all three CARIL3 T cells specifically produced TNFα and IFNγ against CD123-low TF-1 and KG-1 cell lines, though the degree of cytokine release were only marginally above baseline for some CARIL3 vs CD123$^+$ target combinations. Dose-dependent cytotoxicity when co-cultured with KG-1 was greatest with single (K116W) and double (K116W/E22R) CARIL3 constructs. For the lentiviral CARIL3 T cells, only the single mutant construct elicited a significant TNFα response to KG-1, while specific cytotoxicity was observed in the wild type and double mutant constructs. The interpretation is likely confounded by low density of CD123 target antigens on KG-1 cells, with low signal to noise ratio. This is addressed in the subsequent section with artificial CD123-antigen expressing cell lines producing much more consistent results.

Direct comparison between lentiviral and *PiggyBat* constructs are not feasible due to a few reasons including: 1) The lower CAR$^+$ proportions in lentiviral CAR T cultures; 2) Differing levels of co-inhibitory checkpoint molecules in the lentiviral CAR T cells; and 3) Greater proportion of CD4+ T cells in lentiviral CAR T cells. Moreover, due to the differing transgene and manufacturing differences, direct statistical comparison between *PiggyBat* and lentiviral CAR T cells is not attempted. Most transposon-based gene delivery systems utilise anti-CD3 or CD3/CD28 beads, so was not directly comparable to the *PiggyBat* system reported here (Lin *et al.*, 2022, Magnani *et al.*, 2016). Furthermore, the mode of anti-CD3/CD28 activator delivery contributes to differences in CAR T cell phenotype and function (Underwood *et al.*, 2024).

Different gene delivery systems can result in different memory phenotypes even under the same culture conditions, such as higher central memory proportion in *PiggyBac* compared to lentiviral CAR T cells (Lin *et al.*, 2022). The co-stimulatory domain also has significant effects on immunophenotype of CAR T cells. Direct comparison of CD28 and 4-1BB co-stimulatory domains have shown that 4-1BB promotes memory formation and better persistence (Kawalekar *et al.*, 2016), while CD28 CAR T cells had antigen-independent CD3ζ phosphorylation and increased antigen-dependent T cell activation at low antigen levels, leading to exhaustion (Sun *et al.*, 2020), which may have contributed to the lack of significant IFNγ release against $CD123^+$ cell lines. The co-stimulatory domain has major impact on the efficacy, safety and persistence of CAR T cells in the clinic (Zhao *et al.*, 2020). Therefore, the optimal CAR stimulation and expansion protocols must be empirically determined to achieve the desired phenotype.

The co-expressed tEGFRz CAR was convenient for expanding the CAR cultures and as a surrogate for CAR expression. Since *PiggyBat* and LV CARIL3 may be expanded with irradiated PBMC and TransAct respectively and that CARIL3 may be directly detected using a recombinant anti-IL3 antibody (Figure 3.12), it would be possible to delete the tEGFR construct altogether in future CARIL3 T cell cultures. However, tEGFR remains an important tool for the elimination of engineered T cells via ADCC and CDC in the event of adverse reactions *in vivo* (Wang *et al.*, 2011).

Despite the varied responses to CD123-low KG-1, by overexpressing CD123 in $CD123^{neg}$ cell lines, significant TNFα release was observed in all three lentiviral CARIL3 constructs, as well as significant specific lysis by the single and double mutant CARIL3.ΔICD T cells. The higher activity of the single and double mutants could be due to the K116W mutation introduced to increase the affinity of the CAR for CD123. In the *PiggyBat* format, CARIL3sm and CARIL3dm T cells also displayed the higher expansion, enrichment, specific cytokine production and cytotoxicity than wildtype CARIL3. Due to time and resource constraints, only CARIL3dm was chosen for subsequent studies in Chapter 5. The choice was based on the high functional activity and added benefit of disrupting IL-3 signalling. Functional assays were not repeated for *PiggyBat* CARIL3wt and CARIL3sm against CD123 overexpressing cell lines, but they may be conducted in future confirmation experiments.

Ligand-based CARs have several advantages over scFv based CAR designs. One limitation of using scFv as the extracellular antigen binding domain is receptor clustering between scFv of neighbouring CARs, which could lead to tonic signalling, exhaustion and poor persistence (Branella and Spencer, 2021, Long *et al.*, 2015). Moreover, scFv sequences are usually from non-human origin, such as murine antibodies and could trigger adverse immune reaction (Maus *et al.*, 2013) and poor persistence (Turtle *et al.*, 2016a). CARs based on human ligands such as IL-3 may be able to circumvent these issues. A shortcoming of this study is the lack of side-by-side comparison to one of the scFv-based anti-CD123 CARs that have been reported (Arcangeli *et al.*, 2017, Baroni *et al.*, 2020, Cummins *et al.*, 2017, Stevens *et al.*, 2019, Thokala *et al.*, 2016). Although not the main aim of this thesis, this comparison may be made in the future for the assessment of activity and safety of the IL-3 based CARs relative to scFv anti-CD123 CARs.

In vivo animal model studies are needed to establish the safety of IL-3 based CAR prior to clinical translations due to its unknown clinical characteristics. However, the FDA's approval of the diphtheria toxin-IL3 conjugate protein provides some support that IL-3 based therapies may be feasible as it did not cause serious side effects in AML patients at dose levels which induced clinical responses (Frankel *et al.*, 2007). Furthermore, IL-3 with K116W mutation has been used in diphtheria toxin-IL3 fusion protein with proven i*n vitro* and *in vivo* efficacy and tolerability against primary CML stem cells (Frolova *et al.*, 2014).

Besides the safety of the novel IL-3 based CAR construct, the safety of genetic engineering technique is also paramount. Here, I explored two alternative methods to the *PiggyBac* transposase/transposon system which has been associated with cases of CAR T derived lymphoma raising safety concerns (Bishop *et al.*, 2021). The *PiggyBat* transposon/transposase system developed by others in the laboratory is a straight-forward technique which enables rapid generation and screening of potential CAR candidates. The lentiviral system was chosen for its long history in the gene and cell therapy sphere, with its excellent safety track record. Both *PiggyBat* and lentiviral CAR T cells achieved transgene copy numbers of less than 5, which is the regulatory threshold set by the FDA (Chen *et al.*, 2020). The lentiviral transduced CARs have significantly lower copy numbers while still exhibiting *in vitro* specific activity. This may potentially reduce the risk of insertional mutagenesis. Furthermore, both gene delivery techniques resulted in integrations predominantly within intron and intergenic regions, with total of ~2% within transcription start sites and promoters, suggesting that both have low genotoxic potentials. However, the integration site analysis employed in this thesis sequences local genomic regions, and would not detect gross chromosomal changes, which would require techniques such as karyotyping.

Clinical scale of manufacture is also feasible with both methods, with commercial CAR T cell doses easily achievable from a typical buffy coat starting product (approximately 10^9 PBMCs). Out of the three TGA approved CAR T cells (Kymriah, Yescarta and Tecartus), the maximum cell dose is 6 x 10^8 CAR$^+$ T cells per infusion. As an example, *PiggyBat* CARIL3dm, with an average 16-fold expansion and 73.9% CAR positivity after a 15-day culture, and assuming about 50% T cells within a buffy coat PBMC, would require about 10^8 PBMCs, which is well within a typical buffy coat PBMC. The lentiviral CARIL3dm.ΔICD, with a considerable 365.4-fold expansion after a 12-day culture and final CAR proportion of 61%, would only need 5.4 x 10^6 starting PBMCs.

Both *PiggyBat* and lentiviral vectors have their pros and cons. The *PiggyBat* system is straightforward, inexpensive and has a large cargo load capacity. However, so far it has only been used in pre-clinical research settings. If it is to be used in a clinical setting, it would require a much more extensive, safety and efficacy study in small animal models, which would hinder the timely translation of the IL-3 based CAR construct. Lentiviral vectors by nature have a limited transgene size capacity, which was overcome in this thesis by deletion of the intracellular domains of the tEGFR CAR, converting this to a tEGFR-only tag. However, larger cargo capacities are essential for more complicated gene edits which are becoming increasingly prevalent in "armoured" CAR T cells to express selection markers, suicide genes, cytokines and/or additional receptors. Despite the limitations of the lentiviral system, it would likely be faster to translate the novel IL-3 based CAR using this well-characterised vector than using the relatively new *PiggyBat* system with its unknown safety profiles.

Before translation, some optimisations would be required for the lentiviral CARIL3 T cells.. Greater CAR$^+$ proportions could be generated with increasing MOIs or transduction enhancers (Lo Presti *et al.*, 2021, Rajabzadeh *et al.*, 2021). Strategies such as immune checkpoint inhibitors (e.g. CTLA-4, PD-1 and PD-L1 antibodies) and gene editing techniques may be utilised to minimise the suppression by co-inhibitory receptors (Hosseinkhani *et al.*, 2020, Wang *et al.*, 2019a), which would lead to better expansion, activity and persistence (Chong *et al.*, 2017, Li *et al.*, 2018a). CAR T products with defined subsets have been demonstrated to be superior and more consistent in potency compared to unselected CAR T cells (Sommermeyer *et al.*, 2016, Turtle *et al.*, 2016a, Turtle *et al.*, 2016b). Moreover, combining CD4 and CD8 CAR T cells at defined ratios addresses inter-donor variability, which would be valuable for standardising a cell therapy product.

In conclusion, the manufacture of CD123-specific CAR T cells using human IL-3 based antigen recognition domain is feasible using both *PiggyBat* transposon and lentiviral methods. The next steps are to test the most potent CARIL3dm construct in a murine model for safety and efficacy, however, the relatively low efficacy seen *in vitro*, particularly with lentiviral transduction, suggests that further refinement of the construct and manufacturing process will be necessary to achieve meaningful clinical outcomes.

Attractive engineering options include concurrent targeting of other antigens and elimination of co-inhibitory receptors. With these goals in mind, the development of a new CAR targeting TIM-3 and the use of CRISPR/Cas9 mediated knockout and transgene knock-in at the TIM-3 locus is explored in Chapters 4 and 5 of this thesis.

Chapter 4. Anti-TIM-3 CAR T cells

4.1 Introduction

TIM-3 is an overexpressed antigen in AML, whose presence on leukaemic stem cells (LSCs) makes it an attractive target for immunotherapy. The overexpression of TIM-3 on both AML bulk and LSCs is a relatively recent discovery (Kikushige *et al.*, 2010). The self-renewing autocrine loop function of TIM-3/galectin-9 (Kikushige *et al.*, 2015) and the ability of TIM-3^{+} but not TIM-3^{neg} cells to initiate relapse (Jan *et al.*, 2011) suggest the importance of targeting this population of AML cells to combat relapse. Furthermore, TIM-3 is not found on normal CD34^{+} stem cells (Haubner *et al.*, 2019, Jan *et al.*, 2011, Kikushige and Miyamoto, 2013) and TIM-3 antibodies have displayed AML specific activity without affecting CD34^{+} cell engraftment (Kikushige *et al.*, 2010), bolstering its efficacy and safety profile as a target. Moreover, concurrent targeting of TIM-3 along with multiple other AML specific antigens has the potential to reduce the risk of immune escape and treatment failure.

Nevertheless, targeting TIM-3 presents a specific challenge as it is also expressed on activated T cells, and TIM-3 specific CAR T cells are likely to cause fratricide of activated T cells compromising the activity of the product. I aim to address this with CRISPR/Cas9 mediated knockout (KO) of endogenous TIM-3 on the generated anti-TIM-3 CAR T cells akin to CD7 and CD45 KO CAR T cells targeting antigens also present on T cells (Gomes-Silva *et al.*, 2017, Wellhausen *et al.*, 2023). This Chapter demonstrates the utility of CRISPR/Cas9 TIM-3 KO and assesses the antigen-specific activity of two prototype TIM-3 directed CARs. The CRISPR/Cas9 technology also provided an opportunity to test knock-in of the active CAR into the TIM-3 locus for directed insertion to reduce the risk of genetic toxicity. Although the transfection was less efficient, the CRISPR KI method illustrated the feasibility of this approach.

4.2 Materials and methods

4.2.1 Generation of TIM-3 overexpressing cell lines

TIM-3 ectodomain (UniProt Q8TDQ0 amino acids 1 – 202) was overexpressed in HEK293 and K562 using the *PiggyBac* system as described in Chapter 2 with the constructs: pVAX1.PB.Tim3CD8t (Genscript, Figure 4.1) and previously constructed pVAX1.SPBase (GenScript). Transfected cells were stained with anti-TIM-3 APC-Cy7 (BD) for single cell sorting and screening of high-expressing clones.

Figure 4.1 Schematic of gene construct of TIM-3 ectodomain within Super *PiggyBac* transposon vector. TIM-3 leader peptide (UniProt Q8TDQ0 amino acids 1 – 21) and TIM-3 ectodomain (Exons 2-5, UniProt Q8TDQ0 amino acids 22 - 202) were linked with CD8 transmembrane (UniProt P01732 amino acids 183 – 203) and truncated CD8 endodomain (UniProt P01732 amino acids 204 – 222) and synthetised and cloned into pVAX1.PB vector by GenScript.

4.2.2 TIM-3 specific CAR constructs

Two TIM-3 specific CAR constructs were created. The first construct (CARTim3a) is a second-generation 4-1BB CAR identical to the published sequence by Lee *et al* (Lee *et al.*, 2021). The extracellular binding domain scFv is derived from their in-house produced antibody, whose heavy and light chains were joined by a $(G_4S)_3$ linker (composed of 4 glycine residues followed by a serine residue, repeated 3 times). The binding domain is connected to a CD28 transmembrane domain via an IgG1 hinge, followed by 4-1BB intracellular and CD3ζ signalling domains. The final CARTim3a construct also included multi-cistronic expression of truncated EGFR CAR (tEGFRz) and reporter eGFP, separated by P2A and T2A self-cleaving peptides respectively (Figure 4.2a).

The second CAR construct (CARTim3b) was a second-generation CAR with an extracellular scFv sequence derived from an anti-TIM-3 monoclonal antibody (US patent ID 2012/0189617 A1) of which the light and heavy variable chains, LV0 and HV0 were linked by a $(G_4S)_3$ motif. The scFv was connected to a CD28 transmembrane and intracellular domain via another $(G_4S)_3$ linker preceding an IgG1 hinge and followed by a CD3ζ activation domain. The CARTim3b construct was also incorporated into a multi-cistronic plasmid, with tEGFRz CAR and eGFP reporter, separated by P2A and T2A self-cleaving peptides respectively (Figure 4.2b).

Control CAR consisted of tEGFRz and eGFP separated by T2A as previously used in Chapter 3.

(a) CARTim3a

(b) CARTim3b

Figure 4.2 Schematic of transgene constructs for anti-TIM-3 CARs (a) CARTim3a based on a published sequence (Lee *et al.*, 2021), and (b) CARTim3b based on an antibody patent US 2012/0189617 A1, were both incorporated in cis with tEGFRz CAR and eGFP reporter genes in the *PiggyBat* transposon vector. L, light chain; H, heavy chain; TM, transmembrane; ICD, intracellular domain; P2A, T2A, self-cleaving peptides; eGFP, enhanced GFP.

4.2.3 Generation of CARTim3 plasmids

Both CARTim3 constructs were codon optimised, synthesised and cloned into the pVAX1.PBat backbone using BstBI and BspEI restriction sites, replacing the CARIL3wt portion of the pVAX1 expression vector (GenScript) (Figure 4.3).

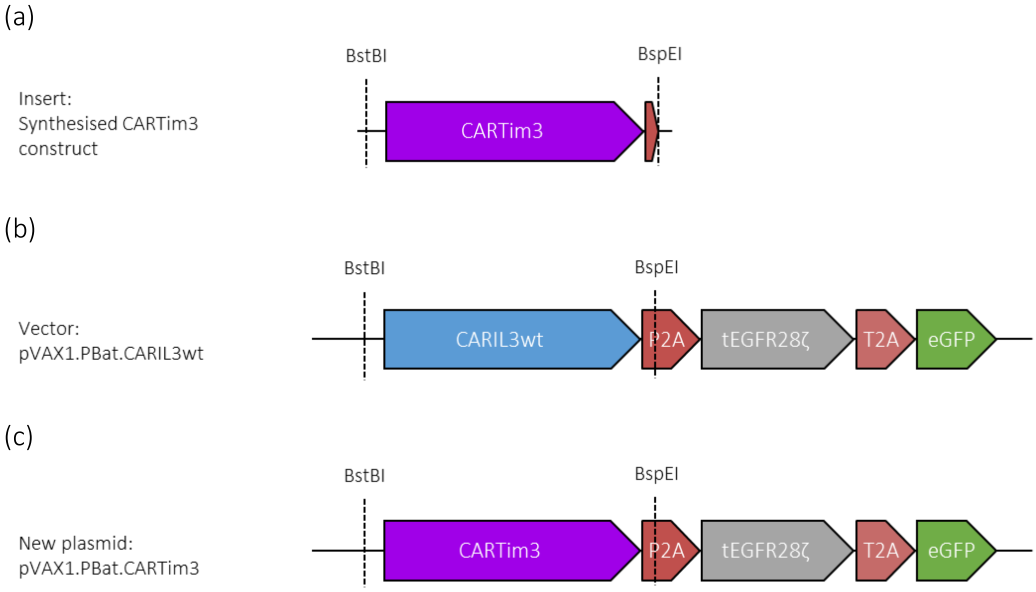


Figure 4.3 Schematic of cloning pVAX1.PBat.CARTim3 plasmids. (a) Inserts were derived from synthesised CARTim3 constructs using BstBI/BspEI digests. (b) *PiggyBat* vector backbone was derived by BstBI/BspEI digest of pVAX1.PBat.CARIL3wt from Chapter 3. (c) Final pVAX1.PBat CARTim3 plasmids were created by ligating (a) and (b).

4.2.4 Donor derived PBMCs

PBMCs were extracted from whole blood of healthy donors by Ficoll density separation and cryopreserved as described in Chapter 2.

4.2.5 Generation and expansion of TIM-3 knockout CAR T cells

TIM-3 knockout (TIM-3^{KO}) CAR T cells were produced from isolated T cells as described in Chapter 2. Briefly, TIM-3 KO was performed using CRISPR/Cas9 with either one synthetic guide RNA (sgRNA) (Assay ID: CRISPR699737_SGM (sg37) or CRISPR699749_SGM (sg49)) or both sgRNAs (ThermoFisher). Cas9 and sgRNA(s) were incubated at room temperature for about 15 minutes before co-electroporation with *PiggyBat* transposase and anti-TIM-3 CAR transposon plasmids into T cells.

All cultures were expanded by weekly exposure to irradiated K562-cetuximab artificial antigen-presenting cells at 0.2x T cell numbers in IL-15 supplemented complete AIM-V. Cultured cells were harvested on day 15 for phenotyping and functional assays (Figure 4.4a).

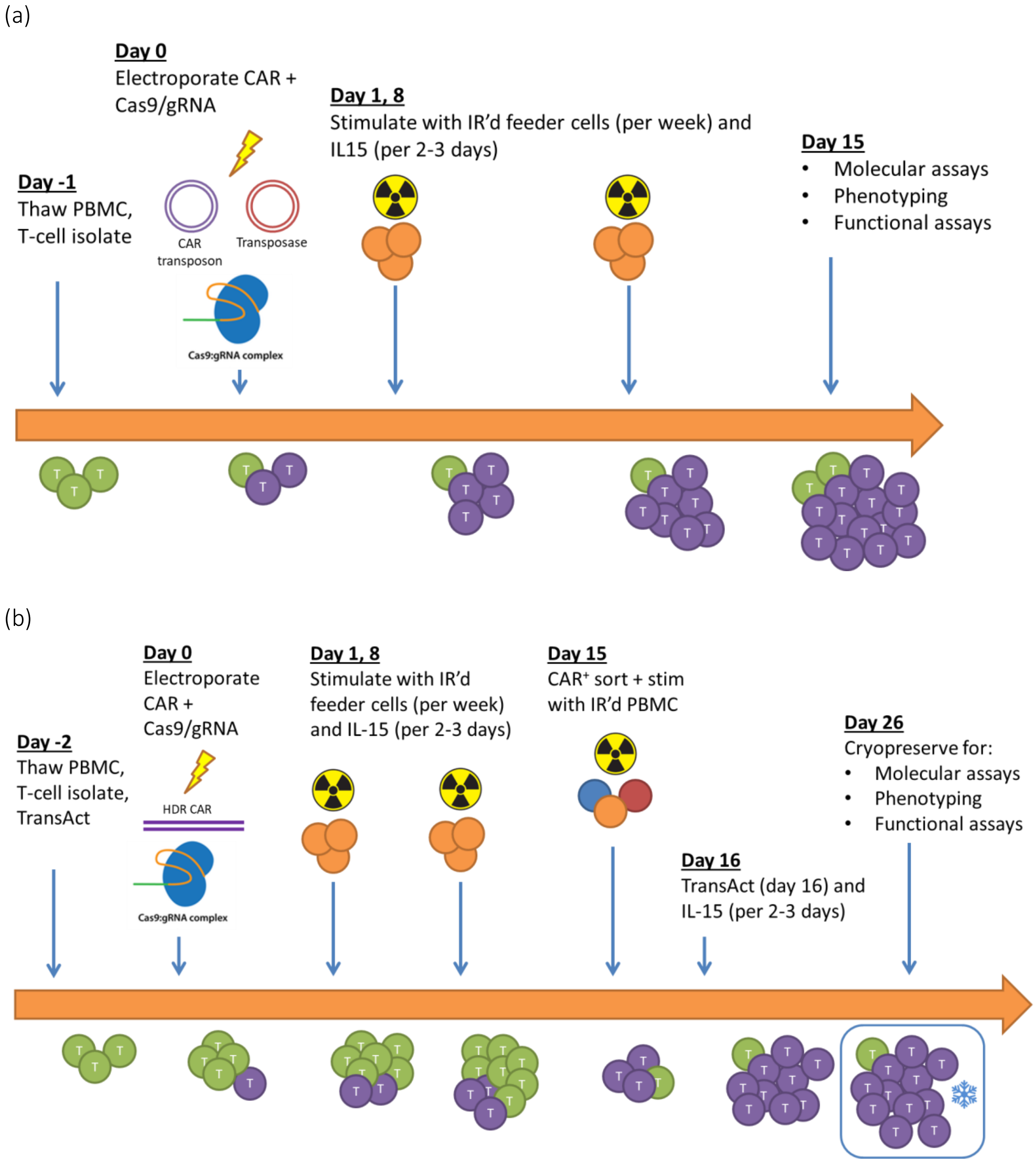
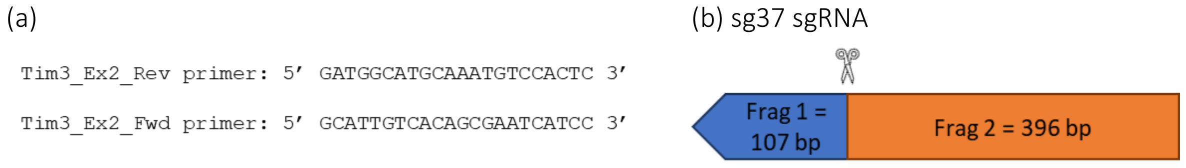


Figure 4.4 **Schematic of TIM-3 KO and KI CAR T cell cultures.** (a) KO CAR T cell culture. (b) KI CAR T cell culture. Untransfected T cells are represented by green circles, transfected CAR T cells as purple circles, other PBMC and feeder cells as other coloured circles; triangle represents antigen of interest. gRNA, guide RNA; HDR, homology directed repair; IL-15, interleukin 15; IR, irradiated; PBMC, peripheral blood mononuclear cells.

4.2.6 Molecular measurement of TIM-3 KO efficiency

TIM-3 KO efficiency was measured semi-quantitatively using the GeneArt Cleavage Efficiency Detection kit (A24372, ThermoFisher) according to manufacturer’s instructions. PCR primers were designed against Exon 2 of TIM-3 which produces PCR products of length approximately 503 bp and 274 bp when one or two CRISPR cuts occurs, respectively (Figure 4.5).

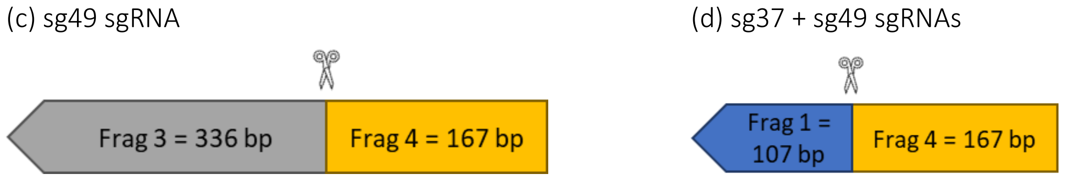
Following PCR, GeneArt enzyme digestion was performed according to manufacturer’s instructions, and cut and uncut PCR products and daughter fragments were visualised on a 2% agarose TBE gel. Relative pixel density of the bands was then used to calculate the proportion of TIM-3 KO in the cultured T cells according to manufacturer’s instructions. Expected band sizes are summarised in the schematic and table below (Figure 4.5 and Table 4.1).



(a)

```
Tim3_Ex2_Rev primer: 5’ GATGGCATGCAAATGTCCACTC 3’
Tim3_Ex2_Fwd primer: 5’ GCATTGTCACAGCGAATCATCC 3’
```

(b) sg37 sgRNA



(c) sg49 sgRNA

(d) sg37 + sg49 sgRNAs

Figure 4.5 Schematic of PCR products and daughter fragments from GeneArt cleavage efficiency detection assay. (a) Primers specific for cleavage sites of TIM-3 exon 2. Schematics of PCR products from CRISPR cuts by sg37 (b), sg49 (c) or both sgRNAs (d). Differently colour fragments represent cleaved DNA fragments after GeneArt enzyme digestion. sgRNA: synthetic guide RNA, ✂ indicates GeneArt enzyme cut sites.

Table 4.1 PCR product sizes and daughter fragment sizes of GeneArt cleavage efficiency assay

sgRNA	Parent PCR product approximate size(s) (bp)	Cleaved daughter fragments' approximate size(s) (bp)
sg37	503	107 396
sg49	503	336 167
sg37 and sg49	503 274	107 396 336 167

4.2.7 Generation and expansion of TIM-3 knock-in CAR T cells

TIM-3 knock-in (TIM-3^{KI}) CAR T cells were produced from isolated T cells as described in Chapter 2. Briefly, activated T cells were electroporated with Cas9, both TIM-3 sgRNAs and the CARTim3b homology directed repair (HDR) template. After 15 days of culture, CAR^+ cells were FACS sorted, cultured further and cryopreserved on day 22 for subsequent phenotyping and functional assays (Figure 4.4b).

4.2.8 Generation of KI HDR templates

CARTim3b HDR templates were PCR products made by amplifying the CAR from *PiggyBat* transposon plasmids using primers with 35 bp overhangs (Figure 4.6a, b, c, d) that were homologous to the TIM-3 gene on both sides of the CRISPR/Cas9 cut sites by CRISPR699737_SGM and CRISPR699749_SGM, following the PCR conditions below (Table 4.2 and Table 4.3). The CAR construct was inserted in anti-sense direction relative to the TIM-3 locus (Figure 4.6 e).

Table 4.2 PCR reaction mixture for generating TIM-3 targeting HDR templates. HA, homology arm.

Reagent	Volume (µL)
Q5 High-Fidelity 2X Master Mix	50
Transposon plasmid (0.25 ng/µL)	4µL (100 ng)
Forward primer, 10 µM (sg37 HA primer)	5
Reverse primer, 10 µM (sg49 HA primer)	5
Nuclease free water	36 (To 50 µL)

Table 4.3 PCR conditions for generating HDR templates

Step	Temperature	Time
Initial denaturation	98℃	30 sec
30 Cycles	98℃	5 sec
	70℃ (by NEB Tm calculator v1.16.5)	10 sec
	72℃	1 min 45 sec (chosen based on longest PCR product, 20 sec/kb)
Final extension	72℃	2 min
Hold	4℃	∞

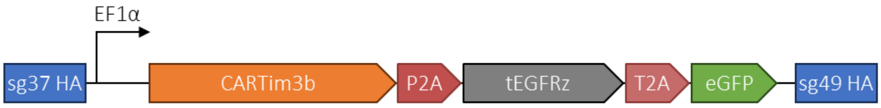
(a) Schematic of overhang PCR

(b) Primers for HDR template PCR (TIM-3 homology arms in cyan)

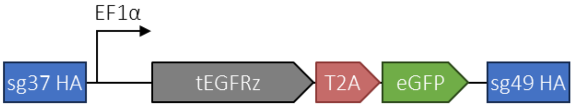
```
sg37 HA primer (5' → 3'):
CTGGGATTTGGATCCGGCAGCAGTAGATCCCACTGACGGGGAAAAGGCCTCCAC

sg49 HA primer (5' → 3'):
TCTCTATGCAGGGTCCTCAGAAGTGGAATACAGAGTCTGAGCACGGTTCCAAGATCG
```

(c) CARTim3b KI template (4687 bp)



(d) tEGFRz KI template (3247 bp)



(e) Schematic of CAR KI by HDR

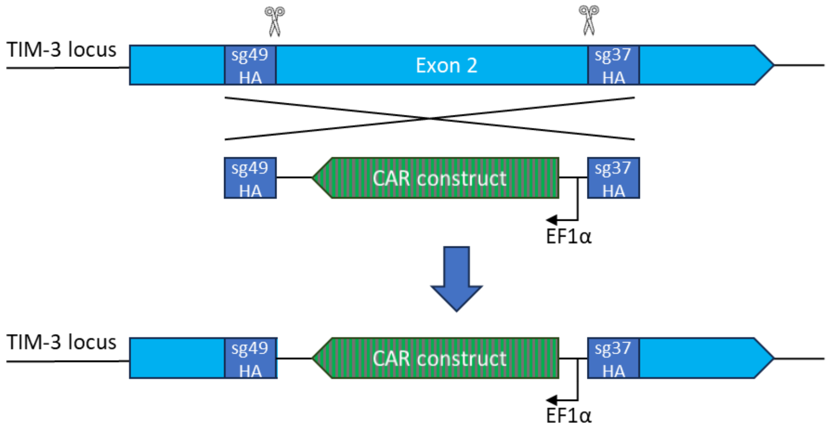


Figure 4.6 Schematic of CAR KI. (a) Schematic of PCR using primers with 35bp 5’ overhangs to generate HDR KI templates from CAR transposons. (b) PCR primer sequences used. Schematic of final PCR products used as HDR KI templates for inserting CAR constructs: (c) CARTim3b and (d) tEGFRz. (e) Schematic of CAR KI by HDR. Cleavage by CRISPR/Cas9 is represented as ✂ scissors symbols, and HDR represented as a large “X”. EF1a, elongation factor 1α promoter; sg37, CRISPR699737_SGM; sg49, CRISPR699749_SGM; HA, homology arm; HDR, homology directed repair.

4.2.9 TIM-3 knock-in confirmation PCR

PCR was performed to confirm the knock-in of TIM-3 directed CAR and control tEGFRz CAR into the TIM-3 locus using primers targeting the sg37 and sg49 HDR junctions according to the protocol in Chapter 2. Following agarose gel electrophoresis of the PCR products, the bands were excised, and the DNA extracted for Sanger sequencing.

4.2.10 Phenotyping flow cytometry

T cell memory phenotyping and exhaustion marker flow cytometry was performed at end of culture following phenotyping protocols in Chapter 2.

4.2.11 Intracellular cytokine flow cytometry

For 5-hour co-incubations of CAR T cells with targets, intracellular cytokine flow cytometry of TNFα and IFNγ release was performed as described in Chapter 2 of the thesis. For overnight/29 hour co-incubation experiments, only GolgiPlug was used for intracellular blockade.

4.2.12 CD107a/b degranulation assay

CD107a/b degranulation assay was performed with CD107a BV786, CD107b BV786 and GolgiStop being added with anti-CD49d and anti-CD28 antibodies and incubated for 4-5 hours with target cells. Subsequently, cells were stained for viability (Zombie NIR), CD3, CD4 and CD8 and analysed by flow cytometry as described in Chapter 2. PMA (50 ng/mL) and ionomycin (1 μg/mL) was used as positive controls, and unstimulated cultured CAR T cells were used as negative controls.

4.2.13 Cytokine bead array assay

Cytokine bead array was performed with effector and target co-incubation as described for intracellular cytokine flow cytometry, but without GolgiPlus or GolgiStop. At the end of incubation, cells were pelleted at 300x *g* for 3 minutes and aliquots of supernatants stored at -30°C for subsequent use. Supernatants were thawed on ice and assessed for human cytokines using LEGENDplex™ Human CD8/NK Panel (13-plex) w/ FP V02 (741186, BioLegend) according to manufacturer's instructions.

4.2.14 Calcein AM release assay

CAR T cell cytotoxicity against TIM-3 overexpressing cell lines were performed according to standard calcein AM assay described in Chapter 2.

4.3 Results

4.3.1 Surface expression of TIM-3 may be disrupted by single or dual sgRNA(s)

Single or dual sgRNA(s) were used in CRISPR/Cas9 KO of TIM-3 along with co-electroporation of CARTim3 constructs in T cells. CAR T cells were then expanded and enriched with weekly exposure to irradiated K562-cetuximab as feeder cells via the co-expressed tEGFRz control CAR.

At day 15 post electroporation, proportions of CD3 cells expressing CAR and TIM-3 were determined by flow cytometry (Figure 4.7a). CAR percentage was measured as CD3 tEGFR$^+$ cells using cetuximab staining, which correlated with GFP expression (Figure 4.7b). All the cultures showed high CAR$^+$ expressions at end of culture (mean > 90% tEGFR$^+$) (Figure 4.7c). Significant TIM-3 KO was achieved with single sgRNA (sg37 or sg49) and dual sgRNA (Mean: sg37, 3.7%; sg49, 4.2%; sg37+sg49, 4.5%; no KO, 39.8%. sg37 vs no KO, $p < 0.01$; sg49 vs no KO, $p < 0.01$; sg37+sg49 vs no KO, $p < 0.01$) (Figure 4.7d). Between the three KO conditions, there was no significant difference in TIM-3 expressions ($p > 0.05$) (Figure 4.7d).

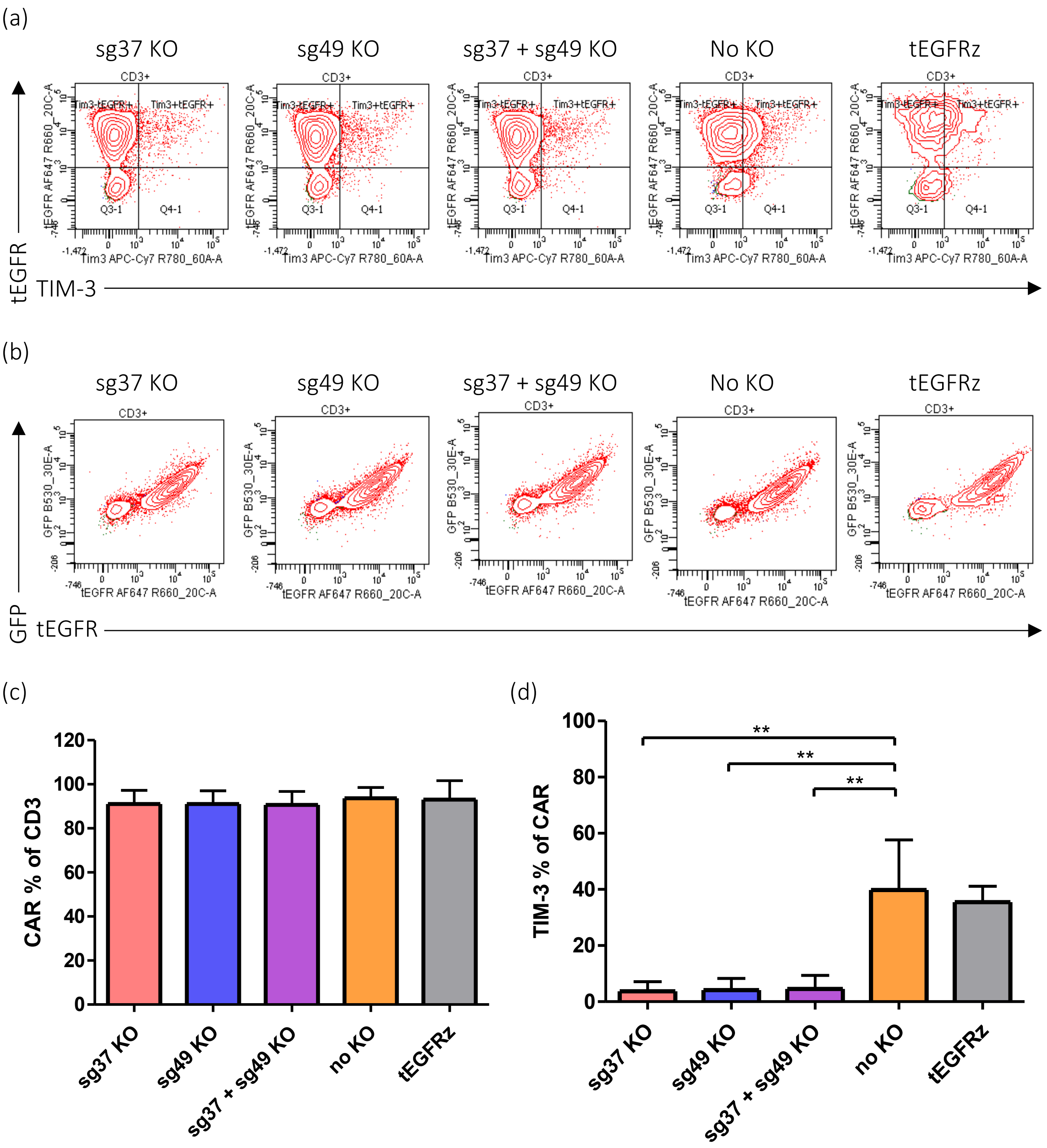


Figure 4.7 Surface expressions of CAR and TIM-3 in single and dual sgRNA TIM-3 KO CAR T cells at end of 15-day culture. Representative flow cytometry plots showing (a) tEGFR$^+$ CAR and TIM-3 expression and (b) dual tEGFR, GFP expressions after 15 days of culture using K562-cetuximab feeders. CAR and TIM-3 expressions are summarised in (c) and (d). CAR expression was determined by tEGFRz CAR surrogate (n = 3 donors, mean ± SD; repeated-measure ANOVA with Bonferroni multiple-comparison post-hoc test; **: $p < 0.01$).

4.3.2 Dual sgRNA was more efficient in TIM-3 knock-out genotypically

TIM-3 gene KO was confirmed using GeneArt Cleavage Efficiency Detection kit according to manufacturer's instructions and semi-quantified using agarose gel densitometry (Figure 4.8a). TIM-3 KO was achieved using both single and dual sgRNA, but the highest efficiency was observed in dual sgRNA KO (mean: no KO – 0%, sg37 – 27.2%, sg49 – 43.8%, sg37+sg49 – 65.2%, p = 0.0002) (Figure 1.7b). Thus, further TIM-3 KOs were all performed with dual sgRNAs in this chapter and Chapter 5.

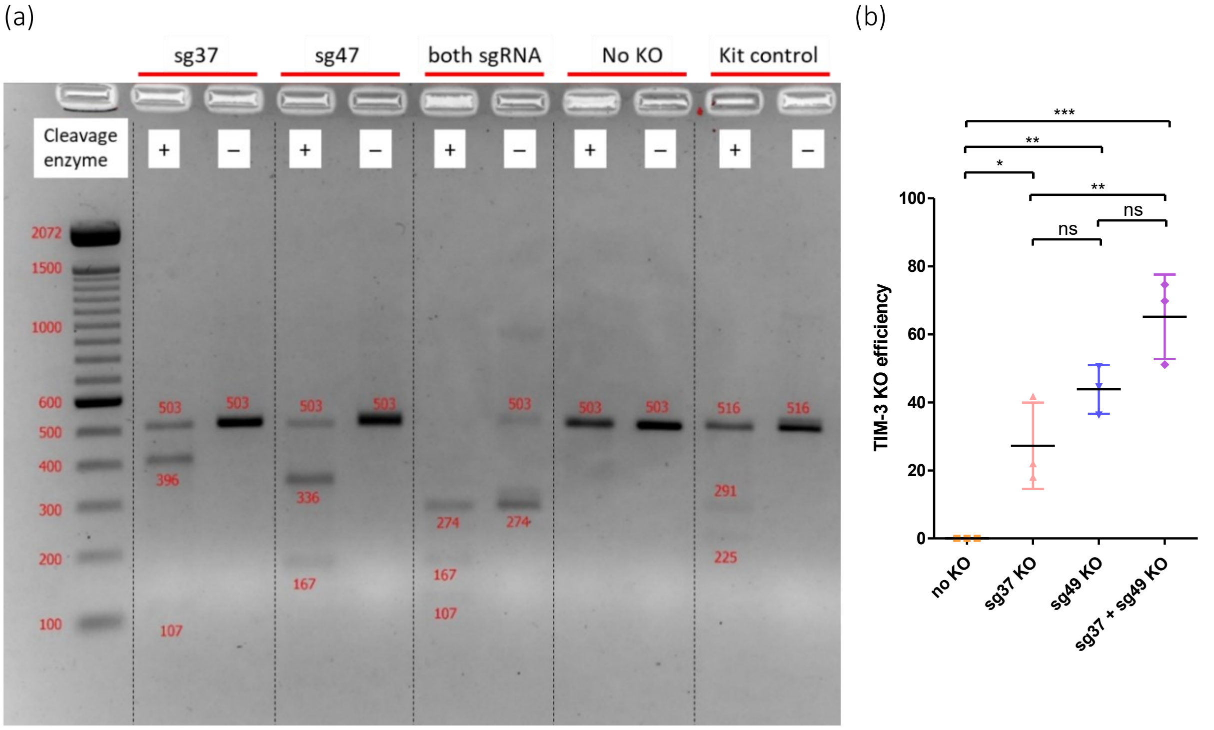


Figure 4.8 Genetic TIM-3 KO efficiencies using single or dual sgRNA after 15-days of culture. (a) Representative 2% agarose gel of cleaved and parental fragments following GeneArt enzyme digestion of PCR products amplified from gDNA extracted from TIM-3 KO CARTim3 T cells at the end of 15-day culture. Control template (kit control) was provided with the kit; +, with cleavage enzyme; -, no cleavage enzyme; sg37, sgRNA CRISPR699737_SGM; sg49, sgRNA CRISPR699749_SGM. Expected band sizes are labelled in red. TIM-3 KO efficiencies were calculated using densitometry of cleaved bands and summarised in (b) (n = 3 donors, repeated measures ANOVA with Bonferroni's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.3.3 TIM-3^{KO} CARTim3a can be expanded with irradiated K562-cetuximab feeders

TIM-3 has been described as a checkpoint receptor expressed on activated and exhausted T cells (Wolf *et al.*, 2020), with limited expression on resting T cells. This was confirmed empirically by flow cytometry, which showed about 10% expression on unstimulated PBMCs (Figure 4.9a). Therefore, CARTim3a T cells were expanded with irradiated K562-cetuximab aAPC via the co-expressed tEGFRz CAR as previously described in Chapter 2. Dual sgRNA TIM-3^{KO} CARTim3a T cells were able to be expanded by irradiated K562-cetuximab, though less than no KO tEGFRz control CAR (mean fold expansion: 6.1 vs 20.6). In the absence of TIM-3 KO, CARTim3a T cells still expanded in numbers following exposure to irradiated K562-cetuximab cells (mean fold expansion: no KO CARTim3a vs no KO tEGFRz, 4.9 vs 20.6, $p < 0.001$ by repeated measures ANOVA, Bonferroni's multiple comparison test) (Figure 4.9b).

A modest enrichment of the CAR expressing population was achieved at the end of 21-day culture for TIM-3^{KO} CARTim3a T cells, which was similar to no KO CARTim3a T cells, but significantly lower than tEGFRz control (mean: KO CARTim3a vs no KO CARTim3a vs no KO tEGFRz, 37.6% vs 45.8% vs 96.5%, $p = 0.0130$ by repeated measures ANOVA) (Figure 4.9c).

There were both CD4 and CD8 CAR T cells present at the end of the 22-day culture, where there was no difference between the three CAR T cultures (CD4 mean range: 28.3 – 41.3%, $p > 0.05$; CD8 mean range: 57.3 – 70.3%, $p > 0.05$) (Figure 4.9d).

There was no significant difference between the constructs in naïve/stem cell memory (mean range: 15.5 – 20.1%), effector memory (mean range: 20.3 – 34.3%) and effector memory RA (mean range: 10 – 26.1%) phenotypes. The three cultures were significantly different only in central memory proportions (mean: TIM-3^{KO} CARTim3a vs no KO CARTim3a vs no KO tEGFRz: 24.5% vs 26.3% vs 54.3%, $p = 0.0064$) (Figure 4.9e).

PD1 expression was not significantly different between the cultures (mean ranges: 31.2 – 60.1%, $p = 0.0799$). LAG3 positive proportions were not significantly different between TIM-3^{KO} CARTim3a and no KO CARTim3a CAR T cells (mean: TIM-3 KO vs no KO: 84.6% vs 77.5%, $p > 0.05$), but no KO CARTim3a was significantly higher than no KO tEGFRz control (mean: no KO CARTim3a vs tEGFRz, 77.5% vs 55.0%, $p < 0.05$). As expected, the TIM-3 expression was significantly lower in the TIM-3 KO cultures than the no KO CARTim3a (mean: KO vs no KO: 2.2% vs 70.2%, $p < 0.0001$), while no KO CARTim3a had more TIM-3 than no KO tEGFRz (mean no KO CARTIM3a vs tEGFRz: 70.2% vs 47.9%, $p < 0.05$) (Figure 4.9f).

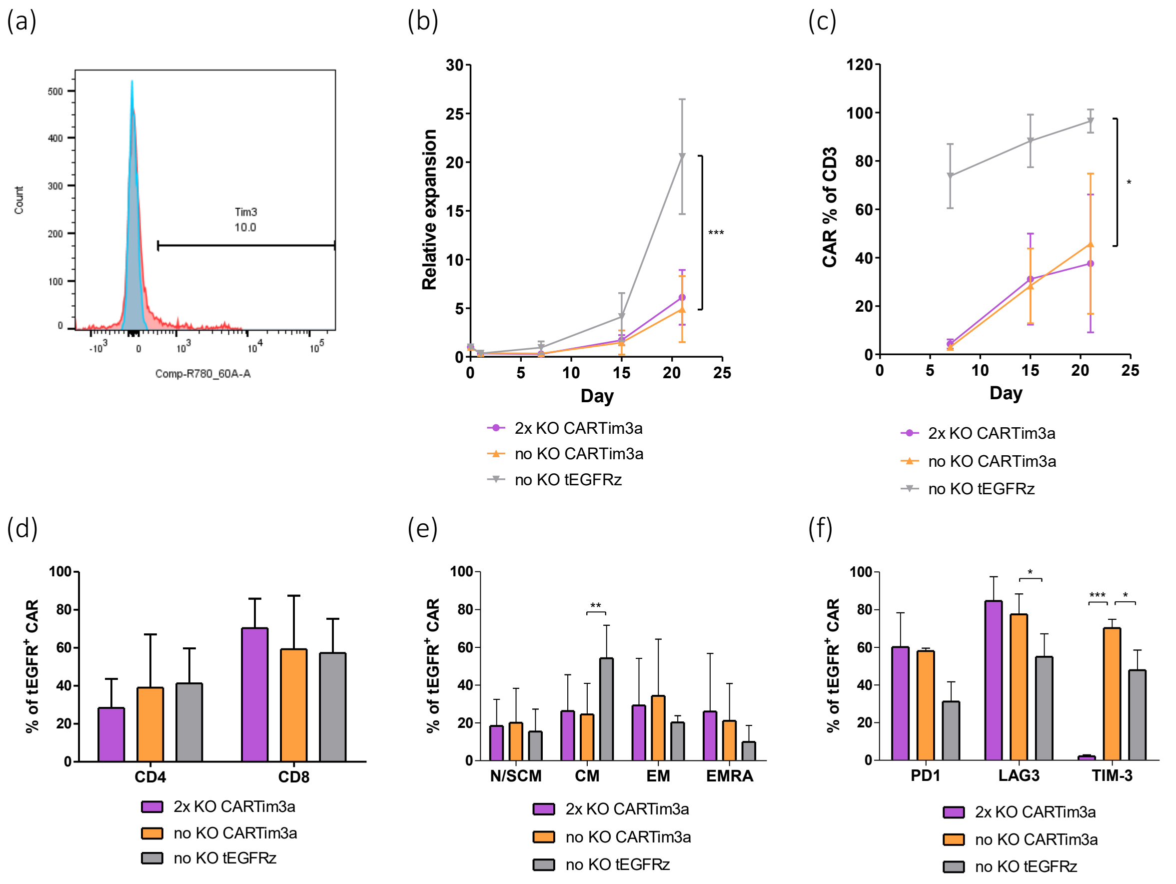


Figure 4.9 CARTim3a T cell fold expansion and phenotypes after 21-day cultures. (a) TIM-3 expression on PBMC as assessed by flow cytometry. Red histogram, stained cells; blue histogram, unstained cells. (b) Relative fold expansion of CARTim3a T cells with weekly exposure to irradiated K562-cetuximab. 2x KO: dual sgRNA TIM-3^{KO}; tEGFRz: truncated EGFR control CAR. (c) Time course of tEGFRz CAR expression as assessed by flow cytometry via surrogate tEGFRz CAR. (d) CD4/CD8 proportions, (e) memory immunophenotype and (f) inhibitory receptor expression of CAR$^+$ T cells were assayed at the end of culture. N/SCM: naïve/stem cell memory (CD62L$^+$CD45RA$^+$); CM: central memory (CD62L$^+$CD45RA^{neg}); EM: effector memory (CD62L^{neg}/CD45RA^{neg}); EMRA, effector memory RA (CD62L^{neg}CD45RA$^+$); PD1: Programmed cell death protein 1; LAG3: Lymphocyte-activation gene 3; Tim3: T cell immunoglobulin and mucin domain-containing protein 3. (n = 3 donors per construct, pooled data represented as mean ± SD. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$)

4.3.4 CARTim3a T cells did not show activity against TIM-3 overexpressing cell lines *in vitro*

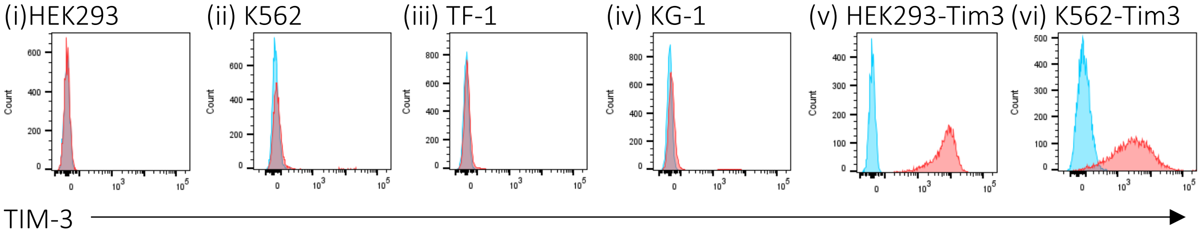
Significant TIM-3 expression on AML cell lines KG-1 and TF-1 were previously reported (Lee *et al.*, 2021). However, in my hands, the expression of TIM-3 by flow cytometry using Tim3-APC/Cy7 antibody (clone F38-2E2) showed only a very small proportion of cells in these cell lines displaying TIM-3 expression. To increase the sensitivity of the functional assays, TIM-3 was overexpressed in TIM-3 negative cell lines (HEK293 and K562) using the *PiggyBac* transposon system. High expressing clones of HEK293-Tim3 and K562-Tim3 were obtained from single cell sorting or limiting dilution (Figure 4.10a).

At the end of 21 days of culture, dual sgRNA TIM-3^{KO} CARTim3a T cells were assessed for TIM-3 specific TNFα and IFNγ production by 5-hour co-culture at a 1:1 effector to target ratio with TIM-3 overexpressing cell lines. No significant release was detected by intracellular cytokine flow cytometry compared to no-target controls (mean range: TNFα: 1.2 – 4.3%, IFNγ: 1.0 – 2.4%). tEGFRz control CAR showed lower range of TNFα and IFNγ releases (mean range: TNFα: 0.2 – 0.5%, IFNγ: 0.1 – 0.3%) (Figure 4.10b).

As maximal cytotoxic activity was demonstrated by Lee *et al.* around 30 hours (Lee *et al.*, 2021), the co-incubation was extended to 29 hours without monensin (to reduce toxicity to CAR T cells). However, there was again no significant increase in TNFα and IFNγ production following prolonged co-culture with TIM-3 overexpressing cell lines (Figure 4.10c).

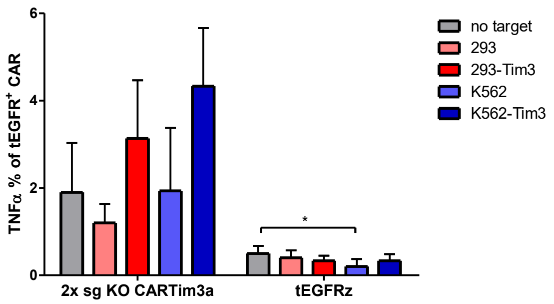
This lack of Tim-3 specific function was confirmed by calcein AM cytotoxicity assay at a range of effector to target (E:T) ratios between 0.625:1 to 20:1. No significant specific lysis of TIM-3 expressing targets was demonstrated at up to 20:1 E:T ratios by CARTim3a or tEGFRz (mean range: CARTim3a: -4.2 to 15.4%, tEGFRz: -17.2 to -1.1%)

(a)

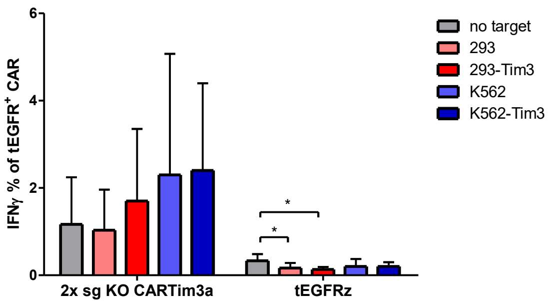


(b) 5hr

(i) TNFα

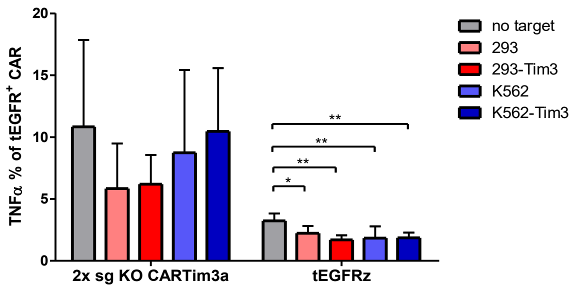


(ii) IFNγ

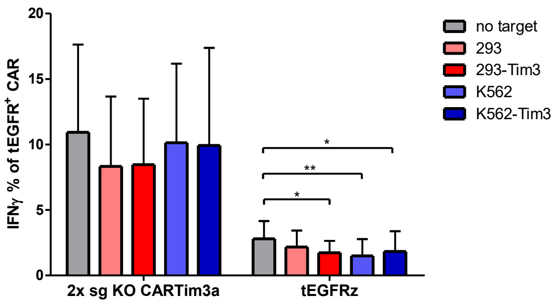


(c) 29hr

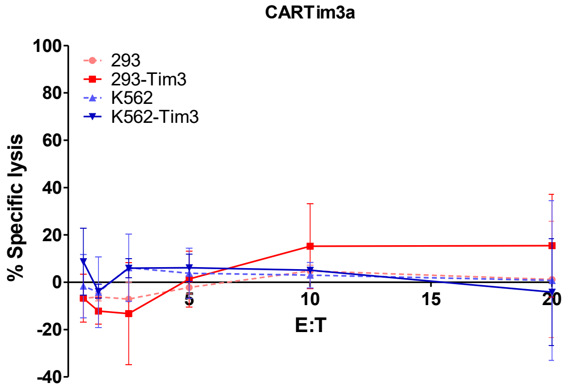
(i) TNFα



(ii) IFNγ



(d)



(e)

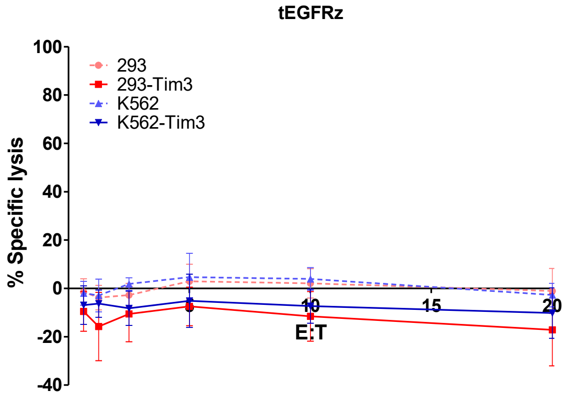


Figure 4.10 Specific *in vitro* cytokine release and cytotoxicity by CARTim3a. (a) TIM-3 expression on negative cell lines: (i) HEK293 and (ii) K562; TIM-3 low cell lines: (iii) TF1 and (iv) KG1; and TIM-3 overexpressing cell lines: (v) HEK293-Tim3 and (vi) K562-Tim3 single cell clones. Blue histograms, unstained cells; red histograms, stained cells. (b) At the end of the 21-day cultures, dual sgRNA TIM-3^{KO} CARTim3a T cells were assayed for TNFα and IFNγ by intracellular cytokine flow cytometry following 5 hours co-incubation with TIM-3 overexpressing cell lines. (c) Longer co-incubation was performed at 29 hours. (n = 3 donors. Repeated-measure ANOVA with Bonferroni's multiple comparison post-hoc test with no target as control, *: $p < 0.05$, **: $p < 0.01$). CAR expression was measured by tEGFRz surrogate CAR. (d) Specific cytotoxicity of TIM-3^{KO} CARTim3a CAR T cells were assessed by calcein-AM release by co-incubating with TIM-3 overexpressing cell lines.

4.3.5 TIM-3^{KO} CARTim3b can be expanded with high CAR expression over 15 days while retaining TIM-3 KO

As the previous manufactured CARTim3a T cells did not possess any functional activity, a second anti-TIM-3 CAR construct was tested, with the antigen recognition domain derived from the scFv sequence of an anti-TIM-3 antibody. Four cultures, CARTim3b and tEGFRz control CAR T cells, both with or without dual sgRNA TIM-3 KO, were expanded by weekly exposure to irradiated K562-cetuximab cells. No significant difference in relative expansion was observed at end of 15-day cultures (mean range: 3.8 – 7.3 fold) (Figure 4.11a).

The cultures were highly enriched for CAR$^+$ cells by day 15 (mean range: 71.8 – 79.5%) (Figure 4.11b), while low TIM-3 proportion was retained in the KO CAR T cells until the end of culture (Figure 4.11c). Both CD4 and CD8 CAR T cells were present, with a predominance of CD8 cells, and no significant differences were observed between the four cultures (mean range: CD4: 25.7 – 30.9%, CD8: 67.3 – 72.4%). The CAR T cell cultures were also not significantly different in terms of memory phenotypes, which consisted of mainly naïve/stem cell memory and central memory T cells (mean range: N/SCM: 46.9 – 57.5%, CM: 29-4 – 31.5%). Most of the CAR T cells expressed PD-1 and LAG3 at the end of culture (mean ranges: PD-1: 72.7 – 89.8%, LAG3: 79 – 92.8%), while TIM-3 expression was lower in the KO cultures than their no KO counterparts as expected (mean: TIM-3 KO vs no KO CARTim3b: 7.5% vs 51.2%, $p < 0.05$; TIM-3 KO vs no KO tEGFRz: 9.6% vs 43%, $p > 0.05$).

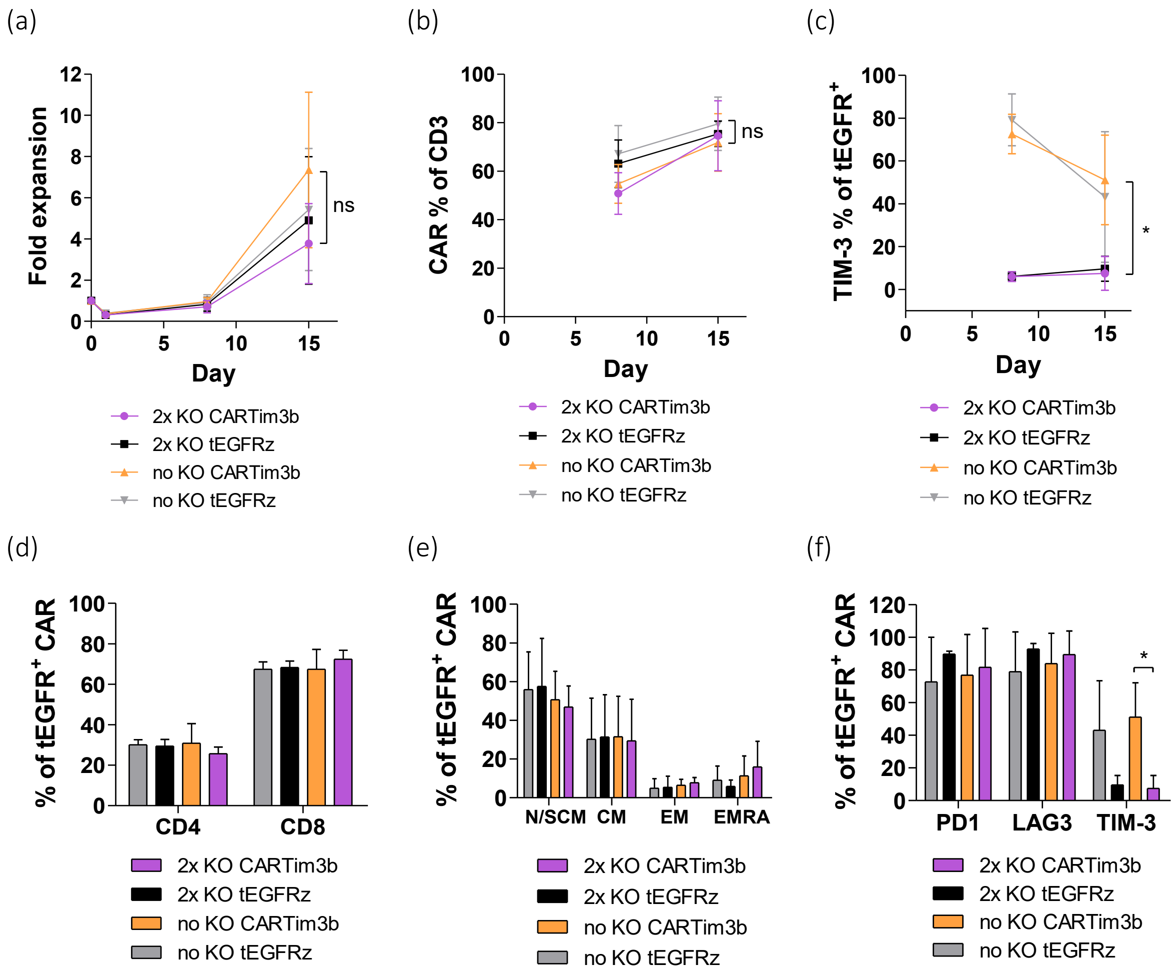


Figure 4.11 CARTim3b T cell expansion and phenotypes after 15-day cultures. (a) Relative fold expansion of CARTim3b T cells with weekly exposure to irradiated K562-cetuximab. Time courses of (b) CAR expression as measured by tEGFRz surrogate CAR and (c) TIM-3^+ expression were assessed by flow cytometry. (d) CD4/CD8 proportions, (e) memory immunophenotype and (f) inhibitory receptor expression of CAR^+ T cells were assayed by flow cytometry. 2x KO, dual sgRNA KO; N/SCM: naïve/stem cell memory ($CD62L^+CD45RA^+$); CM: central memory ($CD62L^+CD45RA^{neg}$); EM: effector memory ($CD62L^{neg}/CD45RA^{neg}$); EMRA, effector memory RA ($CD62L^{neg}CD45RA^+$); PD1: Programmed cell death protein 1; LAG3: Lymphocyte-activation gene 3; TIM-3: T cell immunoglobulin and mucin domain-containing protein 3. (n = 3 per construct, pooled data represented as mean ± SD. *: $p<0.05$)

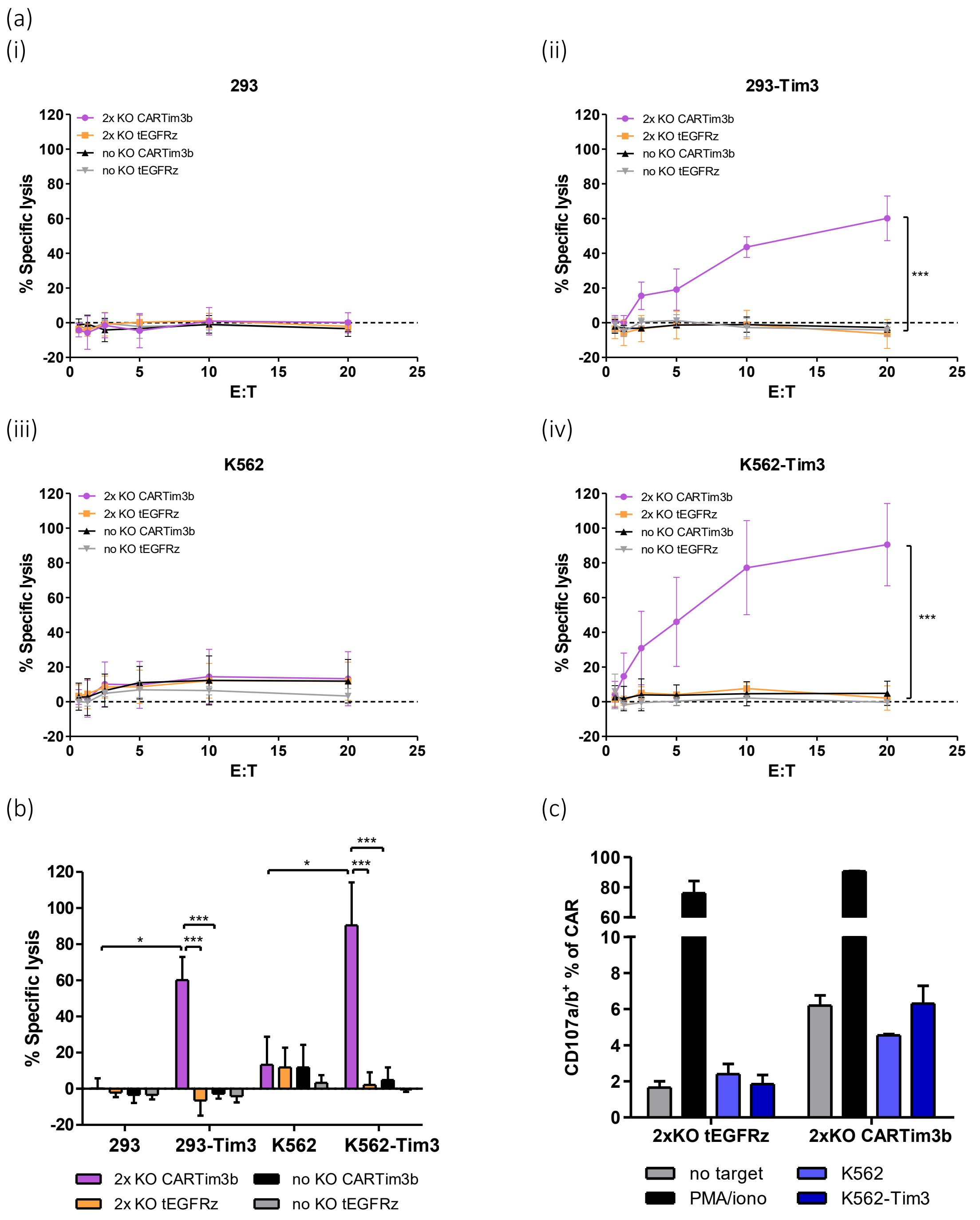
4.3.6 TIM-3^{KO} CARTim3b exhibited specific cytotoxicity against TIM-3 overexpressing cell lines *in vitro*

At the end of the 15-day culture, CARTim3b T cells were examined for specific cytolytic activity by calcein AM assay with TIM-3 overexpressing HEK293 and K562 cell lines as the targets. Cytotoxicity was not observed against TIM-3 negative cell lines HEK293 (mean range: -3.4 to 0.1%) (Figure 4.12a(i)) or K562 (mean range: 3.3 – 13.2%) (Figure 4.12a(iii)) by any of the cultures. Cytotoxicity against HEK293-Tim3 and K562-Tim3 was demonstrated exclusively by the TIM-3^{KO} CARTim3b cells, which were significantly more than the other three cultures, including CARTim3b cells without TIM-3^{KO} (mean lysis at 20:1 E:T against 293-Tim3: TIM-3^{KO} CARTim3b vs TIM-3^{KO} tEGFRz vs no KO CARTim3b vs no KO tEGFRz: 60.1% vs -6.5% vs -2.8% vs -4.1%, $p = 0.0002$; mean lysis at 20:1 E:T against K562-Tim3: TIM-3^{KO} CARTim3b vs no vs TIM-3^{KO} tEGFRz vs no KO CARTim3b vs no KO tEGFRz: 90.5% vs 2.1% vs 4.9% vs -0.4%, $p < 0.0001$) (Figure 4.12a(ii), a(iv), b).

Intracellular cytokine flow cytometry was used to examine specific TNFα and IFNγ production by CARTim3b T cells. No significant increases in TNFα and IFNγ were detected against TIM-3 overexpressing cell lines compared to media only (no target) controls and parental TIM-3 negative cell lines (TNFα mean range: 0.5 – 1.6%; IFNγ mean range: 0.6 – 1.4%) (Figure 4.12d). However, non-specific activation of CAR T cells with PMA/ionomycin consistently showed high levels of TNFα and IFNγ production (TNFα mean range: 64.2 – 73.4%, IFNγ mean range: 59.9 – 76.4%) (Figure 4.12d).

Degranulation assay using flow cytometry to measure the granule-associated proteins CD107a/b expression did not show significant increases either, when TIM-3^{KO} CARTim3b was co-cultured with TIM-3 overexpressing K562 (mean: K562-Tim3: 6.3%, K562: 4.55%, no target: 6.2%). The CAR T cells were capable of degranulation via non-specific activation by PMA/ionomycin (mean: 76.0 – 90.6%) (Figure 4.12c).

To investigate if atypical T cell associated apoptosis induction pathways could explain the observed cytolytic activity of TIM-3^{KO} CARTim3b in the absence of secretion of TH1 cytokines or degranulation, multi-cytokine bead arrays using LegendPlex were performed on supernatants collected after 5-hour and 24-hour co-incubation of TIM-3^{KO} CARTim3b T cells with K562-Tim3. At both time points, there were no significant differences between K562-Tim3, K562 parental cell line and no target controls in the 13 cytokines assayed: granulysin (mean range: 5-hr: 823 – 962, 24-hr: 1940 – 6805 pg/mL), granzyme A (mean range: 5-hr: 9641 – 10867, 24-hr: 13734 – 17409 pg/mL), granzyme B (mean range: 5-hr: 7136 – 8877, 24-hr: 9000 – 21849 pg/mL), perforin (mean range: 5-hr: 118 – 150, 24-hr: 187 – 323 pg/mL), soluble Fas (mean range: 5-hr: 6.9 – 7.1, 24-hr: 12.6 – 15.1 pg/mL), souble Fas ligand (mean range: 5-hr: 65.1 – 85.4, 24-hr: 110 – 138 pg/mL), TNFα (mean range: 5-hr: 8.4 – 15.4, 24-hr: 6.2 – 12.4 pg/mL), IFNγ (mean range: 5-hr: 276 – 662, 24-hr: 1205 – 2814 pg/mL), IL-2 (mean range: 5-hr: 2.9 – 7.5, 24-hr: 2.4 – 12.0 pg/mL), IL-4 (mean range: 5-hr: 3.8 – 4.0, 24-hr: 4.2 – 5.3 pg/mL), IL-6 (mean range: 5-hr: 2.1 – 41.4, 24-hr: 3.1 – 191 pg/mL), IL-10 (mean range: 5-hr: 0.7 – 0.9, 24-hr: 0.9 – 1.6 pg/mL), IL-17A (mean range: 5-hr: 0.3 – 0.3, 24-hr: 0.3 – 0.3 pg/mL (Figure 4.13). Control TIM-3^{KO} tEGFRz CAR T cell also showed similar levels of mediators of target cell lysis (granulysin, granzyme A and B, perforin) as technical PMA/ionomycin positive controls and CARTim3b conditions.



(d)

(i) TNFα

(ii) IFNγ

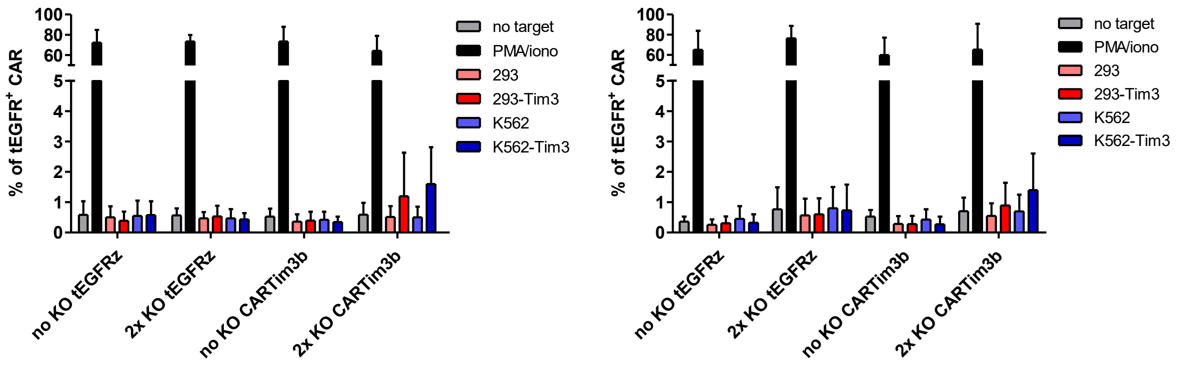
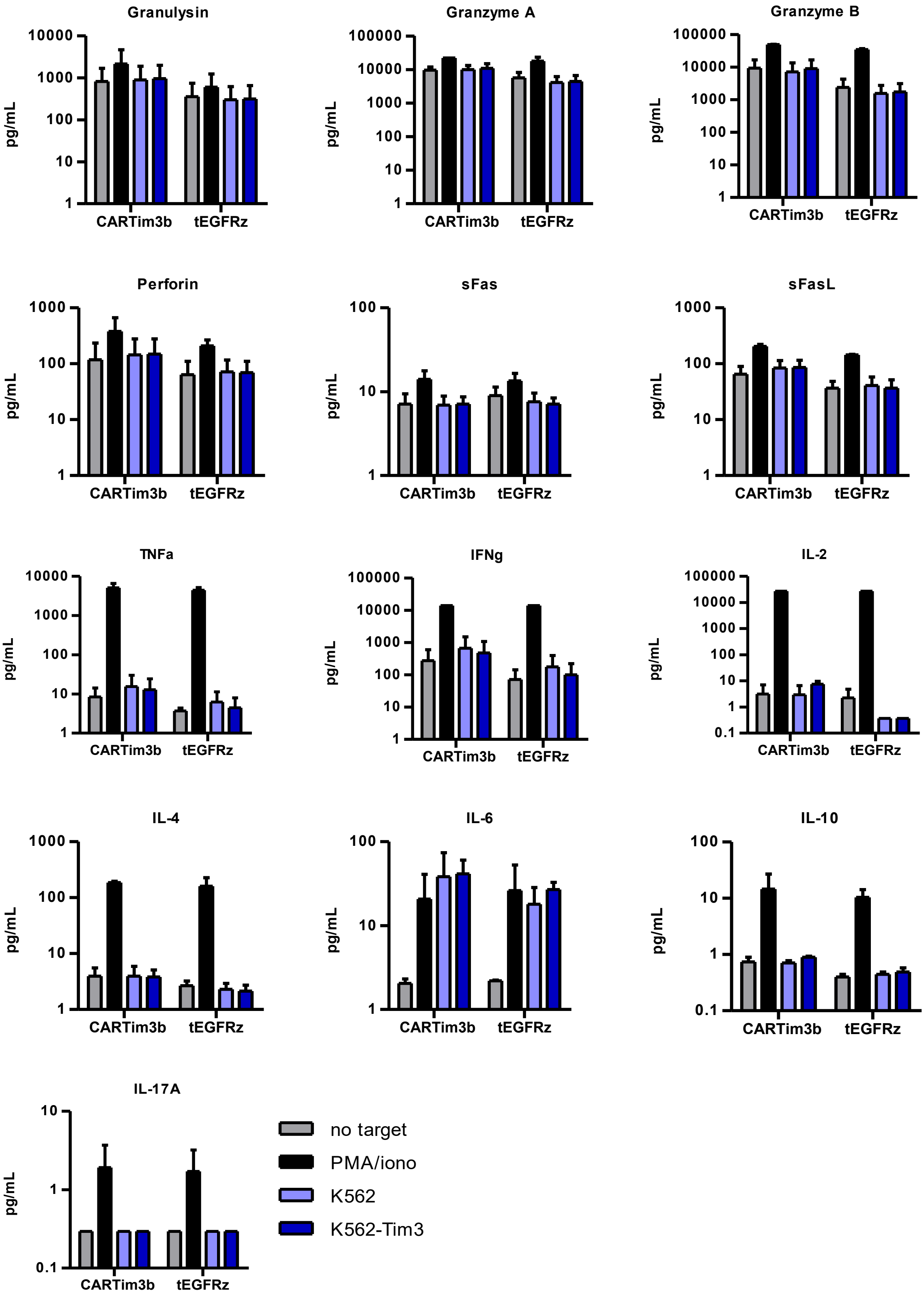


Figure 4.12 ***In vitro* activities of CARTim3b T cells.** (a) At end of 15-day cultures, specific lysis of TIM-3 overexpressing cell lines by CARTim3b and control tEGFRz CAR T cells with and without TIM-3 KO were assessed by calcein AM assay. Specific lysis at 20:1 E:T ratio is summarised in (b) ($n = 3$ donors). (c) Degranulation of TIM-3^{KO} CARTim3b cells was assessed based on CD107a/b expression by flow cytometry after 5-hour co-incubation with targets ($n = 2$ donors). (d) (i) TNFα and (ii) IFNγ production in response to TIM-3 overexpressing cell lines were assayed by intracellular cytokine flow cytometry after 5-hour co-incubation of CAR T cells with target cells ($n \geq 3$ donors). CAR expression was measured via tEGFRz surrogate CAR. Pooled data represented as mean ± SD; repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. PMA/iono, PMA/ionomycin.

(a)


Granulysin
Granzyme A
Granzyme B
Perforin
sFas
sFasL
TNFa
IFNg
IL-2
IL-4
IL-6
IL-10
IL-17A
pg/mL
0.1
1
10
100
1000
10000
100000
CARTim3b
tEGFRz
no target
PMA/iono
K562
K562-Tim3

(b)

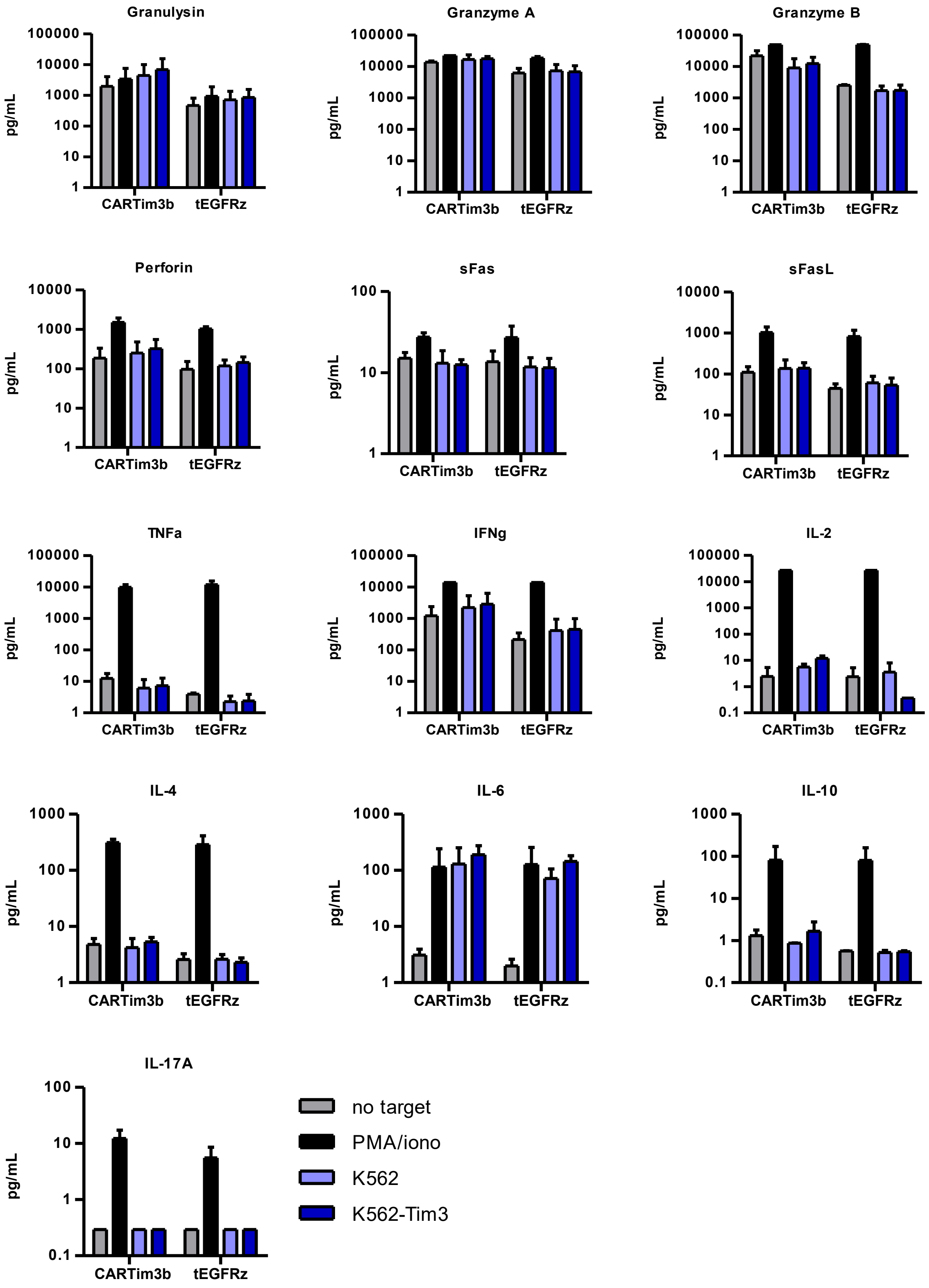

Granulysin
Granzyme A
Granzyme B
Perforin
sFas
sFasL
TNFa
IFNg
IL-2
IL-4
IL-6
IL-10
IL-17A
pg/mL
100000
10000
1000
100
10
1
0.1
CARTim3b
tEGFRz
no target
PMA/iono
K562
K562-Tim3

Figure 4.13 Secreted cytokines by TIM-3^{KO} CARTim3b T cells following co-culture with TIM-3 overexpressing cell line. Cytokine release by TIM-3^{KO} CARTim3b T cells into the supernatant were detected by LegendPlex CD8/NK cytokine bead array after (a) 5 hours and (b) 24 hours co-incubation with TIM-3 overexpressing K562. PMA/iono, PMA/ionomycin; sFas, soluble Fas receptor; sFasL, soluble Fas ligand; (n = 2 donors, pooled data represented as mean ± SD)

4.3.7 CARTim3b T cells may be generated using CRISPR/Cas9 knock-in

Next, CRISPR/Cas9 knock-in (KI) was used to generate CARTim3b T cells with targeted transgene integration into the TIM-3 locus by homology directed repair (HDR) via 35 bp homology arms on both sides of cuts created by sg37 and sg49 sgRNAs. The TransAct stimulated CAR T cells were expanded for 15 days, then FACS sorted for CAR^+ T populations and expanded for another 11 days. Both TIM-3 KI CARTim3b and KI tEGFRz control CAR T cells expanded considerably before cell sorting (mean range: 16.7 – 22.3 fold) (Figure 4.14a). Post sorting, average (± SD) yield of 1.75 ± 0.37 and 1.41 ± 0.58 $x10^6$ CAR T cells were obtained for KI CARTim3b and KI tEGFRz respectively, which expanded further until the end of 26 day culture (mean range: 55.6 – 67.1 fold) (Figure 4.14b).

Insertion into the TIM-3 exon 2 was verified by KI confirmation PCR and Sanger sequencing (Figure 4.15a). However, the sequencing traces show insertion via non-homologous end joining (NHEJ) ligation at the sg37 homology arm (HA) junction for both KI CARTim3b and KI tEGFRz. A mixture of NHEJ and HDR were observed at the sg49 HA junction as sequencing traces past the CRISPR cut point show multiple possible bases at each base position (Figure 4.15b, c).

The proportion of cells in culture with CAR expression before cell sorting was quite low (day 15 mean ± SD: KI CARTim3b: 8 ± 4.2%, KI tEGFRz: 29.5 ± 14.1%). However, after sorting the cultures consisted of high proportions of CAR T cells as expected (day 26 mean ± SD: KI CARTim3b: 88.8 ± 2.4%, KI tEGFRz: 91.8 ± 5.4%) (Figure 4.14c).

At the end of the 26-day culture, there was an almost even distribution of CD4 and CD8 cells (mean ranges: CD4: 49.7 – 51.5%, CD8: 48.5 – 50.1%) (Figure 4.14d). The KI CAR T cells composed of mainly of naïve/stem cell memory and central memory T cells (mean range: N/SCM: 51.9 – 60.9%, CM: 24.8 – 27.3%) (Figure 4.14e). The KI CAR T cells also contained high percentages of PD1 and LAG3 populations, while TIM-3 was low (mean range: PD1: 78.7

– 84.0%, LAG3: 96.1 – 96.5%, TIM-3: 7.7 – 12.1%) (Figure 4.14f). There were no statistical differences between KI CARTim3b and tEGFRz in terms of expansion and phenotype.

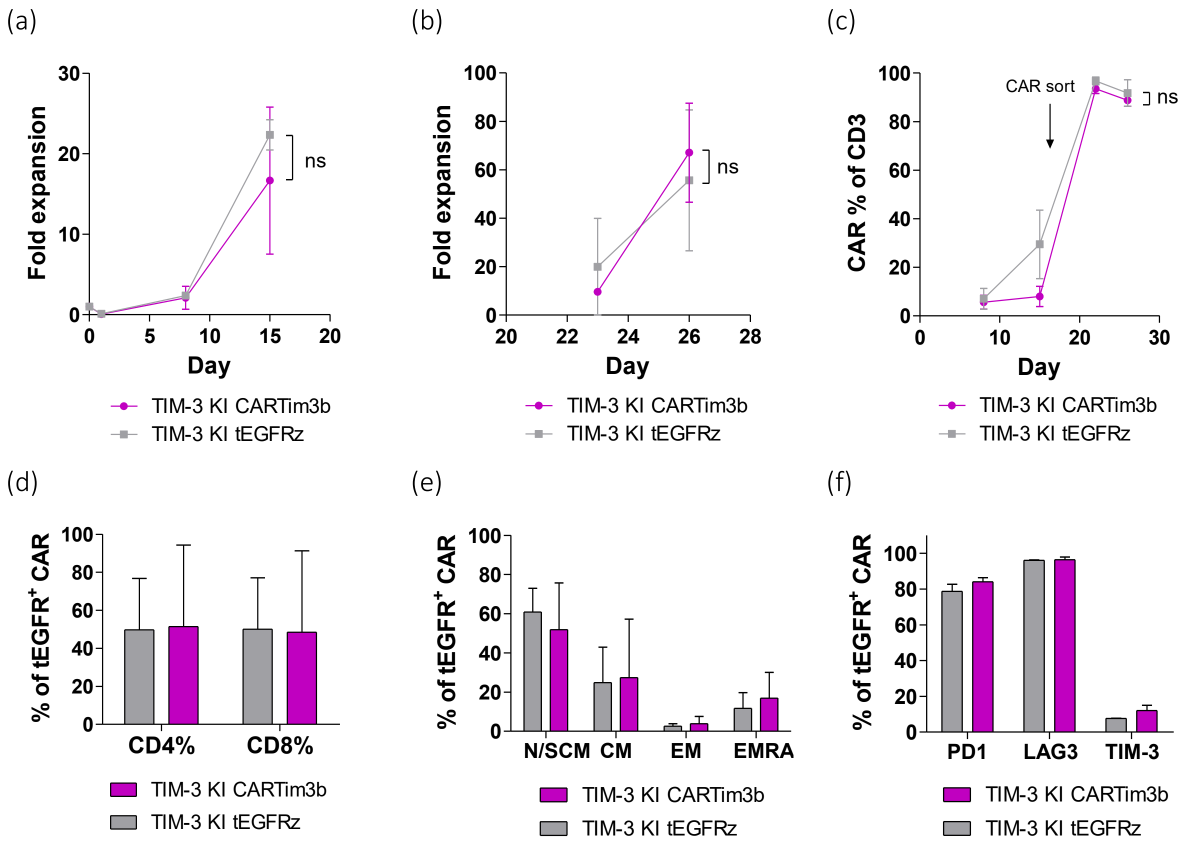
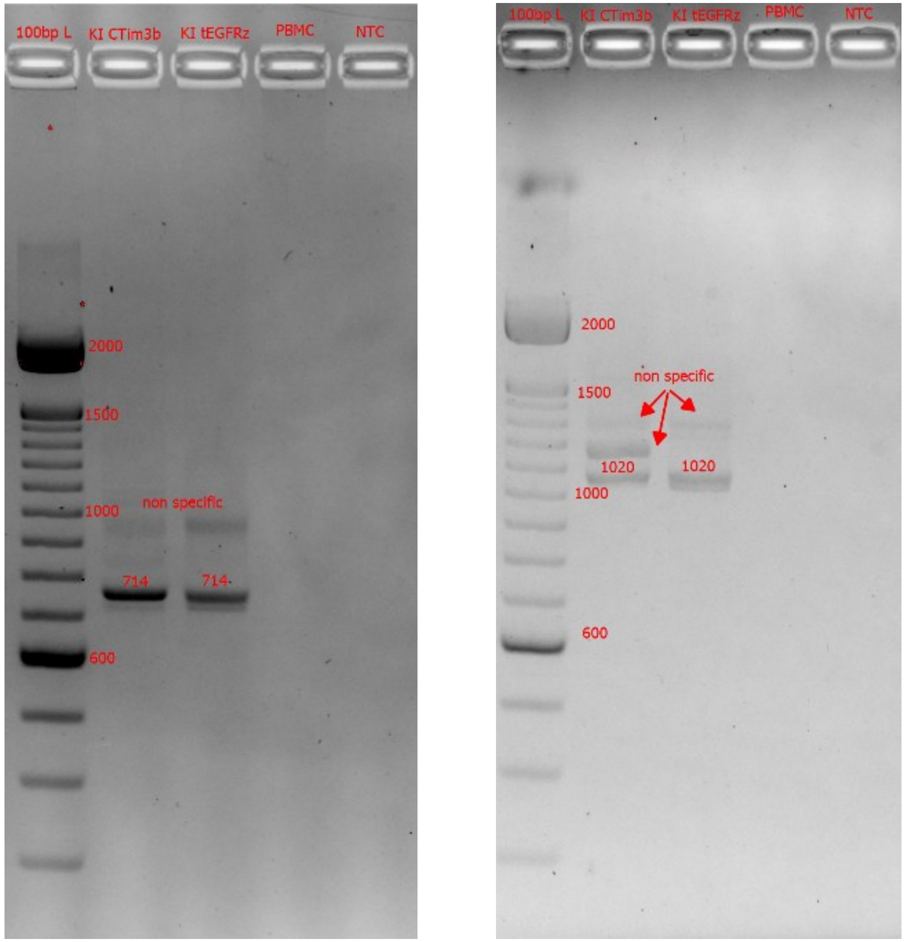


Figure 4.14 Expansion, CAR expression and phenotypes of TIM-3 KI CARTim3b T cells over 26-day culture. (a) Fold expansion of cells following electroporation with CARTim3b HDR templates and weekly exposure to irradiated K562-cetuximab feeder cells until day 15. (b) Further expansion of CAR T cells following fluorescence-activated cell sorting (FACS) on day 15. (c) Time course of CAR expression of KI CAR T cells was tracked by flow cytometry. (d) CD4/CD8 proportions, (e) memory phenotype and (f) co-inhibitory receptor expression of CAR$^+$ T cells were assessed with flow cytometry. CAR expression was measured via tEGFRz surrogate CAR. N/SCM: naïve/stem cell memory (CD62L$^+$CD45RA$^+$); CM: central memory (CD62L$^+$CD45RA^{neg}); EM: effector memory (CD62L^{neg}/CD45RA^{neg}); EMRA, effector memory RA (CD62L^{neg}CD45RA$^+$); PD1: Programmed cell death protein 1; LAG3: Lymphocyte-activation gene 3; Tim3: T cell immunoglobulin and mucin domain-containing protein 3. (n = 3 donors; data represented as mean ± SD. ns: non-significant by repeated-measures ANOVA)

(a)



(i)sg37 junction

(ii) sg49 junction

(b) **CARTim3b** in TIM-3 exon 2, **sg37 junction**

(c) **CARTim3b** in TIM-3 exon 2, **sg49 junction**

(d) **tEGFRz** in TIM-3 exon 2, **sg37 junction**

(e) **tEGFRz** in TIM-3 exon 2, **sg49 junction**

Figure 4.15 TIM-3^{KI} CARTim3b T cells' knock-in confirmation PCR and target bands' sequencing trace alignments. (a) Representative 2% agarose gel image of KI confirmation PCR products. Genomic DNA from end of culture KI CAR T cells were amplified using primers pairs specific against (i) sg37 and (ii) sg49 insert junctions. Target bands are labelled on the gel with expected base pair lengths (714 bp and 1020 bp for sg37 and sg49 junction PCRs respectively). CTim3b, CARTim3b; NTC, no template control; PBMC, peripheral blood mononuclear cells. Target bands from (a) were gel-extracted and Sanger sequenced. Sequence traces are shown in (b) to (e) and compared to reference sequences of different types of insertion (NHEJ or HDR). Reference sequences of before insertion and after insertion (by NHEJ or HDR) are shown above each sequence trace chromatogram. HA, homology arm.

4.3.8 TIM-3 KI CARTim3b T cells demonstrated specific cytotoxicity

The TIM-3 KI CARTim3b T cells demonstrated dose dependent specific lysis activity against TIM-3 overexpressing K562, but not TIM-3 negative K562 parental cell line. Furthermore, CARTim3b T cell's specific activity was significantly higher than KI tEGFRz control CAR (mean lysis at 40:1 E:T ratio: CARTim3b:K562-Tim3, 37.7%; CARTim3b:K562, -7.6%; tEGFRz:K562-Tim3, -2.5%; p = 0.0094) (Figure 4.16a).

However, only small proportions of TIM-3 KI CARTim3b T cells had intracellular TNFα and IFNγ production following co-incubation with TIM-3 overexpressing K562 cells, with no statistical significance detected above media only controls (no target) and TIM-3 negative K562 cells (mean range: TNFα: 0.1 – 1.9%, IFNγ: 0.2 – 0.6%) (Figure 4.16b, c).

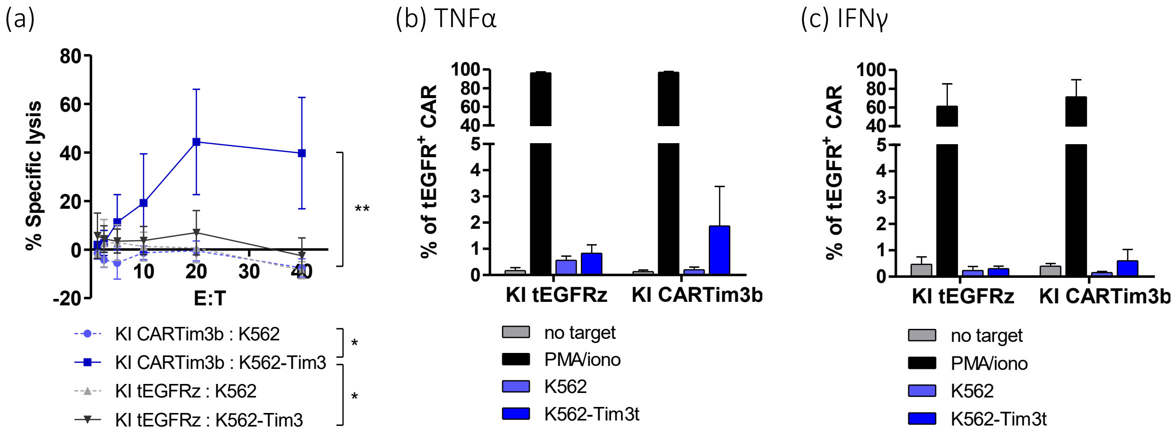


Figure 4.16 Specific cytotoxicity of TIM-3 KI CARTim3b T cells after 26-day culture. (a) Calcein AM assay was used to assess cytotoxic function of KI CARTim3b against K562-Tim3 targets at different effector to target ratios. (b) TNFα and (c) IFNγ production were assessed by intracellular cytokine flow cytometry. CAR expression was measured via tEGFRz surrogate CAR (n = 3 donors, data represented as mean ± SD, *: $p < 0.05$, **: $p < 0.01$. Repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test). E:T, effector to target; PMA/iono, phorbol 12-myristate 13-acetate/ionomycin.

4.4 Discussion

TIM-3 expression was successfully disrupted using single or dual sgRNA CRISPR/Cas9 KO with the intention of preventing fratricide. Although the surface expression of TIM-3 did not differ between the single and dual sgRNA KOs, molecular detection revealed that dual sgRNA was superior in TIM-3 KO on the gene level. The higher KO efficiency of multiple gRNAs has been observed by other groups (Acosta *et al.*, 2018, Wang *et al.*, 2014), and was expected as indels may result from each CRISPR cut site as well as large deletions when both cuts occur. Hence, the dual sgRNA KO approached was used for all subsequent TIM-3 KO in this Chapter and Chapter 5.

Two protype TIM-3 targeting CARs with scFv antigen recognition domains were expressed in TIM-3 KO T cells using the multi-cistronic *PiggyBat* transposons. Both CAR T cells were able to be expanded via the co-expressed tEGFRz CAR. The first CARTim3 construct, CARTim3a, based on a published sequence, lacked any detectable activity by conventional assays, in contrast to the publication by Lee *et al.* from which CAR sequence was replicated (Lee *et al.*, 2021).

A few differences in experimental design and methodologies were noted. Lee *et al*. used either transient mRNA or lentiviral gene delivery systems as opposed to the *PiggyBat* transposon system. In the publication, transient mRNA CAR T cell which were assayed 24 hours post-electroporation at peak CAR expressions of about 70%, while the *PiggyBat* CARTim3a T cells in this Chapter were cultured for much longer (22 days) and the CAR expression was lower (38 – 46%). The lower proportion of CAR positive cells could have contributed to the lower activity of the *PiggyBat* TIM-3^{KO} CARTim3a T cells. Prolonged culture is known to lead to increasing expressions of co-inhibitory receptors such as PD1 and LAG3 (Caruso *et al.*, 2019), which were about 60% and 85% respectively in the *PiggyBat* culture. However, prolonged culture predominantly affects the exhaustion phenotype and *in vivo* function with retention of immediate *in vitro* function as demonstrated by CARs targeting other antigens such as GD2 (Long *et al.*, 2015). Another explanation for this CAR T cell's low activity may be due to its relatively low affinity as higher affinity CARs have been shown to confer stronger cytokine and lytic activities. Affinity maturation and spacer length alterations may be useful in increasing the affinity of the CAR to test this hypothesis in the future (Hudecek *et al.*, 2013, Izgutdina *et al.*, 2023).

Moreover, the assays employed in this Chapter differed from the publication. Calcein AM was used for assaying cytolysis in this thesis, as opposed to flow cytometry and electric impedance-based assays employed by Lee *et al*. The co-incubation was also much longer in the published work (between 24 and 100 hours) compared to this Chapter's study (5-24 hours). In fact, maximal killing was demonstrated after 40 hours by Lee *et al*, possibly due to the use of 4-1BB co-stimulatory domain which is known to be slower acting, but have longer persistence (van der Stegen *et al.*, 2015) or the relatively low affinity of the CAR which contributes to the overall strength of the T cell response (Hudecek *et al.*, 2013). The calcein AM assay's background signal became elevated even at 24 hours, making any longer incubation times impractical. Impedance-based cytotoxicity may be used to confirm the lack of activity by the *PiggyBat* CARTim3a T cells using HEK293-Tim3 as targets in the future. However, the benefits of refining a CAR product whose activity is undetectable by widely used assays was thought to be an inefficient use of time and so a completely new CAR construct was tested.

The second TIM-3 specific CAR, CARTim3b based on another antibody was successfully transfected into T cells. No growth inhibition was detected for the no KO CARTim3b cells, suggesting a lack of fratricide between the CAR T cells, which is dissimilar to the fratricide seen in other CAR T cells (Png *et al.*, 2017). However, despite its growth, the no KO CARTim3b T cells did not possess any cytotoxic activity. Since cell expansion was via stimulation of the co-expressed tEGFRz and CAR expression was also detected using tEGFRz as a surrogate, the actual expression of CARTim3b on the cell surface and possible fratricide impairing activity is uncertain. Another possible cause for lack of function against antigen expressing cell lines is binding of CARTim3b to endogenous T cell TIM-3 either intra/extracellularly, masking or sequestering the CAR (Lu *et al.*, 2022). Direct detection with either engineered tags on the CAR or anti-IgG hinge antibodies could be used to verify its surface expression in resting and activated T cells to assess for which of these mechanisms is contributing to the lack of function in the absence of TIM-3 knockout. A co-precipitation assay could also be used to demonstrate intracellular sequestration.

The fact that CARTim3b T cells demonstrated excellent cytotoxicity against TIM-3 overexpressing targets, but only with TIM-3 KO, confirms that TIM-3 KO was essential for function, similar to knock-out of other target antigens such as CD7 and CD45 present on CAR T cells (Gomes-Silva *et al.*, 2019, Harfmann *et al.*, 2024). Lack of activity of the Tim-3^{KO} CAR T cells against Tim-3 negative controls such as parental cell lines (K562, HEK293) and lack of Tim-3 specific activity of TIM-3 KO tEGFRz control CAR T cells support the conclusion that the activity of the TIM-3^{KO} CAR T cells were TIM-3 specific. To confirm the specificity of the CAR, ELISAs using biotin-tagged TIM-3 and surface plasmon resonance could directly quantitate the affinity of the scFv on which CARTim3b is based.

Despite having excellent lysis activity by TIM-3^{KO} CARTim3b T cells, no significant upregulation of cytokines associated with activation (IL-2, IFNγ and TNFα), extrinsic apoptotic pathway (TNFα, soluble FasL) or perforin/granzyme pathway (Perforin, granzyme A and B) was detected compared to non-activated controls.

It has been reported by some that at high target antigen densities, both high and lower affinity CARs are able to be activated, but at lower target antigen densities, only higher affinity CARs activate (Liu *et al.*, 2015). Furthermore, as some groups have observed, for some CARs, the antigen density threshold for CAR cytotoxicity is lower than cytokine detection (Majzner *et al.*, 2020, Watanabe *et al.*, 2015). The TIM-3 antigen densities/molecules per cell were not formally assessed in this study. Flow cytometry-based methods could be used to enumerate the antigen densities of TIM-3 overexpressing clones and a range of antigen density clones used to determine the threshold for lytic and cytokine activities of CARTim3b.

Cytokine bead array was employed to explore alternative T cell effector molecules which may be involved in CARTim3b T cells' activity. The results showed non-specific release of death inducing granules (granzyme A and B, perforin and granulysin) by both CARTim3b and tEGFRz CAR T cells in the absence of targets, as well as death ligand soluble FasL. This raises the possibility that specific lysis of TIM-3 over-expressing targets in the absence of other evidence of T cell activation could be due to the engagement of CARTim3b with TIM-3 bringing the cells in close enough proximity for the non-specifically secreted death ligands and granules to induce target cell lysis (Benmebarek *et al.*, 2019). This hypothesis may be tested by fluorescent microscopy with cytokine capture (Huse *et al.*, 2006), which could not be completed due to time constraints.

Irrespective of the above potential mechanism of discordant cytotoxicity and activation, it is likely that low affinity of the scFv at least contributed to this phenomenon. This could be assessed for both parent antibody and the scFv by a variety of assays such as surface plasmon resonance or a simple competitive immunoassay (Fischer *et al.*, 2024). If low affinity is confirmed, options to improve CAR function include antibody affinity maturation or development of completely new antibodies or scFv. Other features of the CAR may also be optimised to enhance its activity. CD28 intracellular signalling domain is well known to elicit stronger response than 4-1BB and hence was already employed in CARTim3b. However, given that 4-1BB causes less exhaustion and better persistence *in vivo*, it may be better to use 4-1BB in future CAR designs. The CD28 transmembrane domain of CAR constructs can also heterodimerise with endogenous CD28 molecules, lowering the endogenous CD28 expression

(Muller *et al.*, 2021). It is also conceivable that the CD28 transmembrane domains of the TIM-3 directed CARs and tEGFRz may interact with each other, bringing about unpredictable proliferation or activity. Further investigations using co-immunoprecipitation would illuminate the possible interactions between the CD28 transmembrane domains. In order to prevent these confounding interactions, the CD8 transmembrane domain may be used instead, as this has been shown to not heterodimerise with endogenous CD28 (Muller *et al.*, 2021). Other designs to improve efficacy include altering the origin or length of the extracellular hinge region to optimise scFv-epitope interaction and increasing the number of immunoreceptor tyrosine-based activation motifs (ITAM) in the intracellular domain, which have shown marked enhancements in cytokine production, cytotoxicity and *in vivo* efficacies (Majzner *et al.*, 2020). As T cells are major players of the adaptive immune response, activated CAR T cells and subsequent cytokine product could impact the tumour and the tumour microenvironment. Therefore, new CAR candidates should be tested with immune competent mouse models before clinical translation. However, assessing CAR T cell toxicities such as on-target off-tumour toxicities, CRS and ICANS in models such as humanised or transgenic mice is not perfect, requiring engraftment of human myeloid cells or changes to the CAR construct, limiting the applicability of the findings (Duncan *et al.*, 2022).

A simple method for targeted knock-in of CAR into T cells was developed in this Chapter using HDR templates of linear dsDNA flanked by very short 35 bp homology arms (HAs). The CAR HDR templates were easily and cheaply produced by 5’ extension PCR in-house, in contrast to other published HDR templates with long homology arms that require specialised manufacturing (Roth *et al.*, 2018). The insert length of CARTim3b was 4617 bp, a relatively long insert that would require HA length of between 400 bp – 1.3 kb if delivered via viral vectors and 200 – 800 bp HAs if using dsDNA HDR templates (Shakirova *et al.*, 2023, Yang *et al.*, 2022).

A significant technical issue encountered in this study was the low CAR transfection efficiency and very low viability post electroporation. Cell sorting for CAR$^+$ T cells at day 15 and further culturing was able to compensate for these issues, which drastically enriched the CAR proportion from 8% to about 90%. Clinically, this would entail that the manufacture process would require a high starting number of T cells and substitution of FACS sorting with GMP compliant immunomagnetic enrichment, which adds to the complexity of regulatory approval. Other possible strategies to overcome the low transfection efficiency worth considering include inhibition of cytosolic DNA sensors, enhancing HDR, adding truncated Cas9 target sequences to HDR templates to increase efficiency of nuclear shuttling and stabilising Cas9 ribonuclear proteins into nanoparticles (Kath *et al.*, 2022, Nguyen *et al.*, 2020).

A mixture of insertional mechanisms was also observed at the cleavage sites. As two TIM-3 targeting sgRNAs (sg37 and sg49) were introduced, 8 possible insertion configurations could occur, as each target site may be 1) cut with transgene inserted by non-homologous end joining (NHEJ), 2) cut with transgene inserted via homology directed repair (HDR) or 3) uncut. From the qualitative Sanger sequencing data, it appears CARTim3b knock-in at TIM-3 exon 2 was predominantly by NHEJ at the sg37 HA site and both NHEJ and HDR at the sg49 HA site. Other mechanisms such as microhomology-mediated end-joining (MMEJ) may also occur, confounding the sequencing results (Stadtmauer *et al.*, 2020). The exact proportion of cells which contained HDR may be elucidated with single cell sorting and next-gen sequencing, but was outside the scope of this study. It appears that with the short 35bp HAs, ligation by NHEJ could dominate as the mechanism of repair, which has been utilised by others for gene knock-ins, but this method has an inherent lack of directionality due to the absence of homologous recombination (Geisinger *et al.*, 2016). This will be of concern if the endogenous promoter of the target gene was utilised for expression of the knocked-in CAR, however it is not of concern for this Chapter's CAR construct as the transgene contains an EF1α promoter upstream of the CAR. Other consequences of NHEJ in terms of the transgene persistence through cell divisions, cause of aberrant genetic lesions leading to mutations or its impact on CAR expression remain uncertain. Further cell sorting of different cell generations via CSFE staining, sequencing would be needed to elucidate these questions. These extensive studies would be required for any approval process if this KI approach was to be employed for clinical trials. From a regulatory perspective it would be desirable to optimise the KI to be predominantly HDR and lack of negative consequences from any minor population with NHEJ would have to be excluded.. A whole myriad of strategies to improve the rate of insertion via HDR may be attempted in future experiments, including using longer homology arms (Chai *et al.*, 2024), inhibiting NHEJ (e.g. inhibitor 53, SCR7, adenovirus 4 E1B55K and E4orf6 proteins, siRNA against NHEJ Ku proteins) and promoting HDR (L755507, Brefeldin A and RS-1) by chemical means (Fu *et al.*, 2021, Liu *et al.*, 2018b). Moreover, "all in one" HDR complexes are worth investigating, which tethers single stranded donor oligonucleotides (ssODNs) to the Cas9 ribonucleoprotein and have displayed impressive improvements in HDR efficiency, presumably due to the proximity of the donor DNA to the double-stranded break (Yang *et al.*, 2020).

Off-target effects by CRISPR/Cas9 system are also a potential challenge that need to be investigated. A host of genetic mutations could result as a consequence of off-target cleavages, ranging from benign substitutions, indels, frameshift mutations, translocations and chromothripsis (chromosomal rearrangement and loss due to simultaneous chromosome breaks and repair) (Hunt *et al.*, 2023). CRISPR off-target candidates may be searched *in silico* (Naeem and Alkhnbashi, 2023) and targeted Sanger sequencing may be performed to check for mutations at these candidate sequences. To comprehensively elucidate the genome editing which has occurred in the T cells, more advanced techniques such as CRISPR off-site analysis (e.g. GUIDE-seq, BLESS) and whole genome sequencing would be required (Naeem and Alkhnbashi, 2023). Experimentally, off-target effects can be significantly reduced using novel Cas9 variants such as dCas9-FokI and SpCas9 nickase which produce single stranded breaks to alleviate the genotoxicities associated with traditional CRISPR/Cas9 systems (Naeem *et al.*, 2020).

Despite not being inserted by HDR, the TIM-3^{KI} anti-TIM-3 CAR T cell demonstrated moderate efficacy against TIM-3 overexpressing target cells, albeit at lower cytotoxicity than its KO counterpart. However, due to differences in culture conditions such as TransAct activation, cell sorting and culture time length, the KI CARTim3b T cells cannot be directly compared to KO CAR T cells. Qualitatively both the KI and KO cultures had predominantly naïve/stem cell memory and central memory phenotypes with high PD1 and LAG3 proportions, but the KI CARTim3b T cells had a more even CD4/CD8 distribution. Overall, the feasibility of CRISPR/Cas9 KI CAR T cell culture was demonstrated. However, given its uncertain mechanism of insertion, low transgene insertion efficiency and lower cytotoxicity, it is not yet ready for translation.

Thus, active TIM-3 directed CAR T cells were produced using both random *PiggyBat* integration and site-directed CRISPR knock-in. Of note was the necessity of endogenous TIM-3 ablation for cytotoxic activity. Trends of cytokine production, both specific and non-specific were observed, but none reached statistical significance. This proof-of-concept study established the feasibility of targeting TIM-3 as another potential AML target. Pending *in vivo* mouse studies, one could co-express or co-administer TIM-3 directed CARs in conjunction with CD123-specific CARs as a strategy to avoid risk of antigenic escape by AML cells and improve overall response.

In the next Chapter, the TIM-3 KO and KI technologies established here were applied to CARIL3dm from Chapter 3, with the aim to determine if TIM-3 KO and KI could improve efficacy of CARIL3 and if the technologies developed here were generalisable to other CAR constructs.

Chapter 5. CD123-specific CAR T cells with TIM-3 knock-out or knock-in at the TIM-3 locus

5.1 Introduction

TIM-3 has been shown to play a substantial role in tumour progression through T cell suppression (Acharya *et al.*, 2020). Besides PD-1, it is one of the co-inhibitory/checkpoint receptors which marks the most dysfunctional or terminally exhausted CD8$^+$ T cells in AML (Zhou *et al.*, 2011). Moreover, co-blockade of TIM-3 and PD1 pathways has demonstrated some *in vivo* efficacies in both solid and haematological tumours. Knock-down of TIM-3 expression with short hairpin RNAs (shRNA) have also improved the *in vitro* cytotoxicity, proliferation and cytokine production (TNFα, IFNγ and IL-2) of anti-mesothelin CAR T cells (Jafarzadeh *et al.*, 2021). Another study highlighted the potency of TIM-3 inhibition as triple shRNA knock-down of PD-1, Lag3 and TIM-3 in anti-Her2 CAR T cells significantly improved IFNγ release, survival and tumour control upon rechallenges in mice, while dual knock-down of PD-1 and Lag3 or single knock-down of PD-1 did not show significant improvements (Zou *et al.*, 2019). TIM-3 inhibition using antibodies has also shown clear benefits in increasing the remission induced by CD123-specific CAR T cell in an AML mice model (Kenderian *et al.*, 2015b). These select evidence highlights the potentially significant role TIM-3 plays in CAR T cell activity. Outside of the context of immune effector cell therapy, the role of anti-TIM-3 antibody sabatolimab (MBG453) was tested in a recent clinical trial (STIMULUS-MDS1) in combination with hypomethylating agents, but this study did not show improved remission and survival rates compared to hypomethylating agents alone in patients with MDS (Santini *et al.*, 2022, Zeidan *et al.*, 2024).

In the previous Chapter, it was demonstrated that by incorporating CRISPR/Cas9 mediated TIM-3 locus knockout, a functional TIM-3 targeting CAR T cell, namely CARTim3b, may be generated either by *PiggyBat* gene integration or CRISPR directed knocking-in. It was noted that TIM-3 disruption was essential for specific cytotoxic activity against target cell lines, as without it, CARTim3b T cells had no activity against TIM-3 target cells. Consequently, it was difficult to assess any additional advantages of TIM-3 disruption on CAR function.

In this Chapter, the aim was firstly, to confirm that TIM-3 knockout may be adapted to other CAR T cells, and secondly, to determine the effect of TIM-3 knockout on other normally functional CAR T cells and test whether TIM-3 KO will lead to increased CAR T function. The anti-CD123 CAR, in particular the K116/E22R double mutant, CARIL3dm construct from Chapter 3 was chosen due to it potentially being the best for clinical translation with enhanced binding to CD123 and reduced binding to the common beta subunit responsible for IL3 mediated function, thus decreasing concerns for unwanted effects of receptor activation.

In addition, the CARIL3dm construct was also integrated using the CRISPR knock-in technique from the previous Chapter, into the TIM-3 locus, since it may represent a more specific, safer gene transfer method. Both TIM-3^{KO} and TIM-3^{KI} CARIL3dm T cells were evaluated for *in vitro* activity.

5.2 Materials and methods

5.2.1 Chimeric antigen receptor constructs

The CARIL3dm and tEGFRz constructs in the *PiggyBat* transposon plasmid were previously described in Chapter 3. As illustrated in Chapter 4, the CARIL3dm homology directed repair (HDR) templates (Figure 5.1) for transgene KI was generated using PCR primers with 35bp overhangs that were homologous to the TIM-3 gene on both sides of the CRISPR cut.

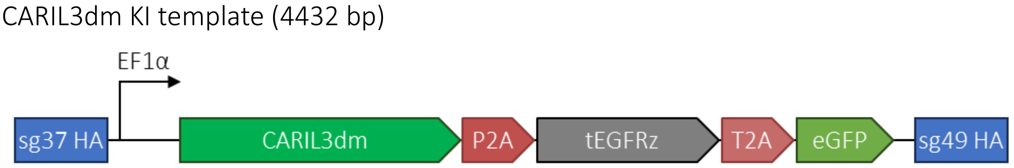


Figure 5.1 Schematic of KI HDR template of CARIL3dm. HDR template was generated by 5' extension PCR of CARIL3dm in the *PiggyBat* transposon plasmid. EF1a, elongation factor 1α promoter; sg37, CRISPR699737_SGM; sg49, CRISPR699749_SGM; HA, homology arm

5.2.2 Generation and expansion of TIM-3^{KO} CARIL3dm CAR T cells

The CARIL3dm T cells with or without TIM-3 KO and TIM-3 KO tEGFRz control were generated as described in Chapter 2, with weekly stimulations using 0.2x IR'd K562-cetuximab feeder cells in cAIM-V media supplemented with 200 U/mL IL-15 every 2-3 days with media changes as required. Culture durations were increased to 26 days, in line with the culture length of TIM-3^{KI} CAR cultures.

5.2.3 Generation and expansion of TIM-3^{KI} CAR T cells

The generation and expansion of TIM-3 KI CARIL3dm T cells were analogous as described in Chapter 2 and Chapter 4. Briefly, it firstly involved the generation of CARIL3dm HDR template by PCR which contained 35bp HAs targeting the sequences flanking the

CRISPR/Cas9 cuts in TIM-3 gene. The HDR templates, Cas9 and sgRNAs (CRISPR699737_SGM and CRISPR699749_SGM) were co-electroporated into pre-activated T cells. Additional flow cytometry activated sorting of CAR$^+$ cells was performed at day 15 and cultured further before cryopreservation at day 26 for use in functional assays at a later date.

5.2.4 TIM-3 knock-in confirmation PCR

PCR was performed to confirm the knock-in of CARIL3dm CAR into the TIM-3 locus using primers targeting the sg37 and sg49 HDR junctions according to the protocol in Chapter 2. Following agarose gel electrophoresis of the PCR products, the bands were excised, and the DNA extracted for Sanger sequencing.

5.2.5 Phenotyping flow cytometry

Thawed CAR T cells were rested in cAIM-V/IL-15 overnight and assayed the next day as described in Chapter 2.

5.2.6 Intracellular flow cytometry

Thawed CAR T cells were assayed for intracellular cytokine production as described in Chapter 2.

5.2.7 Calcein AM release assay

Thawed CAR T cells were rested in cAIM-V/IL-15 overnight and assayed for specific cytotoxicity by co-incubating with target cells the next day as described in Chapter 2.

5.3 Results

5.3.1 TIM-3^{KO} CARIL3dm T cells may be expanded with irradiated K562-cetuximab

CARIL3dm T cells with and without TIM-3 KO were expanded by exposure to irradiated K562-cetuximab artificial feeder cells. TIM-3^{KO} tEGFRz CAR T cells were expanded under the same conditions and used as negative control effectors. The cultures with TIM-3 KO expanded faster than corresponding no KO cultures in the first 2 weeks, but relative cell expansions were not significantly different at the end of day 26 (mean expansions: TIM-3^{KO} CARIL3dm: 7.4-fold, no KO CARIL3dm: 12.9-fold and TIM-3^{KO} tEGFRz: 14.3-fold; $p = 0.1172$ by repeated measures ANOVA) (Figure 5.2a).

The proportion of CAR^+ populations also enriched quickly for all three cultures, increasing to more than 69% by day 15, and then reaching above 89% at the end of the 26-day culture (mean $tEGFR^+$ CAR range at day 26: 89.2 – 97.5%; $p = 0.2758$) (Figure 5.2b). CD4:CD8 proportions were not significantly different between the three CAR constructs (mean ranges: CD4: 36.7 – 63.7%; CD8: 35.3 – 55.7%) (Figure 5.2c).

TIM-3^{KO} CARIL3dm T cells had significantly lower naïve/stem cell memory population compared to no KO CARIL3dm and TIM-3^{KO} tEGFRz control cells (mean: TIM-3^{KO} CARIL3dm vs no KO CARIL3dm: 19% vs 40%, $p < 0.05$; TIM-3^{KO} CARIL3dm vs TIM-3^{KO} tEGFRz: 19% vs 59%, $p < 0.01$; by repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test). Instead, TIM-3^{KO} CARIL3dm T cells were significantly higher in effector memory population than no KO CARIL3dm and TIM-3^{KO} tEGFRz control cells (mean: TIM-3^{KO} CARIL3dm vs no KO CARIL3dm: 21% vs 5%, $p < 0.01$; TIM-3^{KO} CARIL3dm vs TIM-3^{KO} tEGFRz: 21% vs 5%, $p < 0.01$; by repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test). Proportions of central memory and EMRA were not significantly different between TIM-3^{KO} and no KO CARIL3dm T cells. However, EMRA T cells were higher in TIM-3^{KO} CARIL3dm T cells than TIM-3^{KO} tEGFRz control cells (mean: TIM-3^{KO} CARIL3dm vs no KO CARIL3dm: 46% vs 45%, non-significant; TIM-3^{KO} CARIL3dm vs TIM-3^{KO} tEGFRz: 46% vs 20%, $p < 0.05$; by repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test) (Figure 5.2d).

As expected, after 26 days of culture, TIM-3^{KO} CARIL3dm T cells had a significantly lower proportion of TIM-3 cells, as did TIM-3^{KO} tEGFRz control T cells (mean TIM-3^{KO} CARIL3dm vs no KO CARIL3dm: 14% vs 52%, $p < 0.01$; TIM-3^{KO} CARIL3dm vs TIM-3^{KO} tEGFRz: 14% vs 7%, not significant; by repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test). TIM-3^{KO} CARIL3dm T cells had a small but statistically significant increase in PD1 positive population compared with both no KO CARIL3dm and TIM-3^{KO} tEGFRz control T cells (mean TIM-3^{KO} CARIL3dm vs no KO CARIL3dm: 96% vs 85%, $p < 0.05$; TIM-3^{KO} CARIL3dm vs TIM-3^{KO} tEGFRz: 96% vs 86%, $p < 0.05$; by repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test). All three cultures had high proportions of LAG3 positive cells (mean ranges: LAG3: 92 – 96%) (Figure 5.2e).

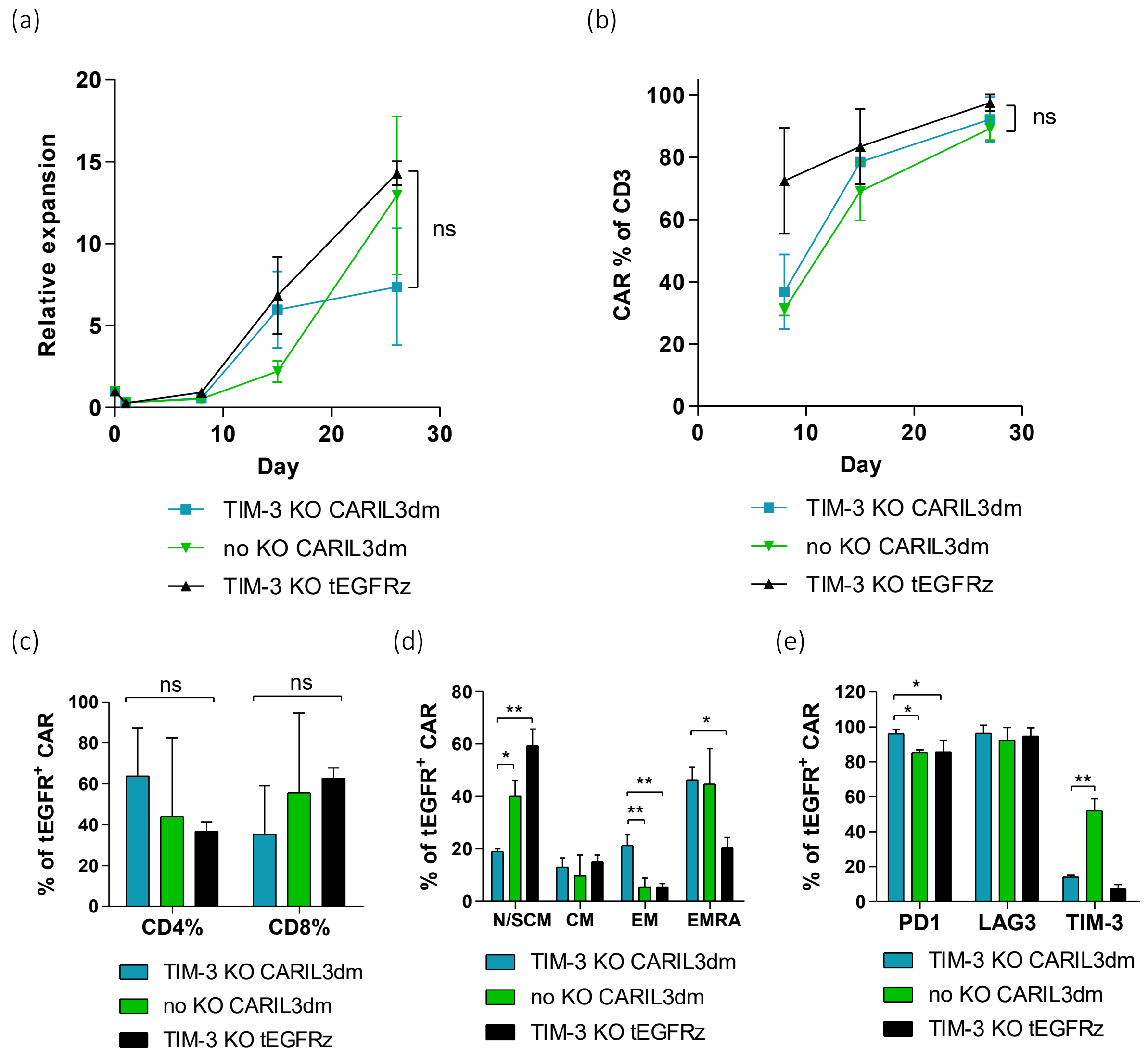


Figure 5.2 Relative cell number expansion and phenotype of TIM-3^{KO} and no KO CARIL3dm T cells. (a) Cell number fold-expansion of three CAR T cell constructs over 26 days following electroporation relative to day 1. (b) Time course of CAR expression as tracked by flow cytometry. (c) CD4/CD8, (d) memory phenotype and (e) co-inhibitory receptors of CAR$^+$ T cells were assessed by flow cytometry at the end of culture (day 26). CAR expression was measured via tEGFRz surrogate CAR. Pooled data (n = 3 per construct) represented as mean ± SD. P-values calculated using repeated measures ANOVA and post-hoc Bonferroni's multiple comparison test. *: < 0.05, **: < 0.01, ns: not significant. N/SCM: naïve/stem cell memory (CD62L$^+$CD45RA$^+$); CM: central memory (CD62L$^+$CD45RA^{neg}); EM: effector memory (CD62L^{neg}/CD45RA^{neg}); EMRA, effector memory RA (CD62L^{neg}CD45RA$^+$); PD1: Programmed cell death protein 1; LAG3: Lymphocyte-activation gene 3; TIM-3: T cell immunoglobulin and mucin domain-containing protein 3.

5.3.2 TIM-3 ligands were expressed in CD123⁺ target cells

Cells with different degrees of CD123 expression, K562-IL3R (CD123^{high}) and KG-1 (CD123^{low}) were used for *in vitro* functional assessment of the CAR T cells (Figure 5.4a). To assess the effect of TIM-3 KO, the presence of TIM-3 ligands, CEACAM-1 and phosphatidylserine (PS) were assessed in the target cell lines. All three cell lines demonstrated presence of CEACAM-1 and PS, though at different levels (Figure 5.4b, c).

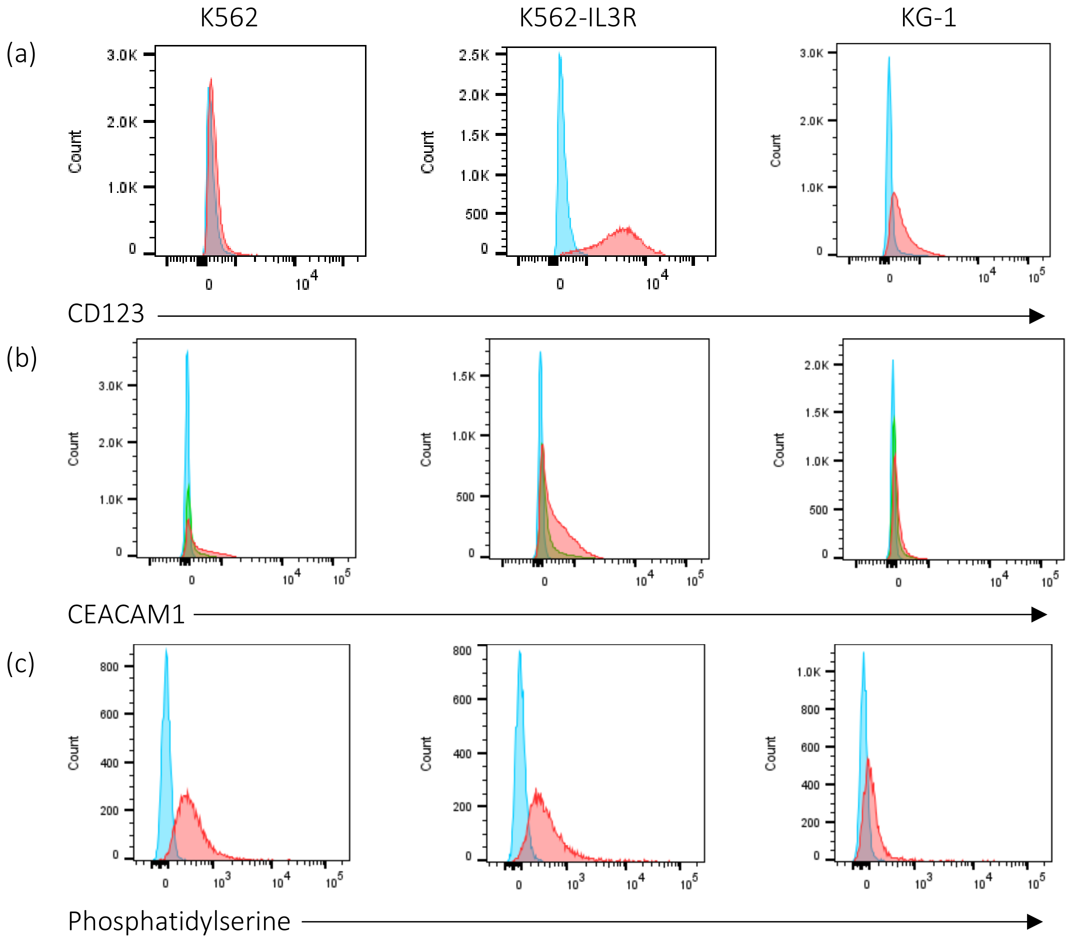


Figure 5.3 CD123 and TIM-3 ligand expressions on target cell lines. Histograms of expressions determined by flow cytometry of (a) CD123; and TIM-3 ligands: (b) CEACAM-1 and (c) phosphatidylserine on target cell lines. In (a), CD123 stained cells are in red. In (b), CEACAM-1 primary antibody and secondary antibody-stained cells are in red, isotype primary antibody and secondary antibody-stained in green, and unstained in blue. In (c), annexin-V stained cells are in red, and unstained in blue.

5.3.3 TIM-3^{KO} CARIL3dm T cells had similar *in vitro* activity compared to no KO CARIL3dm

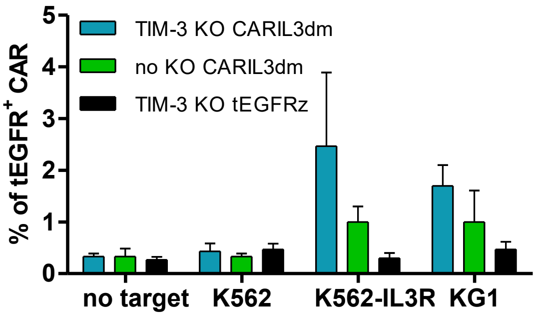
The mean proportions of TIM-3^{KO} CARIL3dm T cells expressing TNFα when co-cultured with either K562-IL3R or KG-1 were numerically higher but did not reach statistical significance compared with no KO CARIL3dm T cells (TIM-3^{KO} vs no KO CARIL3dm: K562-IL3R: 2.5% vs 1.0%, $p > 0.05$; KG-1: 1.7% vs 1.0%, $p > 0.05$; repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test). Similarly, the proportion of the TIM-3^{KO} CARIL3dm T cells expressing IFNγ when co-cultured with CD123 expressing cell lines were also not significantly different to no KO CARIL3dm T cells (TIM-3^{KO} vs no KO CARIL3dm: K562-IL3R: 0.5% vs 0.3%, $p > 0.05$; KG-1: 0.5% vs 0.3%, $p > 0.05$; repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test) (Figure 5.4a, b).

There was no significant expression of TNFα or IFNγ for the TIM-3^{KO} or no KO CARIL3dm T cells when co-cultured with K562 or media only (mean ranges of K562 or media only: TNFα: 0.3 – 0.4%; IFNγ: 0.2 – 0.3%). Similarly, the TIM-3^{KO} tEGFRz control CAR T cells did not have significant expression of TNFα or IFNγ for all co-culture conditions (mean ranges TNFα: 0.3 – 0.5%; IFNγ: 0.2 – 0.4%) (Figure 5.4a, b).

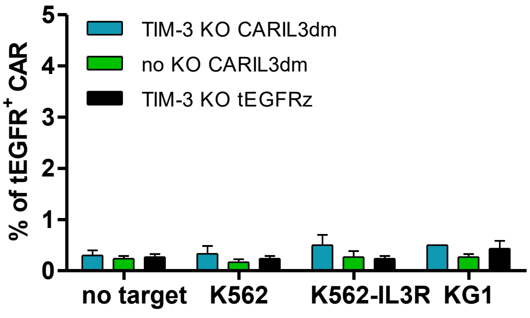
Specific cytotoxicity was assessed by calcein AM assay. Cultures from all three CAR constructs did not show any lysis against CD123^{neg} K562 (Figure 5.4c(i) and (iv)). In contrast, both TIM-3^{KO} and no KO CARIL3dm T cells demonstrated dose-dependent lysis against K562-IL3R and KG-1, which were significantly higher than TIM-3^{KO} tEGFRz control CAR (mean lysis at 20:1 E:T ratio: TIM-3^{KO} CARIL3dm vs no KO CARIl3dm vs TIM-3^{KO} tEGFRz: K562-IL3R: 83.6% vs 70.5% vs 4.5%, $p = 0.0043$; KG-1: 29.8% vs 20.0% vs 6.6%, $p = 0.0068$; repeated measures ANOVA) (Figure 5.4e(ii) and (iii)). TIM-3^{KO} CARIL3dm T cells were not more cytotoxic than no KO CARIL3dm CAR T cells at the highest 20:1 E:T ratio (mean: TIM-3^{KO} vs no KO CARIL3dm: K562-IL3R: 83.6% vs 70.5%, $p > 0.05$; KG-1: 29.8% vs 20.0%,

$p > 0.05$, repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test) (Figure 5.4e(iv)).

(a) TNFα

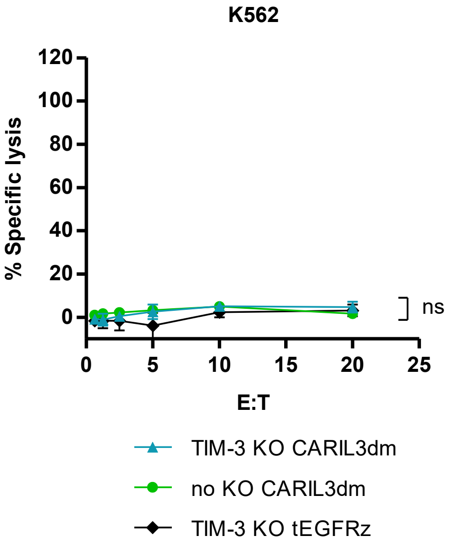


(b) IFNγ

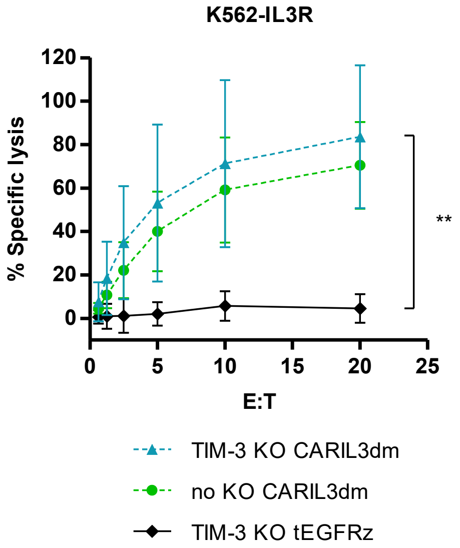


(c)

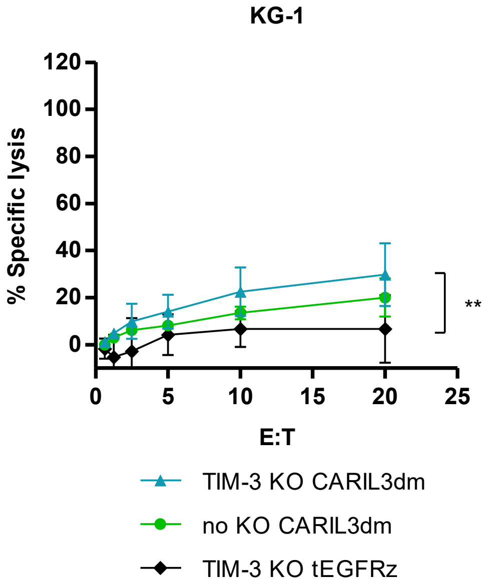
(i)



(ii)



(iii)



(iv)

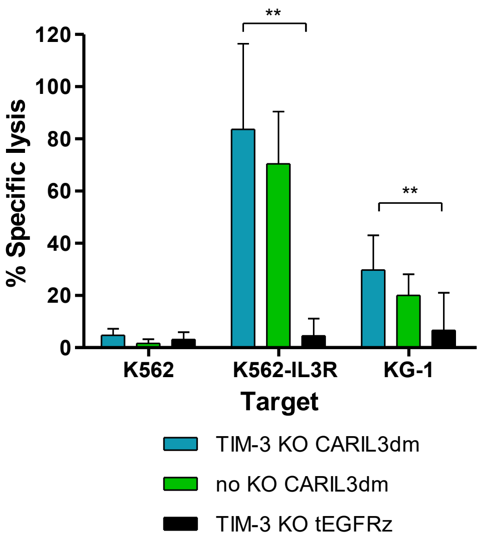


Figure 5.4 ***In vitro* activities of TIM-3^{KO} CARIL3dm T cells.** (a) TNFα and (b) IFNγ productions were assessed by intracellular cytokine flow cytometry after 5-hr co-incubation of CAR T and target cells or media alone (no target). CAR expression was measured via tEGFRz surrogate CAR. (n = 3 donors, pooled data represented as mean ± SD; repeated measures ANOVA). (c) cytotoxicity of CAR T cells after co-incubation with target cells were measured by calcein-AM assay. Specific cytotoxicity at 20:1 E:T ratio is summaries in (iv). (n = 3 donors, pooled data represented as mean ± SD; **: $p < 0.01$, ns: not significant, repeated measures ANOVA at 20:1 E:T ratio with Bonferroni's multiple comparison test).

5.3.4 Enrichment of TIM-3^{KI} CARIL3dm T cells achieved with fluorescence-activated cell sorting

CARIL3dm HDR template was knocked-in to the TIM-3 locus of T cells with or without prior TransAct activation to evaluate the need for T cell pre-activation to allow successful knock-in. Higher expansion with TransAct was observed after 15 days of culture (mean: TransAct vs no TransAct, 33.2-fold vs 0.8-fold, $p = 0.0123$, unpaired t-test) (Figure 5.5a). Therefore, TransAct activation was employed for subsequent knock-in CARIL3dm T cell cultures.

TIM-3^{KI} CARIL3dm T cells expanded up to 24.1-fold by day 15 and was sorted by flow cytometry for CAR expression. After sorting, 0.56 – 1.4 x 10^6 CAR$^+$ cells were obtained and cultured further, which expanded numerically until the end of culture (mean ± SD: 91.8 ± 70.9 fold) (Figure 5.5b). The proportions of CAR$^+$ cells in the cultures were low up to day 15 prior to cell sorting (mean ± SD: 6.6 ± 3.6%). After FACS CAR$^+$ sorting and expansion, a much higher CAR$^+$ population was achieved by the end of culture (mean ± SD: 89.7 + 4.6%) (Figure 5.5c).

At the end of 26 days of culture, CAR T cells' gDNA were extracted and knock-in of CAR was verified using PCR with primers targeting the sg37 and sg49 HA regions (Figure 5.6a). Sanger sequencing was performed on the gel extracted target bands of the PCR products. A mixture of both NHEJ and HDR was evident at both HAs sites, but mostly NHEJ at the sg37 HA, while mostly HDR at the sg49 HA (Figure 5.6b).

The KI CAR T cells were predominantly CD4 (mean CD4: 70.3%, mean CD8: 29.3%) (Figure 5.5d). End of culture memory phenotype was mostly naïve/stem cell memory (mean ± SD: 44 ± 12.6%), followed by EMRA (mean ± SD: 27.7 ± 9.9%) and central memory cells (mean ± SD: 20.7 ± 15.3%) (Figure 5.5e).

High proportions of KI CARIL3dm T cells were PD1 and LAG3 positive (mean ± SD: PD1: 91.7 ± 4.0%, LAG3: 96.7 ± 3.2%). As expected, TIM-3 expression was low in the KI CAR T cells (mean ± SD: 14.7 ± 5.7%) (Figure 5.5f).

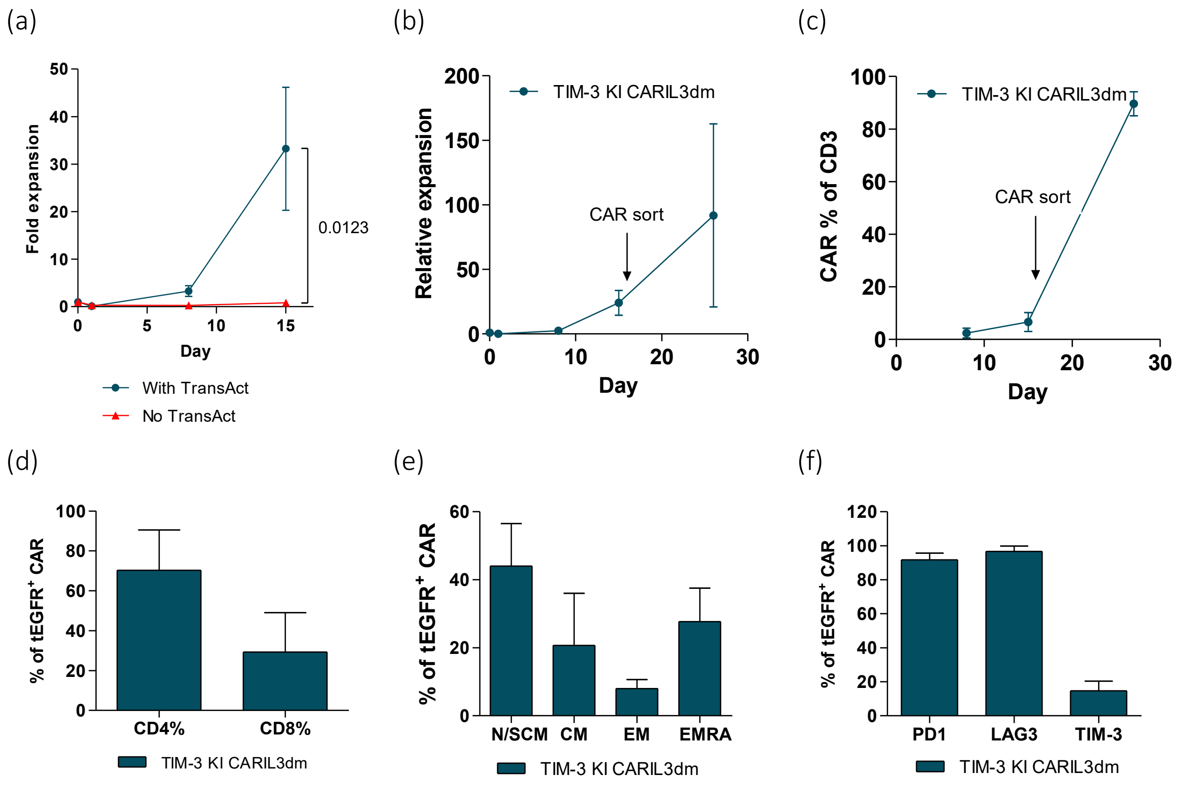
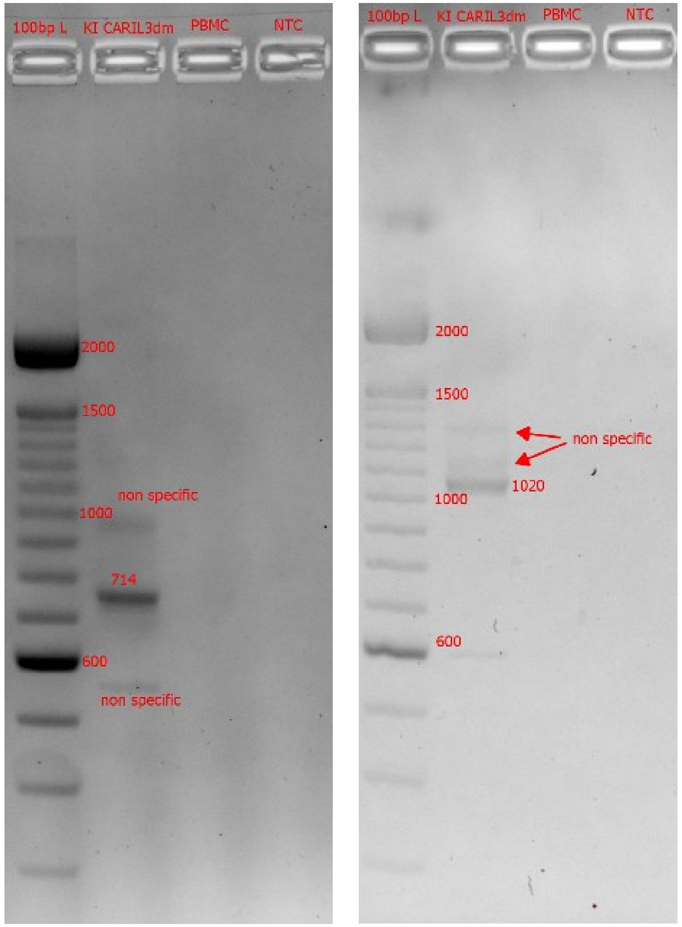


Figure 5.5 TIM-3^{KI} CARIL3dm expansion and phenotype analysis. (a) Cell number expansion of T cells pre-incubated with TransAct or rested prior to electroporation with KI CARIL3dm HDR template. (n =3 donors, data represented as mean ± SD; unpaired t-test). (b) Relative cell number fold-expansion and (c) Time course of CAR expression of TIM-3^{KI} CARIL3dm T cell cultures pre-incubated with TransAct over 26-day culture. (d) CD4/CD8, (e) memory phenotype (f) co-inhibitory receptor phenotypes of CAR T cells were determined at end of cultures by flow cytometry. CAR expression was measured via tEGFRz surrogate CAR. N/SCM: naïve/stem cell memory (CD62L$^+$CD45RA$^+$); CM: central memory (CD62L$^+$CD45RA^{neg}); EM: effector memory (CD62L^{neg}/CD45RA^{neg}); EMRA, effector memory RA (CD62L^{neg}CD45RA$^+$); PD1: Programmed cell death protein 1; LAG3: Lymphocyte-activation gene 3; TIM-3: T cell immunoglobulin and mucin domain-containing protein 3 (n = 3 donors, data represented as mean ± SD).

(a)


(i)sg37 junction

(ii) sg49 junction

(b) **CARIL3dm** in TIM-3 exon 2, **sg37 junction**

Reference sequence: before insertion

Reference sequence: Insertion by NHEJ

Target band forward sequencing trace

(c) **CARIL3dm** in TIM-3 exon 2, **sg49 junction**

Reference sequence: before insertion

Reference sequence: Insertion by HDR

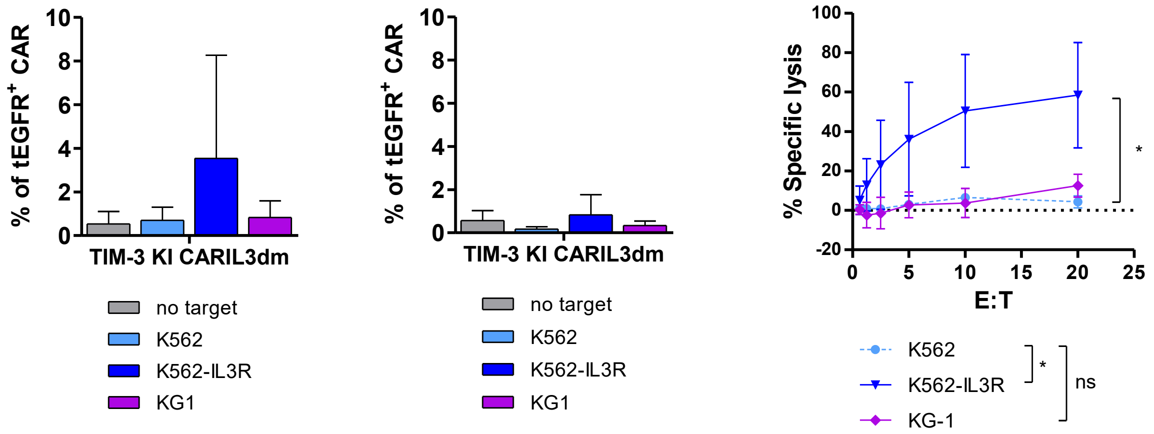
Target band Reverse sequencing trace

Figure 5.6 TIM-3^{KI} CARIL3dm T cells' knock-in confirmation PCR and target bands' sequencing trace alignments. (a) Representative 2% agarose gel image of KI confirmation PCR products. Genomic DNA from end of culture KI CAR T cells were amplified using primers pairs specific against (i) sg37 and (ii) sg49 insert junctions. Target bands are labelled on the gel with expected base pair lengths (714 and 1020 for sg37 and sg49 junction PCRs respectively). CARIL3dm, double mutant (K116W, E22R) IL-3 based CAR; NTC, no template control; PBMC, peripheral blood mononuclear cells. Target bands from (a) were gel-extracted and Sanger sequenced. Sequence traces are shown in (b) and (c) and compared to reference sequences of different types of insertions (NHEJ or HDR). Reference sequences of before insertion and after insertion (by NHEJ or HDR) are shown above each sequence trace chromatogram. HA, homology arm.

5.3.5 TIM-3^{KI} CARIL3dm T cells demonstrated cytotoxicity against CD123-overexpressing cell lines

The *in vitro* cytokine production potentials of the TIM-3^{KI} CARIL3dm T cells were assessed by intracellular flow cytometry after co-incubation with CD123^{+} KG-1 and K562-IL3R targets. No significant increase in TNFα was observed in the TIM-3^{KI} CARIL3dm T cells against CD123 expressing cells when compared to media only controls (mean range: 0.5 – 3.5%, $p = 0.3832$, repeated measures ANOVA) (Figure 5.7a). No significant differences in IFNγ production were found between the cell lines and media only control (no target) as well (mean range: 0.2% - 0.8%; $p = 0.4945$, repeated measures ANOVA) (Figure 5.7b).

The KI CARIL3dm T cells were assessed for specific lysis against CD123^{+} targets by calcein AM assay. Dose-dependent lysis was observed against the CD123 overexpressing K562-IL3R cells up to 20:1 E:T ratio, while little lysis was demonstrated against the CD123^{neg} K562 parental cell line. At 20:1 E:T ratio, significantly higher specific lysis was displayed against K562-IL3R compared to K562, but not between KG-1 and K562 (mean lysis at 20:1 E:T ratio: K562-IL3R vs K562: 58.4% vs 4.3%, $p < 0.05$; KG-1 vs K562: 12.5% vs 4.3%, $p > 0.05$; repeated measures ANOVA with post-hoc Bonferroni's multiple comparison test) (Figure 5.7c).


(a) TNFα

(b) IFNγ

(c)

Figure 5.7 ***In vitro*** **activity of TIM-3^{KI} CARIL3dm T cells.** (a) TNFα and (b) IFNγ productions of KI CARIL3dm T cells against CD123 positive targets (K562-IL3R and KG-1) were assessed by intracellular cytokine flow cytometry. CAR expression was measured via tEGFRz surrogate CAR. (n = 3 donors, data pooled and presented as mean ± SD; repeated measures ANOVA). (c) Specific cytotoxicity of target cells by KI CARIL3dm T cells were measured by calcein AM assay across different E:T ratios (n = 3 donors, data pooled and presented as mean ± SD; *: $p < 0.05$, ns: not significant, repeated measures ANOVA at 20:1 E:T ratio with Bonferroni's multiple comparison test).

5.4 Discussion

This chapter demonstrated that the CD123-specific CAR, CARIL3dm, may be expressed in T cells with disruption of endogenous TIM-3. Furthermore, it was possible to combine TIM-3 KO with directed KI of CARIL3dm into the endogenous TIM-3 locus. Both CRISPR/Cas9 KO and KI were very efficient in TIM-3 ablation, reducing expression to below 15% on average. This is far superior to other reported TIM-3 knock-down methods such as short hairpin RNA (shRNA), which also required multiple targeting molecules to achieve a modest TIM-3 knock-down (25 – 60%) (Jafarzadeh *et al.*, 2021).

Expansion and CAR expression was comparable between the TIM-3^{KO} and no KO CARIL3dm T cells, which underwent the same culturing conditions after *PiggyBat* transfection. For the knock-in of CARIL3dm into TIM-3 locus, the culture method needed to be modified as previously discussed in Chapter 4, including TransAct activation prior to CRISPR/Cas9 electroporation for better expansion of cultured cells (Figure 5.5a). The differences in culturing conditions precludes any meaningful quantitative comparisons between the *PiggyBat* transfected and CRISPR KI CAR T cells.

Qualitatively, the CAR positive population for TIM-3^{KI} CAR T cells was generally much lower than the no KO and TIM-3^{KO} counterparts, as was also previously observed for TIM-3^{KI} CARTim3b in Chapter 4. The low CAR^{+} population was overcome with FACS sorting and cultured further for another 11 days to expand to enough cell numbers for functional assays. The KI transfection efficiency of about 6% is still suboptimal compared to literature (Kruglova and Shepelev, 2024) and would need to be improved via methods discussed in Chapter 4 before translation into a clinically relevant product.

From Chapter 4, CARTim3b without TIM-3 KO did not demonstrate any *in vitro* activities but could lyse TIM-3 expressing target cells in the context of knockout of endogenous TIM-3. It could not be established if TIM-3 KO conferred better *in vitro* activity to CARTim3b T cells due to disabling the co-inhibitory effect of TIM-3, or if it simply removed the TIM-3 on effector cells which could mediate epitope masking, preventing activity against TIM-3 expressing targets. However, in this chapter, I explored knocking out TIM-3 in the context of a known functional CAR T construct (CARIL3dm), which makes it possible to ascertain the impact of TIM-3 KO. Furthermore, target cells expressing TIM-3 ligands CEACAM1 and phosphatidylserine were chosen based on the assumption that a basal level of TIM-3 engagement on effector cells would be required for the effect of TIM-3 KO to be evident.

Specific cytotoxicity against CD123-expressing targets trended higher for TIM-3^{KO} CARIL3dm but did not reach statistical significance. This mirrors observations by others where KO of TIM-3 had a small, non-statistically significant improvement in cytotoxicity and TNFα production in transgenic TCR T cells (Cianciotti *et al.*, 2024). However, the advantages of co-inhibitory receptor KO could become apparent upon long-term chronic antigen stimulation or *in vivo* tumour rechallenge, in which higher degranulation capacity and tumour suppression was demonstrated in TIM-3^{KO} transgenic TCR T cells (Cianciotti *et al.*, 2024). This warrants the exploration of TIM-3^{KO} CARIL3dm T cells' efficacy in an *in vivo* model. Another soluble TIM-3 ligand, galectin-9 is involved in immune escape of AML cells (Gonçalves Silva *et al.*, 2017). Its expression on cells in the tumour microenvironment such as myeloid-derived suppressor cells (Li *et al.*, 2021) and tumour-associated macrophages (Qi *et al.*, 2019) are associated with poor prognosis. The impact of this on CAR T cell efficacy is uncertain, with ligands potentially blocking anti-TIM-3 CAR binding and activity. Conversely, it is conceivable that AML cells that are dependent on the TIM-3/TIM-3 ligand interaction for survival could be more sensitive to TIM-3 specific CAR T cell cytotoxicity. *In vivo* and *in vitro* models using a mix of galectin-9 and TIM-3 overexpressing cell lines could be used to study the interaction of CAR, TIM-3 and its ligands and predict clinical utility of TIM-3 specific CAR T cells.

In viral infection, TIM-3 appears to be a modulator of T cell response. In acute infection, its transient expression is necessary for early T cell activation and effector differentiation. But in chronic infection, TIM-3 expression restricted long-lived memory T cells and contribute to exhaustion (Avery *et al.*, 2018). The shift in memory phenotype by TIM-3 KO was also observed in this Chapter as TIM-3^{KO} CARIL3dm T cells were significantly higher in effector memory phenotype (21% vs 5%), and lower in naïve/stem cell memory population (18% vs 40%) than no KO CARIL3dm. However, the shift to a more differentiated phenotype cannot be explained by TIM-3 KO alone, as the TIM-3^{KO} tEGFRz control CAR had the highest naïve/stem cell memory and the lowest EM and EMRA populations (N/SCM: 59%, EM: 5%, EMRA; 20%).

This shift in memory phenotype was also associated with a higher PD-1 population in the TIM-3^{KO} CARIL3dm T cells. These phenotypes suggest a shift toward a more exhausted phenotype which is characterised by reduced proliferative capacity, persistence and cytotoxic capabilities. It is known that in some anti-PD-1 therapy resistant tumours, alternative co-inhibitory receptors such as TIM-3 are upregulated in tumour-infiltrating T cells (Koyama *et al.*, 2016). Knocking out TIM-3 may have led to the increase of other co-inhibitory receptors such as PD-1. This will have implications for design of future CAR T cell strategies which may require knock-out of multiple co-inhibitory receptor genes, as targeting one or two may not be enough to augment its activity (Zou *et al.*, 2019).

Shorter *ex vivo* culturing times have proven to increase CAR T cell proliferation capacity, limit differentiation, enhance efficacy, anti-leukaemic activity and persistence (Ghassemi *et al.*, 2018). In general, manufactured CAR T cell cultures tend to take between 7 – 10 days long, to avoid exhaustion and terminal differentiation. The longer culture time of 26-days used in this

Chapter likely induced a more exhausted state as shown by a relatively more differentiated phenotype and elevated co-inhibitory receptor expression. Compared to the shorter 15-day culture in Chapter 3, the 26-day no KO CARIL3dm T cells in this Chapter have a higher EMRA and lower central memory proportion (26 days vs 15 days: mean EMRA: 45% vs 21%; mean CM: 10% vs 28%). PD1 and LAG3 expressions were increased in the 26-day cultures as well (26-days vs 15-days culture, mean PD1: 85% vs 54%; mean LAG-3: 92% vs 87%). Inhibition via these receptors may have dampened any enhanced activity from TIM-3 KO which is evident in the qualitatively lower cytotoxicity against KG-1 cells by the no KO CARIL3dm T cells in this Chapter (26-days vs 15-days culture at 20:1 E:T ratio: 20.0% vs 40:6%). Thus, for clinical manufacture, it would be beneficial to reduce the culturing time as much as possible while still obtaining adequate cell doses for infusion. Cytokine supplementations (IL-7 and IL-15) were used in the growth media throughout the thesis to promote a naïve/SCM and CM phenotype and may be compared with other cytokines (e.g. IL-21) and sera (human serum vs foetal bovine serum) to influence the function of the final CAR T cell product (Watanabe *et al.*, 2022).

Functional TIM-3^{KI} CARIL3dm T cells were generated and evaluated for *in vitro* activities against both relatively low CD123 expressing KG-1 and high CD123 expressing K562-IL3R target cells. As demonstrated qualitatively by the Sanger sequencing traces, a mixture of molecular mechanisms of NHEJ and HDR were at play at the CRISPR/Cas9 cut sites, similarly to TIM-3^{KI} CARTim3b and TIM-3^{KI} tEGFRz CAR T cells from the previous Chapter 4. As discussed previously, the exact HDR efficiency may be quantified with single cell sorting and next-generation sequencing. Although outside the scope of this thesis, the efficiency of HDR may be improved in the future with longer homology arms, HDR boosting modules on other types of donor templates (Jin *et al.*, 2024), HDR enhancers, NHEJ inhibitors/silencing and "all in one" HDR complexes (Liu *et al.*, 2018b, Yang *et al.*, 2020).

Off-target double stranded breaks (DSBs) have the potential to cause unwanted genetic abnormalities such as indel mutations, frame-shifts and chromosomal translocations (Hunt *et al.*, 2023). Although there exists many *in silico* methods for predicting off-target cuts, empirical methods for CRISPR off-site analysis such as iGuide (Nobles *et al.*, 2019) and BLISS (breaks labelling in situ and sequencing) (Yan *et al.*, 2017) would provide more clinically relevant information on gRNA specificity. Furthermore, more precise CRISPR/Cas9 gene editing tools are also available such as the programmable addition via site-specific targeting elements (PASTE) system which uses CRISPR/Cas9 nickase fused to reverse transcriptase and serine integrase to knock-in large transgenes up to 36 kilobases without causing double stranded breaks (Ran *et al.*, 2013a, Yarnall *et al.*, 2023). These could substantially reduce the risk of unwanted mutations and gene insertions.

In terms of *in vitro* activity, significant E:T ratio dose-dependent lysis was observed for the KI CAR T cells against K562-IL3R, with almost 60% lysis of target cells at 20:1 E:T ratio, with no significant lysis against KG-which has low CD123 expression. Similarly, a higher percentage of TIM-3^{KI} CARIL3dm T cells expressed TNFα following co-culture with K562-IL3R compared to KG-1, though the difference did not reach statistical significance. However, it was reassuring that the cytotoxicity of TIM-3^{KI} CARIL3dm was comparable to the TIM-3^{KO} and no KO counterparts in this Chapter. The significant specific cytotoxicity of TIM-3^{KO} and TIM-3^{KI} CARIL3dm T cells despite relatively low proportions of specific TNFα and IFNγ expression could relate to the lower CAR affinity threshold required for cytotoxicity as opposed to cytokine release as demonstrated in affinity tuned CAR T cells (Drent *et al.*, 2019, Liu *et al.*, 2015).

The cytokine production of CAR T cells may be increased with different designs such as using scFv derived from higher affinity CARs or modification to the spacer/hinge region. Although a higher affinity CAR could produce higher *in vitro* and *in vivo* activity (Hudecek *et al.*, 2013, Lynn *et al.*, 2016), they carry the inherent greater risk of off-tumour toxicity due to recognition of healthy tissues that possess low level expression of target antigen (Richman *et al.*, 2018). In fact, moderate affinity CARs have produced the best clinical response rates in the context of some solid tumours (Mao *et al.*, 2022). Further efficacy and safety studies, especially using humanised *in vivo* models will be required to determine the optimal affinity for the KI CARIL3dm T cells.

In conclusion, this Chapter demonstrates the feasibility of expressing another CAR, namely CARIL3dm in T cells with either TIM-3 KO or KI to the endogenous TIM-3 locus, with preservation of specific *in vitro* cytotoxicity. Although the TIM-3 disrupted CAR T cells did not demonstrate significantly higher TNFα and IFNγ cytokine release and cytotoxicity compared to the no KO counterparts, TIM-3 disruption did not appear to be detrimental to *in vitro* function. Expansion and CAR expression were similar between the no KO and TIM-3^{KO} CARIL3dm T cells, while TIM-3^{KI} CAR T cells required different culturing conditions. The longer expansion times, T cell activation and cell sorting would not only present logistical challenges in translating the KI CARIL3dm T cells for clinical use, but the longer culturing times and activation could also lead to T cell exhaustion and loss of activity (Wherry and Kurachi, 2015). Furthermore, the exact on-target insertion mechanism and off-target effects of these gRNAs in TIM-3 knock-in need to be elucidated further to ensure minimal genotoxicity.

Chapter 6. General discussion and future directions

CAR T cells are now mainstream therapy for B cell haematological malignancies including B cell ALL, DLBCL and multiple myeloma. Excellent response rates and survival rates have been achieved in both clinical trials and real-world clinics for relapsed/refractory patients. However, CAR T cells have yet to achieve the same success in AML. Difficulty in finding a suitable antigen to target in AML is attributed to multiple factors, including: 1) Heterogeneity of overexpressed antigens between patients, 2) Variable expression of leukaemic antigens in the blast population in each patient – which may provide mechanism for immune escape, and 3) Expression of leukaemic antigens on normal myeloid cells leading to on-tumour off-target toxicities. In this thesis, I explored CD123 and TIM-3 as potential targets for CAR T cell therapy as they are overexpressed in a high proportion of cases of AML and expressed by leukaemic stem cells. These candidate CARs were expressed in T cells using three different gene delivery techniques with the aim to improve the efficacy and safety of the proposed treatment, so as to bring AML targeting CAR T cells closer to the clinic.

6.1 Summary of key findings

The aims of this thesis were to:

1) Explore CARIL3 T cells manufacturing with the *PiggyBat* transposon and lentiviral systems in preparation for future clinical translation.
2) Investigate CRISPR/Cas9 KO and KI at the TIM-3 locus, while concurrently expressing CAR against TIM-3, as a step toward KI CARIL3 T cells.
3) Demonstrate the *in vitro* efficacy of CARIL3 T cells in the context of CRISPR/Cas9 KO and KI at the TIM-3 locus.

In Chapter 3 of the thesis, CARIL3 manufacturing with the *PiggyBat* and lentiviral gene modification system was explored. Three variants of IL-3 ligand-based CAR T cells (wild-type, K116W single mutant and E22R/K116W double mutant) were successfully produced using both *PiggyBat* and lentiviral gene modification systems. Specific activity for all three CAR T constructs was demonstrated in both gene modification system against cell lines with native low-level expression of CD123 and gene-modified cell lines overexpressing CD123. As expected, the CAR-IL3 constructs containing the K116W mutation (CARIL3sm and CARIL3dm) displayed the greatest *in vitro* efficacy. The CARIL3dm construct, which carries the additional point mutation E22R aimed at abrogating potentially counterproductive IL-3 downstream signalling in target cells showed similar *in vitro* efficacy to CARIL3sm in the *PiggyBat* and lentiviral gene modification systems. There were no significant differences in random integration patterns between the lentiviral and *PiggyBat* systems, but the transgene copy number was lower in the lentiviral system.

In Chapter 4, the CRISPR/Cas9 system was explored for generation of TIM-3 specific CAR in the context of TIM-3 KO in CAR T cells to prevent fratricide. The second construct screened (CARTim3b) conferred specific activity, and I confirmed that TIM-3 KO was essential for specific lysis by CARTim3b T cells. Targeted knock-in of anti-TIM-3 CAR transgene to the TIM-3 locus was also undertaken, aimed at reducing the risk of genotoxicity from random integration events. The T cells with knock-in of CARTim3b in the Tim3 locus showed *in vitro* activity despite gene integration into TIM-3 locus via a mixture of NHEJ and HDR.

In Chapter 5, the effect of TIM-3 KO on CAR efficacy was tested in the context of CARIL3dm. Although function was retained in the TIM-3 KO CAR T cells, they were not significantly more efficacious *in vitro*. Furthermore, CRISPR/Cas9 KI was also applied to the CARIL3dm to demonstrate the wide applicability of this technique to other CAR T cells as an alternate gene delivery system for translation of CARIL3dm into the clinic.

6.2 Strength and limitations of the research

6.2.1 Strength

Ligand based CARs, such as the IL-3 based CAR used throughout the thesis, are a relatively novel design with the potential to target multiple cancer types (Murad *et al.*, 2018). Their coding sequences are typically shorter than scFv transgenes, taking less room in the cargo capacity of gene delivery vector (e.g. lentiviral, *PiggyBat*). This potentially allows other transgenes (e.g. another CAR, inducible suicide switches or selection markers) to fit in the gene delivery vector, which is essential as CAR T cell gene manipulations become more complex to combat the challenges of immunosuppressive tumour microenvironment, antigen escape, toxicities and CAR T cell persistence. Furthermore, tonic signalling (spontaneous activation) of CAR T cells due to scFv aggregation remains an issue in some CARs, which reduces anti-tumour function and persistence. While solutions exist, involving computational modelling to identify and substitute the problematic amino acids (Chen *et al.*, 2023a, Landoni *et al.*, 2021), ligand-based CARs could circumvent this issue entirely. CAR binding domains derived from murine antibodies/scFvs also have issues of immune mediated rejection. Non-human sequences of the CAR could be recognised as foreign by both cellular and humoral immunity, leading to early apoptosis of CAR T cells, poor function, short persistence and in severe cases anaphylaxis in patients pre-sensitised to CAR T cells (Wagner *et al.*, 2021). Instead of humanising murine scFvs using labour intensive antibody screening, selection and purification (Sommermeyer *et al.*, 2017), CARs derived from human ligands potentially pose a much lower risk of immune rejection.

Due to heterogeneous target expressions of AML, multi-antigen-targeted CARs in tandem/dual and bicistronic formats or co-administration of multiple CAR T cell products may be required in the clinic. This thesis presented functional CAR T cells targeting two commonly overexpressed antigens in AML (CD123 and TIM-3), demonstrating the feasibility of using these targets for future more complex CAR developments.

This thesis investigated three different gene delivery techniques for CAR expression in T cells, each of which had its strength. *PiggyBat* and lentiviral based methods required only modest starting cell numbers to satisfy clinical cell doses compared to CRISPR knock-in due to a combination of better expansion rates and CAR^+ enrichment. As a result, *PiggyBat* and lentiviral CAR T cells underwent much shorter cultures (12 – 15 days), compared to CRISPR KI CAR T cells (26 days). Shorter CAR T cell cultures are known to improve proliferation capacity, skew toward less differentiated phenotypes, enhance anti-leukaemic activity and persistence (Ghassemi *et al.*, 2018). This could have contributed to the less differentiated phenotype and lower PD-1 expression in the shorter lentiviral and *PiggyBat* CAR T cells cultures.

PiggyBat transposon and lentiviral vectors are considered random integration vectors, and it was confirmed in this thesis that other than an enrichment in rRNA genes by *PiggyBat,* neither had any preference in terms of different genomic features (e.g. exon, intron, intergenic, promoter, untranslated regions, etc).

The vector copy number (VCN) per cell, or the number of integrated CARs per cell, has been theorised to associate with CAR expression and potency of the CAR T cells. However, higher VCN can increase the risk of integration near oncogenes, leading to malignant transformation. Hence, FDA has recommended a limit of 5 VCN per cell for randomly integrating vectors. This limit was satisfied by both *PiggyBat* and lentiviral CAR T cells manufactured under the protocols in this thesis. There was also no clear correlation between CAR copy number and CAR positive proportion, expression level (staining index), cytokine release or cytotoxicity, but this remains to be robustly tested as CAR copies/cell may be titrated up or down depending on the amount of CAR vectors used at electroporation/transduction (Sutrave *et al.*, 2022).

CRISPR/Cas9 targeted integration is much more controlled than the random integration of transposon and lentiviral vectors, so theoretically have a much lower risk of oncogenesis. This would be advantageous in view of recent reports of CAR^{+} T lymphomas in patients infused with lentiviral and transposon CAR T cell products (Harrison *et al.*, 2023, Micklethwaite *et al.*, 2021). However, it should be noted that typical vector induced insertional mutagenesis is extremely rare with only a handful of cases reported to date (Hu and Dunbar, 2024, Perica *et al.*, 2025). As such, the oncogenicity risk of CRISPR KI based CAR T cells could only be apparent after extensive follow up of patients. The TIM-3 locus was chosen as the target integration site, simultaneously disrupting TIM-3 expression to investigate possible functional improvements to the KI CAR T cells. Knock-in to TIM-3 was confirmed by Sanger sequencing in both TIM-3^{KI} CARIL3 and CARTim3b T cells. Reassuringly, both TIM-3^{KI} CAR T cells displayed *in vitro* cytotoxicity against overexpression cell lines despite the mixture of integration mechanisms (HDR and NHEJ) observed at the CRISPR cut sites.

In terms of practicality of clinical translation of CARIL3dm, the *PiggyBat* transposon system presents a simple and cheap method of generating novel CAR T cells utilising only plasmid vectors, with the added advantage of a large transgene cargo capacity providing the option of further genetic engineering to optimise the CAR T cell function, safety and persistence *in vivo*. On the other hand, CAR T cells generated with lentiviral vectors are widely used clinically, with well-characterised toxicity profiles(Labbe *et al.*, 2021). Furthermore, lentiviral CAR T cell cultures required the shortest time to produce clinically relevant T cell numbers, which is a major advantage especially considering that these CAR-T cells are most likely to be initially trialled in patients with relapsed and refractory AML where disease progression is often extremely rapid.

6.2.2 Limitations

Each of the three gene modification systems used in this thesis has its limitations. Transgene insertion via *PiggyBat* transposon and lentiviral vectors are random with an inherent risk of insertion mutagenesis, which are rare but potentially devastating events with case reports recently described (Harrison *et al.*, 2023, Micklethwaite *et al.*, 2021). To my knowledge, CAR T cells manufactured with the *PiggyBat* transposon has not yet been implemented in any clinical trials, so regulatory approval for clinical trials will likely require rigorous testing, delaying clinical translation. Lentiviral vectors have been used extensively in clinical trials and commercial CAR T cell manufacture with a low risk of insertional mutagenesis. While there is an additional risk of inadvertently introducing replication competent lentiviruses (RCL), stringent manufacturing quality control make this theoretical with no known cases reported to date (Cornetta *et al.*, 2018). CRISPR/Cas9 mediated knock-in has the least genotoxicity in the form of insertional mutagenesis, as insertion is targeted via HDR. However, off-target cleavages could still pose a risk and may lead to benign substitutions, indels, frameshift mutations, translocations and other genetic aberrations some of which could lead to malignant transformation (Hunt *et al.*, 2023, Naeem and Alkhnbashi, 2023). Currently, there are only a few CAR T cell clinical trials utilising CRISPR/Cas9 disruption of target genes, mainly for the purpose of preventing product mediated graft versus host disease from allogeneic CAR T cells (universal "off-the-shelf" CAR T cells) (Song *et al.*, 2024) with limited genomic safety data. Moreover, genomic safety data for the CRISPR/Cas9 system cannot be directly extrapolated as differences in gRNA sequences, target genomic region and Cas9 variant dictate the extent of off-target effects. Therefore, the off-target effects by the gRNAs used for disrupting TIM-3 will need to be empirically determined to demonstrate suitability for translation.

Both lentiviral and CRISPR/Cas9 knock-in methods are limited in the cargo size they can deliver, about 12 kb and 10kb respectively. Recent advances in CRISPR technology such as integrase coupled with programmable nuclease and transposase and CRISPR-associated transposon (CAST) could potentially address this issue as the cargo capacity has been increased to over 100kb, although further validation and safety evaluation is still in progress (Chen *et al.*, 2024).

Lentiviral and CRISPR/Cas9 gene modifications are also labour intensive and expensive, especially when GMP grade processing is required. The long culture duration of CRISPR/Cas9 knock-in CAR T cells required in this thesis is also unfavourable for clinical applications.

While *in vitro* cytotoxicity was demonstrated for anti-CD123 CAR T cells (CARIL3wt, CARIL3sm, CARIL3dm), as well as the anti-TIM-3 CAR T cell (CARTim3b), cytokine responses to co-culture with target cells were suboptimal, particularly in the lentiviral constructs and TIM-3^{KO} and TIM-3^{KI} CAR T cells. Further investigations of alternative cytokines and effector molecules is needed to elucidate their mechanisms of target cell killing, in the absence of significant interferon gamma and TNF-alpha release. The final memory and CD4/CD8 distribution of the CAR T cell products were not tightly controlled and have large inter-donor variability, which could lead to variable efficacy in the final products. Studies have shown that defined CD4/CD8 subsets have greater activity (Sommermeyer *et al.*, 2016). The expression of co-inhibitory receptors - PD-1, LAG3 and TIM-3 (when not knocked out) was elevated in the majority of CAR T cell cultures in this thesis, which could potentially impact on persistence *in vivo* (Poorebrahim *et al.*, 2021).

The presence of CD123 and TIM-3 on cells other than AML bulk and leukaemic stem cells could lead to on-target off-tumour toxicity. CD123 and TIM-3 are found at low levels on other myeloid cells, which when targeted by CAR T cells can lead to significant myelosuppression in patients. While rescue haematopoietic stem cell transplants can be performed after remission is achieved, this adds to the cost, complexity and risks of the patient care journey.

As the CAR T cells only target one antigen each, antigenic escape can lead to relapse, (Mishra *et al.*, 2024). *In vivo* models are required to further explore potential mechanisms of treatment failure and to allow mitigating strategies to be developed, such as dual-targeting CARs, combination therapies and switchable CARs. Currently, *in vivo* murine studies are being prepared to ascertain the efficacy and toxicity for the CAR T cells from this thesis.

6.3 Future directions

6.3.1 Improving safety

With the increasing reports of CAR^+ lymphomas and secondary malignancies in patients infused with CAR T cell products, the risk of genetic toxicity has become increasingly scrutinised (Ghilardi *et al.*, 2024, Hamilton *et al.*, 2024, Harrison *et al.*, 2023, Micklethwaite *et al.*, 2021, Ozdemirli *et al.*, 2024, Perica *et al.*, 2025). Cases of CAR T positive lymphoma are exceedingly rare, and could often be difficult to definitively prove. However, a recent case has been published where insertion was within the tumour suppressor gene *TP53* (Perica *et al.*, 2025), adding to the previous reports of disruptions to *TET2*, *KRAS* and *DNMT3A* genes (Hu and Dunbar, 2024). Secondary cancers in general are somewhat more common, with one reporting 25 in 725 (~3%) in patients treated with CAR T cells. However, these patients are often heavily exposed to potentially genotoxic chemotherapy and the increased risked of secondary cancer cannot be attributed to the CAR T cell therapy alone (Hamilton *et al.*, 2024). The CRISPR/Cas9 knock-in system presents a great opportunity to circumvent the problems of random integration using directed knock-in. Theoretically there would only be a maximum of 2 CARs per cell at a predetermined locus if specific knock-in occurs on both chromosomes. However, off-target double stranded breaks (DSB) can occur dependent on the gRNA sequence and the Cas9 variant used, posing a risk of mutagenesis. Non-specific integration of CARs can also theoretically occur at those cut sites, as implied by the NHEJ ligation which was observed at some cut sites in this thesis. Therefore, *in silico* methods may be helpful for predicting off-target cuts and selecting the optimal gRNA sequence (Naeem and Alkhnbashi, 2023). Furthermore, currently other alternative CRISPR/Cas9 systems with lower rates of indels and off-target editing have become available such as single-strand annealing-mediated knock-in and Programmable Addition via Site-specific Targeting Elements (PASTE).

Non-integrating gene transfer methods such as episomal lentiviral vector and bacteria-free DNA mini-circle may also be worthwhile to test as they pose significantly lower risk of insertional mutagenesis (Mulia *et al.*, 2021).

Due to the expression of target antigens on non-tumour cells including normal myeloid cells and other tissues, AML-targeting CAR T cells could cause on-target off-tumour effects in patients. Incorporating safety switches such as inducible caspase-9 (iCasp9) and tag molecules (e.g. tEGFR, CD20) in the CAR T cells could enable targeted apoptosis via chemical inducer of dimerization and antibody-dependent cellular cytotoxicity respectively and may help address prolonged myelosuppression due to prolonged CAR-T persistence (Gargett and Brown, 2014, Philip *et al.*, 2014, Wang *et al.*, 2011). Alternatively, Boolean molecular circuits such as AND, NOT, IF-THEN circuits may also be employed to limit the targeting of CAR to only cells with certain antigen combinations, improving specificity and reducing on-target off-tumour toxicity (Bucher and Feucht, 2023, He *et al.*, 2020, Savanur *et al.*, 2021). CAR constructs may also be cloned under the control of biological or artificial inducers of activation to have temporal control of its expression (Fraessle *et al.*, 2023, Zhang *et al.*, 2019).

Although CRS and ICANS were not the focus of the thesis, they are serious side effects which risk increases with disease burden and CAR T cell dose (Gust *et al.*, 2017). Variable CAR T cell doses could be adopted dependent on antigen expression in individual cases. Additional genetic manipulations such as knock-out of GM-CSF or overexpression of IL1-Rα could also reduce CRS without compromising efficacy, but will require vectors with large cargo capacities (Giavridis *et al.*, 2018, Sterner *et al.*, 2019).

With the advent of allogeneic CAR T cells, gene editing tools such as CRISPR/Cas9 has become indispensable in abrogating molecules such as native TCRs and HLA molecules involved in GvHD and allo-rejection to produce "off-the-shelf" CAR T cells (Castelli *et al.*, 2022).

6.3.2 Improving efficacy

Besides enhancing the safety of CARs, improving their efficacy is also an area of intense research. These improvements may be undertaken for 1) CAR features directly, 2) additional bioengineered features, 3) manufacturing considerations or 4) clinical factors.

6.3.2.1 CAR features

The most obvious way to enhance activity is by altering the design of the CAR. However, as discussed in the introduction, the selection of antigenic target presents the first challenge. CD123 and TIM-3 were chosen as targets in this thesis due to their relative restricted overexpression on AML bulk and LSC, roles in AML developments and prognosis and evidence of pre-clinical and early clinical efficacies. However, they are by no means the only possible targets as other CARs against CD33, CLL1/CLEC12A, FLT3, CD7, GM-CSF receptor and ADGRE2 are under active clinical trials (Saito and Nakazawa, 2024).

Screening for high affinity antibodies and basing scFvs on those are most likely to provide higher activity to the modified T cell (Hudecek *et al.*, 2013, Izgutdina *et al.*, 2023). However, efforts in discovering higher affinity CARs must be tempered as it could lead to on-target, off-tumour toxicity without increasing the maximal activation (Chmielewski *et al.*, 2004). Ligand-based CARs such as CARIL3 explored in this thesis may avoid the problem of tonic signalling for scFv based CARs, their activities may be further improved by mutating key amino acid residues to increase affinity as demonstrated by the K116W mutation in this thesis and by others (Hasegawa *et al.*, 2021). To prevent possible activation of AML blasts from binding of the ligand-based CAR CARIL3, the CAR-IL3dm construct in this thesis includes the point mutation E22R which abrogates IL-3R downstream signalling in target cells, which should remove a potentially counter-productive source of pro-survival signal for the leukaemic blasts. Other domains of the CAR may also be modified to improve its activity, including using CD28 hinge and transmembrane domains, (Majzner *et al.*, 2020), adding G_4S spacer (Hasegawa *et al.*, 2021) using a shorter hinge (Atilla *et al.*, 2019, Hudecek *et al.*, 2013) and increasing the number of CD3ζ ITAMs (Majzner *et al.*, 2020).

6.3.2.2 Additional bioengineering features

Additional bioengineering methods may be employed in CAR T cells to enhance efficacy. Transgenic expression of soluble IL-15, membrane bound IL-15, or tethered IL15-IL15Rα in CAR T cells have also been shown to enhance activities and persistence and may be explored to improve the suboptimal activities of some the CAR T cells in this thesis (Mu *et al.*, 2018, Nguyen *et al.*, 2024, Sánchez-Moreno *et al.*, 2024, Ye *et al.*, 2024). However, its constant expression may have side effects, as exhibited in a case study in which IL-15 expressing 4th generation CAR T cells induced severe and atypical CRS in an AML patient associated with high levels of circulating TNFα (Ataca Atilla *et al.*, 2020). This highlights the need for careful design and testing of new CAR constructs.

Overcoming the immunosuppressive tumour microenvironment (TME) is another hurdle currently under intense investigations. The AML microenvironment comprises of cellular interactions, soluble environmental components and structural factors. AML blasts express inhibitory ligands such as PD-L1 and galectin-9 (a TIM-3 ligand) that interact with co-inhibitory receptors on T cells, including CAR T cells, leading to T cell exhaustion. It is evident that in some tumours, multiple co-inhibitory receptor knockout/knockdown is necessary for an improvement in T cell function, as blockade of one co-inhibitory receptor (e.g. PD-1) can upregulate other co-inhibitory receptors (e.g.TIM-3) (Cianciotti *et al.*, 2024, Koyama *et al.*, 2016, Zou *et al.*, 2019). TIM-3 was readily knocked out using dual sgRNA CRISPR/Cas9 system on two different CAR T cells in this thesis. Although no immediate *in vitro* improvement was observed, it paves the way for multiple knockout of co-inhibitory receptors (e.g. PD-1, LAG-3, 2B4, CTLA-4). Furthermore, inverted cytokine receptors may be co-expressed on CAR T cells to convert inhibitory signals (cytokines/ligands) from AML cells into activation signals, which have exhibited improved persistence, anti-tumour activities and less differentiated phenotype in other solid tumour models (Mohammed *et al.*, 2017, Wang *et al.*, 2019b, Zhou *et al.*, 2023).

6.3.2.3 Manufacturing considerations

CAR expression density on the transgenic T cells must also be balanced in relation to the target antigen density as adequate CAR expression is needed for anti-tumour activity, but excessive CAR density can lead to undesirable loss of selectivity or tonic signalling (Ajina and Maher, 2018, Walker *et al.*, 2017, Weijtens *et al.*, 2000). The CAR expression level may be controlled by titration of transgene delivery vector or using different gene promoters (e.g. PGK vs EF1α) to drive differential CAR expression (Ajina and Maher, 2018).

CAR T products with defined subset ratios have been demonstrated to be superior and more consistent in potency compared to unselected CAR T cells (Sommermeyer *et al.*, 2016, Turtle *et al.*, 2016a, Turtle *et al.*, 2016b). Therefore, combining known ratios of CD4: CD8 or memory phenotype CAR T cells could be another avenue for improving efficacy as well as addressing inter-donor variability, which would make manufactured cell therapy product more consistent. By carefully selecting the cytokine supplementation during manufacture, one can improve the persistence of CAR T cells which is crucial for prevention of relapse. In this thesis, a strategy of IL-7 and IL-15 supplementation instead of IL-2 was used to favour the formation of stem cell like and central memory phenotypes of the final CAR T cell product (Drake *et al.*, 2016, Kaartinen *et al.*, 2017). Metabolite adjustments such as using 2-deoxy-D-glucose, L-arginine and 6-diazo-5-oxo-L-norleucine, other cytokines such as IL-12 and IL-21, and integrating oncolytic viruses with CAR T cells are some novel approaches to improve CAR T cell persistence (Ghorai and Pearson, 2024). Compared to first-generation CAR T cells, second generation CAR T cells such as the ones used in this thesis have better activity and persistence due to inclusion of CD28 or 4-1BB costimulatory domains. Further alterations to CAR designs such as additional costimulatory domains (OX40, ICOS, or CD27) and transgenic cytokine expressions in later generation CARs are also worth exploring for better persistence. Shortening culturing times is also a simple method to improve the function and persistence of autologous CAR T cells (Agliardi *et al.*, 2025), though it could be limited by the number of starting T cells harvested from heavily pre-treated patients.

6.3.2.4 Clinical factors

Furthermore, clinical factors would also play a crucial role in the efficacy of CAR T cell products in patients. Due to the heterogeneity of AML and possibility of antigenic escape, considerations need to be made for addressing resistance such as multi-targeting CAR T cells (Tettamanti *et al.*, 2022). This thesis demonstrated individual functional CARs targeting overexpressed antigens CD123 and TIM-3 on AML, which lays the groundwork for multi-targeting CAR T cell products such as tandem CARs, bicistronic CARs, and co-administration of multiple CAR T cells. This multi-targeting approach is supported by reports of the relatively low response rates by single antigen-targeting BiTEs and DARTs targeting CD123 (vibecotamab (Ravandi *et al.*, 2023) and flotetuzumab (Uy *et al.*, 2021)) and TIM-3 (sabatolimab (Zeidan *et al.*, 2024)). Other overexpressed antigens on AML such as CD33, CLL1/CLEC12A and FLT3 are also explored by other groups as CAR targets, which are also worth considering as additional or alternative targets for multi-targeting CAR T cell products if CD123 and TIM-3 targeting CAR T cells proves to produce less than ideal responses *in vivo*. An example of a multi-targeting CAR is the cooperative CAR co-targeting ADGRE2 and CLEC12A ADCLEC.syn1 which demonstrated preferential *in vivo* efficacy against AML LSCs over normal HSPCs, and a clinical trial (NCT05748197) is underway (Haubner *et al.*, 2023). Nevertheless, targeting more AML associated antigens brings it higher risk of destruction of the closely related normal myeloid cells, which may necessitate salvage therapies including haematopoietic stem cell transplants (HSCTs) that brings with it its own treatment-related risks and costs.

There is emerging evidence that the proinflammatory cytokines in the TME produced following CAR T cell treatment for AML could mediate target cell resistance due to pro-survival signal provided to the leukaemic cells. The phase I study by Bhagwat *et al.* using anti-CD123 CAR T cells resulted in only 25% response in AML patients and were subsequently not durable (Bhagwat *et al.*, 2024). Further investigation revealed an upregulation of cytokines including IL-3, GM-CSF and FLT3L which supported blast survival and resistance to CAR T cell killing. Incorporation of pharmacological agents such as the Janus kinase (JAK) inhibitor, ruxolitinib was able to negate the pro-survival signal from these cytokines *in vitro*. Although this approach still needs to be validated with *in vivo* models, it presents a possible avenue to tackle cytokine-mediated resistance in AML.

In vivo testing of the functional CAR T cells exhibited in this thesis are underway. The anti-tumour activity of CARIL3dm and TIM-3^{KO} CARTim3b T cells will be assessed against patient derived xenografts murine models and AML cell lines. The most likely translatable gene delivery methods of lentiviral and *PiggyBat* vectors will be utilized. Given the relatively low level of *in vitro* activity demonstrated by the CAR constructs tested in this thesis, it is likely that further development such as those described above will be needed before translation of the most promising CAR T cell products for clinical trials using good manufacturing practice (GMP) compliant process developments at our Sydney Cell and Gene Therapy manufacturing facility.

6.4 Conclusion

This thesis presented the successful generation of candidate CAR T cell products targeting overexpressed AML antigens, CD123 and TIM-3, using different gene delivery methods of *PiggyBat* transposon, lentivirus and CRISPR/Cas9 knock-in. Specific *in vitro* activities were demonstrated by the CAR T cells against tumour cell lines.

Balancing different aspects of efficacy and safety, the lentiviral vector is the most acceptable platform for clinical translation for these candidate CARs, given its long-proven record of genetic safety and clinical efficacy. Hence lentiviral CAR T cells will be prioritised for murine model testing.

With the limited cargo capacity of lentiviral vectors, it will be difficult to accommodate more and more complicated genetic manipulations needed to combat antigen heterogeneity, immune evasion and the immunosuppressive TME. Furthermore, to avoid random genetic integrations, lentiviral vectors may be replaced in the future by newer specific methods such as CRISPR/Cas9 knock-in, the feasibility of which was demonstrated in this thesis.

The *PiggyBat* transposon vector system may be accepted as a viable approach for gene delivery in the future following further safety validations. However, for now, this system remains a straightforward, cost-effective and relatively non-labour intensive method which is able to insert large transgenes and facilitate rapid testing of new CARs in a pre-clinical setting.

This thesis presents feasible platforms and CAR candidates for further development with the goal of producing clinically relevant AML-specific CAR T cells, providing pathways to better clinical outcomes for patients of this devastating disease.

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